Physico-chemical, techno-functional and structural properties of native and glycated proteins isolated from black soldier fly (*Hermetia illucens*) larvae

By

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“You have seen by now what education means to us: the identification of ourselves with the masses. Education to us means service to Africa. In whatever branch of learning you are, you are there for Africa. You have a mission; we all have a mission. A nation to build we have, a God to glorify, a contribution clear to make towards the blessing of mankind. We must be the embodiment of our people's aspirations. And all we are required to do is to show the light and the masses will find the way”'

Robert Mangaliso Sobukwe - How Can A Man Die Better (1924 – 1978)

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated) that reproduction and publication thereof by the Cape Peninsula University of Technology will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Extracting the constituents of edible insects such as lipids and proteins for food applications is a promising approach to upturn global consumption in the next 2 – 3 decades. Black soldier fly (*Hermetia illucens*), an edible insect, rich in protein and lipids is one of the most promising insect species for incorporation in food products due to the environmental benefits, coupled with available technology for their rearing. The need to increase the global consumption of edible insects is paramount due to the high demand for protein sources for human consumption.

This study aimed to extract and characterise native BSFL protein and to compare the effect of glycation on the physico-chemical, techno-functional and structural properties of BSFL-Glu (black soldier fly larvae-glucose) protein conjugates as a function of reaction temperature and time with a view to obtain novel proteins with superior functionalities for food application.

The proximate compositions of freeze-dried BSFL (BSFL-FD), defatted BSFL (BSFL-DF), alkali and isoelectric precipitation BSFL protein concentrate (BSFL-PC1), alkaline extraction protein concentrate (BSFL-PC2) was established. BSFL protein concentrates (BSFL-PC1 and BSFL-PC2) displayed significantly higher ($p < 0.05$) protein content and lower ash ($p < 0.05$) compared to BSFL flours (BSFL-FD and BSFL-DF). The essential amino acid content of BSFL-PC1 was superior compared to BSFL-PC2. The high solubility at low pH values for the protein concentrates (BSFL-PC1, 95% and BSFL-PC2, 85%) makes them ideal candidates for use in acidic beverages. The foaming capacity (FC) of BSFL-FD (40%) was not statistically different ($p > 0.05$) than that of BSFL-DF (55%). The highest emulsion capacity (EC) was determined for the protein concentrates BSFL-PC1 (100%) and BSFL-PC2 (100%).

Reaction mixtures containing black soldier fly larvae protein concentrate and glucose (2:1 weight ratio) were wet-heated at 50, 70 and 90°C for 2, 4, 6, 8 and 10 h, respectively, with an initial pH of 9. The $ABTS^{+}$ radical scavenging activity of conjugates produced at 50°C ranged from 10.5 – 16.5% and exhibited the lowest ABTS radical scavenging activity when compared to those heat-treated at 70 and 90°C. Generally, BSFL-Glu conjugates heated at 90°C exhibited higher metal chelation activity compared to those at 50°C and 70°C. For the metal chelating activity of BSFL-Glu conjugates at 90°C, a significant increase was observed until maximum (64.45%) at 6 h, this was then followed by a slight decrease until the end of the heating

period. The emulsion capacity of BSFL-Glu conjugates at 70°C ranged from 54.44 – 59.45%. The emulsion stability increased significantly ($p < 0.05$) as a function of reaction time, with conjugates produced after 8 hrs exhibiting the highest emulsion stability (35.89%).

The zeta-potential (ζ) of BSFL-Glu conjugates heat-treated at 70°C ranged from -10.25 to -25.25 mV while the native BSFL protein ranged from -12.84 to -16.70 mV. The ζ-potential analysis revealed that the glycation reaction modified the surface charge density of the BSFL protein as a function of reaction time and temperature. In addition, an increase in thermal stability of the BSFL-Glu conjugates was observed by utilizing Thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC) was observed for the conjugates compared to the native BSFL protein. The thermal denaturation peak temperature (Tp) for the heat-treated native BSFL protein at 50°C ranged from 72.23 – 73.81°C, with no significant differences ($p > 0.05$) between heating times (2, 4, 6, 8 and 10 h). Fourier transform infrared spectroscopy (FT-IR) analysis indicated that the most apparent structural changes in the BSFL protein were in the amide I and amide II region. The BSFL-Glu conjugates heated at 90°C for 10 h clearly shows an increased intensity in the amide I region (1624 cm^{-1}). Well-separated clusters permitting differentiation between native BSFL and BSFL-Glu conjugates were observed by using principal component analysis (PCA) on FT-IR spectra. At 50, 70 and 90°C the first two principal components (PC1 and PC2) showed an accumulated total variance of 91, 96 and 95%, respectively. Soft independent modelling of class analogy (SIMCA), a supervised chemometric classification tool, was used to establish the best classification model for discrimination between native BSFL protein and BSFL-Glu conjugates based on PCA. The applied model was able to distinguish between native BSFL-Glu and BSFL conjugates with an accuracy of 91%. Taken collectively, the findings of this study clearly illustrate that the degree of glycation results in structural changes to the native insect protein and thus conjugation hold a possibility for delivering novel food components with enhanced functionalities and expand the application of BSFL-Glu conjugates in food applications.

DEDICATION

Emndenini wami, konke lokhu bekungeke kwenzeke ngaphandle kwabo.

Ngiyabonga, Akwande!

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This dissertation is presented in the format of the Department of Food Science and Technology at the Cape Peninsula University of Technology. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introductory chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion. Language, style and referencing format used are in accordance with the requirements of the International *Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

LIST OF CONFERENCE PAPERS PRESENTED

Vusi Mshayisa, Bongisiwe Zozo & Jessy Van Wyk. (2019). Proximal, techno-functional and structural properties of Hermetia illucens and Imbrasia belina edible insect flours. **Oral presentation**, 14 – 16 August 2019. First African Conference on edible insects, Harare, Zimbabwe.

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Vusi Mshayisa & Nanilethu Ngcukayithobi**.** (2019). Comparison of physicochemical properties and oxidative stability of black solider fly larvae (*Hermetia illucens*) oil with sunflower oil. **Poster presentation**, SAAFoST 23rd Biennial congress, 1 – 4 September 2019, Johannesburg, South Africa.

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MANUSCRIPTS SUBMITTED IN ACCREDITED PEER REVIEW JOURNALS

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LIST OF ABBREVIATIONS

Abbreviation/Acronym	Definition
$ABTS^{+}$	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
AO	Alginate oligosaccharides
AOAC	Association of Official Analytical Chemists
AQC	6-aminoquinolyl-n-hydroxysuccinimidyl carbamate
BSFL	Black soldier fly larvae
BSF	Black soldier fly
BSFL-DF	Defatted Black soldier fly larvae
BSFL-FD	Freeze-dried Black soldier fly larvae
BSFL-Glu	Black soldier fly larvae glucose
BSFL-PC	Black soldier fly larvae protein concentrate
DPPH-RS	A-diphenyl-β-picrylhydrazyl-free radical scavenging
EA	Emulsifying activity
EC	Emulsion capacity
EDTA	Ethylenediaminetetraacetic acid
ES	Emulsion stability
FAO	Food and Agriculture Organisation
FE	Foam expansion
FC	Foam capacity
FN	False negative
FP	False positive
FS	Foam stability
FT-IR	Fourier transform infrared spectroscopy
IEP	Isoelectric precipitation
IPP	Ile-pro-pro
Mf	Myofibrillar
min	Minutes
MIR	Mid-infrared
MLPF	Migratory locust protein flour
MR	Maillard reaction

Abbreviation/Acronym	Definition
MRPs	Maillard reaction products
MPC	Milk protein concentrates
NMC	Number of misclassifications
OBC	Oil binding capacity
PC	Principal component
PCA	Principal component analysis
PCs	Principal components
pH	Potential of hydrogen
pI	Isoelectric point
PLS-DA	Partial least squares discriminant analysis
PLS	Partial least squares
RP	Reducing power
SA	South Africa
SEM	Scanning electron microscopy
SET	Single electron transfer
SIMCA	Soft independent modelling of class analogy
SPSS	Statistical Package for the Social Sciences
SVD	Singular value decomposition
TN	True negative
TP	True positive
UATR	Universal attenuated total reflectance
UK	United kingdom
UN	United nations
USA	United States of America
UV-VIS	Ultraviolet–visible
WAC	Water absorption capacity
WBC	Water binding capacity
WHC	Water holding capacity
WHO	World health organisation

CHAPTER 1: Motivation and design of the study

1.1. Introduction

Food systems are at the heart of the 2030 agenda for sustainable development (FAO, 2013a), a global commitment to eradicate poverty and hunger while ensuring the reduction of environmental and socio-economic impact. Amongst the United Nations' sustainable development goals (SDGs), goal 2 (end hunger, achieve food security and improved nutrition and promote sustainable agriculture) and goal 12 (ensure sustainable consumption and production patterns) are the major key focus areas for Food Scientists globally (Van-Huis, 2013). One of the greatest challenges facing Food Scientists and the food industry concerns the steady increase in the World's population and the need to generate sufficient protein to feed the nine billion people anticipated by 2050. At present, more than one billion people are suffering from protein deficiency (Boland *et al.*, 2013). Insects can be considered as an alternative protein source with less environmental impact than conventional livestock. Compared with conventional livestock, insects have several advantages: 1) they have higher reproduction rates and food conversion efficiencies than those of poultry, pigs and beef, 2) thus require less water, 3) they emit low levels of greenhouse gasses and 4) owing to their lack of similarity with humans, they pose a lower risk of producing pathogens that are threatening to human health (Spranghers *et al.*, 2017).

Black soldier fly larvae, *Hermetia illucens* which colonises a wide range of organic matter (animal manure, restaurant waste, residential waste, commercial waste and fermentation straw) have been identified by various researchers in feasibility studies as the most promising species for mass rearing on an industrial scale. Black soldier fly larvae are rich in protein (42%), fat (35%), and minerals (Li *et al.*, 2016). The high protein content makes them an ideal alternative protein source. Moreover, apart from their nutritional significance, proteins, in general, are widely utilized in the food industry due to their ability to add unique techno-functional properties such as emulsifying, foaming, gelling and solubility attributes (Oliver *et al.*, 2006; Yi *et al.*, 2013). These diverse properties depend on both intrinsic (e.g. molecular structure, composition) and extrinsic (e.g. temperature, chemical environment, pH) factors (Oliver *et al.*, 2006). It has been well established that the techno-functional properties

of proteins can be further improved by covalent bonding with polysaccharides and reducing sugars. Conjugation is based on the Amadori rearrangement steps in the Maillard reaction (MR). The MR was first observed by the French chemist, Louis-Camille Maillard in 1912, and involves a complex network of non-enzymatic reactions resulting from the initial condensation between an available amino group and a carbonyl-containing moiety, usually a reducing sugar. The MR is a spontaneous and naturally occurring reaction, in contrast to acetylation, deamination, succinylation, and other chemical methods available to improve the functional properties of proteins. The MR is greatly accelerated by heat and no extraneous chemicals are required. Hence the MR is probably the most promising approach to improve the functional properties of proteins for food purposes. Protein glycation during the early stages of the MR has been shown to increase emulsifying activity (EA) (Darewicz *et al.*, 2001; Diftis *et al.*, 2004), improve foaming properties (Chobert *et al.*, 2006; Achouri *et al.*, 2010), increase food protein solubility (Sato *et al.*, 2000; Shepherd *et al.*, 2000; Katayama *et al.*, 2002; Maitena *et al.*, 2004), enhance antimicrobial activity (Takahashi *et al.*, 2000; Chevalier *et al.*, 2001;Chevalier *et al.*, 2001), increase heat stability (Chevalier *et al.*, 2001; Sato *et al.*, 2005), and increase antioxidant activity (Sun *et al.*, 2006; Lertittikul *et al.*, 2007).

Mahran *et al*. (2011) showed improved foaming stability of buffalo milk casein due to the Maillard reaction when reacted with glucose, galactose, lactose, and ribose, and displayed the highest emulsion stability after glucose treatment, compared to other types of reducing sugars. Glucose is most frequently used to conjugate with proteins to improve solubility and emulsifying properties. Moreover, the advanced stages of the MR have been shown to produce compounds that possess antioxidant activity (Vhangani & Van-Wyk, 2013a). Furthermore, Sato *et al.* (2003) reported that glycation with alginate oligosaccharide (AO) using the MR provides myofibrillar protein (Mf) with excellent emulsion-forming ability. Omotoso (2005) investigated the functional properties of *Cirina forda* larvae powder and obtained high oil and water absorption capacities and concluded that the larvae powder could be applied in baked products. These findings further demonstrate that protein glycation is useful for improving the functional properties of food proteins.

Although it is known that BSFL is rich in protein, fat and minerals, there are still knowledge gaps regarding the techno-functional properties of native and glycated proteins derived from BSFL. It is necessary to obtain a clearer understanding of the

physiochemical and techno-functional properties of these proteins. Therefore, the aim of this study was to evaluate the physico-chemical, antioxidant, structural and techno-functional properties of black soldier fly larvae protein conjugates for food application.

1.2. Statement of the research problem

To meet the increasing protein demand of the growing world population in a sustainable way and achieve the 2030 Agenda for SDGs, notably SDG-2 Zero Hunger, insects have emerged as promising alternative protein sources. BSFL in particular is known to be rich in proteins and lipids. Despite research from an entomological and zoo-biological sciences point of view on intact edible insects, there is still little information from a food science point of view on the application of insect protein in particular BSFL in food products. Therefore, an in-depth insight into the structural, techno-functional and antioxidant properties of native and glycated proteins derived from BSFL for food application and how these can be improved by the Maillard reaction is crucial.

1.3. Objectives of the study

1.3.1. Broad objective

The aim of this study was to extract and characterise native BSFL protein and to compare the effect of glycation on the physico-chemical, techno-functional and structural properties of BSFL-Glu (black soldier fly larvae-glucose) protein conjugates as a function of reaction temperature and time with a view to obtain novel proteins with superior functionalities for food application.

1.3.2. Objectives of the research

The specific objectives of this research were to:

1. Extract the proteins from BSFL (alkaline extraction and alkaline and acid precipitation extraction), establish the proximal composition (moisture, crude fibre, crude protein, crude fat and carbohydrates) and describe protein amino acid composition of the obtained BSFL flours and protein fractions.
2. Establish the techno-functional (solubility, water and oil binding, foam capacity and stability, emulsifying capacity and stability) properties, colour and bulk density of BSFL flours and protein concentrates.

3. Determine and describe the structural properties [surface charge (zeta potential, surface morphology (scanning electron microscopy) and secondary/tertiary structure (Fourier transform infrared)] of BSFL flours and protein concentrates.
4. Compare the non-specific browning indicators (pH, and browning index) of native BSFL protein and BSFL-Glu conjugates as a function of reaction time (2, 4, 6, 8 and 10 h) and temperature (50, 70, 90 °C).
5. Compare the antioxidant indices (DPPH-RS, ABTS-RS, reducing power, and Iron chelation) of BSFL-Glu conjugates as a function of reaction time (2, 4, 6, 8 and 10 h) and temperature (50, 70, 90 °C).
6. Compare the techno-functional properties of native BSFL protein and BSFL-Glu protein conjugates (foaming capacity and stability and emulsion capacity and stability) as a function of reaction time (2, 4, 6, 8 and 10 h) and temperature (50, 70, 90 °C).
7. Determine and characterize the structural modifications of native BSFL protein and BSFL-Glu conjugates using novel analytical techniques, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FT-IR) coupled with chemometric approach (principal component analysis and soft independent modelling of class analogy), surface charge and surface morphology as a function of reaction time (2, 4, 6, 8 and 10 h) and temperature (50, 70, 90 °C).

1.4. Hypotheses

The following hypothesis were tested in this study:

1. Protein extraction using alkaline and acid precipitation will yield protein fractions with high crude protein content and exhibit superior functional properties.
2. Native BSFL protein will exhibit lower emulsifying activity, antioxidant activity and solubility compared to glycated BSFL-Glu conjugates. The DPPH-RS, reducing power, and Iron chelation are expected to increase with an increase in reaction time.
3. The heat treatment (conjugation via Maillard reaction) of black soldier fly larvae protein with glucose will induce structural changes and yield conjugates with enhanced techno-functional properties.

1.5. Delineation of the research

Proteins were extracted from Black soldier fly larvae reared on a standard feed (formulation). A single protein-sugar (BSFL-Glu), three reaction temperatures (50, 70 and 90 °C), and three reaction times (2, 4, 6, 8 and 10 h) were used to synthesize BSFL-Glu conjugates. An aqueous model system was used to measure the antioxidant indices of the BSFL-Glu protein conjugates.

1.6. Significance of the research

The world population is increasing, which requires the production of vast amounts of foods. However, it might be difficult to increase the productivity of food to a level that satisfies food demand due to the limited availability of new farm land. This may lead to shortages of food, especially animal protein (Akpossan *et al.*, 2015). Therefore, it is necessary to look for new sources of animal protein such as insects, which are rich in nutrients, thus resulting in the practice of eating insects known as entomophagy. Entomophagy is exercised traditionally in about 113 countries all over the world, including Mexico, Japan, Thailand, Asia, Brazil and Africa. In addition, there are more than 2 000 insect species that are considered edible counted to date (Rumpold & Schlüter, 2013). Insects are healthy and nutritious alternatives to mainstream staples such as chicken, pork, beef and sea food. Insects are rich in protein and good fats and high in calcium, iron, and zinc and already form part of a traditional diet for many regions. Insects are considered as having the potential to contribute to the World's food security (FAO, 2013a).

Besides improving the nutrition status, insects also have positive effects on the environment. They play an important role in waste biodegradation and as pollinators in plant reproduction (FAO, 2013a). Furthermore, they have high feed conversion efficiency because they are cold-blooded e.g. crickets need 12 times less feed than cattle, four times less feed than sheep, and half as much feed as pigs and broiler chickens to produce the same amount of protein (FAO, 2013a). Insect harvesting/rearing is a low-tech, low-capital investment option that offers opportunities even to the poorest sections of society, such as women and those without land, as their production is less land-dependent compared to conventional livestock. Insect rearing requires less food and feed and produces less greenhouse gases and significantly uses less water than conventional livestock (FAO, 2013a; Rumpold &

Schlüter, 2013). Glycation is an effective method for improving the functional properties of food proteins and even endows them with novel functionality (Oliver *et al.*, 2006). Studies on glycated BSFL protein are scarce. Therefore, it is essential to elucidate the techno-functional, structural and antioxidant properties of these glycoconjugates with the view to confirm BSFL as an alternative protein source, in particular glycoconjugates, as functional food ingredients.

1.7. Dissertation Overview

This dissertation consists of six chapters and was structured in article format where each chapter is an individual entity. Figure 1.1 depicts the structure of the dissertation. Chapter one introduces the research overview, including the research problem, objectives anticipated significance of the research. Chapter two is the literature review which expands on the background of the research topic. The nutritional aspects and processing technologies of edible insects were reviewed, followed by the current research to date on the extraction methods and techno-functional properties of edible insect proteins. Finally, the commercialization of edible insects was explored and the role of the Maillard reaction conjugates was highlighted as a potential mechanism to modify edible insect proteins was discussed.

Chapter three is the first research chapter, focussing on the extraction of BSFL protein using two techniques, alkaline extraction and alkaline extraction with and acid precipitation. In this chapter the Freeze-dried (BSFL-FD), defatted (BSFL-DF) flours and protein concentrates (BSFL-PC1 and BSFL-PC2) evaluated for some physicochemical, techno-functional and structural properties. Chapter four is the second research chapter and focused on the conjugation of BSFL protein with glucose. The antioxidant and techno-functional properties of BSFL-Glu conjugates as a function of reaction time and temperature were established.

Chapter five is the final research chapter and focused on the structural and thermal properties of heat-treated native BSFL protein and glycated BSFL-Glu conjugates. In terms of structural properties, the surface charge and surface morphology were assessed using novel analytical techniques. In addition, a multivariate classification model was used to discriminate and classify heat-treated native BSFL protein and BSFL-Glu conjugates as a function of reaction temperate and time.

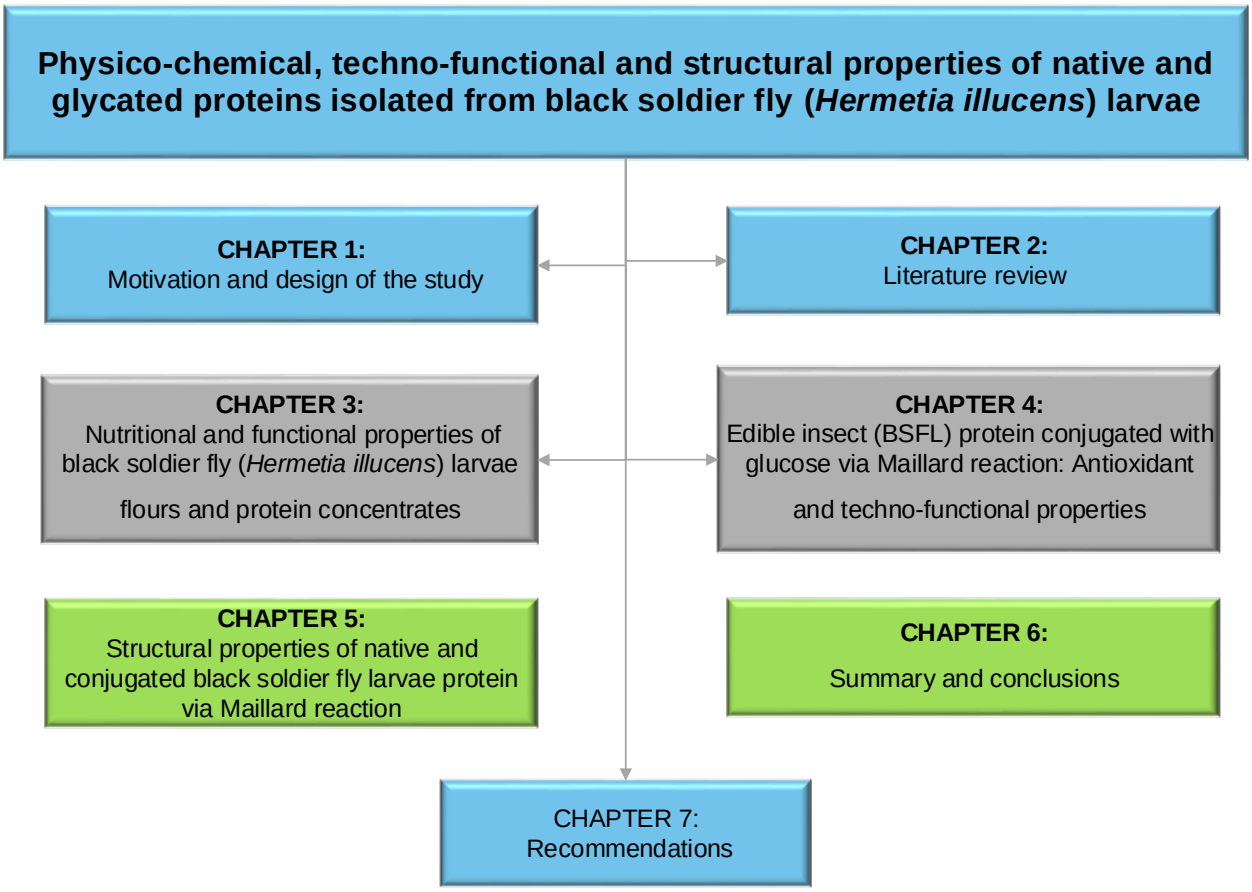

Figure 1.1 Dissertation overview

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CHAPTER 2: Literature review

2.1. Introduction

Anthropological literature, as well as historic reference, demonstrate the use of insects as food, medicines and other use throughout history (Costa-Neto & Dunkel, 2016; Williams *et al.*, 2016). Entomophagy, the practice of consuming insects has been practised by humans on every inhabited continent, not only historically but up until this present day (Wang & Shelomi, 2017). Approximately 113 countries worldwide practice entomophagy predominantly in Africa, Asia, and Latin America (FAO, 2013a; Sun-Waterhouse *et al.*, 2016; Bessa *et al.*, 2018). In addition, examples of traditional cheeses such as, Casu marzu and Milbenkäse, which contain maggots of the cheese fly (*Piophila casei*) and cheese mites or their digestive juices are produced in Sardinia and Germany (Nongonierma & FitzGerald, 2017). Entomophagy is not a strange practice in Africa, especially in rural households. *Imbrasia belina* (commonly known as Mopani worm) is consumed as a dry snack due to its protein content by people in semi-arid environments of Botswana, Namibia, Zimbabwe and South Africa (Baiyegunhi & Oppong, 2016). In Nigeria, four popular edible insect species (*Imbrasia belina, Rhynchophorus phoenicis, Oryctes rhinoceros, Macrotermes bellicosus*) are consumed in various forms due to their nutritional content (Omotoso, 2005; Van-Huis, 2013). The most consumed insects comprise beetle, caterpillar, bee, wasp, ant, grasshopper, locust and cricket varieties (Van-Huis, 2013; Anankware *et al.*, 2015). It is estimated that two billion people worldwide consume insects and over 2 000 species have been deemed edible since 2012 (Rumpold & Schlüter, 2013; Hall *et al.*, 2017). There are three ways in which insects are consumed: whole insects, whole insects processed into a granular powder or paste, or an extract such as a protein isolate (Bußler *et al.*, 2016a; Williams *et al.*, 2016). However, in the western world and some urban areas in African countries, today insects are not considered as a protein source. In addition, insects as food are regarded as a cultural “taboo”, with the general attitude of being disgust and reluctance (Hall *et al.*, 2017).

By 2050, the global population is predicted to grow to nine billion people (FAO, 2013a), and the demand for animal-derived protein is expected to increase at an even higher rate (Van-Huis, 2013; Dunkel & Payne, 2016). Moreover, the amount of available arable land is not sufficient to cater for this increasing population which will

require an increase in food production and conventional protein sources may be insufficient (Yen, 2009; FAO, 2013a). There is a need for a paradigm shift as far as alternative sources of proteins for food application is concerned. Currently, there are approximately 870 million undernourished people in the world (FAO, 2012). Approximately 178 million children under the age of five years are stunted from malnutrition and most of those live in sub-Saharan Africa (Dunkel & Payne, 2016). In addition, more than one billion people are suffering from protein deficiency (Azagoh *et al.*, 2016b). Edible Insects have been proposed as sources of alternative proteins to feed the growing population and to combat the challenge of poverty and malnutrition, particularly in developing countries (FAO, 2013a; Wang & Shelomi, 2017). In comparison to conventional livestock in general, insects have a higher feed conversion efficiency. This is due to the fact that they are poikilothermic and spend much lower amounts of food energy and nutrients but produce more animal protein than warm-blooded animals (Van-Huis, 2013; Ortiz *et al.*, 2016; Oibiokpa *et al.*, 2018). It has been indicated that insects might contribute less greenhouse gases (GHG) than pigs and cattle (Rumpold & Schlüter, 2013). According to Van-Huis (2013), the feed conversion of the house cricket (*A. domestica*) is twice that of chickens, four times higher than in pigs and more than twelve times higher than in cattle. Many insects naturally feed on organic wastes, converting biomass nutrients into their own biomass and reducing the amount of waste material and thus they are regarded as environmentally friendly (Caligiani *et al.*, 2018).

In terms of nutritional value, insects have been assessed as an interesting food and feed alternative to legumes (soya) and algae (Azagoh *et al.*, 2016b; Gaffigan, 2017; Smetana *et al.*, 2018; Gravel & Doyen, 2020). According to several studies, insects offer an important source of minerals, vitamins, antioxidants, lipids and above all proteins (Rumpold & Schlüter, 2013; Kouřimská & Adámková, 2016; Sun-Waterhouse *et al.*, 2016). Insect proteins possess nutritional advantages in total protein level and/or essential amino acid profiles over plant proteins such as cereals, beans, lentils or soybeans (Rumpold & Schlüter, 2013; Anankware *et al.*, 2015). The possible use of insect protein as a functional food ingredient is dependent on its physical, chemical and techno-functional properties. Protein functionality as it relates to food products is a complex concept (Wouters *et al.*, 2016). It covers techno-functional properties such as solubility, gelation, and surface activity, but also bio-active, health-related, and even basic nutritional properties. In this review, the term

protein functionality is reserved for all properties contributing to the structure and texture of food products. Apart from sensorial properties, the techno-functional properties of ingredients directly or indirectly affect the processing applications, food quality and ultimately their acceptance and utilisation in food and food formulations. To date, commercially reared insects for human consumption include the house cricket, the palm weevil, the giant water bug, and water beetles (Rumpold & Schlüter, 2015). AgriProtein in South Africa, one of the biggest companies in the world produces black soldier fly larvae under strict hygienic conditions for feed and food consumption. Although various studies have reported the functional properties of insects and preparations thereof (Zhao *et al.*, 2016; Purschke *et al.*, 2018b), a general image of the current knowledge on the topic is limited. This literature review focused on addressing knowledge gaps in relation to insect protein extraction methods and techno-functional properties of edible insect proteins. Next to extraction methods and techno-functional properties, we also reviewed consumer perceptions towards edible insect and insect-based food products, provided an overview of commercially available insect-based products and addressed the possible use of insect protein as an alternative to sources of priority allergens such as eggs, gluten, soybean and nuts.

2.2. Nutritional aspects

Various studies have been reported addressing the nutritive value and nutrient composition of various insects (Omotoso, 2005; Tzompa-Sosa *et al.*, 2014; Hall *et al.*, 2017; Caligiani *et al.*, 2018; Purschke *et al.*, 2018b). A comprehensive review on the nutritional properties of common edible insect has been written by Williams *et al.,* (2016). In general, edible insects were found to be good sources of proteins, fat, energy, vitamins and minerals. The nutritional value of edible insects is very diverse mainly due to the large number and variability of species, diet, environmental factors and the stage of metamorphosis (Kouřimská & Adámková, 2016; Spranghers *et al.*, 2017). Similarly, the nutritional value changes based on the preparation and processing prior to consumption (e.g. boiling, frying, drying and cooking) (Manditsera *et al.*, 2019). The energy value of edible insects depends on their composition, mainly on fat content. Larvae or pupae are usually richer in energy compared to adults. Conversely, high protein insect species have lower energy content (Rumpold & Schlüter, 2013; Kouřimská & Adámková, 2016). The energy content of insects is on

average comparable to that of meat (on a fresh weight basis), except for pork because of its particularly high-fat content (Williams *et al.*, 2016). The total protein content of seven edible insect species was investigated by Bednárová *et al.* (2013). In this study, the protein content for species such as *T. molitor* and African migratory locust (*L. migratoria*) was 50.7 and 62.2%, respectively. Up until 2017, the standard nitrogen factor (Kp) of 6.25 was used in protein analysis; however, it was constantly under debate whether it was an accurate reflection of the actual protein content, as reports suggested that the chitin content affects the protein results (Janssen *et al.*, 2017; Jonas-levi & Martinez, 2017). Chitin makes up the exo-skeleton of insects and comprises a long-chain polymer, which contributes towards the insoluble fibre content of insects. Chitin only contributes about 5 – 20% of an insect's biomass, depending on the species, and studies done on the *in vitro* digestibility of insect protein showed that 77.9 – 98.9% of insect protein is readily digested by humans (Liland *et al.*, 2017; Caligiani *et al.*, 2018). Subsequently, a study has indicated that a Kp of 4.76 should be used for whole insects and a Kp of 5.6 for insect protein extracts, but it is suggested that validation studies be done to define and standardize the Kp factor for insects (Janssen *et al.*, 2017).

Considering the amino acid composition of edible insects, they contain several nutritionally valuable amino acids including high levels of tyrosine and phenylalanine. Huang *et al.* (2018) investigated the influence of conventional drying and microwave drying methods on the amino acid composition of the black soldier larvae protein. The authors reported that aspartic acid was predominant in conventionally dried BSFL protein, while glutamic acid was higher in microwave-dried BSFL protein. The high levels of glutamic acid and aspartic acid were reported to be responsible for the special flavour and taste. In another study, Caligiani *et al.* (2018) reported that the amino acid content of BSFL is comparable to the animal and vegetable protein sources such as egg protein and soybean. Moreover, BSFL contained higher amounts of tyrosine, phenylalanine and histidine and comparable amounts of valine, lysine and threonine. When BSF essential amino acids were compared to the amino acids requirements for human adults calculated in the FAO/WHO reference protein, it appears that BSF proteins contain all the essential amino acids in the required amounts (Caligiani *et al.*, 2018). This could be of great importance for the incorporation of BSFL in food products especially in poor communities in Africa and Asia. Fatty acid analysis shows that BSFL fat is dominated by lauric acid (46.7 ± 0.6%), followed by oleic acid (15.1 ± 0.9%),

myristic (8.3 ± 0.1%), palmitic (8.4 ± 0.2%) and palmitoleic (7.6 ± 0.4%) acids. Minor amounts of decanoic acid (1.9 ± 0.2%), stearic (2.5 ± 0.1%) and linoleic acid (1.8 ± 0.4%) were also observed (Yoong *et al.*, 2016; Liland *et al.*, 2017). Edible insects are interesting in terms of the nutritional content of minerals such as iron, zinc, potassium, sodium, calcium, phosphorus, magnesium, manganese and copper (Omotoso, 2005; Zielińska *et al.*, 2015; Kouřimská & Adámková, 2016). The successful adoption and application of edible insect in food products by the food industry depend largely on processing technologies available to guarantee safe food for human consumption. The section below details the current production and processing of edible insects which can be applied in the food industry.

2.3. Production and processing of insect flours, protein concentrates and isolates

There are different methods that can be used to obtain protein-rich ingredients such as milk protein concentrates (MPC), soy protein isolates, pea protein concentrates and even edible insect's protein concentrates and isolates. These techniques depend largely on the raw material and its proximate composition. Nevertheless, it is possible to describe four different processing steps (Figure 2.1).

a) Pre-treatment
b) Defatting
c) Protein solubilisation
d) Drying

The following sections describe these steps in detail.

a) Pre-treatment

The initial preparation step is highly dependent on the raw material. For example, in the food industry milk is pasteurized to ensure product safety and quality. Legumes such as lentils, peas, and soybean are usually dried and dehulled in order to make the subsequent milling or grinding process easier. This process also increases the protein content by removing hulls from the cotyledon. For insects such as black solider fly, the initial step involves the removal of gastrointestinal contents. This is achieved by fasting the insects for 24 hours prior to protein extraction. Yi *et al.* (2013) and Zielińska *et al.*

(Zielińska *et al.*, 2018) applied thermal treatment such as freezing which was followed by freeze-drying. Another approach was freezing followed by pasteurisation (Hall *et al.*, 2017) or hot air drying at 60°C then milling (Zhou *et al.*, 2017), prior to protein extraction to prevent enzymatic and microbial degradation during storage.

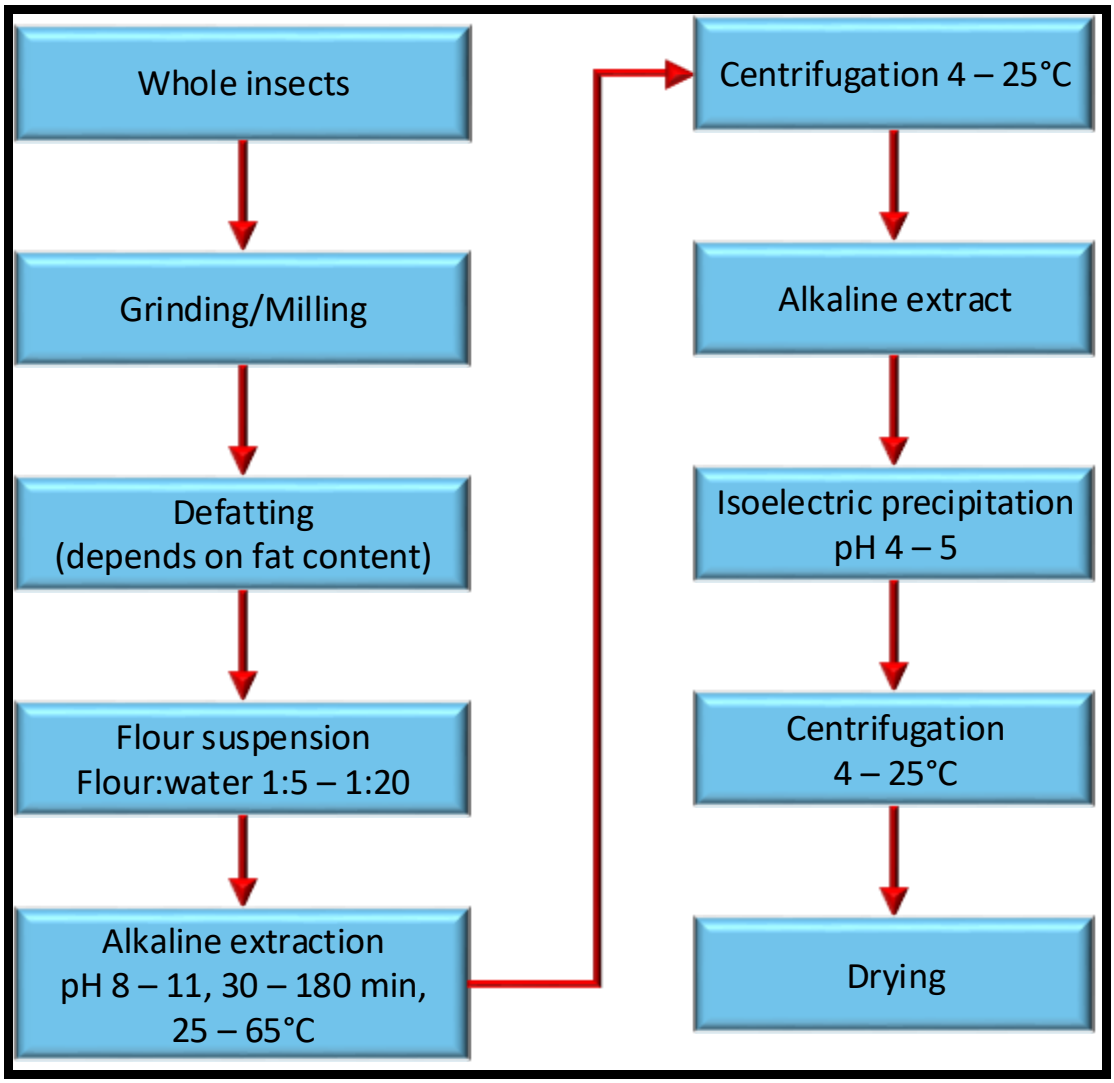

Figure 2.1 Schematic representation of alkaline and isoelectric precipitation extraction of insect protein.

b) Defatting

The defatting step is also highly dependent on the form and fat content of the starting insect material. This step may be omitted for insects that already contain low levels of lipids. The most frequently used method of fat removal for edible insects is the use of solvents. Zhou *et al.* (2017) described the use of ethanol or hexane:isopropanol [3:2 $v.v^{-1}$)] during defatting of *T. molitor* and no significant differences were noted between the samples. Petroleum ether was used to remove lipids from *T. molitor* larvae (Dai *et al.*, 2013) and *B. mori* powders (Jia *et al.*, 2015). Yi *et al.* (2013) used hexane as a defatting solvent in a study conducted to extract soluble proteins in five different insect species, *T. molitor, Z. morio, A. diaperinus, A. domesticus and B. dubia*. The hexane defatting step allowed Bußler *et al.* (2016b) to increase the protein content of both *H. illucens* and *T. molitor* from 34.7% to 44.9% and 57.8% to 64.6%, respectively. Similar results were also reported by Ndiritu *et al.* (2017) with hexane extraction. Other defatting methods which avoid the limitations posed by organic solvents include supercritical CO_2 (Purschke *et al.*, 2017), mechanical processing (Purschke *et al.*, 2018a) and aqueous extraction (Leni *et al.*, 2019). Tzompa-Sosa *et al.* (2019) reported that aqueous extraction of yellow mealworm (*T. molitor*), lesser mealworm (*A. diaperinus*) and house cricket (*A. domesticus*) gave the lowest oil yield, compared to other methods of defatting. Removing fat from the sample impacts the final product protein recovery and purity due to the hydrophobic nature of lipids, protein-lipid interaction and solubility.

c) Protein solubilisation

Aqueous alkaline extraction followed by isoelectric precipitation (IEP) is a frequently used technique for the extraction of insect proteins (Azagoh *et al.*, 2016b; Bußler *et al.*, 2016b; Caligiani *et al.*, 2018). This technique takes advantage of the solubility of insect proteins which is high at alkaline pH and low at pH values close to their isoelectric point (pI) (pH 4 – 5). The unit operations for this process are quite simple and are presented in Figure 2.1. Generally, ground insect flour (with or without integument) is dispersed in water using flour: water ratios ranging from 1:5 to 1:40 (Zhao *et al.*, 2016; Ndiritu *et al.*, 2017; Mishyna *et al.*, 2019). The pH of the mixture is adjusted to alkaline (pH 8 – 11) and the mixture is allowed to stand for periods varying from 30 to 180 min to maximize solubilisation of the proteins (Boye *et al.*, 2010).

Different precipitation pHs have been used depending on the insect species, i.e. around pH 3.0 for *G. sigillatus* (Hall *et al.*, 2017), between pH 4.0 – 5.0 for *T. molitor* (Yi *et al.*, 2013; Zhao *et al.*, 2016), pH 4.0 (Jia *et al.*, 2015), and pH 4.5 (Wang *et al.*, 2011b) and pH 4.5 (Wu *et al.,* 2015) for *B. mori* pupae, and pH 5.0 for *B. mori* chrysalises (Zhou *et al.*, 2017). During this time the pH is maintained at the desired value and the temperature may be elevated (up to 55 – 65°C) to further enhance protein solubilisation and extraction. The mixture is subsequently filtered to remove any insoluble material and the pH of the extract is adjusted to the isoelectric point to induce protein precipitation, followed by centrifugation to recover the protein, washing to remove salts, neutralization and drying. Protein contents of insect fractions extracted with this technique can be variable, probably due to differences in the processing conditions (Boye *et al.*, 2010). Surprisingly, very few studies have reported on further characterisation (e.g. yield and purity) of the insect protein isolates.

Edible insect proteins may also be directly extracted with water without the subsequent acid precipitation step. Yi *et al.* (2013) described protein extraction from five different edible insects *T. molitor*, Z. morio, *A. diaperinus*, *A. domesticus* and *B. dubia* and reported protein purity and yield ranging from 17% to 23% and 50% to 61%, respectively, while Ndiritu *et al.* (2017) reported extraction yields of 32 – 66% for *A. domesticus.* In both these studies, the authors reported that the whole insects were initially frozen using liquid nitrogen and ground into powder using a blender. The insect mixture was further freeze-dried. Freeze-dried insect powder (400 g) was mixed with 1 200 mL demineralized water containing 2 g ascorbic acid. A ratio of 1:3 solids to solvent was employed. The mixture was blended for 1 min using a laboratory blender. Then the obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 500 µm. The filtrates and residues were collected. The filtrate was further centrifuged at 15 000 x g for 30 min at 4°C. Three fractions were obtained from the filtrate: the supernatant, the pellet, and the fat fraction. The residue (from initial centrifugation), the pellet and the supernatant fractions were freeze-dried. The freeze-dried residue, supernatant and pellet fractions of all insect species were characterised for protein content. All three fractions were found to contain protein. However, the highest protein content was found in the pellet and residue.

Another protein extraction method on *Imbrasia oyemensis (I. oyemensis)* larvae has been reported by Akpossan *et al*. (2015). Dried caterpillars were initially milled into powder using a porcelain mortar and pestle. The powder was further defatted

using hexane by continuous stirring for 16 h followed by filtration. The defatted and non-defatted larvae powder was mixed with four different solvents for protein extraction namely; deionized distilled water, 0. 5 M NaCl (30 mL: 1 g), 70% Ethanol ($w.v^{-1}$) and 0.1 M NaOH (25 mL: 1 g). The full-fat and defatted powder were extracted by simply stirring, using a solid powder to solvent ratio of 1:10 ($w.v^{-1}$), for 14 – 16 h at 4°C. The insoluble residue was removed by centrifugation at 6 000 rpm for 30 min. Extraction with each solvent was repeated three times, and all supernatants for each solvent were pooled to obtain a representative of each solubility fraction. Each fraction then was dialyzed against its own solvent followed by deionized water. Dialysates were stored in a freezer at about -20°C. Distilled water-, NaCl-, Ethanol- and NaOH-soluble proteins were designated “albumin”, “globulin”, “prolamine” and “glutelin”, respectively. Defatting was found to significantly increase glutelin and albumin.

d) Drying

Drying can act as both the initial step for insect flour production, as well as the last step to obtain protein concentrates or isolates. Many existing techniques from the food industry can be used depending on the sample's form. For instance, oven-drying, fluidized bed drying and microwave drying can be used to dry solid samples and are therefore used for flour production from whole insects, before the grinding process. Sun-drying (Kinyuru *et al.*, 2013), freeze-drying (Ndiritu *et al.*, 2017; Huang *et al.*, 2018; Köhler *et al.*, 2019), and oven-drying are the preferred technologies for drying whole edible insects, while freeze-drying, oven-drying, and nonconventional (Vandeweyer *et al.*, 2017; Huang *et al.*, 2018) drying techniques are used primarily for insect flours and powders. Drying and grinding whole, perfectly recognizable edible insects into unrecognizable powders is one of the preferred technologies for increasing human consumption of insects, mostly in Western countries (Menozzi *et al.*, 2017; Janssen *et al.*, 2019b). Drying also increases product shelf-life during distribution and storage. However, regardless of the blanching and drying treatments applied to insects, they should be reheated before consumption to eliminate residual microorganisms.

In a study conducted by Purschke *et al.* (2018a), dry fractionation was used successfully with *T. molitor* to increase the yield of protein-enriched fractions, but results were dependent on the pre-treatments and the drying treatment used before

fractionation. Experimental setup and parameters can vary widely depending on the study. For example, in a review by Melgar-Lalanne *et al.* (2019), oven-drying parameters of different insects ranged from 40 – 80 °C for 7 to 48 h, or until a constant weight was attained (Melgar-Lalanne *et al.*, 2019). A few papers, comparing various drying methods reported using microwaves to dry *T. molit*or or *H. illucens,* have been published (Huang *et al.*, 2018; Kröncke *et al.*, 2018; Melis *et al.*, 2018). The microwave drying parameters usually ranged from 0.5 to 2 kW for 30 s or up to 20 min, depending on the experimental setup (Huang *et al.*, 2018; Kröncke *et al.*, 2018). Although less commonly used, fluidized bed drying was also documented as an alternative drying method for insect flour production (Kröncke *et al.*, 2018; Purschke *et al.*, 2018a).

2.4. Techno-functional properties

It is essential to examine the techno-functional properties of edible insect proteins in order to ascertain if they are suitable for food applications. Depending on the origin and once processed, protein concentrates and isolates have desirable techno-functional properties. However, food systems are complex and insect proteins could interact with other components such as lipids, carbohydrates and minerals. Therefore, the functional properties of edible insect proteins presented in the following section cannot be exclusively attributed to their protein content, but rather the whole system of which they are a part.

2.4.1. Solubility

The solubility of most insect proteins is highest at low acidic and high alkaline pH values. Solubility markedly decreases near the isoelectric point, generally between pH 4 and pH 6 for most insects. The protein solubility of the yellow mealworm (*T. molitor*) protein extract in distilled water was at a minimum between pH 4 and 5 (Zhao *et al.*, 2016). In a study conducted by Zielińska *et al.* (Zielińska *et al.*, 2018), the protein solubility of all samples showed minimum values at around pH 5 with values of 3% for *T. molitor*, 4% *for G. sigillatus*, and 8% for *S. gregaria*. *H. illucens* protein flours had a higher solubility in the alkaline region at pH 12 and also in the acidic region at pH 2. Purschke *et al*. (2018b) reported protein solubility of enzymatically treated migratory locust protein flour (MLPF) which ranged from, 10 to 22% with its minimum and

maximum at pH 5 and pH 9, respectively. *Imbrasia oyemensis* had a lower solubility in alkaline media. Higher solubility values were obtained in acid media and the isoelectric point (IEP) values are 4 and 7.6 for full-fat and defatted powders (Akpossan *et al.*, 2015). Solubility is one of the most important physicochemical and functional properties of protein and depends on hydration and the degree of hydrophobicity of protein molecules. Good solubility of proteins is important in many uses, mainly for the formation of emulsions, foams, and gels in designed food products. As a result, molecules in colloidal systems are homogeneously dispersed, which improves the interfacial properties.

2.4.2. Water and fat binding capacity

Water binding capacity (WBC) sometimes also referred to as water absorption capacity (WAC) may be defined as the amount of water that can be absorbed per gram of protein material. The methods used for these measurements are sometimes different. WBC/WAC measurements are important for food processing applications. Materials that have low WBC/WAC may not be able to hold water effectively while materials having high WBC/WAC may render food products brittle and dry, especially during storage. Although different terms have been used to describe the concept of water holding capacity (WHC), researchers agree that it is a measure of the total amount of water that can be bound or retained by proteins. A distinction can be made between the water bound to the individual protein molecules and that physically trapped by the protein matrix (Kostić *et al.*, 2015; Wouters *et al.*, 2016). Omotoso (2005) studied the functional properties of *Cirina forda* (*C. forda*) – one of the most widely eaten insects in Southern Nigeria. The WBC of the dried ground insect was 300% and this value is the most similar to the WHC of the *G. sigillatus* protein preparation (3.44 $g.g^{-1}$). In turn, Zhao *et al*. (2016) reported the WHC for a *T. molitor* protein extract to be 1.87 $mL.g^{-1}$ and the WHC of yellow mealworm larvae was found to be 2.33 ± 0.03 ($g.g^{-1}$). The differences can be attributed to the different protein extraction methods used by authors as well as the insects' origin. Overall, this makes the larvae powder suitable for use in baked products due to its high water absorption capacity, as a flavour retainer and to improve the mouthfeel of food products because of its high oil absorption capacity.

2.4.3. Foam capacity and foam stability

The most frequently used indices for measuring foaming properties are foam expansion (FE), foam capacity (FC) and foam stability (FS). Foams are formed when proteins unfold to form an interfacial skin that keeps air bubbles in suspension and prevents their collapse. Foam formation is important in food applications such as beverages, mousses, meringue cakes and whipped toppings. Different protocols are reported in the literature for measuring the foaming properties of proteins. In general, for measuring FE and FC, protein dispersions at specified concentrations are homogenized at high speed to induce foam formation. FC or FE is expressed as the volume (%) increase due to whipping, whereas foam stability is measured as the change in the volume of foam over a specified period (0 – 30 min). Zielińska *et al.* (Zielińska *et al.*, 2018) reported higher values of foaming capacity in both protein preparations and whole insects (99% and 41%, respectively) for *G. sigillatus*. Yi *et al.* (2013) reported poor or no foam capacity, over a range of pH, for acid-extracted protein fractions from five different insect species, including a cricket (*A. domesticus*) protein. Currently, research is directed towards the exploration of alternatives for eggs, a common foaming agent in food products. Insect proteins that exhibit higher foaming properties have the potential to be used for such food applications.

2.4.4. Emulsifying properties

Emulsifying activity (EA) and emulsifying stability (ES) are two indices often used to evaluate the emulsifying properties of protein flours. Proteins act as emulsifiers by forming a film or skin around oil droplets dispersed in an aqueous medium, thereby preventing structural changes such as coalescence, creaming, flocculation or sedimentation. Emulsifying properties of proteins are, therefore, affected by their hydrophobicity/hydrophilicity ratio and structural constraints which determine the ease with which they can unfold to form a film or skin around dispersed oil droplets. In a simplified system, EA measures the amount of oil that can be emulsified per unit of protein, whereas ES measures the ability of the emulsion to resist changes to its structure over a defined period. There are limited studies on the emulsification properties of edible insect flour and protein concentrates due to the field being in its infancy stages. The EA of *I. oyemensis* was 80.85 and 86.46%, respectively, from full-

fat and defatted powders (Akpossan *et al.*, 2015). Kim *et al.* (2017) studied the emulsifying capacity of house cricket flour in meat emulsions. The authors reported that no significant differences ($p > 0.05$) in the emulsifying capacity of house cricket flour was observed between 0 and 2.10 M NaCl concentration, which ranged from 39.17% to 45.00%. Hydrolysis of migratory locust (*Locusta migratoria L*.) resulted in improved emulsifying properties compared to the control samples (Purschke *et al.*, 2018b). Further research is required to investigate the emulsification properties of edible insect flours and protein concentrates. In order to commercialise production and processing, it is imperative to understand the chemical and physical characteristics of edible insects such as BSFL, with the view to optimise processing for various applications.

2.5. Edible insect flours and proteins fractions in food systems

The previous section dealt with the potential of edible insects as an alternative protein source using model systems. However, the techno-functional properties of edible insects in “real” or complex food systems remain largely unexplored. The following section highlights information currently available on the incorporation of insect flours, defatted flours, protein concentrates and isolates in complex food systems.

Recently, González *et al.* (2019) studied the addition of 5% insect flour (*H. illucens, T. molitor and A. domesticus*) in bakery goods. The authors reported that *A. domestica* flour showed similar specific volume and texture parameters when compared with wheat bread, but with higher content of proteins and fibre. Moreover, in comparison with *H. illucens* and *T. molitor* flours, *A. domesticus* flour was found to be the most suitable for bread application since it exhibited the best functional properties and flavour (González *et al.*, 2019). In another study, de Oliveira *et al.* (de Oliveira *et al.*, 2017) investigated the addition of cinereous cockroach (*Nauphoeta cinerea*) in bread and observed a linear correlation between the incorporation of insect flour and an increase in bread volume. The flour exhibited a good nutritional profile due to the amino acid and fatty acid composition and was successfully used to enriched wheat bread without any noticeable changes in sensorial quality.

Azzollini *et al.* (2018) produced extruded snacks containing 10 and 20% *T. molitor* flour. The nutritional content, microstructure, texture and digestibility were evaluated. The authors concluded that the addition of 10% *T. molitor* flour significantly

improved the microstructure, in terms of expansion and pore structure, delivering acceptable textural qualities. In a similar study, Kinyuru *et al.* (2015) used sun-dried termites to formulate extruded complimentary foods containing amaranth grain, white maize, soybean oil and sugar, with the view to combating child malnutrition in Kenya. It was reported that the products met the recommended limits for energy, macronutrients, iron and zinc for complementary foods. Moreover, the shelf-life of these food products was six months and had the potential to be sold at prices lower than most commercially processed products produced for young children in Kenya (Kinyuru *et al.*, 2015).

Bread and snack products are not the only food products where the incorporation of insects has been explored. Recently Smetana *et al.* (2018) successfully recreated the texture and moisture of meat analogues by partially replacing its soy content with 40% *A. diaperinus* protein concentrate. Moreover, Kim *et al.* (2016) replaced 10% of lean pork in emulsions with mealworm larvae (*T. molitor*) and silkworm pupae (*B. mori*) full-fat flour, defatted flour and protein hydrolysates. The addition of the protein-enriched flour leads to a significant increase in cooking yield and hardness to a similar extent, regardless of pre-treating methods and insect types. Another study by Kim *et al.* (2017) examined the effect of house cricket (*A. domesticus*) flour addition on physicochemical and textural properties of meat emulsion under various formulations. The authors reported that the replacement of lean meat/fat portion with house cricket flour within a 10% level could fortify in terms of protein and some micronutrients (phosphorus, potassium, and magnesium) in meat emulsions, without negative impacts on cooking yield and textural properties (Kim *et al.*, 2017). Lastly, Duda *et al.* (2019) demonstrated that the incorporation of 5% cricket powder into wheat pasta influenced the cooking weight and cooking loss (reducing losses and increasing water absorption), as well as the colour of the pasta. Due to the complexity, of food systems, the functional properties of edible insect proteins presented in this section cannot be attributed solely to their protein content, but rather the whole system of which they are a part. Current research, as illustrated in this section, has focused more on fortifying traditional forms of food such as bread, sausages, pastas and cookies with edible insects, thereby increasing their nutritional value.

2.6. Commercialization of edible Insects

Some products have been commercialised and have opened new avenues for entrepreneurs and other role players in the food industry. Insects are already consumed as traditional food or snacks in Africa, Asia and South America. Edible insects have traditionally been sold dried and/or ground and are sometimes marketed as flours, heat-dried larvae, pupae, or whole adult insects. In a review by Melgar-Lalanne *et al.* (2019), it is stated that approximately 113 start-up companies have been created in the past decade. Figure 2.2 depicts some of the commercially available insect-based food products. The Netherlands paved the way by releasing its first insect-based product (mealworm burgers) into commercial stores in 2014. They were followed by countries such as Belgium and Switzerland, which legalised the sale of insects for human food in the commercial market in 2015 and 2017, respectively (Bessa *et al.*, 2018). In Belgium, the Goffard sisters produce wheat pasta enriched with a flour of mealworms (*T. molitor* or *A. Diaperinus*) with a brand name of Aldento (www.goffardsisters.com). Mopani Queens, a company in South Africa, sells flavoured Mopani caterpillars or Mopani worms. Barbeque, chilli, chutney, and the original salted flavour are part of their product basket. Some insect-based ingredients have substantial potential owing to their nutritional characteristics and functional properties in food, pharmaceutical, and cosmetic products. For instance, protein concentrates and/or isolates derived from insects have high foaming and emulsifying properties, as well as other techno-functional capacities.

2.7. Consumer attitudes: disgust or food neophobia?

One major challenge with consumption of edible insects in Southern Africa isthat many consumers have adopted the western diet or lifestyle and that makes them ashamed or ignorant of consuming insects. The more educated population are reluctant to admit that entomophagy still exists and in some cases, it is associated with a perception of poverty or poor standard of living. The problem is even worse in some urban areas and African countries including Botswana, Zimbabwe and Nigeria are reporting that young people are rejecting entomophagy (Baiyegunhi & Oppong, 2016; Manditsera *et al.*, 2018; Oibiokpa *et al.*, 2018). In contrast, studies are emerging revealing that in the West, edible insects are being promoted as an environmentally sustainable protein

source to young people in higher income brackets (Emory *et al.*, 2018; House, 2018; La Barbera *et al.*, 2018).

Figure 2.2 Examples of commercially available insect-based products. (Source https://www.goffardsisters.com, https://grazingfoods.com, https://www.absoluteorganix.co.za).

Although edible insects are promoted as a delicacy in some experimental restaurants, most consumers are still repelled by the idea of consuming them. In an attempt to understand this phenomenon, a few studies have identified two major barriers or impediments to insect consumption: food neophobia and disgust (Gmuer *et al.*, 2016; Menozzi *et al.*, 2017; Chan, 2019; Jensen & Lieberoth, 2019; Mancini *et al.*, 2019). Food tastes reflect a spectrum of attitudes, from neophobia, fear of tasting new foods to neophobia, just as a curiosity about and desire to try novel food sources. Neophobia is strongly associated with the fear of the unknown for sanitary, cultural and even religious reasons and neophilia is associated with the development of cuisine which allows the incorporation of new food items into the diet to satisfy the needs for variety. The majority of the studies agree that one of the best tools to achieve regular consumption of insect-based products is educating the consumers and incorporating insect in foods in the form of powders or pastes (Menozzi *et al.*, 2017; Gere *et al.*,

2018; Imathiu, 2020). For the purpose of this research, only insects from the *Stratiomyidae* family will be discussed further

2.8. Current study

The high protein content of edible insects makes them an ideal alternative protein source. Moreover, apart from their nutritional significance, proteins, in general, are widely utilized in the food industry due to their ability to add unique techno-functional properties such as emulsifying, foaming, gelling and solubility attributes (Oliver *et al.*, 2006; Yi *et al.*, 2013). These diverse properties depend on both intrinsic (e.g. molecular structure, composition) and extrinsic (e.g. temperature, chemical environment, pH) factors (Oliver *et al.*, 2006). It has been well established that the techno-functional properties of proteins can be further improved by covalent bonding with reducing sugars and polysaccharides. Conjugation is based on the Amadori rearrangement steps in the Maillard reaction (MR). The MR was first observed by the French chemist, Louis-Camille Maillard in 1912, and involves a complex network of non-enzymatic reactions resulting from the initial condensation between an available amino group and a carbonyl-containing moiety, usually a reducing sugar. The MR is a spontaneous and naturally occurring reaction, in contrast to acetylation, deamidation, succinylation, and other chemical methods available to improve the functional properties of proteins. The MR is greatly accelerated by heat and no extraneous chemicals are required. Hence the MR is probably the most promising approach to improve the functional properties of proteins for food purposes. Protein glycation during the early stages of the MR has been shown to increase emulsifying activity (EA) (Darewicz *et al.*, 2001; Diftis *et al.*, 2004), improve foaming properties (Chobert *et al.*, 2006; Achouri *et al.*, 2010), increase food protein solubility (Sato *et al.*, 2000; Shepherd *et al.*, 2000; Katayama *et al.*, 2002; Maitena *et al.*, 2004), enhance antimicrobial activity (Takahashi *et al.*, 2000; Chevalier *et al.*, 2001;Chevalier *et al.*, 2001), increase heat stability (Chevalier *et al.*, 2001; Sato *et al.*, 2005), and increase antioxidant activity (Sun *et al.*, 2006; Lertittikul *et al.*, 2007).

Mahran *et al*. (2011) showed improved foaming stability of buffalo casein due to the MR when reacted with glucose, galactose, lactose, and ribose, and displayed the highest emulsion stability after glucose treatment, compared to other types of reducing sugars. Glucose is most frequently used to conjugate with proteins to

improve solubility and emulsifying properties. Moreover, the advanced stages of the MR have been shown to produce compounds that possess antioxidant activity (Shepherd *et al.*, 2000; Vhangani & Van-Wyk, 2013a, 2016; Anzani *et al.*, 2020; Jia *et al.*, 2020; Nooshkam *et al.*, 2020). Furthermore, Sato *et al.* (2003) reported that glycation with alginate oligosaccharides (AO) using the MR provides myofibrillar protein (Mf) with excellent emulsion-forming ability. Omotoso (2005) investigated the functional properties of *Cirina forda* larvae powder and obtained high oil and water absorption capacities and concluded that the larvae powder could be applied in baked products. These findings further demonstrate that protein glycation is useful for improving the functional properties of food proteins.

Although it is known that BSFL is rich in protein, fat and minerals, there are still knowledge gaps regarding the techno-functional properties of native and glycated proteins derived from BSFL. It is necessary to obtain a clearer understanding of the physico-chemical and techno-functional properties of these proteins. Therefore, the aim of this study was to evaluate the physico-chemical, techno-functional and structural properties of native and glycated proteins isolated from black soldier fly (*Hermetia illucens*) larvae with the view to explore alternative proteins for food application.

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CHAPTER 3: Nutritional and functional properties of black soldier fly (*Hermetia illucens*) larvae flours and protein concentrates

3.1 Abstract

Food proteins play a vital role in determining the structure and texture of numerous food products. Edible insects have been identified as an excellent alternative source of protein due to their protein content. Edible insects such as black soldier fly larvae (BSFL) can contribute to food security as the global population continues to increase. The effect of different protein extraction methods i.e. Alkaline solution and acid precipitation (BSFL-PC1) and extraction with an alkali (BSFL-PC2) on the nutritional and functional properties of BSFL flours and protein concentrates were studied. The highest protein content (73.35%) was obtained under alkaline and acid precipitation extraction (BSFL-PC1). The essential amino acid levels in all insect flours and fractions were comparable with egg and cow's milk. Protein solubility was significantly higher in protein concentrates (85 – 97%) compared with flours (30 – 35%) at pH 2. The emulsion capacity (EC) was significantly higher ($p < 0.05$) in the protein concentrates (BSFL-PC1 and BSFL-PC2) compared to the freeze-dried and defatted BSFL flours, while the emulsion stability (ES) was significantly ($p < 0.05$) higher in BSFL-PC1 (100%) compared with BSFL-PC2 (49.8%). No significant differences ($p > 0.05$) were observed in foaming stability (FS) between freeze-dried and defatted BSFL flours. Fourier transform infrared spectroscopy (FT-IR) analysis revealed distinct structural changes between BSFL flours and protein concentrates. This was supported by surfaces morphology through scanning electron microscopy (SEM) images which showed that the protein extraction method had an influence on the structural properties of the protein concentrates. Therefore, based on the nutritional and techno-functional properties, BSFL flour fractions and protein concentrates show promise as novel functional ingredients for use in food applications.

Keywords:

Functional properties, black soldier fly larvae, protein extraction, foaming, insect protein, novel food.

3.2 Introduction

The United Nations (UN) has predicted that the world population will increase from 7 to 9 Billion people by the year 2030 (Van-Huis *et al.*, 2013) and with this increase, about 60% of people are expected to migrate and live in cities (Gmuer *et al.*, 2016). Animal protein is expensive and getting beyond the reach of many people, especially in developing countries. To feed this growing population, a paradigm shift towards producing sustainable and cost-effective food products is required now more than ever before. Entomophagy, consuming insects, has been practised by humans on every inhabited continent, not only historically but up until this present day. However, the development of the scientific basis for insect production and utilisation began in the last decade (Van-Huis *et al.*, 2013; Wang & Shelomi, 2017). Over 2 000 species have been deemed edible since 2012 in 116 countries and there has been an increase of industrial insect rearing companies in recent years (Rumpold & Schlüter, 2013; Van-Huis, 2013). Examples of large industrial-scale insect farming companies include AgriProtein in South Africa, Ynsect in France, Enviroflight in Ohio, USA, and HaoCheng Mealworms Inc. in China (Rumpold & Schlüter, 2015). Recently it has been suggested that edible insects could be a food source that may address the economic, environmental and health concerns as the global population surges on. With the growing interest in edible insect consumption, industrial farming of edible insect such as black soldier fly larvae (*Hermetia illucens*) in particular, has been expanding due to its stable supply, cost-effectiveness and hygienic production. Currently, edible insects are primarily marketed as whole insects, ground pastes or flours, protein powders and oil fractions which can further be used as ingredients in food applications (Gmuer *et al.*, 2016; Kim *et al.*, 2016, 2017). Edible insects offer an essential source of vitamins and minerals, fat and, above all proteins. According to La Barbera *et al.* (2018), consumers may be willing to consume insects when added as an ingredient in an unrecognisable form. Further processing of insects as an alternative protein source and acceptance by the food industry depends largely on their ability to fulfil tailored techno-functional properties required in food systems. The potential use of an insect ingredient as techno-functional food ingredients is dependent on chemical, physical and techno-functional and structural properties (Azagoh *et al.*, 2016a).

An ingredient's techno-functionality has been described as any food property, excluding its nutritional value, affecting its utilisation (Boye *et al.*, 2010; Kostić *et al.*,

2015). Emulsification, solubility, water and oil binding, foam capacity and stability, gelation and viscosity are among the techno-functional properties of most interest in food processing. Yi *et al*. (2013) studied five different acid-extracted insect proteins and reported that these exhibited poor foam and gelling properties. Protein-enriched fractions of *Schistocerca gregaria* (*S. gregaria)* and *Apis melifera* (*A. mellifera)* were reported to have higher foam stability after alkaline and sonication extraction, respectively. Moreover, Akpossan *et al.* (2015) demonstrated that *Imbrasia oyemensis* (*I. oyemensis*) protein fractions from full fat and defatted flours possess poor solubility due to their isoelectric point. However, both studied flours exhibited good water absorption and emulsification characteristics. According to Omotoso (2005), *Cirina forda* (*C. forda*) dried powders possess good emulsion and solubility properties. Kim *et al.* (2016), evaluated the effects of added defatted mealworm larvae (*Tenebrio molitor*) and silkworm pupae (*Bombyx mori*) in sausages. The addition of edible insect flours resulted in increased cooking yield and firmness in emulsion-based sausages. For the successful application of insects in food formulations, it is essential to understand their nutritional and techno-functional properties and how these are affected by processing.

To date, an in-depth analysis of the nutritional value of commercially available *H. illucens* available in South Africa has not been reported. For the possible use of *H. illucens* flours and protein concentrates as foodstuffs, information on the nutritional value and techno-functional properties are extremely essential. Therefore, the aim of this study was to determine the effect of different extraction methods on the nutritional, techno-functional and structural properties of BSFL flour and protein concentrates with the view to find alternative protein sources for human nutrition.

3.3 Materials and Methods

3.3.1 Chemicals

All chemicals were purchased from either Merck (Modderfontein, South Africa) or Sigma-Aldrich (Aston Manor, South Africa) if not stated otherwise. All the chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical laboratory procedures. Prepared reagents were stored under conditions that prevented contamination or deterioration. The water used

in the study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, Cape Town, South Africa).

3.3.2 Edible insects flour preparation

Previously fasted *Hermetia illucens* (black soldier fly – BSF) in the larval stage were obtained from AgriProtein, Cape Town, South Africa. They were immediately blanched in boiling water for two minutes to prevent browning and washed with cold water (Figure 3.1). The clean larvae were frozen at -75°C in a blast freezer prior to grinding in a laboratory blender to obtain a paste. This paste was then freeze-dried (Genesis, Virtis, USA) to obtain a stable powder. Some of the freeze-dried (BSFL-FD) powder was ground in a laboratory blender (Kenwood, Titanium, South Africa) and was passed through a 40-mesh US Standard sieve (Endecotts, London, UK) to separate the integument and stored at -20°C until further analysis (Appendix A). The sieved BSFL flour was defatted using hexane and isopropanol (3:2) mixture. One part of the insect flour and five parts of defatting solvent were stirred in a magnetic stirrer for six hours. Following sedimentation of the solids, the solvent-fat mixture was decanted. The procedure was repeated twice. Residual hexane was removed by evaporation overnight in a fume hood and the defatted flour (BSFL-DF) was stored at -20°C until further analysis.

3.3.3 Preparation of insect proteins

Two chemical techniques, alkaline solution and isoelectric precipitation (IEP) and alkaline extraction were used in this study to extract protein concentrates from defatted BSFL flour (Figure 3.2).

Alkaline and Isoelectric precipitation

Defatted BSFL flour (BSFL-DF) was mixed with Milli-Q water at a ratio of 1:10 ($w.w^{-1}$) and the pH of the mixture was adjusted immediately to pH 10 using 0.1 or 1 N NaOH solution (Figure 3.2). The mixture was stirred on a laboratory magnetic stirrer at a rate designed to prevent the formation of a vortex, for 30 min at ambient temperature (25°C ± 1.0), subsequently, the samples were centrifuged at 10 000 *g* for 20 min at 4°C. Next, the pH of the supernatants was adjusted to 4.5 with 1 M HCl and then the

suspension was left at 4°C overnight to facilitate protein precipitation. The precipitated proteins were recovered by centrifugation at 10 000 *g* for 30 min at 4°C. The BSFL protein concentrate (BSFL-PC1) was then freeze-dried and stored at -20°C for further analysis.

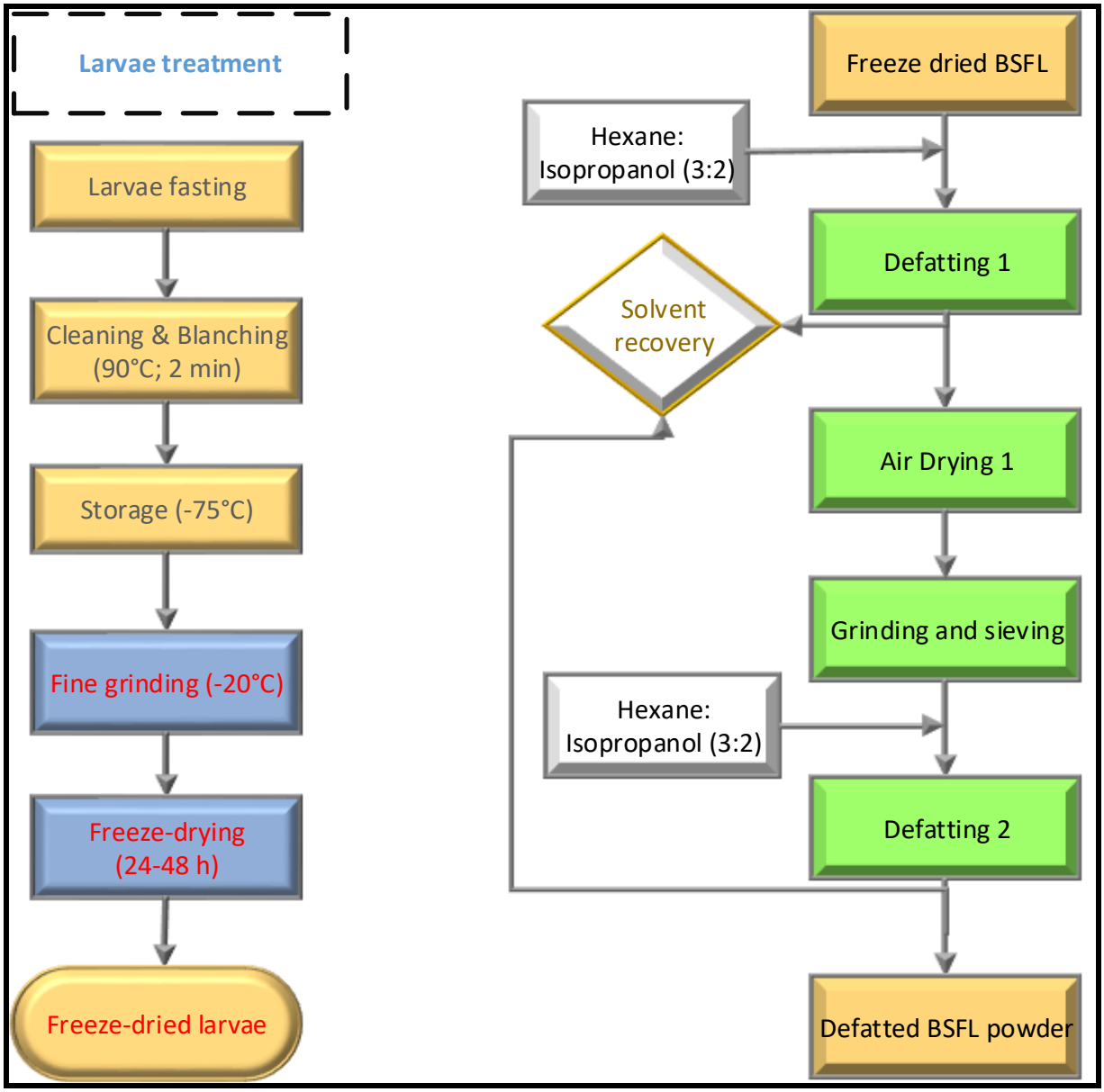

Figure 3.1 BSFL pre-treatment and defatting.

Alkaline extraction

Following the method of Azagoh *et al*. (2016b), with some modifications, BSFL-PC2 were extracted by mixing defatted BSFL flour with Milli-Q water at a ratio of 1:40 ($w.w^{-1}$) and the pH of the mixture was adjusted immediately to pH 10 using an 0.1 or 1 N NaOH solution (Figure 3.2). The mixture was stirred at a rate designed to prevent the formation of a vortex, for 2 hours at 40°C. The pH was monitored intermittently and maintained at 10 throughout the stirring period. The mixture was centrifuged at 10 000 *g* for 30 min at 4°C. The BSFL protein concentrate (BSFL-PC2) supernatants were then freeze-dried and stored at -20°C before use in subsequent experiments.

3.3.4 Proximate composition analysis

Proximate composition, i.e. moisture (925.10), crude protein (920.87), crude fat (932.06), and ash content (923.03) of the insect flour and protein concentrates were determined following standard methods recommended by the Association of Official Analytical Chemists (AOAC, 2015). Moisture percentage was calculated by drying the sample in an oven at 100°C for two hours. The dried sample was placed into a desiccator, allowed to cool and then re- weighed. The process was repeated until a constant weight was obtained. Crude protein content was analysed by the high-temperature combustion process according to the Dumas combustion method (TruSpec-N Leco, St. Joseph, MI) using a protein-to-nitrogen conversion factor of 5.60as recommended by Janssen *et al.* (2017). Ethylenediaminetetraacetic acid (EDTA) was used as a standard. Crude fat was calculated by drying fats after extraction in a Soxhlet assembly using diethyl ether. The ash percentage was calculated by combusting the samples in a silica crucible placed in a muffle furnace at 550°C. The percentage of carbohydrate on a dry basis was determined by subtracting all of the components (moisture, crude protein, crude lipid and ash) from 100.

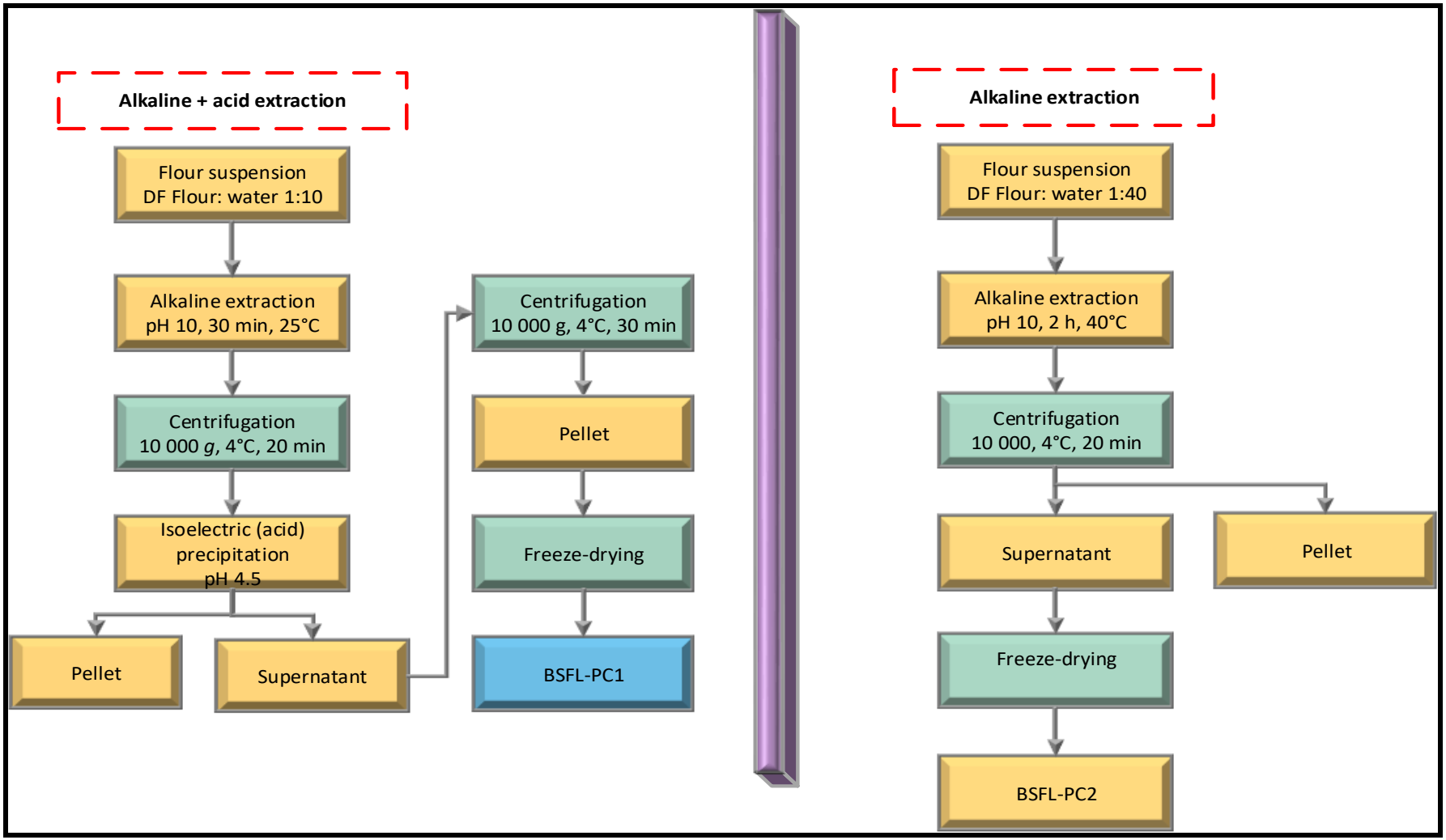

Figure 3.2 Alkaline and acid precipitation extraction (BSFL-PC1) and alkaline extraction (BSFL-PC2) of protein concentrates.

3.3.5 Amino acid analysis

Five hundred milligrams of each insect flour and protein concentrate were hydrolysed with 6 mL of 6 N HCl at 110 °C for 23 h. Then the internal standard (7.5 mL of 5 mM norleucine in water) was added. The hydrolysed samples were analysed by High Performance Liquid Chromatography with a fluorescence detector (HPLC/FLD, Waters Alliance 2695) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The amino acid profiles of insect flour and protein concentrates were compared with data in the literature on egg white and cow's milk proteins used for human consumption.

3.3.6 Bulk density

Bulk density was measured as a ratio of mass to volume. A graduated cylinder, previously tared, was gently filled up to the ten mL mark with BSFL flour or protein. The sample was then packed by gently tapping the cylinder on the bench-top from a height of five cm until there was no further diminution of the sample level and the volume was noted. The weight of the filled cylinder was taken and the bulk density was calculated as the weight of sample per unit volume ($kg.L^{-1}$).

3.3.7 Colour

Colour was determined by the method as described by Diedericks & Jideani (2015) and Bußler *et al.* (2016b), with minor modifications. The colour of the insect flours and protein concentrates was measured with a spectrophotometer (Model CM-5; Konica Minolta Sensing, Japan) using the CIEL* a* b* colour space system. The instrument was calibrated by using the white calibration plate followed by zero calibration. Powdered samples were placed evenly in the provided cuvette (diameter 30 mm) covering the bottom of the dish, to allow for reflectance measurement. Measurements for each sample were performed in triplicate at 3 different positions in the samples (one reading = average of 3 readings per rotated position), with the results recorded in L∗ (lightness), a* (+a* = red and –a* = green), b* (+b* = yellow and –b* = blue). The change in colour (ΔE) was calculated, whereas the indices 0 and s indicate measured values of unprocessed (larvae) and processed insects (flour fractions), respectively.

$$\Delta E = \sqrt{(L_0 - L_s)^2 + (a_0 - a_s)^2 + (b_0 - b_s)^2}$$

Where: L – is L* (lightness), a – a* (difference in green and red), b – b* (difference in yellow and blue), o – indicates measured values of BSFL-FD, s - indicates measured values of processed flour or protein concentrates.

3.3.8 Determination of techno-functional properties

Water and oil binding capacity

The water binding capacity (WBC) was determined according to Purschke *et al*. (2018), with slight modifications. Briefly, 0.5 g insect flour or protein concentrate sample was mixed with 2.5 mL deionised water at ambient temperature (25°C), vortexed for 1 min (Vortex-Genie 2, Scientific Industry Inc., USA) and centrifuged for 2 min at 3 330 *g*. The supernatant was removed by decantation and drainage of the residual non-bound water by placing the centrifugation tube upside-down on a Whatman No 1 filter paper for one hour. WBC was calculated as:

$$WBC = \frac{W_b - W_a}{W_{a,DM}}$$

Where: W_a is the initial weight, W_b is the final weight and $W_{a,\,DM}$ is the initial weight of the sample based on dry matter. The oil binding capacity (OBC) was analysed using commercial sunflower oil instead of Milli-Q water. Except for the vortexing step (2 min), the experimental procedure was performed in analogy to the WBC assay and OBC was similarly calculated.

Emulsion capacity and stability

Emulsifying properties were determined according to the method of Coelho and Salas-Mellado (2018) and Zielińska *et al.* (2018) with slight modifications. The powder

sample was dispersed in distilled water (5% w.v^{-1}) and centrifuged (Thermo Electron Corporation Jouan MR1812) at 9 000 rpm speed for 15 min, 15 mL of the supernatant were homogenized (Polytron PT 2500 E) with 15 mL of commercial sunflower oil at a speed of 18 000 rpm for 1 min. Next, the samples were centrifuged at 3 000 *g* for five min and the volume of the individual layers were read using a 50 mL centrifuge tube. Emulsion stability was evaluated by heating the emulsion in a water bath set at 80°C for 30 min. Then, the samples were centrifuged at 3 000 *g* for five min. Emulsion capacity and emulsion stability were calculated from the formula:

$$\text{Emulsion capacity (EC)} = \frac{V_{el}}{V} \times 100$$

$$\text{Emulsion stability (ES)} = \frac{V_{30}}{V_{el}} \times 100$$

Where: V – total volume of tube contents, V_{el} – volume of the emulsified layer, V_{30} – volume of the emulsified layer after heating.

Solubility

The solubility of the insect flours and protein concentrates was determined using a modified version of the method by Hall *et al*. (2017). Each sample (400 mg) was dispersed in 20 mL phosphate buffers of pH 2 – 10, respectively. Each buffer mixture was stirred with a magnetic stirrer bar at room temperature for 30 min and centrifuged at 7 500 *g* for 20 min at 4°C. The protein content of the supernatant and total protein in the samples were determined using the bicinchoninic acid protein assay (BCA) method with bovine serum albumin as a standard, following the manufacturer's protocol (Sigma, St. Louis, USA). Protein solubility was expressed as a percentage and calculated as follows:

$$\text{Solubility (ES)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}_e} \times 100$$

Foaming capacity and stability

Foaming capacity (FC) and foam stability (FS) were determined according to the method of Zielińska *et al* (2018) with minor modifications. The powder sample was dispersed in distilled water (5% w.v^{-1}) and centrifuged (Thermo Electron Corporation Jouan MR1812) at 10 000 rpm for 15 min. Then 20 mL of the supernatant was homogenized in a high shear homogenizer mixer (Polytron PT 2500E) at a speed of 16 000 rpm for 3 min. The homogenized sample was immediately transferred into a cylinder. The total volume was read at time zero and 30 min after homogenization. The foaming capacity and foam stability were calculated from the formula:

$$\text{Foaming Capacity (FC)} = \frac{V_a - V}{V} \times 100$$

$$\text{Foam Stability (FS)} = \frac{V_{30}}{V_a} \times 100$$

Where: V – volume before whipping (mL), V_a – volume after whipping (mL), V_{30} – volume after standing (mL).

3.3.9 Surface charge (Zeta potential)

The ζ-Potential of BSFL-PC1 and BSFL-PC2 protein concentrates were determined using a Zetasizer Nano Series (Malvern Instruments, Malvern, Worcestershire, UK) as described by Ladjal-Ettoumi *et al.* (2016). Samples were diluted to 1% (w.v^{-1}) at protein concentration with Milli-Q water before measurement. Data was collected over at least five sequential readings and processed using the Smoluchowski mode (Schwenzfeier *et al.*, 2013).

3.3.10 Scanning electron microscopy (SEM)

The surface morphology of the native BSFL protein and BSFL-Glu conjugates was examined using scanning electron microscopy (TM-3000, Hitachi Corporation, Tokyo, Japan). The freeze-dried sample was placed onto double-sided carbon adhesive tape attached to the specimen stubs. The surface structure of the sample was observed at 320 X magnification and in secondary electron mode at 15.0 kV.

3.3.11 Fourier transform infrared spectroscopy (FT-IR)

All flour and protein concentrate samples were analysed using a Perkin Elmer Fourier transform infrared spectroscope (FT-IR) equipped with a universal attenuated total reflectance (UATR) polarization accessory for spectra. Prior to data collection of each sample, a background spectrum was collected and then the sample powders obtained by grinding in a mortar were placed directly covering the surface of the ATR crystal. All spectra were acquired by co-addition of 32 scans at a resolution of 4 cm^{-1} in the range of 400 – 4000 cm^{-1}. The UATR crystal was cleaned with acetone to remove any residual contribution of the previous samples.

3.3.12 Data analysis

All data were subjected to multivariate analysis of variance (MANOVA) to ascertain whether the main effects resulted in significant differences in response variables. The Duncan's multiple comparison post hoc test was used to test significant differences ($p < 0.05$) between individual means. SPSS 25.0 for Windows® was used for the statistical analyses and the level of confidence required for significance was selected at $p < 0.05$.

3.4 Results and discussion

3.4.1 Nutritional properties

The proximate compositions of freeze-dried BSFL (BSFL-FD), defatted BSFL (BSFL-DF), alkali and isoelectric precipitation BSFL protein concentrate (BSFL-PC1), alkaline extraction protein concentrate (BSFL-PC2) are depicted in Table 3.1. The ash content of all samples ranged between 2.08 – 10.81% with BSFL-FD having the highest ($p < 0.05$) content. BSFL-PC1 had the highest protein (73.35%) content and BSFL-FD (44.47%) had the lowest protein content ($p < 0.05$), while BSFL-PC1 had a significantly ($p < 0.05$) higher protein content compared to BSFL-PC2. This signifies that the protein extraction method had an effect on the protein content. The protein content obtained for BSFL-FD was within a similar range to that reported by Huang *et al.* (2018) for oven-dried BSFL (40 – 44%). The protein contents measured in this study were much higher than those reported by Bußler *et al.* (2016b) for fresh and freeze-fried BSFL (black soldier fly larvae) namely, 31.7% and 34.7%, respectively. The differences in

protein content can be attributed to the different feeding regimes (diets), age and size of the black soldier fly larvae prior to analysis. Moreover, in this study, the conversion factor of 5.6 0 as recommended by Janssen *et al.* (2017) was used instead of 6.25 which has been reported to lead to an overestimation of the protein content since edible insects contain chitin which contributes to the nitrogen content. The protein and lipid composition of BSFL is highly impacted by what they consume (Wang & Shelomi, 2017). Edible insects reported in this study were fed on clean standardised feed and were fasted and blanched prior to further processing. The protein content of all the fractions investigated in this study is higher than that of common food products such as cow's milk (3.5%), eggs (13%), fish (18.3%) and chicken (22%) (Yi *et al.*, 2013; Zhao *et al.*, 2016).

As can be seen in Table 3.1, the defatting step significantly ($p < 0.05$) decreased the crude fat present in BSFL from 22.60 to 0.83% while protein concentrates contained 0.27 – 0.37 % fat. The fat content of BSFL-FD (22.60%) was comparable to the data reported by Bußler *et al.* (2016b) for *H. illucens* (21.1%) and *T. molitor* (20.0%). The current practice in the food industry is geared towards the extensive use of soy protein products as a non-meat protein in processed meat products. These are classified based on their protein concentration as soy flour (50 – 54%), concentrated soy protein (62 – 69%), and isolated protein (86 – 87%) (Kim *et al.*, 2016). The results of this study clearly demonstrate that BSFL flours and protein concentrates were comparable to that of soy flour and soy concentrated protein, both of which are widely used as food ingredients commercially.

3.4.2 Bulk density and Colour

The bulk density of BSFL flour and protein concentrates is shown in Table 3.2. Bulk density can be described as the weight of powder per unit volume (expressed as $g.mL^{-1}$). The bulk density of BSFL flours and protein concentrates ranged from 0.83 – 1.04 $g.mL^{-1}$. No significant differences were observed between the freeze-dried and defatted BSFL flours ($p > 0.05$). Akpossan *et al.* (2015) reported similar bulk density values in full-fat and defatted ground *I. oyemensis* flours. In terms of the protein concentrates, a significant ($p < 0.05$) reduction in bulk density was observed compared to the flour fractions signifying that the extraction process employed affected bulk density. In assessing packaging requirements, material handling and application in

wet manufacturing, bulk density is an essential parameter for consideration by food processors (Wani *et al.*, 2013).

Table 3.1 Proximate composition of BSFL flour and protein concentrates.

Sample	Crude Protein (%)	Crude Fat (%)	Carbohydrates (%)	Moisture (%)	Ash (%)
BSFL-FD	44.47 ± 1.77^{a}	22.60 ± 1.39^{b}	21.13 ± 0.57^{a}	9.48 ± 0.34^{c}	10.81 ± 0.26^{d}
BSFL-DF	50.12 ± 0.66^{b}	0.83 ± 0.06^{a}	40.80 ± 0.39^{d}	6.07 ± 0.25^{b}	8.25 ± 0.33^{c}
BSFL-PC1	73.35 ± 0.88^{d}	0.37 ± 0.12^{a}	22.92 ± 1.02^{b}	1.48 ± 0.01^{a}	3.36 ±0.28^{b}
BSFL-PC2	68.47 ± 0.93^{c}	0.27 ± 0.06^{a}	29.19 ± 0.92^{c}	1.57 ± 0.03^{a}	2.08 ±0.01^{a}

Freeze-dried BSFL flour – BSFL-FD, defatted BSFL flour – BSFL-DF, alkaline and acid precipitation extraction BSFL protein concentrate – BSFL-PC1, alkaline extraction BSFL protein concentrate – BSFL-PC2. [1]Results are reported as Mean ± Standard deviation. Different superscripts in the column indicate significant differences between treatments ($p \leq 0.05$).

Processing of the BSFL affected the visual appearance of the BSFL flour and protein concentrates produced. The colour changes are summarised in Table 3.2. Defatting with the hexane:isopropanol mixture led to a significantly higher ($p < 0.05$) L-value compared to the freeze-dried BSFL flour. Similar results were reported by Mishyna *et al.* (2019) in *A. mellifera* defatted with hexane. In this study, the defatting process significantly increased lightness ($p < 0.05$), redness ($p < 0.05$) and yellowness ($p < 0.05$). in terms of a* (red/green), only BSFL-PC fell within the greener quadrant while no significant differences were observed in the yellowness of the samples. In this study, the ΔE was in the range 11.95 – 23.87 which indicates perceptible colour differences at a glance, in other words, the colour changes could be clearly perceived without closer inspection. The high ΔE value for BSFL-PC2 can be partly attributed to phenolic compounds located in the insect cuticle or integument that can undergo oxidation and protein-polyphenol interaction and enzymatic browning catalysed by phenol oxidase (Kim *et al.*, 2016; Janssen *et al.*, 2019a). The bright colour of the defatted BSFL flours could therefore be due to the removal of these compounds after the defatting process, resulting in browning (Appendix B). However, the lightness as

well as the redness decreased significantly after further extraction. The reaction mechanism resulting in the dark and brown colour of extracted protein fractions is still not yet well understood. Nonetheless, edible insect flours can also be used in baked goods where colour or visual appeal may not be a critical problem.

3.4.3 Amino acids

The amino acid composition of BSFL flours and protein concentrates in comparison to that of cow's milk, egg, as well as the FAO protein intake recommendations for adults, are shown in Table 3.3. The essential amino acids, leucine and lysine content was predominant in BSFL-DF compared with BSFL-FD. Lysine is regarded as a limiting amino acid in staple cereals such as maize, rice and wheat (Boye *et al.*, 2010; Wang & Shelomi, 2017). Thus, incorporation of BSFL-DF (6.76% lysine) and BSFL-PC1 (9.16% lysine) in food products can serve as a source of lysine, especially in developing countries. Histidine, which is considered an essential amino acid for toddlers and infants, was significantly higher in BSFL-DF (3.84%) and BSFL-PC1 (3.64%) than that in cow's milk and egg (2.70% and 2.44%, respectively). The sum of essential amino acids increased as a result of defatting from 24.98% to 38.20%. Moreover, BSFL-PC1 had a considerably higher sum of amino acids compared to BSFL-PC2. These values are in line with those of Leni *et al.* (2019) and Huang *et al*. (2018). Among the non-essential amino acids in Table 3.3, glutamic acid had the highest concentration in BSFL-DF (13.2%), BSFL-PC1 (12.4 %) and BSFL-PC2 (12.13%), respectively. These results are in agreement with the work conducted by Köhler *et al*. (2019) who reported high levels of glutamic acid in whole house cricket (*Acheta domesticus*) flour. The content of essential amino acids for BSFL flours and protein concentrates were comparable to or exceed the recommended by FAO (2013b) as a basic human dietary requirement. In the case of BSFL-PC1, the sum of essential amino acid for BSFL was more than double (45.52%) the FAO requirements. The results of this study confirm the claims of other studies that in general, edible insects are good sources of amino acids (Table 3.3), proteins and lipids (Table 3.1) (Adebowale *et al.*, 2005; Yi *et al.*, 2013; Kouřimská & Adámková, 2016; Köhler *et al.*, 2019).

Table 3.2 Colour measurement of colour values (L, a and b), change in colour(ΔE) and bulk density of flour fractions from black soldier fly larvae[1].

Fractions	Bulk density (g/ml)	L*	a*	b*	ΔE
BSFL-FD	1.01 ± 0.02 [b]	51.24 ± 0.34[c]	0.84 ± 1.34[ab]	17.99 ± 1.70[a]	Control
BSFL-DF	1.04 ± 0.02 [b]	62.95 ± 1.01[d]	1.67 ± 0.12[b]	18.43 ± 2.67[a]	11.95 ± 1.16[a]
BSFL-PC1	0.84 ± 0.01[a]	44.56 ± 0.63[b]	0.85 ± 1.35[ab]	17.99 ± 1.70[a]	16.16 ± 1.27[a]
BSFL-PC2	0.86 ± 0.01 [a]	35.20 ± 1.87[a]	-2.14 ± 3.28[b]	18.78 ± 6.56[a]	23.87 ± 4.16[b]

Freeze-dried BSFL flour – BSFL-FD, defatted BSFL flour – BSFL-DF, alkaline and acid precipitation extraction BSFL protein concentrate – BSFL-PC1, alkaline extraction BSFL protein concentrate – BSFL-PC2. [1]Results are reported as Mean ± Standard deviation. Different superscripts in the column indicate significant differences between treatments ($p \leq 0.05$).

Table 3.3 Amino acid composition (g.100g^{-1}) of BSFL flour fractions and protein concentrates compared to cow's milk, egg protein and FAO requirements for human consumption.

Amino acid	**BSFL-FD**	**BSFL-DF**	**BSFL-PC1**	**BSFL-PC2**	**Cow's Milk**	**Egg Protein**	**FAO t**(2013)
Essential							
Histidine	1.82 ± 0.01^{a}	3.84 ± 0.01^{d}	3.64 ± 0.02^{c}	2.48 ± 0.00^{b}	2.70	2.40	1.50
Isoleucine	2.49 ± 0.01^{a}	4.32 ± 0.44^{bc}	5.18 ± 0.78^{c}	3.99 ± 0.45^{b}	4.90	5.60	3.00
Leucine	4.01 ± 0.12^{a}	7.29 ± 0.15^{c}	7.99 ± 0.29^{d}	4.61 ± 0.17^{b}	9.10	8.30	5.90
Lysine	3.73 ± 0.01^{a}	6.79 ± 0.19^{c}	9.16 ± 0.34^{d}	5.37 ± 0.20^{b}	7.40	6.30	4.5
Methionine	2.53 ± 0.00^{a}	1.72 ± 0.45^{a}	2.53 ± 0.00^{a}	2.67 ± 0.08^{a}	2.60	3.20	1.60
Phenylalanine	4.14 ± 0.01^{a}	4.36 ± 0.20^{a}	7.18 ± 0.05^{c}	5.41 ± 0.21^{b}	4.90	5.10	Not supplied
Threonine	2.87 ± 0.53^{a}	4.09 ± 0.49^{b}	4.95 ± 0.12^{bc}	4.65 ± 0.00^{b}	4.40	5.10	2.30
Valine	3.39 ± 0.01^{a}	5.80 ± 0.51^{b}	5.61 ± 0.89^{c}	5.09 ± 0.51^{b}	6.60	7.60	3.90
Sum	**24.98 ± 0.80**	**38.20 ± 2.44**	**46.52 ± 2.48**	**34.27 ± 2.19**	**42.60**	**43.60**	**22.70**
Non-essential							
Alanine	3.58 ± 0.01^{a}	5.95 ± 0.48^{c}	4.66 ± 0.04^{b}	5.41 ± 0.48^{bc}	3.60	5.40	-
Arginine	3.38 ± 0.00^{a}	4.85 ± 0.36^{b}	4.57 ± 0.02^{b}	5.72 ± 0.02^{c}	3.60	6.10	-
Aspartic acid	5.89 ± 0.01^{a}	10.38 ± 0.39^{b}	12.56 ± 0.69^{c}	9.62 ± 0.40^{a}	7.70	10.70	-
Glycine	3.08 ± 0.06^{a}	4.80 ± 0.13^{c}	3.88 ± 0.06^{b}	4.97 ± 0.15^{c}	3.00	2.00	-
Glutamic acid	6.89 ± 0.05^{a}	13.62 ± 0.05^{d}	12.13 ± 0.09^{b}	12.40 ± 0.05^{c}	12.0	20.60	-
Proline	2.57 ± 0.01^{a}	5.71 ± 0.23^{d}	4.38 ± 0.01^{c}	3.38 ± 0.24^{b}	8.50	3.80	-
Serine	2.13 ± 0.04^{a}	4.42 ±0.10^{d}	3.99 ± 0.28^{c}	3.13 ± 0.17^{b}	5.20	7.90	-
Tyrosine	6.89 ± 0.00^{c}	6.47 ± 0.07^{b}	6.28 ± 0.15^{a}	9.34 ± 0.09^{b}	4.10	7.60	-
Sum	**34.41 ± 0.21**	**56.21 ± 1.74**	**52.45 ± 1.85**	**53.98 ± 2.00**	**55.30**	**56.50**	-

Freeze-dried BSFL flour – BSFL-FD, defatted BSFL flour – BSFL-DF, alkaline and acid precipitation extraction BSFL protein concentrate – BSFL-PC1, alkaline extraction BSFL protein concentrate – BSFL-PC2. Results are reported as Mean ± Standard deviation of triplicate analysis. Different letters indicate significant ($p < 0.05$) differences between means across rows.

The high levels of essential amino acids are particularly pertinent as they cannot be synthesised by the human body in sufficient quantities and thus should be provided by the diet. Moreover, protein functionality and bioavailability is governed by the amino acid composition, as well as the amino acid sequence.

3.4.4 Protein solubility

In order to provide knowledge on the successful use of insect-derived ingredients in different food applications, the protein solubility of edible insect flours and protein concentrates were investigated. Solubility at different pH values, as well as the level of protein denaturation due to heat or chemical treatment, serve as a measure of how well insect flours and protein concentrate can perform when integrated into food systems (Ma *et al.*, 2018).

In general, BSFL-FD and BSFL-DF flours had low protein solubility at pH 2 – 3 compared to the protein concentrates (Figure 3.3). The high solubility at low pH values for the protein concentrates (BSFL-PC1, 95% and BSFL-PC2, 85%) makes them ideal candidates for use in acidic beverages. The protein solubility of all BSFL samples ranged from a minimum at isoelectric point (pI) to its maximum at pH 11 (Figure 3.3). For all the insect flours the pI was found to be in the region of pH 4. 0 – 4.5. During the process of extraction, protein solubility was highly dependent on the pH. The results of this study resemble the pI of common major food proteins such as casein (4.6), soybean (4.5) and meat products (5.0) (Boye *et al.*, 2010; Yi *et al.*, 2013; Ma *et al.*, 2018). These results can be attributed to the reduced interaction between protein and water at pH 4.5 – 5.0 and this phenomenon enhances protein-protein interaction of foods resulting in protein aggregation and precipitation. The protein solubility profiles of BSFL flours and protein concentrates against pH were generally similar to each other and consistent with previously published data for insect species (Akpossan *et al.*, 2015; Omotoso, 2015; Hall *et al.*, 2017) and plant legumes such as pea, Kabuli chickpea and kidney beans (Boye *et al.*, 2010; Wani *et al.*, 2013). The findings from this study further highlight the potential of using BSFL protein concentrates in acidic foods such as acidified sauces and sports drinks. These findings further support the possible application of BSFL flour and BSFL protein concentrates over a broad pH spectrum, in addition to acidic foods.

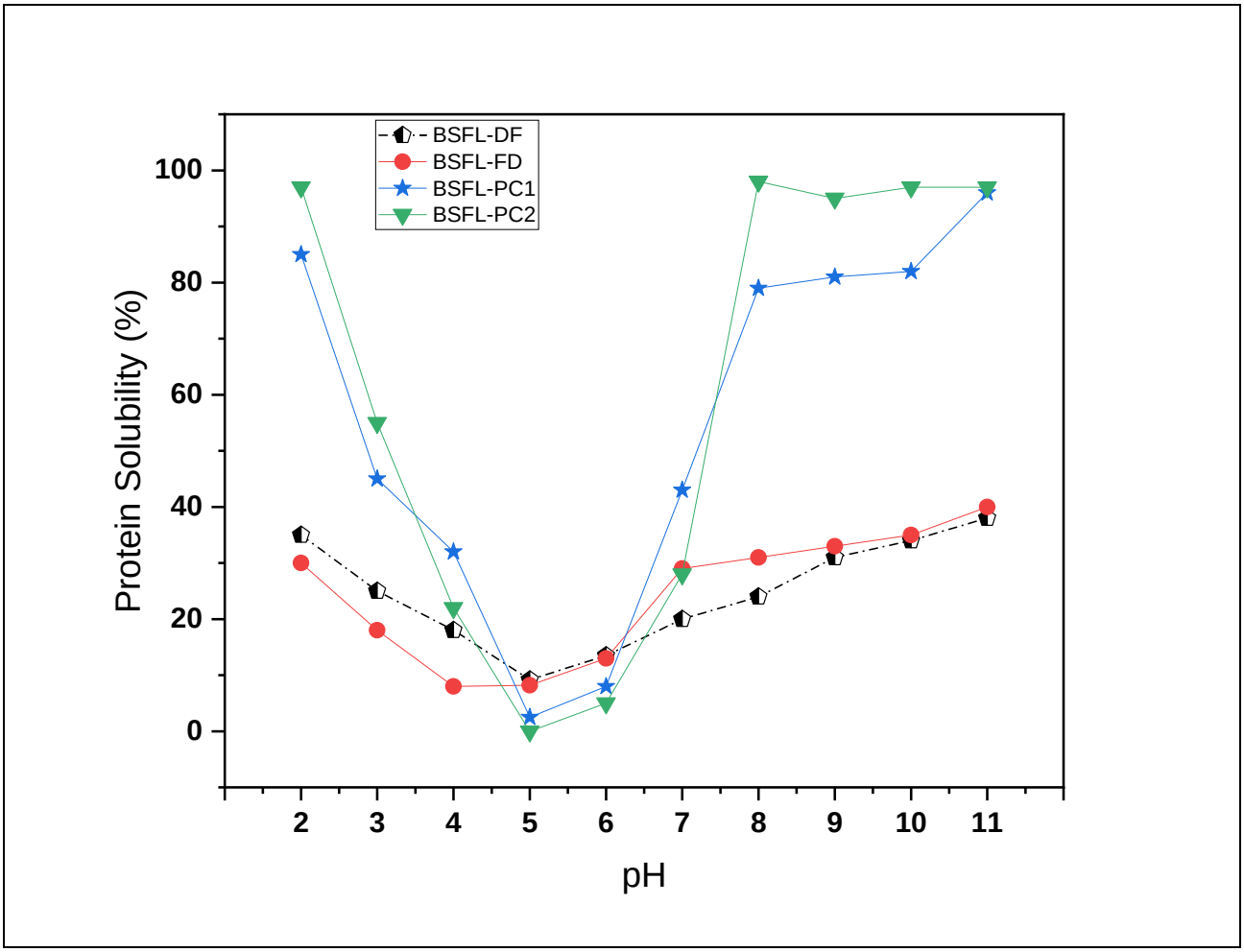

Figure 3.3 Solubility profile of BSFL flour fractions and protein concentrates as a function of pH. Freeze-dried BSFL flour (pentagon), Defatted BSFL flour (circle), Alkaline and acid precipitation extraction BSFL protein concentrate (star), alkaline extraction BSFL protein concentrate (triangle).

3.4.5 Water and oil binding capacity

Owing to their effects on flavour and textural properties of food products, water and oil interactions with proteins play significant roles in food systems. WBC and OBC of protein ingredients may be influenced by intrinsic factors such as amino acid sequence, protein conformation, hydrophobicity and polarity. The ability of protein ingredients to interact with water under restricted conditions is expressed by its water binding capacity. The WBC of BSFL flour and protein fractions is displayed in Figure 3.4. No significant ($p > 0.05$) impact of the defatting step on WBC was observed, whereas the BSFL-PC1 showed a higher ($p < 0.05$) WBC compared to BSFL-PC2. Few studies have been conducted on the WBC of BSFL flours and protein fractions extracted using different chemical techniques. The results of this study are higher than the WBC values described for BSFL flour fractions (0.4 – 0.8 $g.g^{-1}$) by Bußler *et al.* (2016b). The differences in WBC values of BSFL flours and protein concentrates can be ascribed to the differences in methods of extraction used by the authors and the insect origin and diet. Among the BSFL flour and protein fractions, the highest WBC was observed in BSFL-PC1 (5.6 $g.g^{-1}$). This value is comparatively higher than the WBC of other insect protein fractions reported in the literature such as *T. molitor* (1.87 $g.g^{-1}$) (Zhao *et al.*, 2016), and *S. gregaria* (2.18 $g.g^{-1}$) (Zielińska *et al.*, 2018). A high water binding capacity value for a protein aids to maintain moist mouthfeel and freshness of baked goods and is associated with reduced moisture loss in bakery products. Thus, information about WBC of insect-derived ingredients is essential for future application in food systems.

The OBC is another crucial property of food ingredients used in formulated foods. High OBC is desirable in improving the palatability and flavour retention of foods. Figure 3.4 depicts the result of the OBC. Similar to the WBC, the defatting step did not have a significant effect on the OBC of BSFL flours, but for the BSFL protein concentrates it was significantly lower. The OBC of BSFL-FD flour in this study was (4.1 $mL.g^{-1}$). This compared favourably to the OBC value obtained for hydrolysed migratory locust protein flour (1.5 $mL.g^{-1}$) (Purschke *et al.*, 2018b) and *T. molitor* (1.71 $mL.g^{-1}$) (Zielińska *et al.*, 2018). The ability of a protein to bind oil or fats is essential in meat replacers and extenders and it is a requisite for the formulation of cake batters, sausages, mayonnaise and salad dressings (Gravel & Doyen, 2020). In terms of the protein fractions, the lowest ($p < 0.05$) OBC was observed in BSFL-PC1 compared to BSFL-PC2 (Figure 3.4).

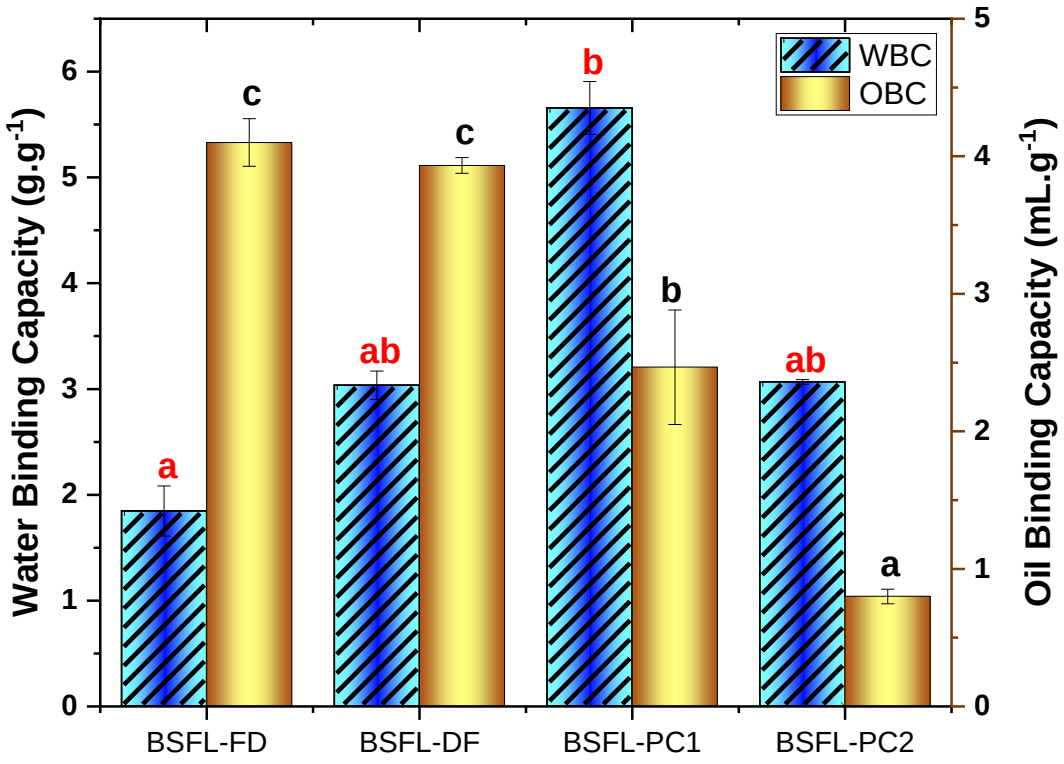

Figure 3.4 Water and Oil binding capacity of flour and protein fractions. Different letters indicate significant ($p < 0.05$) differences between means across columns.

The differences in OBC are possibly due to different surface hydrophobicity or hydrophilicity of these proteins which may be influenced by the extraction method. The WBC and OBC values obtained in this study demonstrated that BSFL flour and protein concentrates can be useful for multiple food applications such as improving the palatability and texture of formulated foods.

3.4.6 Foaming capacity and foam stability

Foams can be defined as air bubbles dispersed in a liquid and stabilised by protein at the air-liquid interface. Proteins in foams contribute to the uniform distribution of fine air cells in the structure of the foods, improving its smoothness, lightness and allowing the volatilization of flavours that enhance the palatability of the food products (Garcia-Vaquero *et al.*, 2017). The results of the foam capacity and stability for BSFL flour and protein concentrates are exhibited in Figure 3.5. The FC of BSFL-FD (40%) was not statistically different ($p > 0.05$) than that of BSFL-DF (55%). The foaming capacity of BSFL-PC2 (78.43%) after alkaline extraction was significantly higher ($p < 0.05$) than BSFL-PC1(75.97%). Overall, the protein concentrates exhibited improved foaming capacity. The high foamability may be attributed to increased protein content and the possibility of changes in protein characteristics after the extraction. The FC results of this study show that BSFL-FD and BSFL-DF flours are higher than those reported by Adebowale *et al.* (2005) for the large African cricket (*Grylllidae sp.*) that had an FC of only 6%. Previous studies on freeze-dried *A. mellifera* and *S. gregaria* conducted by Mishyna *et al.* (2019) also exhibited low FC of 5.8% and 45%, respectively. These results are consistent with the observation of Akpossan *et al*. (2015) who reported poor FC of the edible full-fat insect *I. oyemensis* flour. The differences in FC of edible insect proteins may be due to their different conformational characteristics.

The stabilization of foam is mostly dependent on the formation of a thick cohesive viscoelastic film involving each gas bubble. The present study supported earlier findings that, with the removal of fat, foam stability increases (Yi *et al.*, 2013; Zielińska *et al.*, 2018). Most notably, this is the first study to our knowledge to investigate the effect of protein extraction methods on the FC and FS of BSFL protein concentrates. The high FC and FS of BSFL-PC1 and BSFL-PC2 reported in this study suggest that they can be used in bakery and confectionery products. Currently, the food industry utilises wheat, soy and dairy-based protein concentrate and isolates as

ingredients. However, consumers and food processors are in search of novel protein sources to alleviate the allergenicity challenges posed by the common eight priority allergens (Wheat, peanut, soy, fish, dairy, tree nuts, crustaceans and egg).

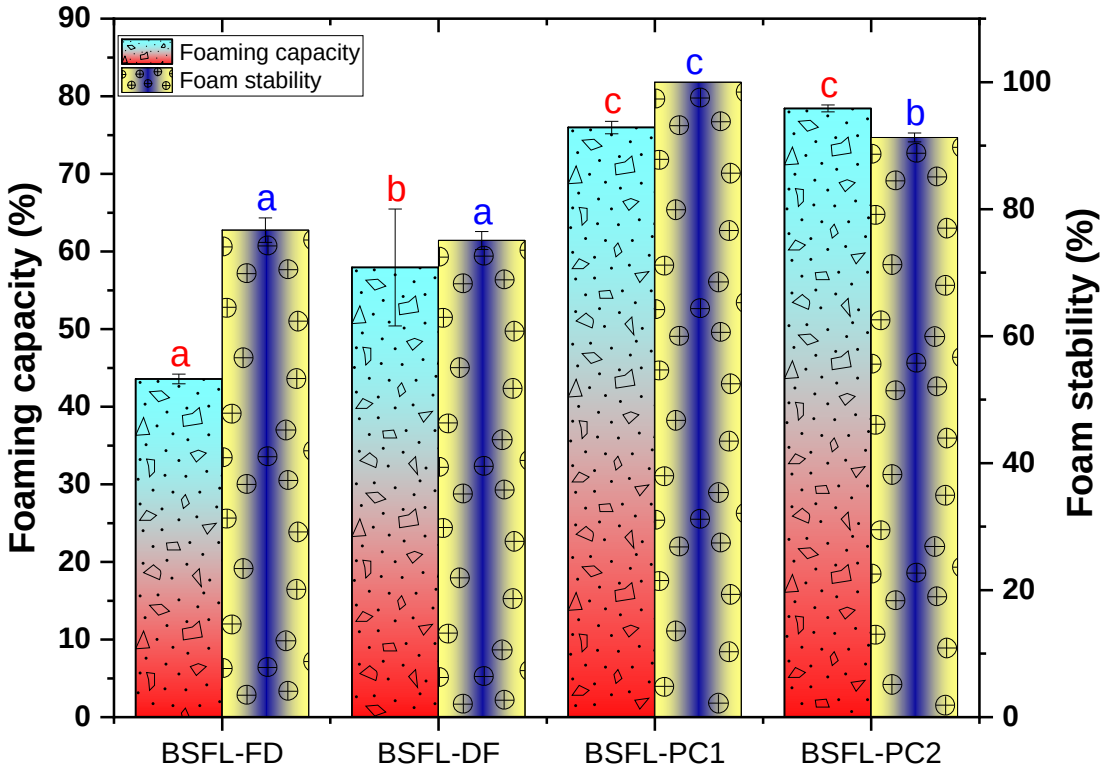

Figure 3.5 Foaming capacity and stability of BSFL flour and protein concentrates.. Results are reported as Mean ± Standard deviation. Different superscripts in the column indicate significant differences between treatments ($p \leq 0.05$) across columns.

3.4.7 Emulsifying capacity (EC) and stability (ES)

The amphiphilic nature of proteins allows them to form and stabilise food emulsions. Emulsifying property is one of the most vital properties in the manufacturing of formulated foods and good emulsifying properties are desired to produce meat analogues and milk-like beverages (Garcia-Vaquero *et al.*, 2017). Figure 3.6 exhibits the emulsion capacity and stability of the BSFL flour and protein concentrates. Emulsion capacity of defatted BSFL flour (BSFL-DF, 78.73%) was significantly higher ($p < 0.05$) than the full-fat flour (BSFL-FD, 76.80%). Similar results were reported for EC of *I. oyemensis* by Akpossan *et al.* (2015), while Kim *et al*. (Kim *et al.*, 2017) observed lower EC of cricket flour (39.17 – 45%). In a study conducted by Mishyna *et al.* (2019), no significant differences were observed in raw and defatted *S. gregaria* flours. The variations in EC of edible insect flours could be attributed to differences in protein content and molecular structure. In general, the EC of a food protein is based on the protein-oil and protein-water interactions. The highest EC was determined for the protein concentrates BSFL-PC1 (100%) and BSFL-PC2 (100%). These results show that the processes used to obtain protein concentrates did not have a significant ($p > 0.05$) impact on the ability of BSFL-PC1 and BSFL-PC2 to aid in the creation of emulsions.

In terms of ES, BSFL-FD (25.27%) formed emulsions with significantly ($p < 0.05$) lower ES compared with BSFL-DF (33.73%) signifying that its protein did not effectively interact at the interface to form a strong interfacial membrane (Figure 3.6). The ES of the protein concentrates was significantly higher ($p < 0.05$) than the flour fractions. The results of the BSFL protein concentrate suggest that these novel proteins could be suitable for its use in the formulation of a wide variety of food products. Currently, there is a paucity of studies investigating the emulsification properties of insect protein concentrates obtained using different extraction techniques which makes the comparison of the results difficult. Proteins currently used by the food industry due to their emulsifying abilities are mostly derived from soybean, milk (whey or casein) and egg. These are widely used in various food formulations due to their commercial availability and good functional properties (Burger & Zhang, 2019). However, the major drawback is that they all have been identified as common food allergens. Therefore, further studies on the emulsifying properties of edible insect concentrates are required, especially using different extraction methods.

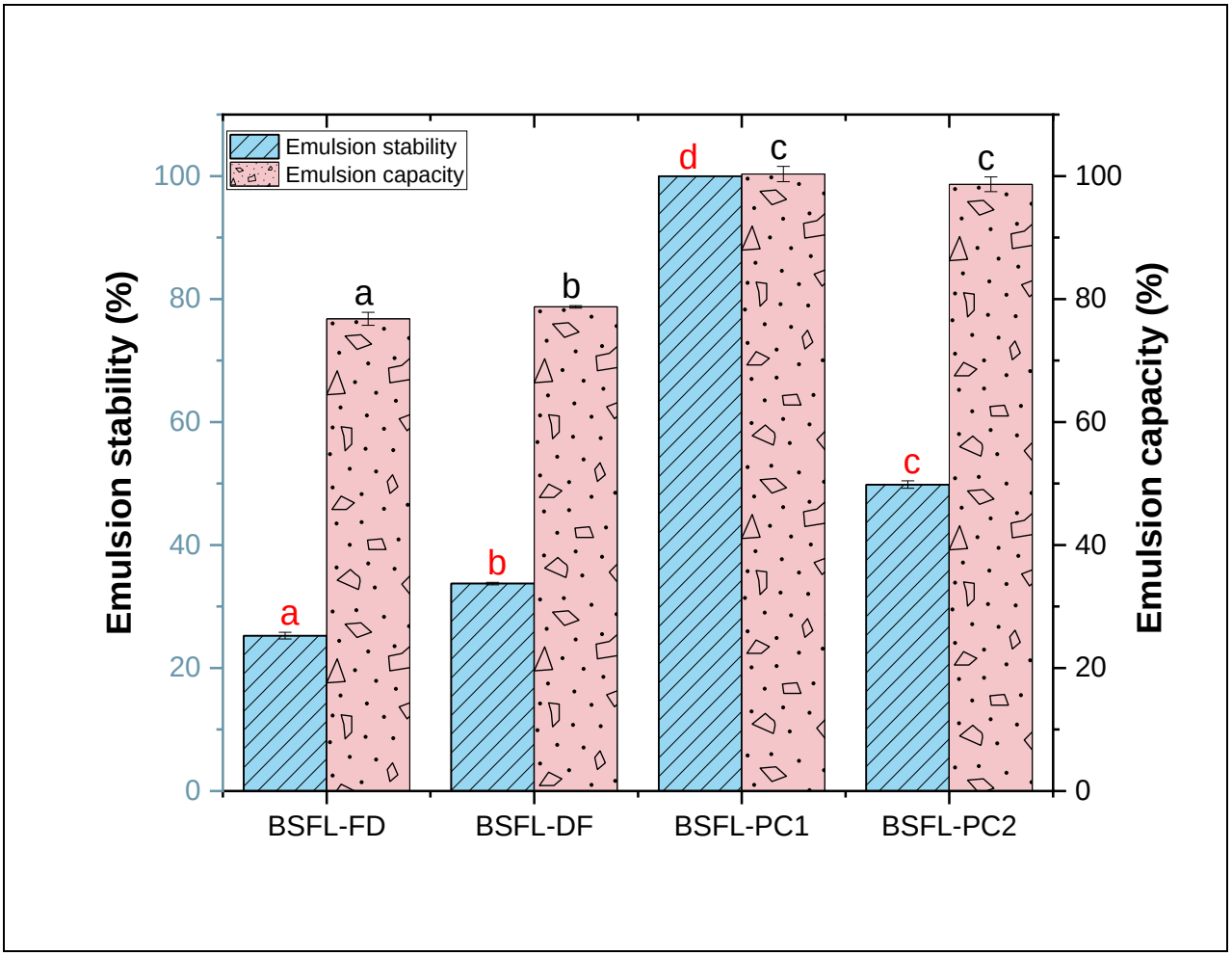

Figure 3.6 Emulsification capacity and stability of BSFL flour fractions and protein concentrates. Results are reported as Mean ± Standard deviation. Different superscripts in the column indicate significant differences between treatments ($p \leq 0.05$) across columns.

Based on the results of this study, it could be concluded that BSFL flours and protein concentrates possess emulsifying properties.

3.4.8 Effect of pH on z-potential of BSFL protein concentrates

A significant feature of proteins, deciding their functional properties, is the surface charge. Zeta (ζ) potential measurements of protein dispersions for different pH values provide information about the isoelectric point. The ζ-potential of BSFL protein concentrates is displayed in Figure 3.7. The apparent pI of all proteins together was determined at zero net charge. The pH-dependent droplet ζ-potential of BSFL proteins extracted with both methods is zero at pH 4.5 indicating the isoelectric point of the isolate at this pH. These results are in agreement with previously reported data for BSFL (Janssen, 2018). Typically, proteins (e.g. from milk, soy, egg) used as ingredients in the food industry have an isoelectric point in the pH range 4 – 6 (Burger & Zhang, 2019). Therefore, the respective droplet ζ-potentials converge to zero in this pH range to finally change from positive to negative values at pH values above the pI. The IEP tends to coincide with the minimum solubility, as previously shown in Figure 3.3.

3.4.9 Scanning electron microscopy (SEM)

The study of the microstructure of BSFL flours and protein concentrates provided further information to the results of physicochemical and functional parameters and allowed a more complete interpretation of the effects produced by the different treatments. The surface morphology and microstructure of BSFL flours and protein concentrates are shown in Figure 3.8. The BSFL freeze-dried and defatted flours showed differences in particle size and distribution with BSFL-DF exhibiting large particles. The microstructure of BSFL-FD flour was less dense and had a smooth surface although some irregular, cracked or shrunk particles can also be observed, while the BSFL-DF was largely irregularly shaped. The BSFL-PC1 microstructure exhibited thin flaky plate-like surface morphology; whereas the surface morphology of BSFL-PC2 appeared large and blocky. There were distinct differences in the microstructure of the protein concentrates.

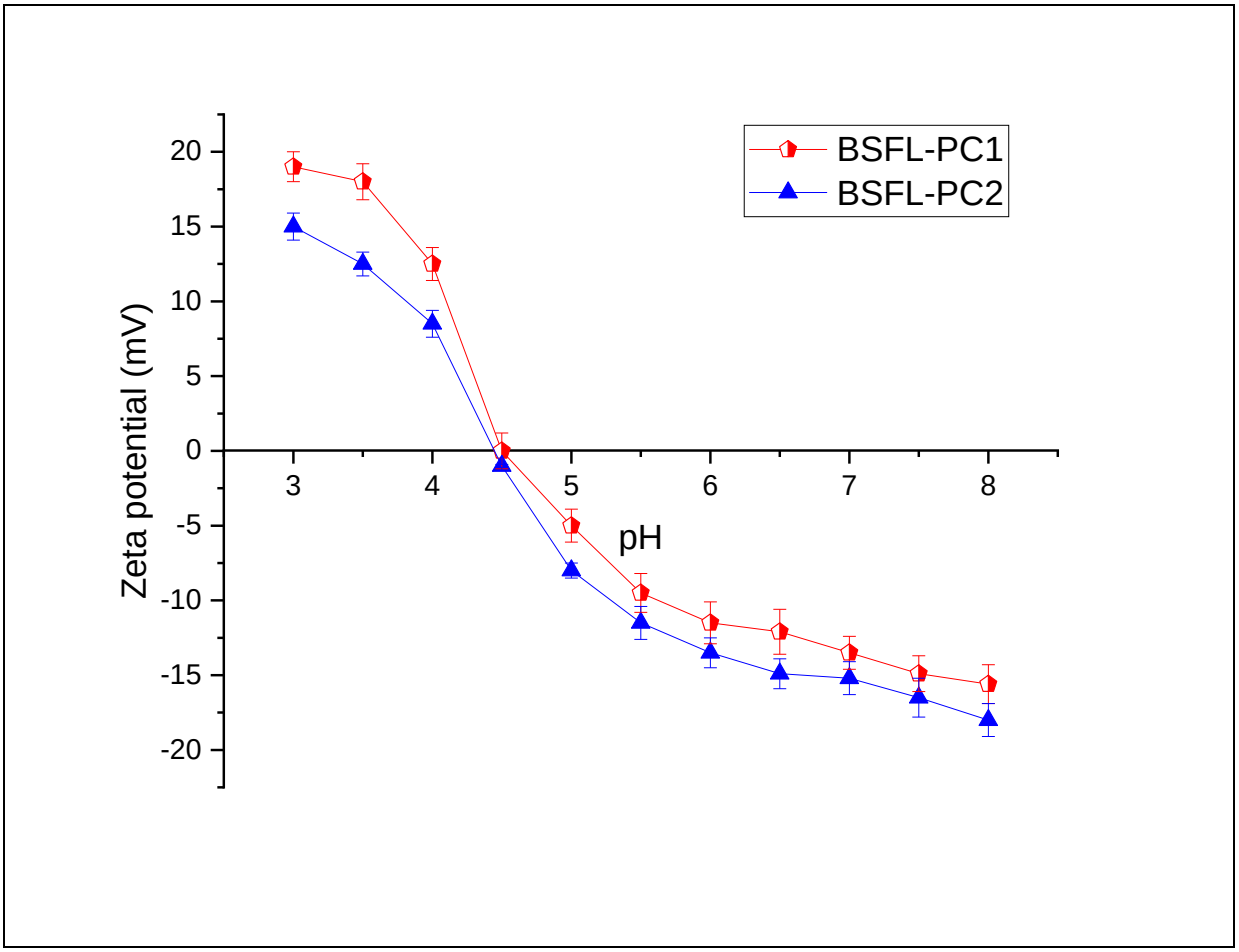

Figure 3.7 Effect of pH on zeta potential of BSFL protein concentrates. The point where the line crosses the x-axis represents the apparent pI of the protein solution (prepared in MilliQ water). Error bars indicate standard deviations.

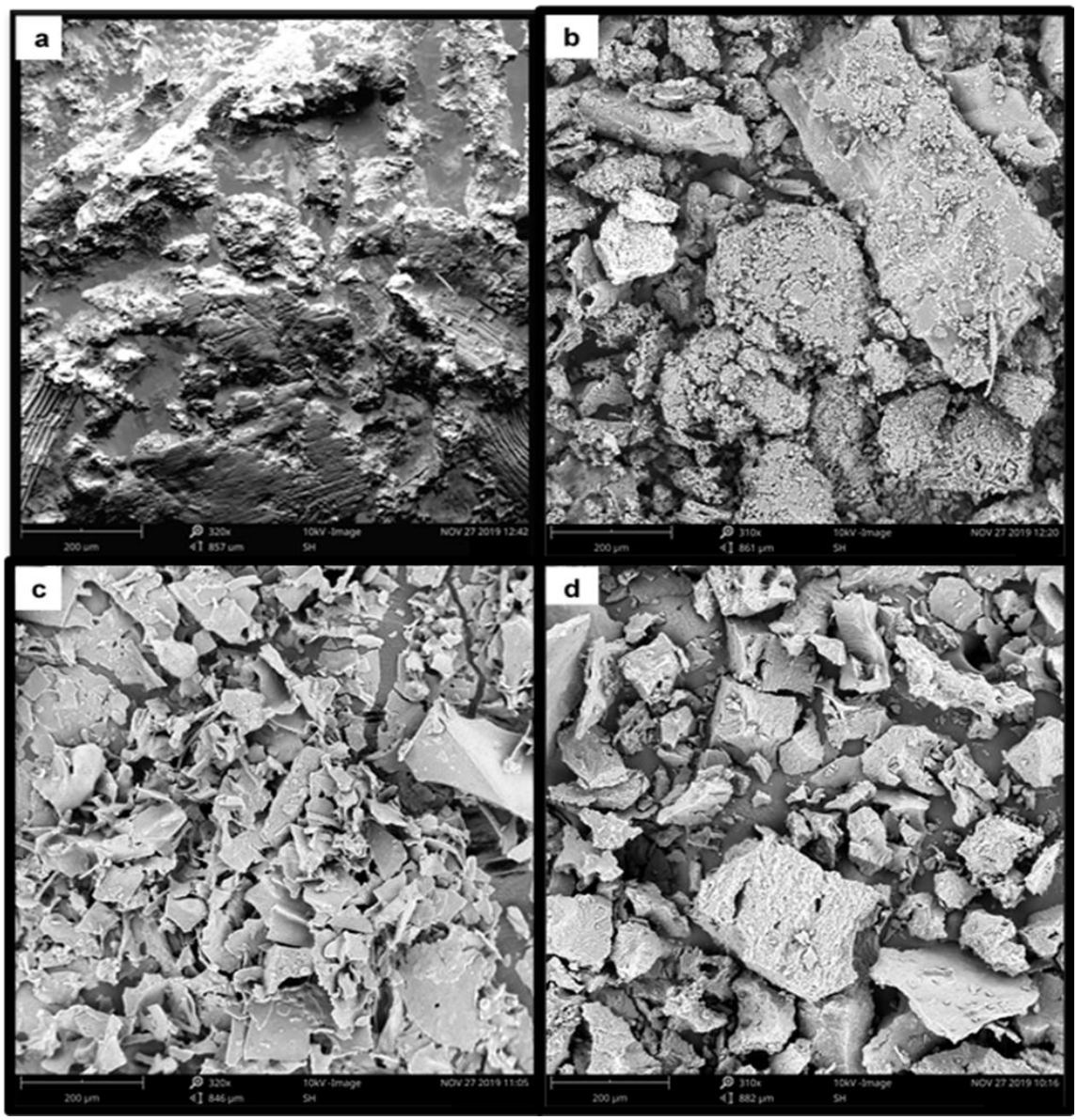

Figure 3.8 Scanning electron micrographs of a) BSFL-FD flour, b) BSFL-DF flour, c) BSFL-PC1 and d) BSFL-PC2.

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These findings suggest that the protein extraction methods changed or modified the microstructure of the BSFL proteins and further explains the observed differences in the functional and physiochemical and properties. Moreover, more work on higher resolution or magnification should be further conducted with the view to fully characterize the BSFL flours and protein concentrates

3.4.10 Fourier transform infrared (FT-IR) spectrometer analysis

FT-IR, a precise, low-cost and non-destructive analytical technique was employed to examine the effect of the protein extraction technique on the protein secondary structure (functional groups). Figure 3.9 present representative FT-IR absorption spectra for BSFL flours and protein concentrates in the 400 – 4000 cm^{-1} region. The results showed that BSFL flours and protein concentrates had five characteristic amide bands representing amide A (3200 – 3300 cm^{-1}), amide B (2900 – 3200 cm^{-1}), amide I (1600 – 1700 cm^{-1}), amide II (1500 – 1600 cm^{-1}) and amide III (1200 – 1400 cm^{-1}), which confirms those previously reported in water-soluble proteins extracted from grasshoppers, *Patanga succincta* and *Chondracris roseapbrunner* (Chatsuwan *et al.*, 2018). The major peaks for BSFL samples in this study were found at wavenumbers 3278, 2931, 2580, 1742, 1627, 1534 cm^{-1} for amide A, amide B, amide I, amide II and amide III, respectively. The absorption peaks of all samples at 2931 and 2850 cm^{-1} represent the functional groups O-H and C-H, respectively. After defatting and protein extraction the intensity of these peaks decreased significantly. This can be attributed to the chemical treatment applied. Vital information on the protein secondary structure is provided by the Amide I band (1650 – 1800 cm^{-1}) resulting from the stretching of the C=O of amide in protein. Its intensity decreased as a function of protein extraction and thus indicates alteration of the protein structure. The bands Amide I and II are the most important. The sensitivity of C = O peptide bonds to the different conformations of protein secondary structures is mainly due to these bands. The protein concentrate (BSFL-PC1 and BSFL-PC2) samples showed significantly lower peak intensity at 1627 cm^{-1} according to the FT-IR spectrum presented in Figure 3.9, which corresponds to native intramolecular β-sheets and had marginally lower α-helices intensities (1652 cm^{-1}) compared to the BSFL-FD sample (Hadnađev *et al.*, 2018; Wang *et al.*, 2020). The IR spectra of BSFL-FD and BSFL-DF were composed of the characteristic regions

that correspond to the amide I band (1600 – 1700 cm^{-1}) which is mostly carbonyl stretching (C=O) vibrations and amide II which is essentially the combination of the N-H plane boundary and C-N stretching vibrations. It is widely accepted that the functional and digestive properties of proteins are directly related to their molecular structure, wherein the configurations of the α-helix and β-sheet are associated with their performance in food systems, in particular their absorption of water and digestion in vitro (Hadnađev *et al.*, 2018; Wang *et al.*, 2020). The FT-IR spectra of BSFL flours and protein concentrates were generally similar but the differences in a few characteristic peaks and intensities were detected (Figure 3.9), indicating slight differences in the structure, amino acids and functional groups of proteins. Due to distinctly different functional properties, it could be expected that the two BSFL protein concentrates could be used in different food systems. In addition, prolonged exposure of proteins during freeze-drying to low temperatures may have caused an increase in disordered structures. Thus, the extraction process influences the secondary structure of the proteins.

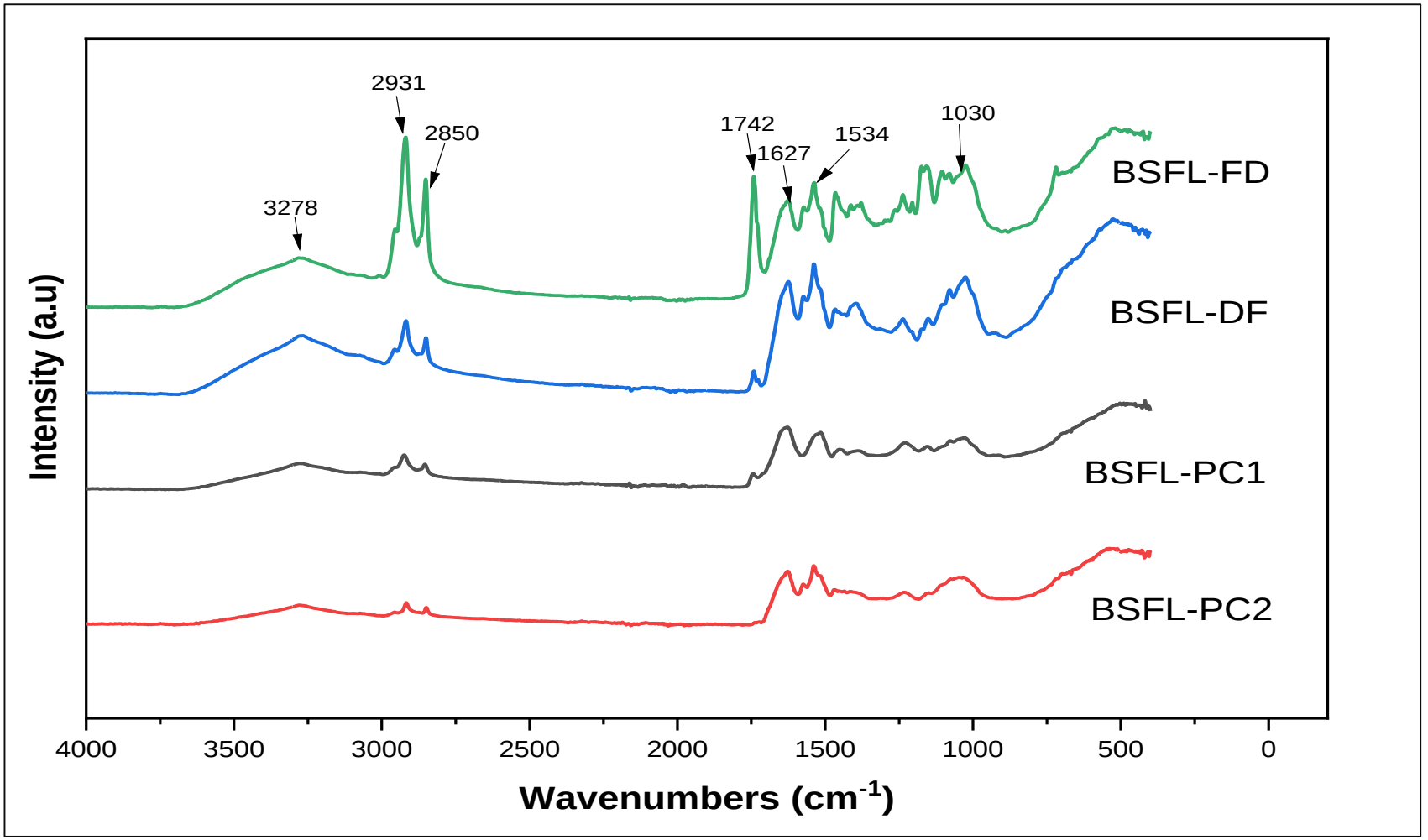

Figure 3.9. FT-IR Spectra of BSFL flours and protein concentrates.

3.5 Conclusion

In this study, the nutritional properties of BSFL flour fractions and protein concentrates were established. All fractions met the recommended FAO requirements for a well-balanced essential and non-essential amino acid content for human consumption. The overall results indicated that alkaline and acid precipitation extraction of BSFL protein concentrate resulted in enhanced nutritional and functional properties. The protein extraction method appeared to have altered the molecular structure and characteristics of proteins such as surface charge, hydrophobicity and thus contributed to improved functionality of protein fractions. As a general trend, an improvement in water binding capacity, solubility and emulsifying capacity were observed. Protein concentrates (BSFL-PC1 and BSFL-PC2) extracted from BSFL exhibited high emulsion capacity. Since functional properties are known to strongly depend on protein conformation, the structure of the BSFL flours and protein concentrates extracted are therefore are inferred to be different. This opens up the possibility of these edible insect protein concentrates may be manufactured industrially and be used in suitable commercial food applications or tailor-made for different food formulations. However, more research is still required on the interaction of edible insect ingredients with other food components, microbiological, rheological and sensory properties of new insect-based food proteins.

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CHAPTER 4: Edible insect (BSFL) protein conjugated with glucose via Maillard reaction: Antioxidant and techno-functional properties

4.1 Abstract

The food industry is considering novel sources of proteins with enhanced functionalities to meet the increasing demand and population growth. Edible insect proteins have emerged as an alternative that is environmentally friendly, economically viable and thus could make a significant contribution to global food security. The aim of this study was to establish the effect of conjugation via the Maillard reaction on the antioxidant and techno-functional properties of black soldier fly larvae protein concentrate. Reaction mixtures containing black soldier fly larvae protein concentrate and glucose (2:1 weight ratio) were wet-heated at 50, 70 and 90°C for 2, 4, 6, 8 and 10 h, respectively, with an initial pH of 9. The results showed that the browning indices of the black soldier fly larvae-glucose (BSFL-Glu) model system increased with an increase in reaction time and temperature, with conjugates formed at 90°C exhibiting the highest browning intensity at 420 nm. At 50°C, the DPPH-RS of the conjugates ranged from 15.47 – 32.37%. The $ABTS^{+}$ radical scavenging activity of BSFL-Glu conjugates produced at 90°C exhibited significantly ($p < 0.05$) higher scavenging activity as a function of reaction time. The foaming capacity of BSFL-Glu conjugates produced at 70°C showed a significant increase ($p < 0.05$) as a function of reaction time. Principal component analysis was applied to browning and antioxidant indices. Component 1 of the score plot accounted for 89% while component 2 accounted for 8% of the observed variability and allowed discrimination/differentiation of the samples based on the heating temperature. These findings provide a practical means to improve the functionality of novel edible insect proteins for food application.

Keywords

Antioxidant activity, Maillard reaction, conjugates, techno-functional properties, Foaming, edible insects, black soldier fly larvae.

4.2 Introduction

The predicted population increase to 9 billion people by the year 2050 presents a considerable threat to global food security. This is exacerbated by climate change, energy crises and the consumption and demand patterns for good quality protein for human consumption (Van-Huis, 2013; Kouřimská & Adámková, 2016). Edible insects have been proposed as an alternative food protein source that can address the current and future nutritional, health, economic and food security concerns (Ortiz *et al.*, 2016; Van-Huis & Dunkel, 2016; Gravel & Doyen, 2020). With the growing interest in edible insect protein consumption, industrial farming of species such as mealworm larvae (*Tenebrio molitor*) and black soldier fly larvae (*Hermetia illucens*), which are high in protein content, has been expanding due to investment in research and development, leading to a stable supply, consistent quality, cost-effectiveness and hygienic production. Proteins extracted from edible insects and used in food applications as functional ingredients (in powder form or paste) may have greater success in terms of acceptance for human consumption since the willingness to consume whole insects is low (Tan *et al.*, 2016; Mancini *et al.*, 2019).

Apart from the nutritional value, food proteins provide unique techno-functional properties which affect their behaviour in food systems during preparation, processing, storage and consumption and contribute to the quality and sensory attributes of food. To further promote the application of edible insects proteins in food applications, physical (Hu *et al.*, 2013), chemical (Zhou *et al.*, 2017) and enzymatic (Purschke *et al.*, 2018b) methods have been explored to enhance their techno-functional properties. Due to the potential health hazards brought in by chemical modifications, most of these methods cannot be applied in food processing. Meanwhile, with the requirement of mechanical forces such as high pressure or shear, physical modification is difficult for wide application (Son *et al.*, 2019).

In the last decade, the Maillard reaction – a non-enzymatic chemical reaction between a carbonyl compound (usually a reducing sugar) and protein amino groups has been reported to remarkably improve the techno-functional properties of proteins and even endows them with novel functionality. The Maillard reaction (MR) leads to the formation of protein-sugar conjugates with enhanced emulsifying ability, solubility, antibacterial, antioxidant properties and even alleviates allergenicity of proteins (Jian *et al.*, 2016; Liu *et al.*, 2016; Zha *et al.*, 2019). As reported by Medrano *et al.* (2009), glycation of β-lactoglobulin with glucose exhibited improved foaming stability, suggesting increased

adsorption to the air/water interface. Conjugates with superior emulsion properties were also reported by Diftis & Kiosseoglou (2003).

There is now extensive scientific evidence proving that protein functionality can be significantly improved by covalent coupling with saccharides through the MR without the use of any chemical reagents and thus rendering this technique safe (Chevalier *et al.*, 2001; Oliver *et al.*, 2006; Zeng *et al.*, 2011; Dong *et al.*, 2012). Different factors such as temperature, time, relative humidity (RH), pH, and reactants' molar ratio affect the rate and extent of the MR, hence, the nature of the products formed and their functional properties. Recently, various scientific publications have studied the effect of heating temperature on the functional properties of conjugates generated from fructose-lysine and ribose-lysine (Vhangani & Van-Wyk, 2013b), coconut sap (Karseno *et al.*, 2018), bovine serum albumin-glucose (Jian *et al.*, 2016) and whey protein isolate with xylo-oligosaccharide (Jia *et al.*, 2020) model systems. However, there is a paucity of information about the effect of conjugation on the antioxidant activity and techno-functional properties of edible insect proteins extracted from black soldier fly larvae (BSFL). Since the Maillard glycation between reducing sugars and amino acids/proteins is inevitable during thermal food processing and manufacturing, it is imperative to understand its effect on novel proteins extracted from edible insects.

Therefore, the aim of this study was to investigate the effect of conjugation time and temperature on the antioxidant activity and techno-functional properties of black soldier fly larvae (BSFL) protein with the view to find alternative protein sources for food application. This study will extend current knowledge of the functionality changes of insect proteins due to the Maillard reaction.

4.3 Materials and methods

4.3.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Aston Manor, South Africa). Prepared reagents were stored under conditions that prevented contamination or deterioration. The water used in the study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, South Africa).

4.3.2 BSFL protein extraction

Defatted BSFL flour (BSFL-DF) was mixed with Milli-Q water at a ratio of 1:10 ($w.w^{-1}$) and the pH of the mixture was adjusted immediately to pH 10 using an 0.1 or 1 N NaOH solution. The mixture was stirred on a laboratory magnetic stirrer at a rate designed to prevent the formation of a vortex, for 30 min at ambient temperature (25°C ± 1.0). Subsequently, the samples were centrifuged at 10 000 *g* for 20 min at 4°C. Next, the pH of the supernatants was adjusted to 4.5 with 1 M HCl and then the suspension was left at 4°C overnight to facilitate protein precipitation. The precipitated proteins were recovered by centrifugation at 10 000 *g* for 30 min at 4°C. The BSFL protein concentrate was then freeze-dried and stored at -20°C for further analysis.

4.3.3 Synthesis of BSFL-Glu conjugates, pH and Browning index

Black soldier fly larvae protein and glucose (BSFL-Glu) conjugates were prepared according to the method of Vhangani & Van Wyk (2013b) and Mshayisa (2016) with slight modifications. BSFL protein concentrate and glucose (2:1 $w.w^{-1}$) were dissolved in 100 mL of 0.1 M phosphate buffer at pH 9. The samples were transferred into 250 mL Schott bottles and heated at 50, 70 and 90°C in a water bath for 2, 4, 6, 8 and 10 h, respectively. After the heating period had elapsed, the resulting BSFL-Glu conjugates were immediately cooled in an ice bath. An unheated BSFL-Glu solution (0 min) was prepared as the control. A portion of the BSFL-Glu conjugates in solution was retained for pH measurements (pH meter: Metrohm, Switzerland). The remainder of the solutions were freeze-dried (Virtis, Wizard 2.0, NY, USA) and stored in air-tight screw-capped glass bottles at -80°C until analysis. Before use, the powder was reconstituted to the required concentration with Milli-Q water and browning intensity was measured with a spectrophotometer (Lambda 25, Perkin Elmer, Singapore) at 294 and 420 nm. The pH and browning intensity (BI) measurements were used as non-specific indicators of the MR. For all direct antioxidant assays, the same concentration (10 $mg.mL^{-1}$) of BSFL-Glu conjugates was consistently used as determined in trials as the ideal concentration.

4.3.4 Determination of antioxidant activity of BSFL-Glu conjugates

4.3.4.1 DPPH radical scavenging activity

DPPH radical scavenging (DPPH-RS) of BSFL-Glu conjugates was determined according to the method of Lertittikul *et al.*(2007) and (Mshayisa, 2016) with slight modifications. A

0.12 mM solution of DPPH in ethanol was prepared daily and protected from light. A 4 mL aliquot of DPPH solution was added to 2 mL of BSFL-Glu conjugate (10 mg.mL^{-1}) samples. The mixture was vortexed (Vortex-Genie 2, Scientific Industry Inc., USA) and allowed to stand at ambient temperature in the dark for 30 min. The absorbance of the mixtures was measured at 517 nm with a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). A reaction mixture containing 2 mL distilled water and 4 mL ethanolic DPPH solution was used as control. All measurements were performed in triplicate. The percentage of DPPH-RS radical scavenging activity was calculated using the equation:

$$\%\mathrm{DPPH} - \mathrm{RS} = 1 - \frac{\mathrm{A}_{\mathrm{sample\ (517nm)}}}{\mathrm{A}_{\mathrm{control\ (517nm)}}} \times 100$$

Where $A_{control}$ is the absorbance of the control at 517 nm and A_{sample} is the absorbance of the sample at 517 nm.

4.3.4.2 ABTS+ radical scavenging activity

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity of BSFL-Glu conjugates was determined according to a method as described by Yu *et al.* (2012) and (Mshayisa, 2016) with slight modifications. The ABTS radical was prepared by reacting 7 mmol.L^{-1} ABTS solution and 2.45 mmol.L^{-1} potassium persulphate solution in equal volume and the mixture was allowed to stand overnight in the dark at ambient temperature. The ABTS solution was diluted twenty-fold (20-fold) with Milli-Q water to obtain an absorbance of 1.5 – 1.6 at 730 nm. Fresh ABTS was prepared daily. A 4 mL aliquot of diluted ABTS solution was added to 200 µL of aqueous BSFL-Glu conjugates solution (10 mg.mL^{-1}) and the mixture was allowed to stand at room temperature for one hour. The absorbance was then measured at 730 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). The control was prepared in the same manner with the substitution of distilled water for the sample. All measurements were performed in triplicate. The percentage of ABTS radical scavenging activity was calculated according to the following equation:

$$\%\mathrm{ABTS} - \mathrm{RS} = \frac{\mathrm{A}_{\mathrm{control\ (730\ nm)}} - \mathrm{A}_{\mathrm{sample\ (730\ nm)}}}{\mathrm{A}_{\mathrm{control\ (730\ nm)}}} \times 100$$

Where $A_{control}$ is the absorbance of the control at 730 nm and A_{sample} is the absorbance of the sample at 730 nm.

4.3.4.3 Reducing power

The reducing power (RP) was determined according to the method of Vhangani and Van Wyk (2013b) and Mshayisa (2016) with slight modifications. A one millilitre aliquot of each BSFL-Glu conjugates sample (10 $mg.mL^{-1}$) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1.0% potassium ferricyanide. The reaction mixtures were incubated in a temperature-controlled water bath at 50°C (Memmert, Germany) for 30 min, followed by the addition of 2.5 mL of 10% trichloro-acetic acid after cooling at room temperature. The mixture was centrifuged at 1 750 *g* for 10 min at 25°C. The supernatant obtained (2.5 mL), was treated with 1 mL of Milli-Q water and 0.5 mL of 0.1% Ferric chloride. The absorbance of the reaction mixture was measured at 700 nm in a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). All measurements were performed in triplicate. The RP was expressed as an increase in absorbance at 700 nm.

4.3.4.4 Determination of Iron chelation activity

The chelating activity of BSFL-Glu conjugates was determined according to the method of Gu *et al.* (2010) with slight modifications. One millilitre BSFL-Glu conjugate sample was mixed with 1.85 mL of Milli-Q water and 0.05 mL 2.0 mM $FeCl_2$, and the mixture was allowed to stand at room temperature for 30 s. The reaction mixture thus obtained was added to 0.1 mL of 0.5 mM ferrozine and mixed; the absorbance was measured at 562 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore) after 10 min resting time and 5 min centrifugation at 3 000 *g*. The control was prepared in a similar manner, except that BSFL-Glu conjugates were replaced with Milli-Q water. All measurements were performed in triplicate. The percentage of chelating activity was calculated as follows:

$$\%\ \text{Chelating activity} = 1 - \frac{\text{A}_{\text{sample (562 nm)}}}{\text{A}_{\text{control (562 nm)}}} \times 100$$

Where $A_{control}$ is the absorbance of the control at 562 nm and A_{sample} is the absorbance of the sample at 562 nm.

4.3.5 Analysis of techno-functional properties

4.3.5.1 Foaming property evaluation

Foaming capacity (FC) and foam stability (FS) were determined according to the method of Zielińska *et al.* (2018) with minor modifications. The freeze-dried BSFL-Glu conjugates sample was dispersed in distilled water (5% $w.v^{-1}$) and centrifuged (Thermo Electron Corporation Jouan MR1812) at 10 000 rpm for 15 min. Then 20 mL of the supernatant was homogenized in a high shear homogenizer mixer (Polytron PT 2500E) at a speed of 16 000 rpm for 3 min. The whipped sample was immediately transferred into a 50 mL cylinder. The total volume was read at time zero and 30 min after homogenization. The foaming capacity and foam stability were calculated from the formula:

$$\text{Foaming Capacity (FC)} = \frac{V_a - V}{V} \times 100$$

$$\text{Foaming stability (FS)} = \frac{V_{30}}{V_a} \times 100$$

Where: V – volume before whipping (mL), V_a – volume after whipping (mL), V_{30} – volume after standing (mL).

4.3.5.2 Emulsion capacity and stability of conjugates

Emulsifying properties were determined according to the method of Coelho & Salas-Mellado (2018) and Anzani *et al.* (2020) with slight modifications. The sample was dispersed in distilled water (5% $w.v^{-1}$) and centrifuged (Thermo Electron Corporation Jouan MR1812) at 9 000 rpm speed for 15 min, 15 mL of the supernatant were homogenized (Polytron PT 2500 E) with 15 mL of commercial sunflower oil at a speed of 18 000 rpm for 1 min. Next, the samples were centrifuged at 3 000 *g* for five min and the volume of the individual layers were read. Emulsion stability was evaluated by heating the emulsion in a water bath set at 80°C for 30 min. Then, the samples were centrifuged at 3 000 *g* for five min. Emulsion capacity and emulsion stability were calculated from the formula:

$$\text{Emulsion capacity (EC)} = \frac{V_{el}}{V} \times 100$$

$$\text{Emulsion stability (ES)} = \frac{V_{30}}{V_{el}} \times 100$$

Where: V – total volume of tube contents, V_{el} – volume of the emulsified layer, V_{30} – volume of the emulsified layer after heating.

4.3.6 Data analysis

All data were subjected to multivariate analysis of variance (MANOVA) using SPSS for Windows®, version 26.0 (IBM Corp, New York, USA) to ascertain whether the main effects resulted in significant differences in response variables. Duncan's multiple comparison post-hoc test was used to test significant differences ($p < 0.05$) between individual means. Principal component analysis (PCA) was performed on aggregated mean centred data for antioxidant indices and techno-functional properties using Singular Value Decomposition (SVD) algorithm and cross-validation methods with 18 segments to determine potential clusters with the use of The Unscrambler software, version 11 (CAMO Software, Oslo, Norway). Graphs and figures were generated using Origin software, version 9.60 (Origin labs, Northampton, MA, USA) and The Unscrambler software.

4.4 Results and discussion

4.4.1 The extent of Maillard reaction

During the Maillard reaction (MR), the initial reactants are consumed which consequently results in the formation of initial, intermediate and advanced brown polymers. The initial condensation step of the MR is facilitated by higher pH values and thus it is vital to monitor the pH of the model system. Therefore, change in pH values during the MR was monitored to evaluate its significance during the reaction. The pH of BSFL-Glu conjugate model systems at 50, 70 and 90°C as a function of reaction time is shown in Figure 4.1. The MANOVA with Duncan's multiple range tests showed that the decrease in pH observed as the reaction temperature and time increased was significant ($p < 0.05$) for each model system. This showed that higher reaction temperature and time combinations resulted in the highest pH reduction. As shown in Figure 4.1, the final pH values of BSFL-Glu conjugates reacted at 90°C were lower than those at 50°C and 70°C, respectively. These results are due to the fact that the degree of the Maillard reaction was greater at high

temperatures than at low temperatures. Vhangani & Van Wyk (2013b) also observed a decrease in pH of MRPs from fructose-lysine and ribose-lysine model systems. In addition, Benjakul *et al.* (2005) also reported a decrease in pH of porcine plasma protein-sugar model systems heated up to 5 hours. The decrease in pH can be attributed to the formation of formic and acetic acid during the MR (You *et al.*, 2011; Xu *et al.*, 2019) and the free amino groups of BSFL protein consumed in the early stages of the reaction. In addition, higher pH was further away from the isoelectric point of the BSFL protein concentrate and therefore could cause stronger intramolecular electrostatic repulsions which may result in more extensive unfolding, more reactant amino groups exposed and higher solubility, all these contribute to the improvement of the conjugation reaction.

4.4.2 Browning intensity

The UV–VIS absorbance of Maillard reaction products (MRPs) at 294 nm and 420 nm have been widely accepted as non-specific markers to monitor the development of early to intermediate stage Amadori compounds and final stage melanoidins, respectively. In the early stage of the MR, intermediate compounds are formed. The early-stage Amadori reaction products with characteristic absorption at 294 nm and advanced stage melanoidins which represent brown pigments at 420 nm are usually measured as an indicator of the extent of the MR in model systems. As shown in Figure 4.2 A – C, a gradual increase in absorbance at 294 nm was observed as the reaction time increased at the same reaction temperature up to 10 h, indicating that the increase of the reaction time increases the formation of intermediate Maillard reaction products. BSFL-Glu conjugates prepared at higher reaction temperature (90°C) exhibited the highest increase ($p < 0.05$) in absorbance as a function of reaction time. The absorbance at 294 nm was used to determine the intermediate compounds of the MR (Benjakul *et al.*, 2005). From these results, the increase in absorbance at 294 nm, irrespective of heating temperature, suggests that the early intermediate compounds were dominant in all BSFL-Glu conjugates.

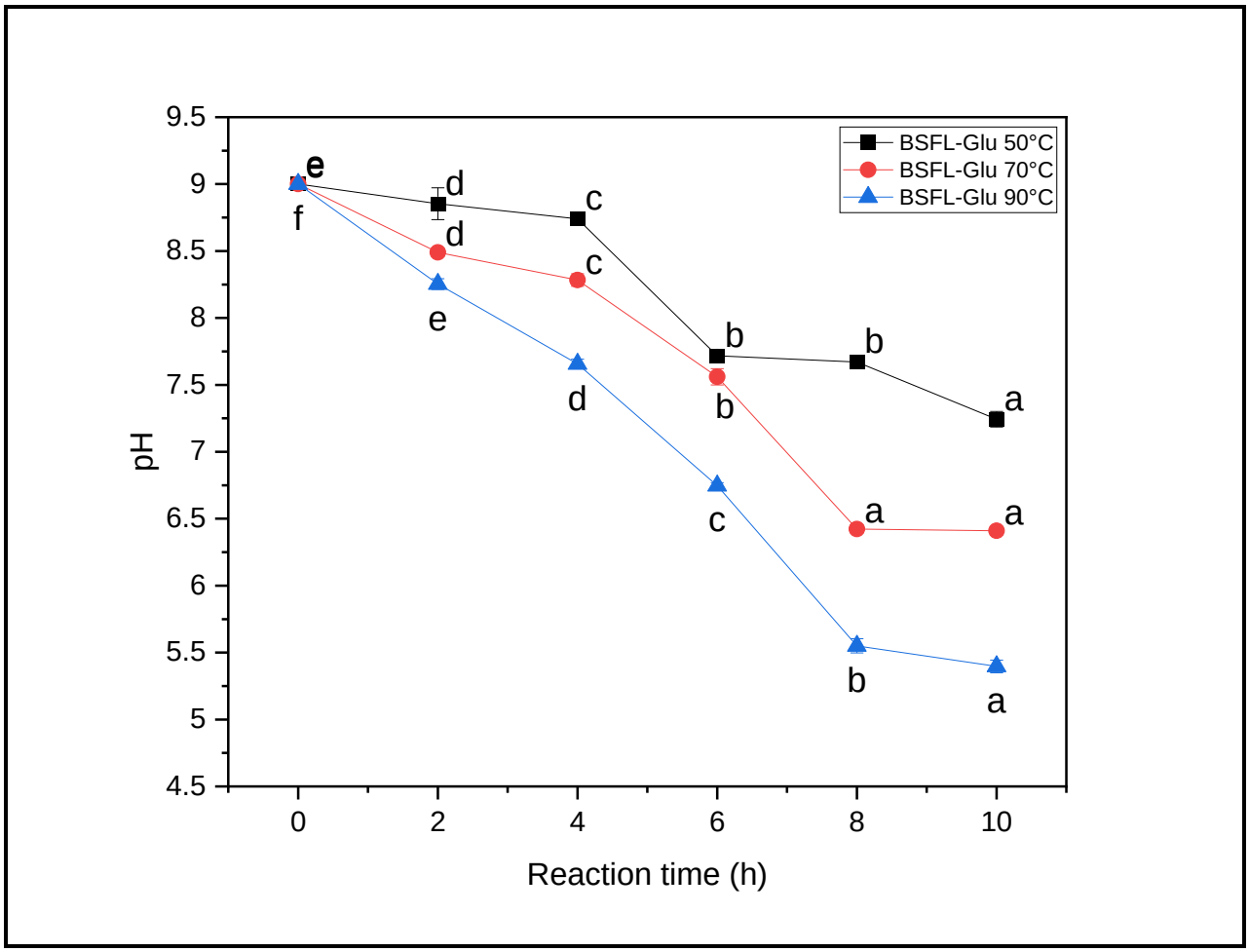

Figure 4.1 Changes in pH of BSFL-GLu conjugates model system as a function of reaction time. Values are mean ± standard deviation; means with different superscript are significantly different at the same heating temperature.

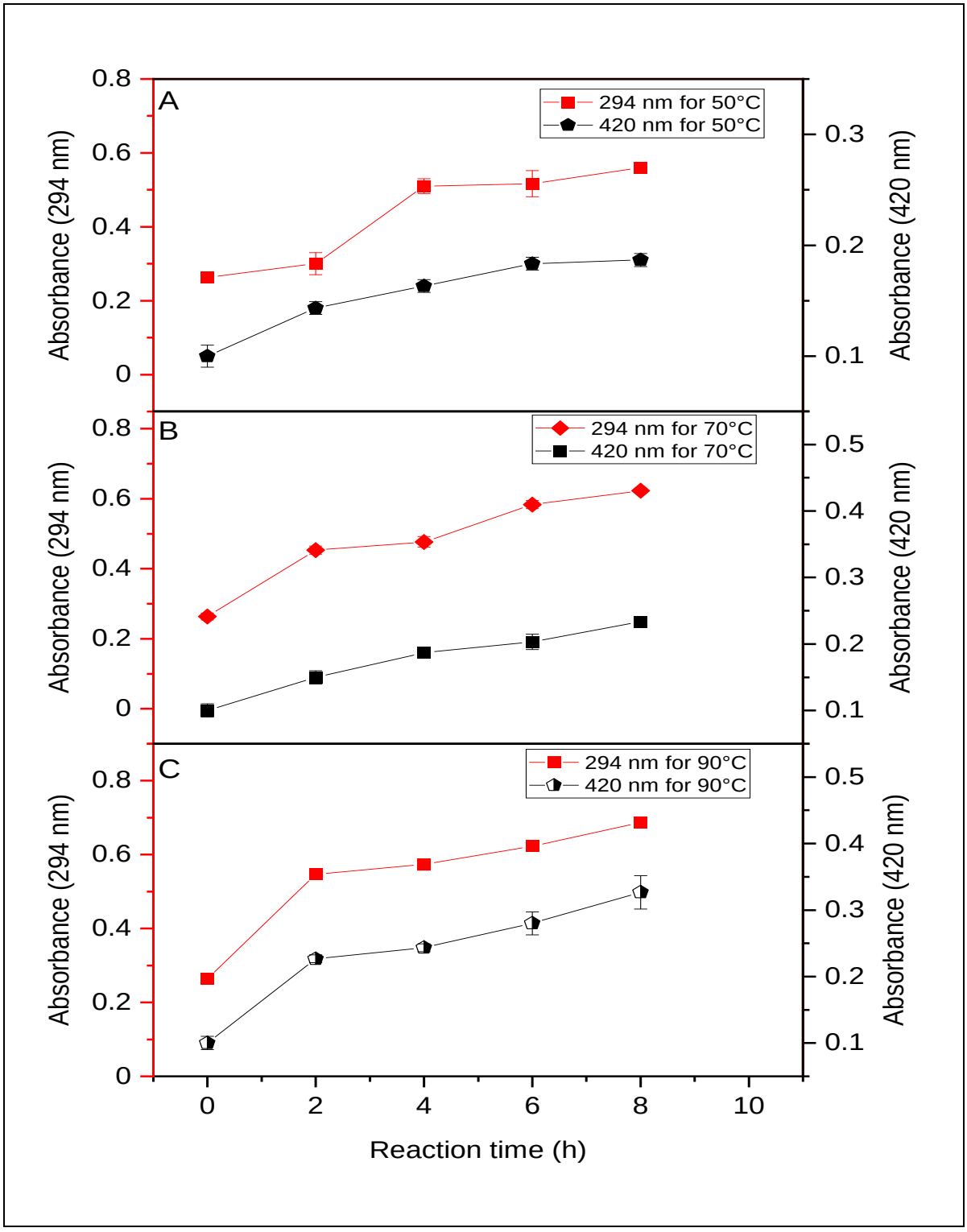

Figure 4.2 Changes in absorbance at 294 nm and 420 nm of BSFL-Glu conjugates as a function of reaction time. A) 50°C, B) 70°C and C) 90°C.

The most commonly used indicator of the browning pigment is the spectrophotometric measurement at 420 nm (Matmaroh & Benjakul, 2006). An increase in browning of BSFL-Glu conjugates, as measured by absorbance at 420 nm, was observed as the heating time increased ($p < 0.05$) irrespective of the heating temperature (Figure 4.2 A – C). The increase in absorbance at 420 nm indicates the development of browning pigment in the final stage of the Maillard reaction (Wang *et al.*, 2013). The reaction consists of the condensation of an amino compound and sugar fragments into polymerised glycoproteins and the brown pigment melanoidin. The increase in brown pigment development was coincidental with an increase in colourless intermediate formation, as evidenced by the increased absorbance at 294 nm and this suggests that brown pigments were formed proportionally with the intermediate products generated (Figure 4.2 A – C). These results indicate that the formation of intermediate Amadori compounds occurs prior to the coloured melanoidins under mild incubation conditions.

4.5 Antioxidant properties

When evaluating the antioxidant activity of MR products, it is crucial to implement more than one assay, due to the complexity of these compounds (Vhangani & Van-Wyk, 2016) and the fact that they proceed by different mechanisms. Thus, the antioxidant activity of heat-treated BSFL-Glu conjugates was assayed in terms of their free radical-scavenging activity, reducing power and metal chelation.

4.5.1 DPPH radical scavenging

The DPPH radical scavenging activity indicates the hydrogen-donating ability of antioxidants. The DPPH-RS of BSFL-Glu conjugates is presented in Figure 4.3. In general, the DPPH-RS of BSFL-Glu conjugates increased significantly ($p < 0.05$) with an increase in reaction time at 50, 70 and 90°C, respectively. At 50°C, the DPPH-RS of the conjugates ranged from 15.47 – 32.37% and it was significantly higher than the control ($t = 0$). Compared with the lower temperatures (50 and 70°C), conjugates produced at 90°C had a significantly higher DPPH radical scavenging activity ($p < 0.05$). BSFL protein concentrate heated alone exhibited a weak radical scavenging activity with only 3% radical activity and there was no significant difference ($p > 0.05$) over the reaction conditions (time and temperature) examined in this study (results not shown). This signifies that BSFL

protein conjugated with glucose can significantly improve the antioxidant activity of BSFL protein.

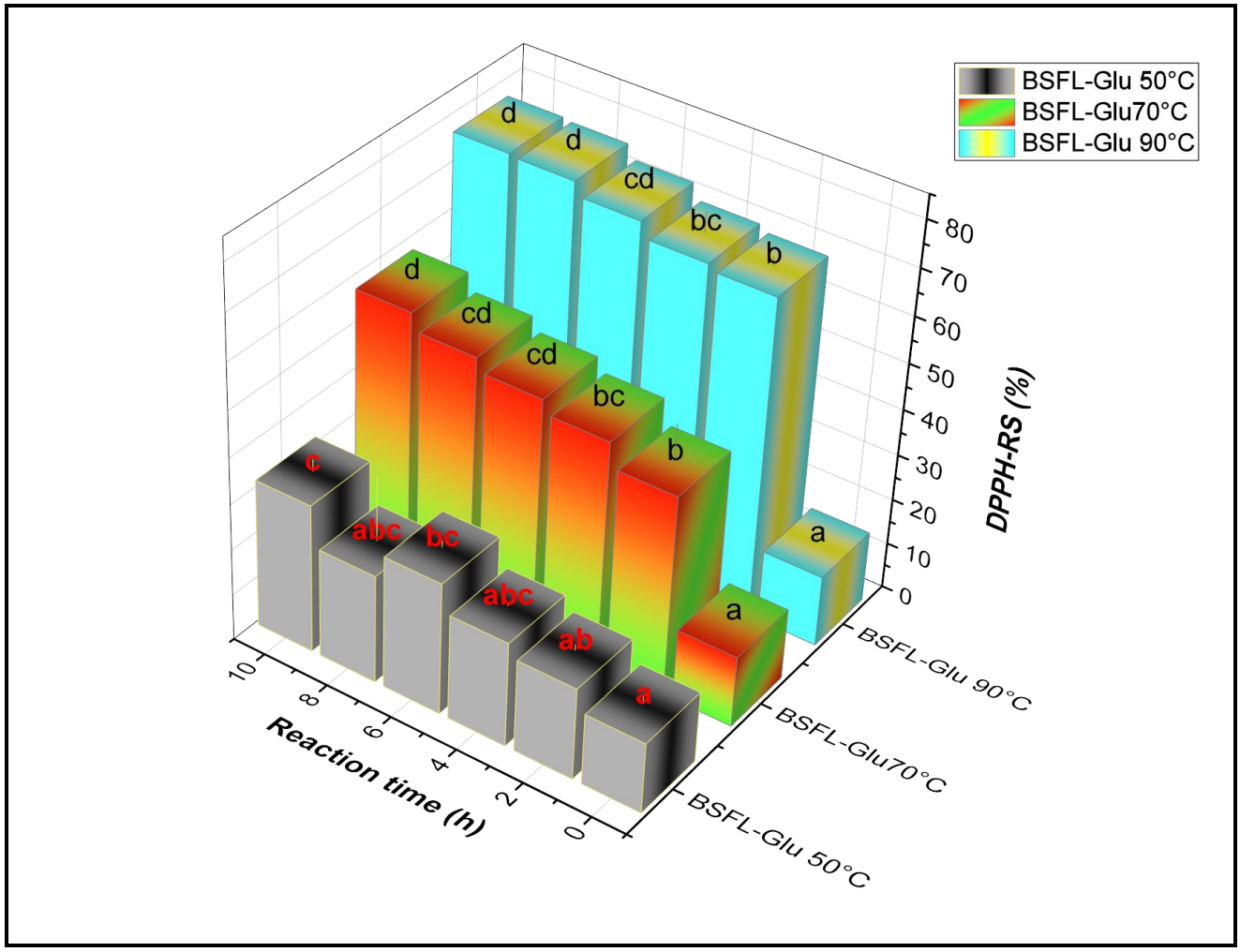

Figure 4.3 DPPH radical scavenging activity of BSFL-Glu conjugates as a function of reaction temperature. Values are mean ± standard deviation; means with different superscripts are significantly ($p < 0.05$) different at the same heating temperature.

The results of this study are in agreement with Jiang *et al.* (2013) who observed an increase in DPPH-RS of the tripeptide Ile-Pro-Pro (IPP) MRPs as a function of reaction time. Moreover, ultra-filtered casein-glucose MRPs model systems showed strong DPPH radical scavenging (Gu *et al.*, 2009). Taken collectively, the results of this study show that conjugates prepared from insect protein concentrate extracted from BSFL possess the hydrogen-donating ability, which suggests that these conjugates exhibit potency to react with free radicals. This opens up new avenues or possibilities for the application of insect proteins as novel functional ingredients in food formulations.

4.5.2 ABTS radical scavenging

The $ABTS^+$ radical scavenging activity was determined in order to assess the antioxidant potential of BSFL-Glu conjugates. As depicted in Figure 4.4, the $ABTS^+$ radical scavenging activity of conjugates produced at 50°C ranged from 10.5 – 16.5% and exhibited the lowest ABTS radical scavenging activity. This can be attributed to the electron-donating ability of some of the amino acids of BSFL protein, which reduces $ABTS^+$ radicals to offer weak antioxidant activity. However, increasing the reaction temperature to 70°C increased significantly ($p < 0.05$) the $ABTS^+$ radical scavenging activity as a function of reaction time. The results of this study concur with You *et al*. (2011) who reported that the $ABTS^+$ radical scavenging activity of a silver carp protein hydrolysate-glucose system obtained at 60°C was significantly higher than that obtained at 50°C.

The $ABTS^+$ radical scavenging activity of BSFL-Glu conjugates produced at 90°C exhibited significantly ($p < 0.05$) higher scavenging activity as a function of reaction time (Figure 4.4). Sun *et al.* (2006) found that the MRPs formed by α-lactalbumin and various types of reducing sugars exhibited greater ABTS-RS than intact α-lactalbumin. It has been reported that the browning compounds formed during the Maillard reaction, which are primarily composed of melanoidins, are major contributors to the radical scavenging capacity (Wang *et al.*, 2011a; You *et al.*, 2011). The results of this study indicate that glycation could induce the antioxidant activity of the edible insect protein, the intensity of which depends on the duration of reaction time and temperature. To the best of our knowledge, this is the first study to explore the effect of reaction temperature and time on the antioxidant properties of BSFL-Glu conjugates. Therefore, it is proposed that food ingredients with strong antioxidant potential, which is derived from edible insect protein and reducing sugar, might act as new functional ingredients.

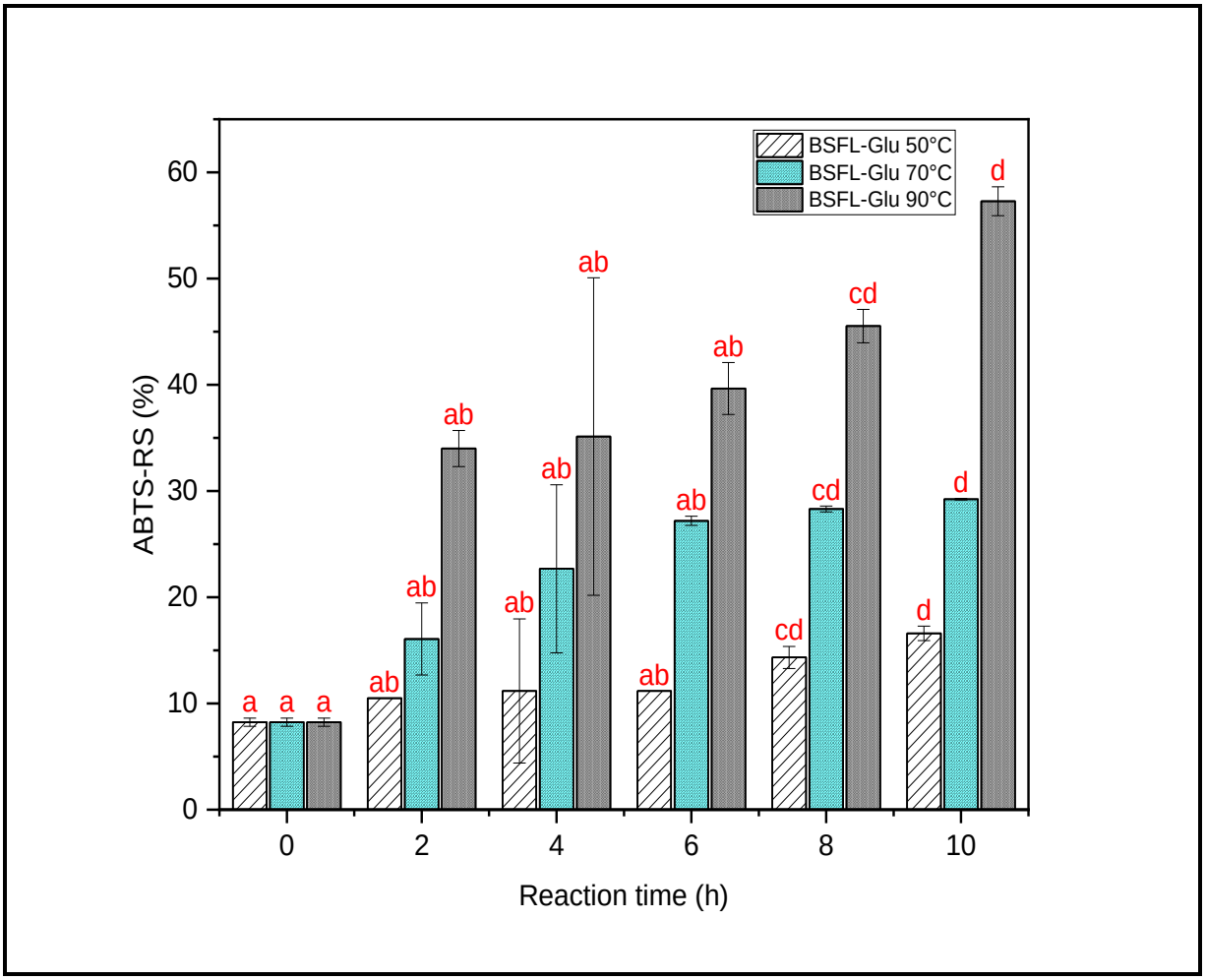

Figure 4.4 ABTS radical scavenging activity of BSFL-Glu MRPs as a function of reaction temperature. Values are mean ± standard deviation; means with different superscript are significantly different at the same heating temperature.

4.5.3 Changes in reducing power

Reducing power has been used to determine the antioxidant effect of MRPs and it is regarded as a good indicator of antioxidant activity of food components (Nooshkam *et al.*, 2019). This assay measures particularly the antioxidant activity of MRP conjugates with the hydroxyl groups of conjugates playing a role in the reducing activity through their redox potential of transferring electrons (Vhangani & Van-Wyk, 2013b; Nooshkam *et al.*, 2019). In this assay, the ferric chloride/ferricyanide complex is reduced to the ferrous form (Fe^{2+}) in the presence of antioxidants and therefore the Fe^{2+} concentration can be monitored spectrophotometrically by measurement of Perl's Prussian blue colour measured at 700 nm.

The reducing power of BSFL-Glu conjugates increased significantly ($p < 0.05$) with an increase in reaction time, with conjugates derived at 50°C and 70°C (Table 1) having the lowest and highest reducing power, respectively. The increased reducing power of BSFL-Glu conjugates as a function of time was attributed to the electron donation capacity of the conjugate and heat-induced protein denaturation, unmasking more amino acid residues that were able to donate electrons. Heat-induced MRPs from ribose-lysine (Vhangani & Van-Wyk, 2013b), glucose-glycine (Yoshimura *et al.*, 1997) and porcine plasma protein-glucose (Benjakul *et al.*, 2005; Lertittikul *et al.*, 2007) model systems also possessed reducing power. From these results, the reducing power of BSFL-Glu conjugates increases as a function of reaction time at all temperatures correlated well with browning intensity (Figure 4.3). The increase in reducing power at higher temperatures (90°C) can be attributed to the fact that the protein structure was unravelled (denatured) by heating, enhancing the Maillard reaction rate, and producing more intermediate and advanced products, with higher reducing power. Moreover, the results of DPPH and ABTS assays together with the reducing power results indicated that the difference in the antioxidant activity of the conjugates from this edible insect protein was mainly attributable to the mechanism of single electron transfer (SET). Conjugation of BSFL protein with glucose via the Maillard reaction may induce structural changes in the BSFL-Glu system, which result in the formation of products or compounds that contribute to the reducing power.

Table 4.1: Changes in reducing power of BSFL-Glu conjugates as a function of reaction time and temperature (700 nm).

Reaction time (h)	Reaction temperature		
	50°C	**70°C**	**90°C**
0	0.02 ± 0.01[a]	0.12 ± 0.01[a]	0.41 ± 0.03[a]
2	0.04 ±0.00[b]	0.33 ± 0.02[b]	0.76 ± 0.04[b]
4	0.05 ±0.00[b]	0.34 ± 0.01[b]	0.80 ± 0.03[b]
6	0.06 ±0.00[c]	0.38 ± 0.01[c]	0.83 ± 0.03[b,c]
8	0.07 ±0.01[d]	0.41 ± 0.01[d]	0.81 ± 0.04[b]
10	0.11 ±0.01[e]	0.42 ± 0.01[d]	0.89 ± 0.06[c]

[a] Values are the mean ± SD (n = 3); values with different superscript in the same column indicate significant differences ($p < 0.05$).

4.5.4 Fe^{2+} chelating activity

Transition metal ions, such as Fe^{2+} and Cu^{2+}, can catalyze the generation of reactive oxygen species, such as hydroxyl radical ($^{\bullet}OH$) and superoxide anion ($O_2^{\bullet-}$). In particular, Fe^{2+} generates •OH by the Fenton reaction that accelerates the lipid peroxidation chain reaction. Chelators can form complexes with metal ions and inhibit the Fenton reaction. Therefore, the chelation of metal ions contributes to antioxidant activity. MRPs are known metal chelators (Gu *et al.*, 2010; Vhangani & Van-Wyk, 2013b; Mshayisa, 2016) and their metal-ion binding affinity has been proposed as a possible mechanism to explain their antioxidant activity (Jing & Kitts, 2004) because transition metals, especially iron and copper, are involved in the generation of free radicals by Fenton reaction. The chelating activity of BSFL-Glu MRPs is shown in Figure 4.5. The chelating activity of BSFL-Glu conjugates increased significantly ($p < 0.05$) as a function of reaction time and temperature. Chelating activity significantly increased from 17.20 – 28.20% for BSFL-Glu conjugates heated at 50°C. BSFL-Glu conjugates heat treated at 50°C for 2 and 6 hours were significantly different that those at 8 h with the latter exhibiting higher chelating ability.

Generally, BSFL-Glu conjugates heated at 90°C exhibited higher metal chelation activity compared to those at 50°C and 70°C. For the metal chelating activity of BSFL-Glu conjugates at 90°C, a significant increase was observed until maximum (64.45%) at 6 h, this was then followed by a slight decrease until the end of the heating period. The results of this study coincide with the findings of Mshayisa (2016) and Zeng *et al.* (2011). This decrease of metal chelation after 6 h can be attributed to the formation of compounds with high molecular weight at higher reaction temperatures and time. The results of this study are in agreement with Gu *et al.* (2009), who observed high metal chelation of MRPs with higher molecular weight in casein-glucose model systems. The chelating activity can possibly be attributed to hydroxyl groups originating from BSFL-Glu conjugates. Metal chelation activity plays an important role in antioxidant activity as it results in reducing the concentration of the transition metal which catalyses lipid oxidation (Delgado-Andrade *et al.*, 2004).

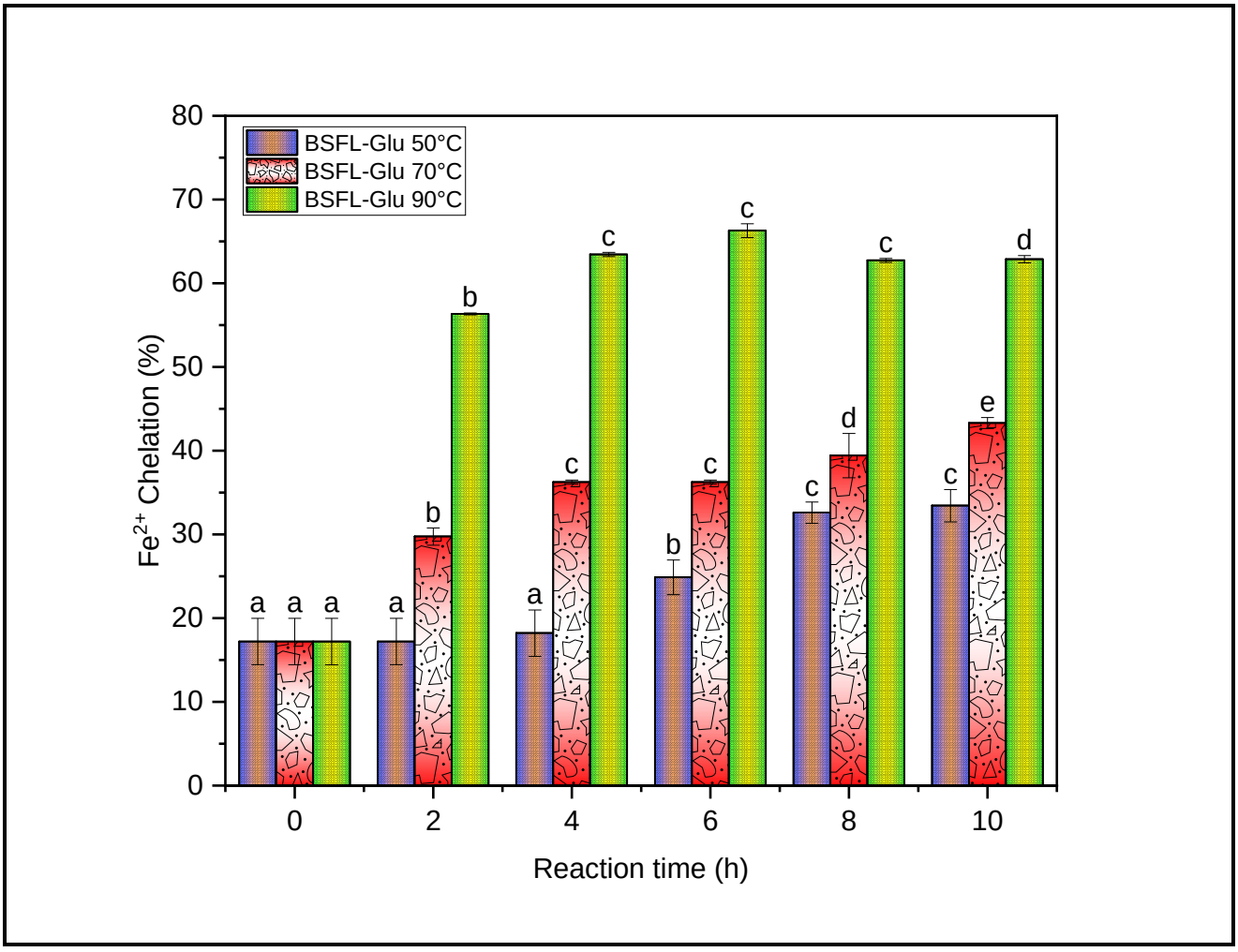

Figure 4.5 Iron chelating activity of BSFL-Glu conjugates as a function of reaction time. Values are mean ± standard deviation; means with different superscript are significantly different at the same heating temperature.

4.6 Techno-functional properties

4.6.1 Foaming capacity and stability

Foaming property is an important attribute in food ingredients for products such as desserts, baked products or ice creams. Figure 4.6 A – C illustrates the percentage of foam capacity (FC) and foam stability (FS) of conjugates derived from BSFL-Glu conjugates at 50°C, 70°C and 90°C. All BSFL-Glu conjugates had the ability to form foams. In the case of BSFL-Glu conjugates heat-treated at 50°C (Figure 6 – A), the FC was not significantly ($p > 0.05$) different between 2 and 4 h. The FC increased significantly ($p < 0.05$) after 6 h to a maximum of 30.67% and then decreased. However, the FS increased ($p < 0.05$) gradually during the heating period to a maximum of 32.64%. Jian *et al.* (2016) reported a decrease in foaming capacity of bovine serum albumin (BSA) conjugated with glucose at 45°C. This apparent contradiction may be attributed to the differences in the protein hydrophobicity in the respective studies.

The foaming capacity of BSFL-Glu conjugates produced at 70°C showed a significant gradual increase ($p < 0.05$) from 2 h to 10 h (Figure 4.6 – B). In addition, the FS increased after 6 h to 42.66%. The conjugates prepared at 70°C performed better in terms of FC and FS compared to those at 50°C. BSFL-Glu conjugates treated at 90°C for 10 h showed superior foaming capacity (44%) compared to the conjugates heated for shorter times. A similar trend was also observed in the foam stability at 90°C (Figure 4.6 – C). This can be attributed to the formation of a viscoelastic thick and dense layer around the entrapped air bubble interface and preventing the foam from collapsing. In general, the results of this study reveal that the foams derived from BSFL-Glu conjugates heated for longer periods (e.g. 10 h) were considerably more stable, compared to those obtained at shorter reaction times, irrespective of the temperature. This is due to the modulation of the hydrophilic-lipophilic balance of proteins and the creation of conjugates with the inherent capability to entrap air bubbles during foam formation and retain them for longer periods by the Maillard reaction. These results were consistent with several previous reports showing that foaming capacity increased after glycation (Chevalier *et al.*, 2001; Achouri *et al.*, 2010; Garcia-Amezquita *et al.*, 2014) due to changed hydrophobicity and conformation after glycation. This work further demonstrates that glycation improved the techno-functional properties of proteins. Thus, modified BSFL protein via glycation could be used as a functional ingredient in food applications where foaming properties are desirable.

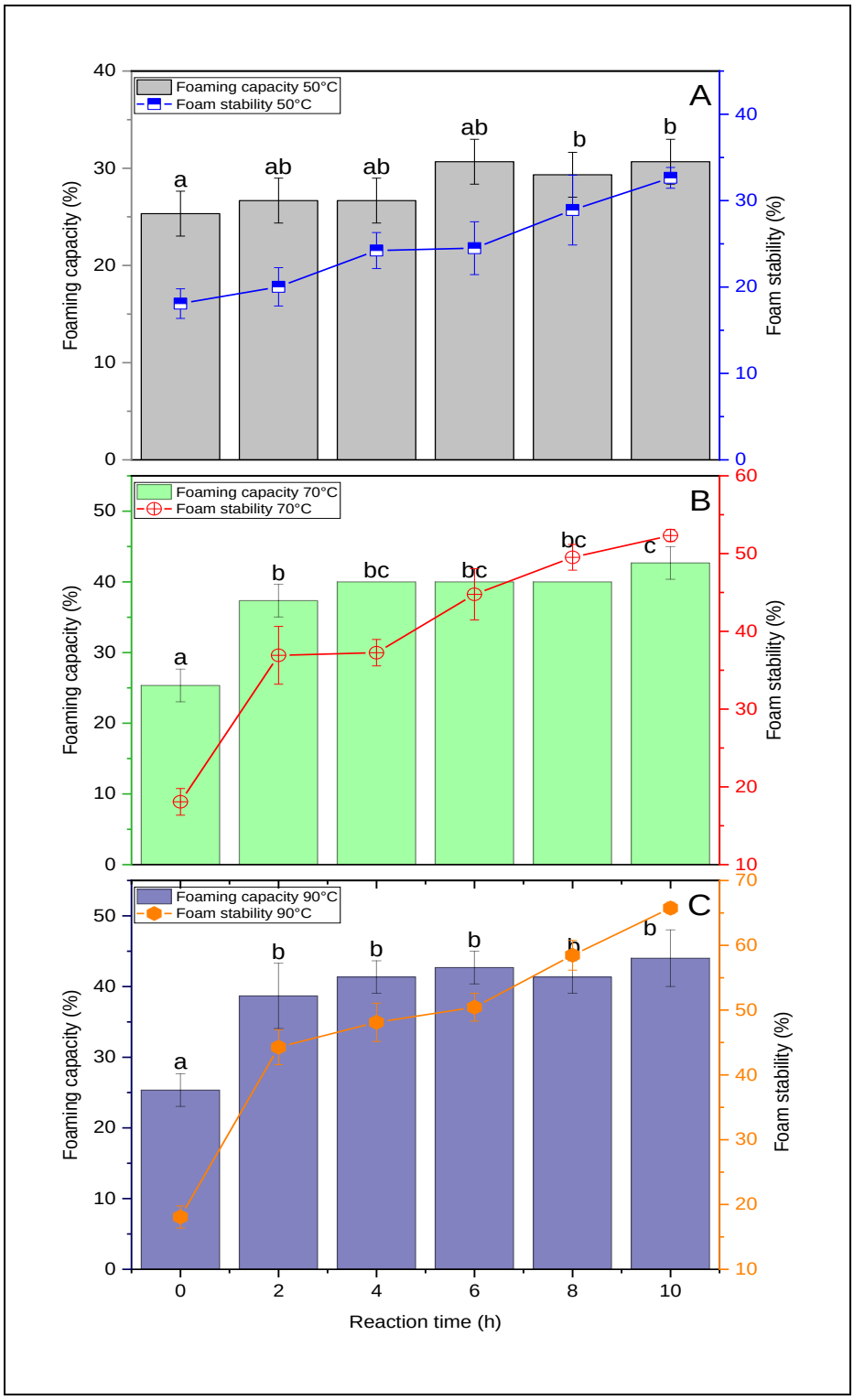

Figure 4.6 Foaming capacity and foam stability of BSFL-Glu conjugates as function of reaction time. A) 50°C B) 70°C and C) 90°C. Values are mean ± standard deviation; means with different superscripts are significantly different at the same heating temperature.

4.6.2 Emulsion capacity and stability

Emulsifying properties play an important role in food systems, as they contribute directly to the texture and organoleptic properties of food. The emulsion capacity and stability of BSFL-Glu conjugates are depicted in Figure 4.7 A – C. The emulsifying capacity of BSFL-Glu conjugates derived at 50°C decreased significantly ($p < 0.05$) as the reaction time increased. The control ($t = 0$) had a significantly higher ($p < 0.05$) emulsion capacity compared to the conjugates. However, the emulsion stability increased significantly ($p < 0.05$) as the reaction time increased. These observations were inconsistent with those reported by Rangsansarid *et al.* (2008), namely that no improvements in emulsion stability were observed for BSA-sugar (glucose, allose, and 6-O-octanoyl-D- glucose) conjugates. On the contrary, BSFL-conjugates prepared at higher temperatures, for example, 70 and 90°C had significantly higher emulsion capacity compared with the control ($t = 0$). The emulsion capacity of BSFL-Glu conjugates at 70°C ranged from 54.44 – 59.45%. The emulsion stability increased significantly ($p < 0.05$) as a function of reaction time, with conjugates produced after 8 h exhibiting the highest emulsion stability (35.89%). In general, the emulsion stability of the conjugates increased significantly ($p < 0.05$) as a function of time, irrespective of glycation temperature. The reduction in interfacial tension by the emulsifier is mainly achieved by directing the hydrophobic and hydrophilic parts of the emulsifier toward the nonpolar fraction (oil phase) and the polar component (water phase), respectively. To the knowledge of the authors, the data presented in Figure 4.7 A – C is a first for BSFL-based conjugates and thus enriches the current literature on the potential incorporation of edible insect-derived ingredients in the food supply chain.

In this study, the BSFL-Glu conjugated at 90°C showed higher emulsion capacity and stability due to the Maillard reaction. This increase can be attributed not only to an increase in reactivity between the carbonyl group and amino group but also to a greater unfolding of the BSFL protein structure, thereby exhibiting a greater number of reactive functional groups (lysine residues) which is favoured by increasing temperature. Most notably, this is the first study, to our knowledge, to examine the effect of Maillard conjugation on insect protein, in particular black soldier fly larvae. The high emulsion stability and capacity of BSFL-Glu conjugates suggest that they have the potential to be used as novel functional ingredients in food processing.

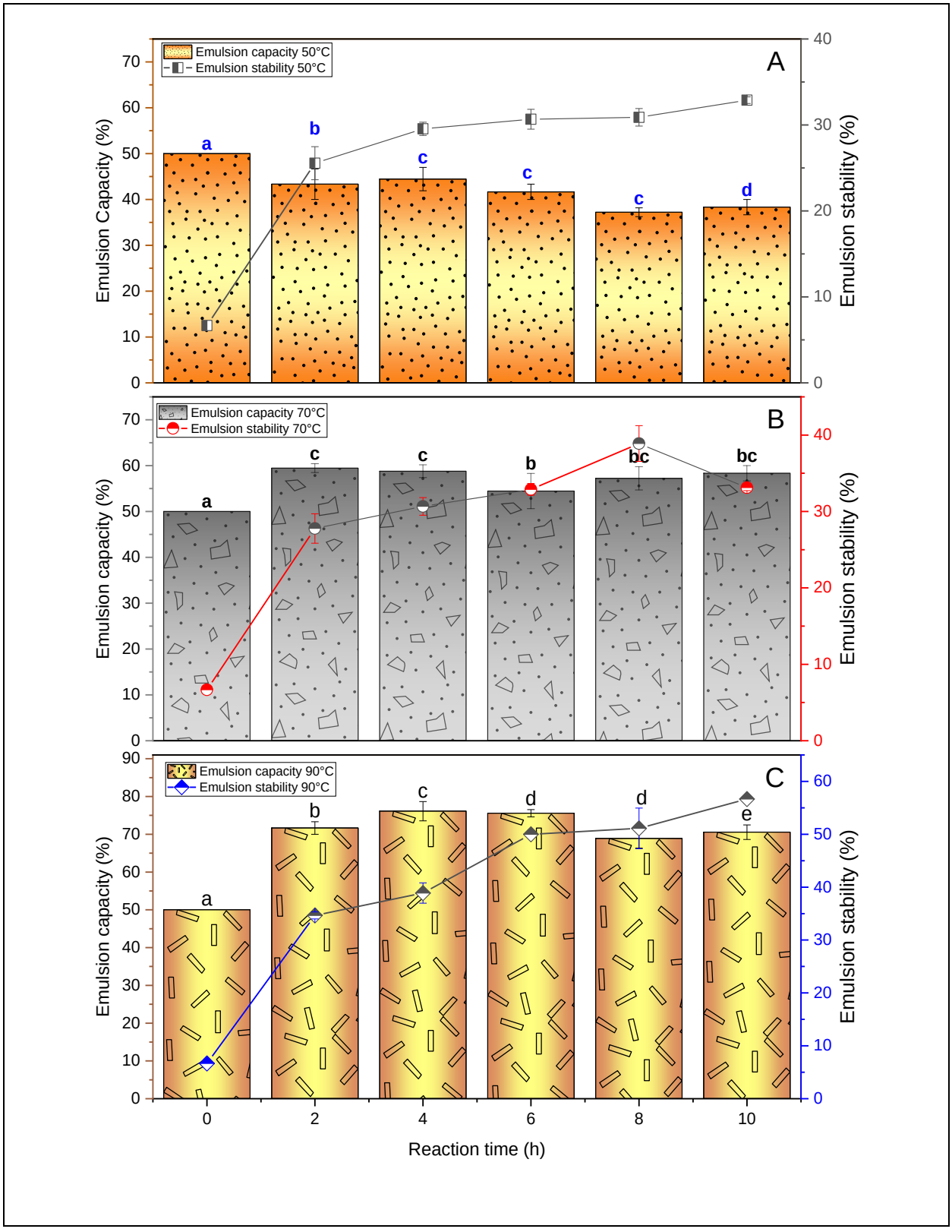

Figure 4.7 Emulsion capacity and stability of BSFL-Glu conjugates A) 50°C B) 70°C and C) 90°C. Values are mean ± standard deviation; means with different superscript are significantly different at the same heating temperature.

4.7 Principal component analysis

4.7.1 Principal components explaining the variability in BSFL-Glu browning index and antioxidant activity

Principal component analysis (PCA) was used to reduce the variability of data among the heat-treated conjugates. In principal component analysis, the original data matrix is converted to loadings and scores (tested parameters) vectors, whereby new variables – the principal components were obtained. PCA was, therefore, applied to the browning indicators, pH reduction and antioxidant properties of BSFL-Glu conjugates. The PCA exhibiting score, correlation loadings, explained variance and influence plots are shown in Figure 4.8. The explained variance plot gives an indication of how much variation in the data is described by the components. In this study, there is no clear break in the explained variance plot, that is the calibration is closer to the validation plot. The variability in the data could be explained by two PCs where PC1 contributes 89% of the variability and PC2 contributes 8% while PC3 explains 2%. The cumulative variation explained by PC1 and PC2 was 97% (Figure 4.8). Therefore, the first two PCs are sufficient to describe the variability in terms of BSFL-Glu conjugates in terms of the browning index and antioxidant properties.

Score plots were used for outlier identification, identification of groups or trends, exploration, of replicate similarities and more. The score plot showed that PC1 describes the heating temperature since samples are distributed from right to left according to the heating temperatures. Clusters of BSFL-Glu as a function of heating temperature could be clearly observed from the score plot. Moreover, the correlation loading matrix (Figure 4.8) shows that the outer ellipse explained 100% of the variability and while the inner ellipse indicates 50% of the explained variance. As can be seen from Figure 4.8 (top right), the browning index and antioxidant properties (DPPH-RS, ABTS-RS, Fe chelation and reducing power) were close to each other reflecting a high positive correlation with each other and overwhelmingly explained the PC1 direction. The pH reduction was diametrically opposed to the browning index and antioxidant properties. Therefore, these two components (PC1 and PC2) would be adequate for the prediction of browning and antioxidant indices of BSFL-Glu conjugates. The three-dimensional score plots showing PC1 vs. PC2 vs. PC3 further exhibited clear paten separating the samples based on temperature (appendix B).

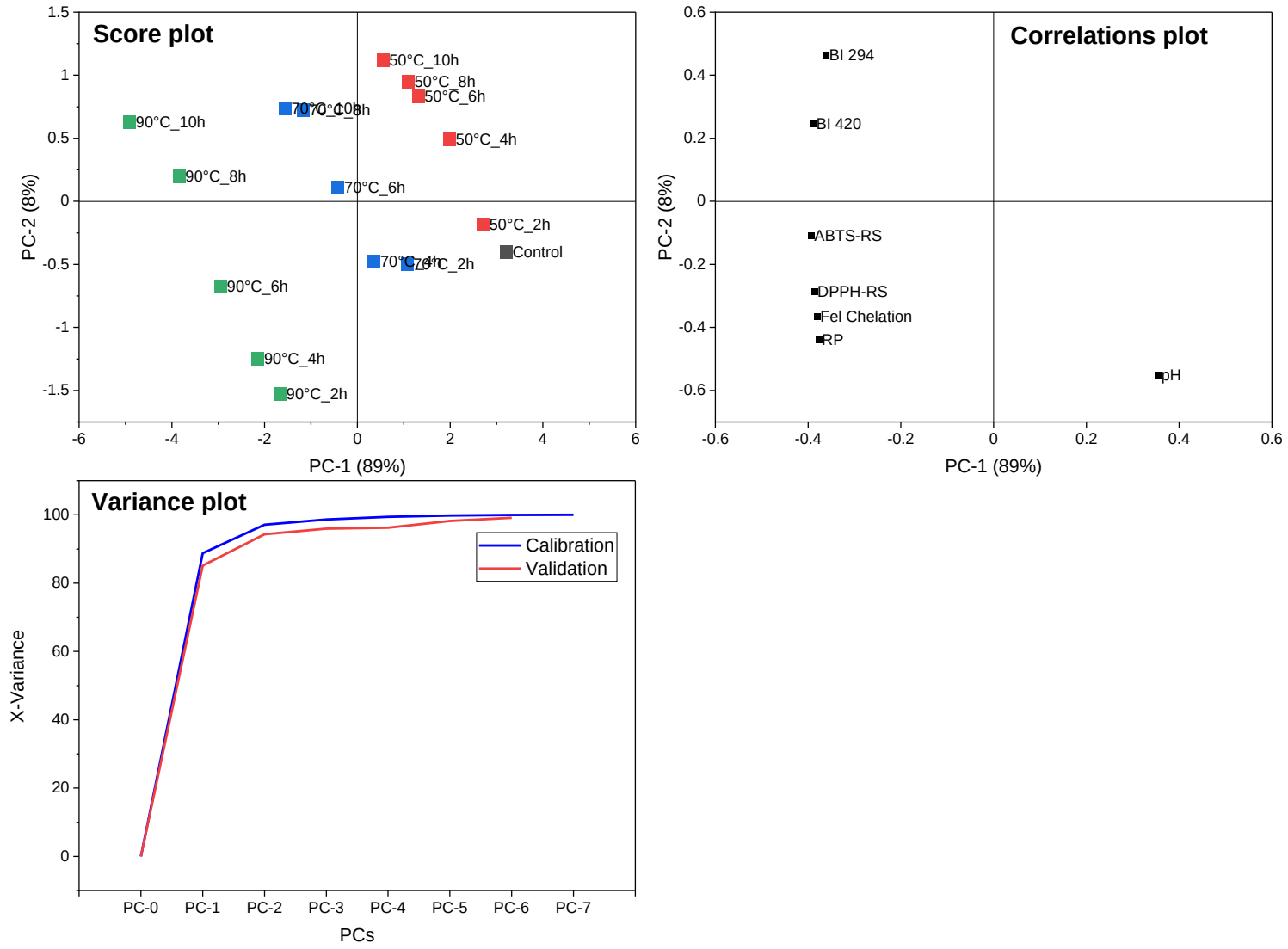

Figure 4.8 PCA plots of independent (scores) and dependent (loadings) variables for BSFL-Glu antioxidant indices.

4.7.2 Principal components explaining the variability in BSFL-Glu techno-functional properties.

Principal component analysis was further used to determine the clusters or sample groupings with respect to the techno-functional properties. From the explained variance plot it can be deduced that two PCs are sufficient to explain the observed variance (Figure 4.9). The score plot indicates that cumulative variance explained by PC1 and PC2 were 85% and 11%, respectively, with a total of 96%. PC1 showed positive loadings in FC and FS while PC 2 showed negative loading for EC. The PCA confirms the results obtained with regards to the heating temperature. From the model applied, the BSFL-Glu samples could be clearly separated into clusters based on the heating temperature. The three-dimensional score plots showing PC1 vs. PC2 vs. PC3 further exhibited clear paten separating the samples based on temperature (appendix D). An important implication of these findings is that conjugated BSFL with glucose possesses enhanced techno-functional properties and thus can potentially bed used as a novel functional ingredient in food formulations.

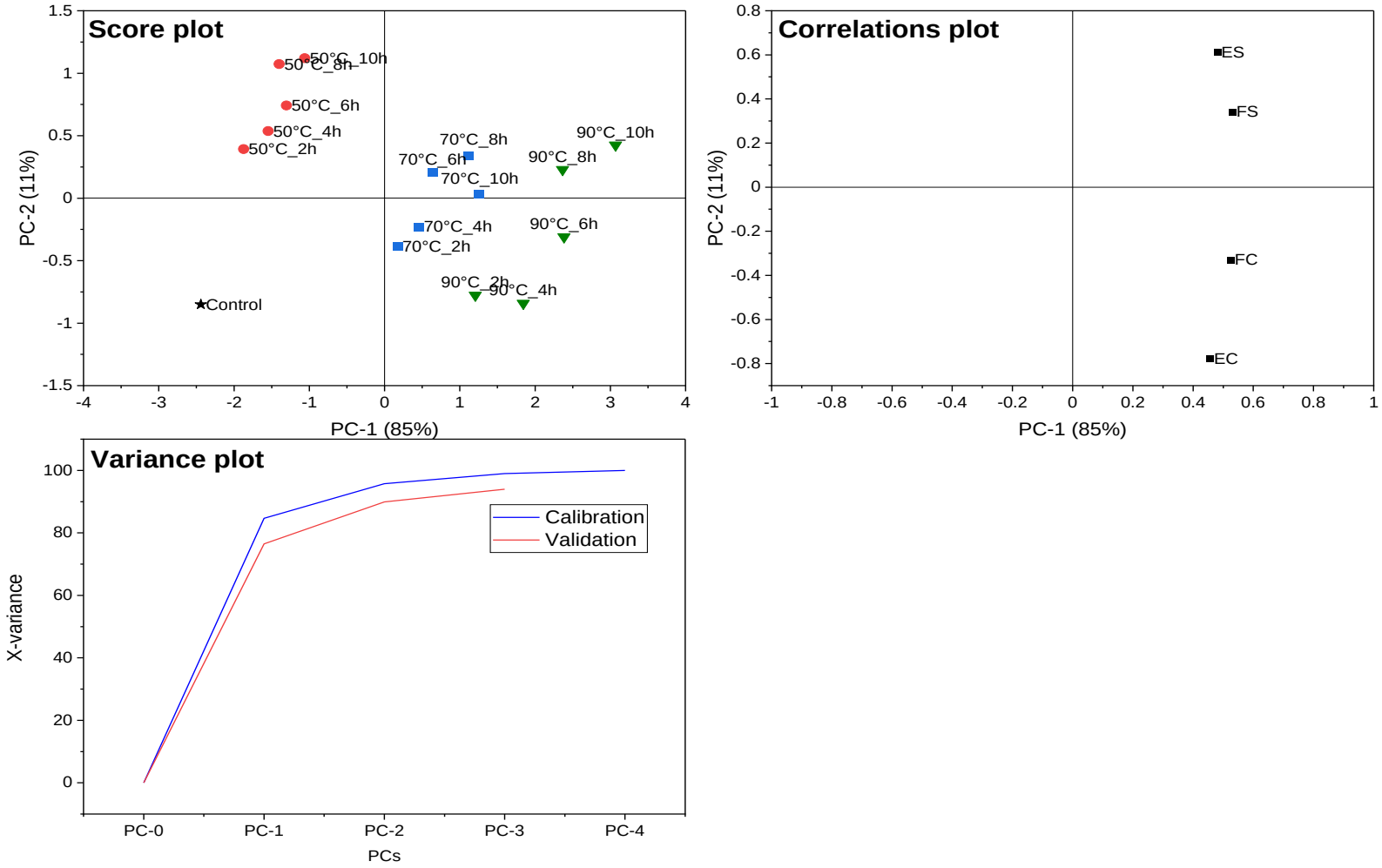

Figure 4.9 PCA plots of independent (scores) and dependent (loadings) variables for techno-functional properties.

4.8 Conclusion

In order to create new products and to enhance existing ones, awareness of protein functionality is important. The present study demonstrated the impact of heating temperature and time on the antioxidant and techno-functional properties of BSFL-Glu conjugates. Browning intensity, DPPH-RS and ABTS-RS radical scavenging activity consistently increased with an increase in reaction temperature and time. BSFL-Glu conjugates exhibited enhanced techno-functional properties, especially foaming stability and emulsion stability. The presented results indicate the utilisation of conjugation via Maillard reaction as a way to improve protein functionality, which is especially relevant for highly sought-after edible insect proteins. The performance of the BSFL-Glu conjugates in applications such as foams and emulsions need to be explored in future studies via more time-temperature combinations since it was established that heating time and temperature influence the extent of the glycation reaction. The results of the present study suggested that edible insect (BSFL) protein conjugates have considerable potential application as functional ingredients for the production of a stable foam and emulsion structure. However, the effects of variation in pH and salts on these conjugates need to be investigated in future work.

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CHAPTER 5: Structural properties of native and conjugated black soldier fly larvae protein via Maillard reaction

5.1. Abstract

Black soldier fly (Hermetia illucens) has received considerable interest as an alternative protein source. Aqueous solutions of black soldier fly larvae (BSFL) protein and glucose (1:2 $w.w^{-1}$, pH 9) were heated at 50, 70 and 90°C, for 2 – 10 h at 2 h intervals, respectively. The zeta potential analysis revealed that the glycation reaction modified the surface charge density of the BSFL protein as a function of reaction time and temperature. In addition, an increase in thermal stability of the BSFL-Glu conjugates was observed by means of Thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC). Fourier transform infrared spectroscopy (FT-IR) analysis indicated that the most apparent structural changes in the BSFL protein were in the amide I and amide II region. Well-separated clusters permitting differentiation between native BSFL and BSFL-Glu conjugates were observed by using principal component analysis (PCA) on FT-IR spectra. At 50, 70 and 90°C the first two principal components (PC1 and PC2) showed an accumulated total variance of 91, 96 and 95%, respectively. A classification efficiency of 91% was obtained when using soft independent modelling of class analogy (SIMCA). The findings of this study clearly illustrate that the degree of glycation results in structural changes to the native insect protein and could potentially alter the techno-functional properties, thus promoting the utilisation of edible insects as functional ingredients in the food industry.

Keywords

Chemometric method, conjugation, Maillard reaction, soft independent modelling of class analogy, principal component analysis, glycation.

5.2. Introduction

The food industry is exploring alternative protein sources for human consumption in response to the global concerns about food security and protein malnutrition due to the growing population. Novel protein sources such as algae, pea, rapeseed, duckweed and insects have received a great deal of research interest in the past decade (Boland *et al.*, 2013; Van-der Spiegel *et al.*, 2013; Kim *et al.*, 2016; Leni *et al.*, 2019). Edible insects such as crickets (*Acheta domesticus*), mealworm (*Tenebrio molitor*), Mopani worm (*Imbrasia. Belina*) and black soldier fly larvae (*Hermetia illucens*) have emerged as promising alternatives since they have a high fat and protein content, high feed conversion ratio, lower environmental footprint and higher economic value (Wang & Shelomi, 2017; Gould & Wolf, 2018; Huang *et al.*, 2018). In particular, proteins extracted from black soldier fly larvae and used as novel food ingredients in different food applications might have greater success in terms of consumer acceptance since some consumers show aversion towards consuming whole insects (Patel *et al.*, 2019).

To further promote the application of black soldier fly larvae (BSFL) proteins in the food industry, it is imperative to understand the structural and thermal properties which influence their techno-functional behaviour such as solubility, emulsification, oil and water binding capacity and gelation in food systems. In order to enhance the techno-functionality of proteins, food manufacturers attempt by all means to avoid chemical agents that may be potentially toxic and harmful to consumers (Chevalier *et al.*, 2001; Medrano *et al.*, 2009; Corzo-martínez *et al.*, 2017).

Therefore, conjugation via the Maillard reaction has been deemed as a generally regarded as safe (GRAS) process since it occurs spontaneously and does not involve extraneous chemicals. The Maillard glycation between amino groups of proteins and the carbonyl groups of reducing sugars is inevitable during food processing and storage. The initial stage of this reaction involves the condensation of the carbonyl group with the available ε-amino group which results in Amadori reaction products being produced through the formation of a Schiff base with the release of water (O'Mahony *et al.*, 2017; Pirestani *et al.*, 2018). The reaction is greatly accelerated by heat and can be induced under both wet and dry states. However, the latter is not feasible from an industrial point of view since it requires extended reaction times up to several days or weeks. Moreover, factors such as reaction pH, temperature and time can influence the physico-chemical and techno-functional properties of the

formed conjugates. These conjugates are diverse, complex, not fully understood and thus assessment of the reaction with a single analysis is not effective.

Studies have shown that FT-IR (Fourier transform infrared spectroscopy) can be used to evaluate the structural properties of proteins induced by conjugation in wet or dry state (Oliver *et al.*, 2009; Wang *et al.*, 2013; Mellado-Carretero *et al.*, 2019). FT-IR is a powerful technique for rapid, sensitive, low cost, reliable, precise and non-destructive analysis of chemical compounds in food matrices and provides an alternative to wet-chemical and time-consuming techniques (Rodríguez *et al.*, 2019). It is considered as a green analytical technique since it eliminates the use of hazardous chemicals. To further enhance the use of FT-IR and apply a multi-pronged approach, it is often used in combination with multivariate statistical methods known as chemometrics. Chemometric methods such as Partial least squares discriminant analysis (PLS-DA), Principal component analysis (PCA), Cluster analysis (CA) or Soft independent class modelling analogy (SIMCA) allows researchers to elicit vital information from big sets of data (Balan *et al.*, 2020). Mellado-Carretero *et al.* (2019) used pairwise SIMCA models to show significant chemical differences in protein-polysaccharide conjugates. SIMCA showed 100% classification efficiency when applied to detect adulteration of milk samples with formalin. In addition, Oliver *et al.* (2009) reported that FT-IR was able to discriminate between non-glycated and glycated sodium caseinate conjugates when PCA and SIMCA were applied. To the best of our knowledge, there is a paucity of information on the application of FT-IR with chemometric methods on the conjugation of BSFL protein via the Maillard reaction and the knowledge underpinning insect protein-sugar interactions is still rather limited.

The aim of this study was to characterise the native BSFL protein and BSFL-Glu conjugates using novel analytical techniques, attenuated total reflection (ATR) FT-IR coupled with chemometrics (PCA and SIMCA). The complementary analysis techniques, thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC), zeta potential and scanning electron microscopy (SEM) were also performed to further probe the structural modifications to the native BSFL protein and BSFL-Glu conjugates due to the Maillard reaction.

5.3. Materials and methods

5.3.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Aston Manor, South Africa). Prepared reagents were stored under conditions that prevented contamination or deterioration. The water used in the study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, South Africa).

5.3.2. Synthesis of Maillard conjugates, pH and Browning index

BSFL-Glu conjugates were prepared according to the method of Vhangani and Van Wyk (2013b) and Mshayisa (2016) with slight modifications. BSFL protein concentrate and glucose (2:1 $w.w^{-1}$) were dispersed in 100 mL of 0.1 M phosphate buffer at pH 9. The samples were transferred into 250 mL Schott bottles and heated at 50, 70 and 90°C in a water bath for 0, 2, 4, 6, 8 and 10 h, respectively. After the heating period had elapsed, the resulting BSFL-Glu conjugates were immediately cooled in an ice bath. The native (as is or non-glycated) BSFL protein was also heated at the same temperature and time intervals as described above. In addition, unheated BSFL-Glu conjugates served as a control. The samples were freeze-dried and stored in air-tight screw-capped glass bottles at -80°C until analysis.

5.3.3. Zeta potential Surface charge (Zeta potential)

The ζ-Potential of native BSFL protein and BSFL-Glu conjugates of protein concentrates were determined using a Zetasizer Nano Series (Malvern Instruments, Malvern, Worcestershire, UK) as described by Ladjal-Ettoumi *et al.* (2016). Samples were diluted to 1% ($w.v^{-1}$) protein concentration with Milli-Q water before measurement. Data was collected over at least five sequential readings and processed using the Smoluchowski mode (Schwenzfeier *et al.*, 2013).

5.3.4. Scanning electron microscopy (SEM)

The surface morphology of the freeze-dried samples of the native BSFL protein and BSFL-Glu conjugates was examined using scanning electron microscopy (TM-3000, Hitachi Corporation, Tokyo, Japan). The sample was placed onto double-sided carbon

adhesive tape attached to the specimen stubs. The surface structure of the sample was observed at 320 X magnification and in secondary electron mode at 15.0 kV.

5.3.5. Thermal analysis

The thermal properties that include differential scanning calorimetry (DSC) analysis and thermo-gravimetric analysis (TGA) were evaluated on the freeze-dried heat-treated native BSFL protein and BSFL-Glu conjugates. In both, analyses, approximately 4.0 – 6.0 mg of sample was weighed into an aluminium pan. A sealed empty pan was used as a reference. The DSC profile of the samples was evaluated using a thermal analyser (Q2000, Perkin Elmer, Singapore). The pans were sealed and heated from 30 to 180°C at a heating rate of 20°C min^{-1}. The thermal stability behaviour of native BSFL protein and BSFL-Glu conjugates was evaluated using a TGA system (TGA-7, Perkin Elmer, Singapore). The analysis was performed from 30 to 180°C at a heating rate of 20°C min^{-1} with nitrogen as carrier gas at a flow rate of 50 $mL.min^{-1}$.

5.3.6. FT-IR spectra acquisition

All heat-treated native BSFL protein and BSFL-Glu conjugate samples in powder form were analysed using a Fourier transform infrared spectroscopy (FT-IR) equipped with a universal attenuated total reflectance (UATR) polarization accessory for spectrums (Spectrum 400, Perkin Elmer, Singapore). Prior to data collection of each sample, a background spectrum was collected and then the sample powders obtained by grinding in a mortar were placed directly covering the surface of the ATR crystal. All spectra were acquired by co-addition of 32 scans at a resolution of 4 cm^{-1} in the range of 400 – 4000 cm^{-1}. The UATR crystal was cleaned with acetone to remove any residual contribution of the previous samples. To ensure reproducibility, spectra of each sample was acquired in triplicate.

5.3.7. Chemometric analysis

All the acquired sample spectra were analysed using the Unscrambler X software version 11 (CAMO software, Oslo, Norway) (CAMO analytics – www.camo.com). For SIMCA, the samples were divided into a training set (60 samples) and prediction set

(30 samples). Every spectrum was base-line corrected, transformed into absorbance units and normalized. Characteristic fingerprinting regions were recognized by exploring various peaks in the plotted raw spectra.

5.3.7.1. Principal component analysis

PCA was carried out to classify the samples based on spectral differences. This data reduction method crafts new uncorrelated variables called principal components (PCs) from the original set of variables. It is a linear projection method that minimizes a high dimension data matrix based on variance and thus retaining maximal variability. Each component of the PCA model is characterized by scores, containing new coordinates of calculated PCs and loadings, with the weight of each wavenumber in the score of that PC. PCA helped to identify the most important variables in the data set that can contribute to a more robust and less complex model. PCA was performed with the constant weight and full cross-validation option assigned to all samples to explore the clustering of samples. It also helps in the detection of any outliers in the dataset. The non-linear iterative partial least square algorithm (NIPALS) algorithm was used to build the PCA model.

5.3.7.2. Sample classification using SIMCA

SIMCA is a classification approach based on PCA in which each class is independently modelled. SIMCA is applied to predict the class membership of the test set at a significance level of 5%. Primarily, a global model was developed. In the next step, class models for heated BSFL protein and BSFL-Glu conjugates based on the heating temperature and time were developed using a calibration data set (60 samples) and test or prediction set (30 samples). The best fit test set samples were classified to one of the developed class models. The statistical significance level for the SIMCA classification was set at 5%. This assumed that there was a 5% risk that a particular test sample would fall outside the class, even if it actually belonged to the class; while 95% of the test samples which truly belonged to the class would fall inside the class. The performance criterion is evaluated by analysing the designated class of each sample with its true class membership. True positive identifications (TP) represent samples of a given class that are correctly classified by the respective class whereas, in true negative identifications (TN), samples are correctly refused by the

respective class model. Samples that belong to a given class but were misclassified by the respective class are referred to as false negative identifications. In false positive identifications, samples do not belong to a given class but were incorrectly recognized by the established class model. Sensitivity, specificity, and precision are the evaluation parameters that determine the classification performance of each model. Sensitivity (Eq. 1) represents the percentage of samples in the prediction set which are recognized by the model class while specificity (Eq. 2) represents the percentage of samples from other categories which are correctly refused by the model class. Precision is basically the geometrical mean of specificity and sensitivity (Eq. 3) (Balan *et al.*, 2020). The efficiency may vary between 0 (sensitivity or specificity values are zero) and 1 (sensitivity or specificity values are 1). SIMCA class model interpretations were based on interclass distance, discrimination power and class projections.

$$\text{Sensitivity}\ (\%) = \frac{\text{TP}}{\text{TP} + \text{FN}} \times 100 \qquad \text{Equation 1}$$

$$\text{Specificity}\ (\%) = \frac{\text{TN}}{\text{TN} + \text{FP}} \times 100 \qquad \text{Equation 2}$$

$$\text{Precision}\ (\%) = \sqrt{\frac{\text{TP} \times \text{TN}}{(\text{TP} + \text{FN}) \times (\text{TN} + \text{FP})}} \times 100 \qquad \text{Equation 3}$$

5.3.8. Data analysis

All data were subjected to multivariate analysis of variance (MANOVA) to ascertain whether the main effects resulted in significant differences in response variables. Duncan's multiple comparison *post hoc* test was used to test significant differences ($p < 0.05$) between individual means. SPSS 26.0 for Windows® was used for the statistical analyses and the level of confidence required for significance was selected at $p < 0.05$. The Unscrambler software, version 11 (CAMO Software, Oslo, Norway) was used to perform PCA and SIMCA. Graphs and figures were generated using Origin software, version 9.60 (Origin labs, Northampton, MA, USA) and The Unscrambler software.

5.4. Results and discussion

5.4.1. Zeta potential

Protein molecules in most cases carry a charge and this plays a significant role in interaction with other components in food matrices. The zeta potential (ζ) of proteins is a vital analysis that can be applied to optimise food product formulations for novel ingredients, predict interactions with surfaces and prediction of long-term stability. The ζ-potential of the native BSFL protein and BSFL-Glu conjugates heated at 50, 70 and 90°C, respectively, is illustrated in Figure 5.1 A – C. Since most colloidal dispersions in aqueous media carries an electric charge, a negative ζ-potential indicates that the particles in suspension carry an overall negative charge. The ζ-potential of native BSFL protein heated at 50°C ranged from -10.25 to -10.55 mV while BSFL-Glu conjugates ranged from -10.80 to -13.05 mV (Figure 5.1 A). The BSFL conjugates heated at 70 and 90°C exhibited lower ζ-potentials compared to the native BSFL proteins at the same heating temperature (Figure 5.1 B and C). This is might be because conjugation with glucose altered the surface charge distribution of BSFL protein-making more negatively charged groups such as –COOH and –OH more accessible due to the heat treatment. Moreover, the ζ-potential of BSFL-Glu conjugates prepared at 90°C decreased from -27.15 to -37.11 mV as a function of reaction time. The linkages of the glucose to the BSFL protein during conjugation might lead to electrostatic screening and modifications of the surface charge of BSFL protein and thereby contributes to the observed lower ζ-potential. These results are in agreement with previous studies on pea protein isolate-maltodextrin conjugates (Kutzli *et al.*, 2020). Proteins with ζ-potentials greater than 30 mV absolute value are usually considered stable, exhibiting no flocculation (Mellado-Carretero *et al.*, 2019). Therefore, BSFL-Glu conjugates heated at 90°C are expected to be more stable compared to those heated at 50 and 70°C. Generally, the ζ-potential of all samples remained negative at all reaction times tested. The covalent bond formed between the amine group of proteins and the carbonyl compounds of reducing sugars during the Maillard reaction results in a shift in the isoelectric point (pI) of proteins to a lower pH value. Therefore, the differences in the ζ-potential of the native BSFL protein and BSFL-Glu conjugates can be attributed to the structural modifications of the proteins induced by the heat treatment and to the unfolding of the protein leading to the exposure of more negative charges on the surface of the BSFL protein i.e. more stable

to flocculation. Therefore, conjugated BSFL protein has the potential to be used in dressings and beverages which requires stability against flocculation.

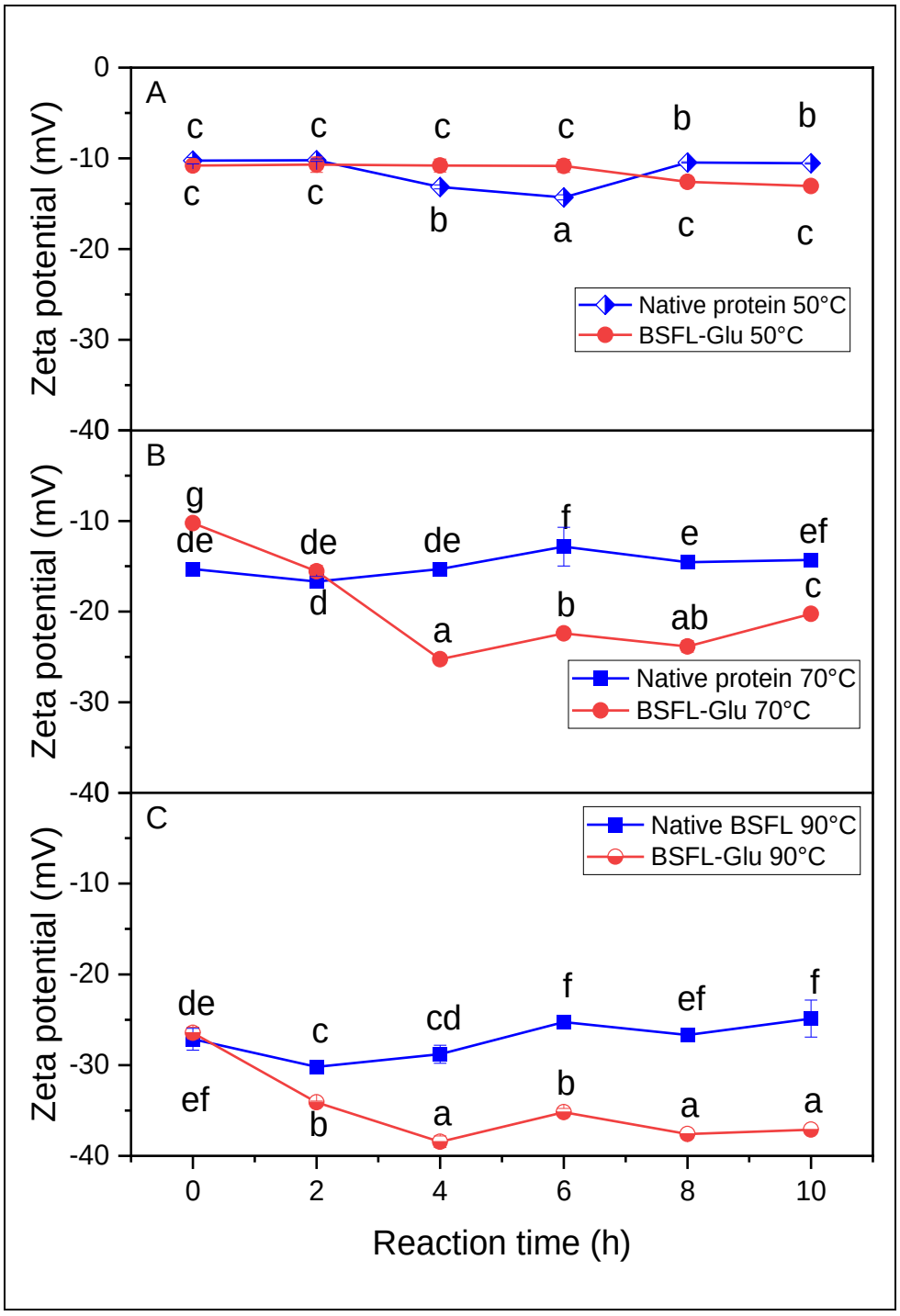


Figure 5.1 Zeta potential of native BSFL protein and BSFL-Glu conjugates heated at A) 50°C, B) 70°C and C) 90°C as a function of time. Comparisons were carried out between values of the same series and values without same letter(s) indicate significant difference at $p < 0.05$.

5.4.2. Thermo-gravimetric analysis (TGA)

TGA is an analytical technique which has been widely used to monitor the mass (weight) of a substance as a function of temperature as the sample is subjected to a controlled environment and temperature increase program. The thermal stability of native BSFL protein and BSFL-Glu conjugates synthesised by heating at 50, 70 and 90°C, for 2 – 10 h was assessed utilising TGA in a temperature range between 25 and 650°C. The TGA results of native BSFL protein and BSFL-Glu conjugates are shown in Figure 5.5 – 5.7. By selecting the reaction temperature of 90°C as a representative, it can be seen that the native BSFL protein undergoes three stages of thermal decomposition. The first stage of decomposition was attributed to the evaporation of free and loosely bound water and other volatiles in the temperature range 60 – 150°C (Figure 5.3 A). The second stage of the weight loss displayed by the heated native BSFL protein was the decomposition or degradation at approximately 150 – 300°C. All the curves in this region are closer together signifying that the rate of decomposition is similar. The third stage from 450°C – 550°C may be associated with the polypeptide decomposition in the native BSFL protein. It was observed that native protein heated at 10 h had the lowest residual mass after the heating process. In terms of the BSFL-Glu conjugates prepared at 90°C (Figure 5.4 B), the TGA thermograms revealed that the BSFL-Glu conjugates had approximately 10% weight loss due to moisture loss in the temperature range 0 – 150°C.

BSFL-Glu conjugates prepared at 90°C (6 h and 10 h) started to decompose at about 150°C – 210°C and its weight loss sharply increased in the temperature range 250 – 500°C (Figure 5.4 A – B). The sharp decrease in the weight is due to the decomposition of conjugation or glycation products and the pyrolysis of other compounds. Compared with the native BSFL protein, the BSFL-Glu conjugates TGA thermograms exhibited a superimposed pattern in terms of the samples (6 h and 10 h) between 100 – 400°C which were further away from the rest of the samples (Figure 5.4). Conjugates at 6 and 10 h had the most gradual slope of the weight versus temperature plot. The remaining products at this heating range (500 – 650°C) could be other pyrolysis residues and ash (Figure 5.3 B). This implies that these conjugates (6 h and 10 h) exhibit greater thermal stability compared to the other samples. The enhanced thermal stability of the BSFL-Glu can be ascribed to crosslinking and Maillard reaction between BSFL protein and glucose.

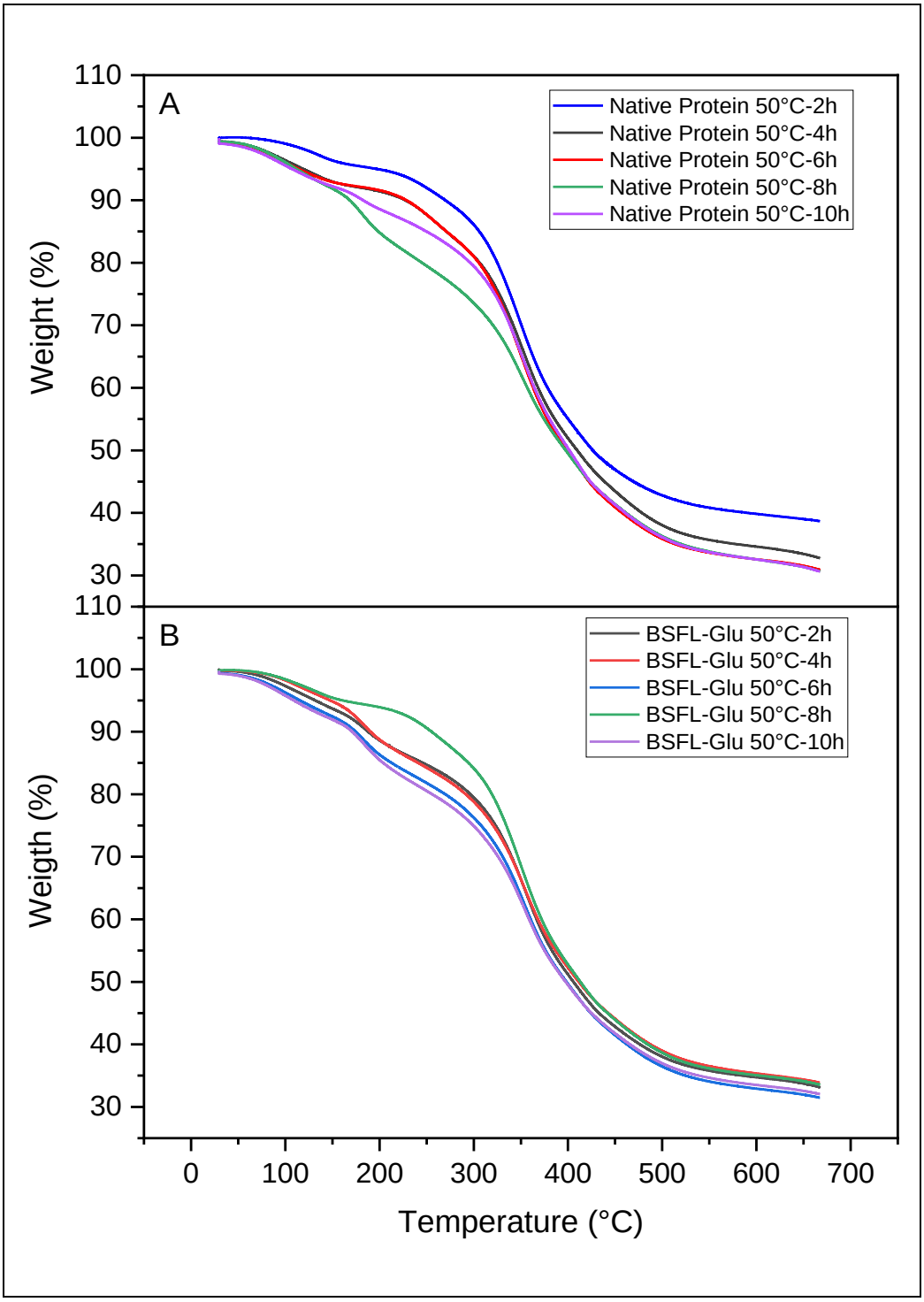


Figure 5.2 TGA scans of A) native BSFL protein and B) BSFL-Glu conjugates heated at 50°C.

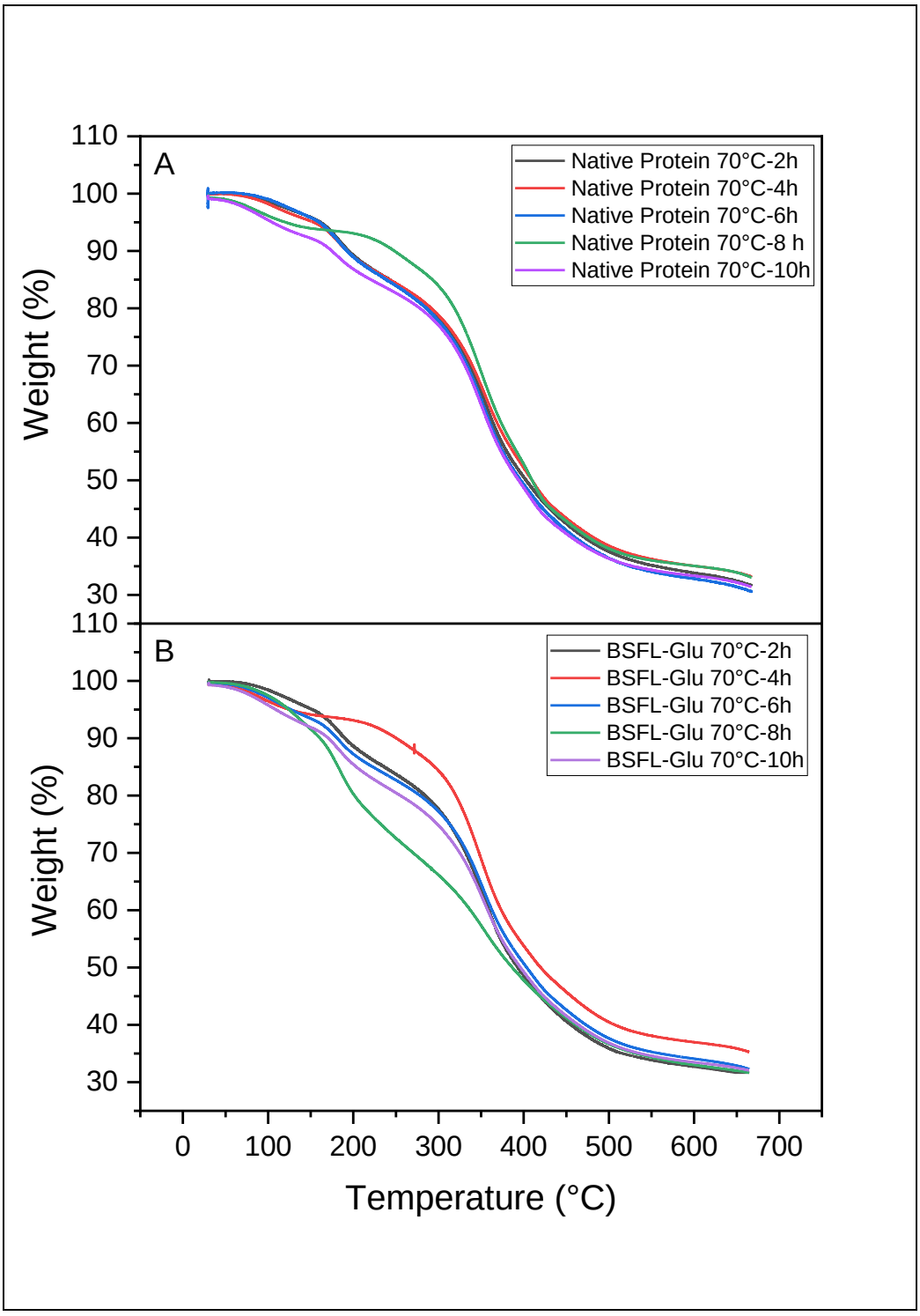


Figure 5.3 TGA scans of A) native BSFL protein and B) BSFL-Glu conjugates heated at 70°C.

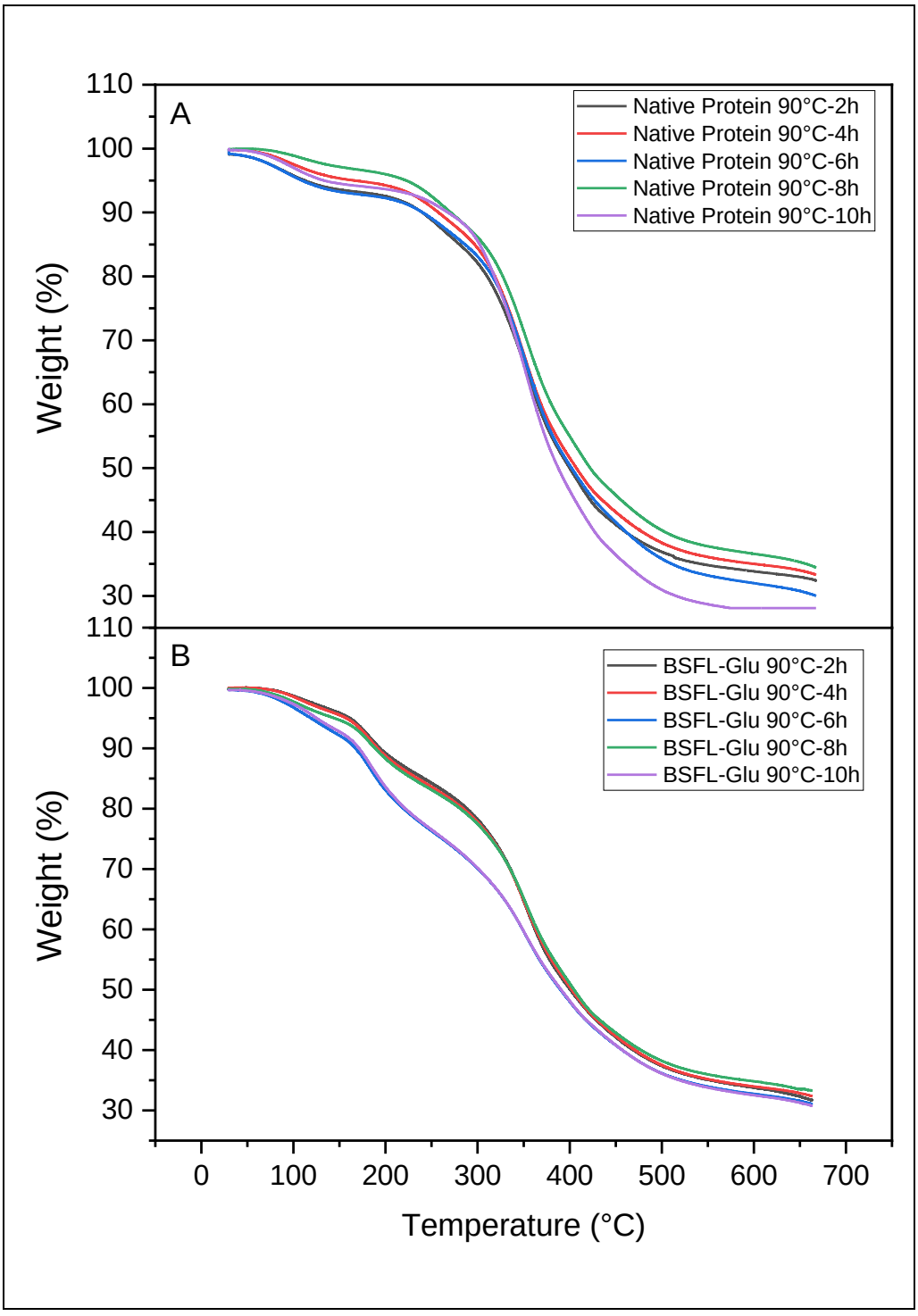


Figure 5.4 TGA scans of A) native BSFL protein and B) BSFL-Glu conjugates heated at 90°C.

The addition of glucose to the native BSFL protein via the Maillard reaction resulted in structural changes in the BSFL protein, becoming more unfolded and this was observed by the SEM results.

5.4.3. Differential scanning calorimetry (DSC)

After glycation, the tertiary/quaternary structure of food protein also changes, resulting in the functional properties being altered. It is vital to investigate such structural modifications since they are closely related to the functional properties of the protein in food. DSC can directly quantify heat changes in the sample during monitored temperature increases or decreases and can provide very important information about the sample's thermal stability (Liu *et al.*, 2012).

The changes in thermodynamic parameters of heat-treated native BSFL protein and BSFL- Glu conjugates are presented in Table 5.1. Critical information on heat-induced protein unfolding and protein thermal stability is given by T_{on} and T_p, respectively. The enthalpy changes (ΔH) correspond to the energy requirement for transforming the ordered structure of proteins from native compact architectures to the unfolded state. The present results indicate that the thermal denaturation peak temperature (T_p) for the heat-treated native BSFL protein at 50°C ranged from 72.23 – 73.81°C, with no significant differences ($p > 0.05$) between heating times (2, 4, 6, 8 and 10 h). On the contrary, at the same heating temperature (50°C) BSFL-Glu conjugates exhibited significantly higher T_p in the range 78.37 and 78.92°C.

Furthermore, the enthalpy (ΔH) of BSFL-Glu conjugates significantly decreased ($p < 0.05$) with increasing duration of heating. After the conjugation reaction, the decreased magnitude of the enthalpy (ΔH) can be attributed to the disturbance of the intramolecular forces of the BSFL protein when it is covalently bound to glucose. In this study, the steric spacers between the protein molecules can also be decreased by glucose molecules, which would facilitate aggregation by increasing the interactions of the surface hydrophobic binding sites. At 70°C, no significant differences ($p > 0.05$) were observed in the onset temperature (T_o) between the native BSFL protein and BSFL-Glu conjugates. Similar findings were reported by Tang *et al*. (2011) who showed that the T_p of glycated kidney bean vicilin (phaseolin) with glucose was significantly higher compared to the native samples.

Generally, at 90°C the T_p for the BSFL-Glu conjugates ranged from 90.17 – 90.73°C and was significantly higher ($p < 0.05$) compared to the heat-treated native BSFL protein (74. 31 – 75.25°C). These results suggest a remarkable increase in thermal stability of the BSFL-Glu conjugates which might be due to the unfolding of the protein structure which was initially ordered. Moreover, the denaturation temperature T_p of the conjugates increasedis with an increase in heat treatment (90 > 70 > 50°C) which implied that the conjugates interfere with the process of native protein denaturation thus enhancing the thermal stability of BSFL-Glu conjugates. Throughout the food processing process, heat treatment may result in irreversible protein modifications due to the destruction of hydrogen and hydrophobic bonds or bridges, electrostatic and disulfide bonds interactions that stabilize the native conformations. The high thermostability of BSFL-Glu conjugates via the Maillard reaction under controlled conditions could make them good functional ingredients in heat-treated food products and expand their applications. These findings have significant implications for the understanding of how glycation of conjugation of BSFL protein with glucose influence thermal stability and future application of glycation with edible insect proteins.

Table 5.1 Thermal properties of heat-treated native BSFL protein and BSFL-Glu conjugates.

Test parameters	T_{on} (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Heat treatment at 50°C				
Native BSFL 50°C_2h	35.86 ± 0.40 ^{e}	72.23 ± 0.04 ^{c}	142.06 ± 0.00 ^{e}	117.15 ± 0.04 ^{de}
Native BSFL 50°C_4h	34.65 ± 0.22 ^{d}	73.02 ± 0.75 ^{c}	144.45 ± 0.71 ^{f}	116.54 ± 0.04 ^{d}
Native BSFL 50°C_6h	34.62 ± 0.18 ^{d}	73.06 ± 0.04 ^{c}	139.23 ± 0.64 ^{d}	117.67 ± 0.75 ^{de}
Native BSFL 50°C_8h	38.94 ± 0.18 ^{g}	73.92 ± 0.64 ^{c}	136.78 ± 0.21 ^{d}	118.07 ± 0.04 ^{de}
Native BSFL 50°C_10h	37.24 ± 0.00 ^{f}	73.81 ± 0.01 ^{c}	137.04 ± 0.71 ^{f}	119.48 ± 0.75 ^{de}
BSFL-Glu 50°C_2h	32.06 ± 0.00 ^{a}	78.63 ± 0.35 ^{a}	129.31 ± 0.00 ^{a}	113.26 ± 0.71 ^{c}
BSFL-Glu 50°C_4h	33.03 ± 0.00 ^{b}	78.70 ± 0.24 ^{ab}	131.08 ± 0.00 ^{b}	112.84 ± 2.83 ^{c}
BSFL-Glu 50°C_6h	32.07 ± 0.0 ^{a}0	78.92 ± 0.02 ^{ab}	129.92 ± 0.00 ^{a}	110.12 ± 0.71 ^{b}
BSFL-Glu 50°C_8h	33.05 ± 0.00 ^{b}	78.37 ± 0.45 ^{b}	139.74 ± 0.00 ^{c}	108.80 ± 0.35 ^{b}
BSFL-Glu 50°C_10h	33.66 ± 0.00 ^{c}	78.71 ± 0.92 ^{b}	143.73 ± 0.00 ^{c}	106.25 ± 0.28 ^{a}
Heat treatment at 70°C				
Native BSFL 70°C_2h	36.80 ± 0.04 ^{a}	74.76 ± 0.28 ^{c}	128.41 ± 0.00 ^{e}	124.52 ± 0.71 ^{ef}
Native BSFL 70°C_4h	35.06 ± 0.04 ^{a}	74.52 ± 0.04 ^{d}	102.78 ± 0.00 ^{a}	124.99 ± 0.04 ^{f}
Native BSFL 70°C_6h	36.82 ± 0.04 ^{a}	74.36 ± 0.38 ^{e}	125.63 ± 0.00 ^{d}	122.04 ± 1.41 ^{e}
Native BSFL 70°C_8h	41.43 ± 0.04 ^{a}	73.92 ± 0.64 ^{c}	130.30 ± 0.00 ^{f}	122.07 ± 0.59 ^{e}
Native BSFL 70°C_10h	53.11 ± 0.27 ^{a}	74.31 ± 0.69 ^{d}	125.54 ± 0.00 ^{bc}	118.15 ± 1.07 ^{d}

Test parameters	T_{on} (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
BSFL-Glu 70°C_2h	35.09 ± 0.00 [a]	81.62 ± 0.00 [b]	102.75 ± 0.04 [a]	74.30 ± 0.04 [a]
BSFL-Glu 70°C_4h	41.43 ± 0.04 [a]	86.97 ± 0.01 [ab]	130.27 ± 0.04 [f]	102.27 ± 0.31 [d]
BSFL-Glu 70°C_6h	33.99 ± 0.04 [a]	88.28 ± 0.00 [ab]	125.51 ± 0.04 [b]	98.87 ± 0.04 [b]
BSFL-Glu 70°C_8h	36.83 ± 0.00 [a]	82.16 ± 0.00 [a]	128.38 ± 0.04 [e]	76.33 ± 2.83 [a]
BSFL-Glu 70°C_10h	36.82 ± 0.04 [a]	86.84 ± 0.00 [ab]	125.60 ± 0.04 [cd]	101.01 ± 0.04 [bc]
Heat treatment at 90°C				
Native BSFL 90°C_2h	30.47 ± 0.23 [j]	75.26 ± 0.42 [b]	126.42 ± 0.42 [d]	105.43 ± 0.71 [f]
Native BSFL 90°C_4h	31.63 ± 0.14 [e]	75.02 ± 0.75 [b]	124.11 ± 0.15 [b]	111.43 ± 0.42 [g]
Native BSFL 90°C_6h	31.25 ± 0.00 [h]	74.86 ± 1.09 [b]	125.61 ± 0.53 [c]	117.70 ± 0.28 [h]
Native BSFL 90°C_8h	32.72 ± 0.00 [i]	74.42 ± 1.34 [b]	125.59 ± 0.40 [c]	131.47 ± 0.64 [j]
Native BSFL 90°C_10h	33.47 ± 0.00 [g]	74.31 ± 0.69 [b]	133.34 ± 0.29 [e]	128.22 ± 0.03 [i]
BSFL-Glu 90°C_2h	40.96 ± 0.00 [a]	90.17 ± 0.05 [a]	126.39 ± 0.07 [d]	96.77 ± 0.01 [d]
BSFL-Glu 90°C_4h	33.06 ± 0.00 [c]	90.37 ± 0.01 [a]	124.43 ± 0.04 [b]	97.68 ± 0.37 [e]
BSFL-Glu 90°C_6h	37.47 ± 0.00 [b]	90.46 ± 0.01 [a]	132.79 ± 0.29 [e]	95.37 ± 0.35 [c]
BSFL-Glu 90°C_8h	40.75 ± 0.00 [d]	90.57 ± 0.03 [a]	77.89 ± 0.00 [a]	71.29 ± 0.00 [a]
BSFL-Glu 90°C_10h	36.18 ± 0.00 [f]	90.73 ± 0.05 [a]	136.10 ± 0.00 [f]	92.52 ± 0.01 [b]

Onset temperature (T_{on}), denaturation peak temperature (T_p), conclusion temperature (T_c) Enthalpy (ΔH). Data in the same column in the same heat treatment indicate significant ($p < 0.05$) difference. Values reported as mean values (n=2) and standard deviation.

5.4.4. ATR-FTIR spectral analysis

The spectroscopic investigation of polymeric particles, including protein, is complex due to the atomic vibrations emerging from various molecules. FT-IR spectroscopy is a useful non-destructive, precise and reliable analytical tool that can be used to probe structural modifications in protein-sugar systems since there are several readily identifiable regions of the mid-infrared spectrum where the chemical fingerprints of carbohydrates and proteins do not overlap significantly. Compared with other analytical techniques, FT-IR can detect changes in the protein secondary structure and hydrogen bonding force. The FT-IR spectra of glucose (unheated), native BSFL, BSFL-Glu (unheated, control sample) and BSFL conjugates (90°C, 10 h) are depicted in Figure 5.8. The glucose spectrum reveals all normal features of glucose as previously described (Oliver *et al.*, 2009) with no absorption in the 1600 – 1700 cm^{-1} range. The strong absorption (C – O stretching) in the range 900 – 1100 cm^{-1} with a maximum of 1028 cm^{-1} is typical for carbohydrates. As can be seen in Figure 5.5, the spectra for the native BSFL protein reveals all the normal features of a protein spectrum with amide I and II in the 1624 and 1513 cm^{-1} region, respectively. These bands are due to stretching vibrations of the peptide linkages (C – N and C = O). The spectra of the conjugates (unheated and heated at 90°C, 10 h) show that when the glucose was reacted with the native BSFL protein, the IR band associated with carbohydrates (1028 cm^{-1}, C – O stretching) was modified and increased in the resulting conjugates. Distinct conformational changes that occur as a result of interactions between the BSFL and glucose at a molecular or structural level are evident through the use of FT-IR. The BSFL-Glu conjugates heated at 90°C for 10 h clearly shows an increased intensity in the amide I region (1624 cm^{-1}). This is due to the covalent combination of BSFL protein with glucose via the Mallard reaction mechanism led to the loss of $-NH_2$ groups, particularly for the lysine. Moreover, the carbohydrate region 1022 cm^{-1} had an increased intensity in the conjugates compared to the native protein, indicating that glycation has taken place. After 10 h of heating the conjugates at 90°C, the IR bands 1634 and 1536, which belong to C = O and C-N stretching from amide I and amide II, were modified by the MR (Wang *et al.*, 2013). Thus, the FT-IR spectrum verified the formation of BSFL-Glu conjugates, mainly by C-N covalent bonding. Edible insect protein incubated for 10 h had undergone a significant unfolding which caused a change in the structure of the conjugates. It is

expected that the chemical changes accompanying the MR in BSFL-Glu conjugates would lead to several changes in the mid-IR spectrum as a result of depletion or consumption of some functional groups, including NH_2, especially from lysine and the formation of others. These changes in molecular structure as influenced by the heating temperature and time are expected to cause a change in the functional properties of the resulting conjugates. The observations of this study are in agreement and further supports previous findings that FT-IR can be used as a tool to probe the structural modifications of protein-sugar systems due to Maillard conjugations (Wang *et al.*, 2013; Jia *et al.*, 2020; Zhang *et al.*, 2020). Therefore, the changes in the protein structure following glycation corresponded with the results obtained from thermal stability measurements. Most notably, this is the first study to our best knowledge to investigate the conjugation of BSFL protein with glucose via the Maillard reaction and the analysis thereof with FT-IR, and thus provide valuable and unique information towards the ultimate adoption of conjugated edible insect proteins by the food industry as alternative functional food ingredients with beneficial techno-functional properties.

5.4.5. Principal component analysis

Principal component analysis (PCA), a commonly used unsupervised pattern recognition chemometric tool, was performed to assess the similarities and differences between heat-treated native BSFL protein and BSFL-Glu conjugates. In addition, it was used to further extract the spectral changes due to the Maillard reaction as a function of reaction temperature and time using a multivariate statistical approach. In this case, PCA essentially breaks the large dataset down into orthogonal components that describe the variance in the dataset and allows one to examine only the spectral differences. Figure 5.6 illustrates the PCA score plots (top) and loadings plots (bottom) of native BSFL and BSFL-Glu conjugates. PCA of the spectral region between proteins and sugars (1800 – 600 cm^{-1}) was performed on the native BSFL and BSFL-Glu synthesised by heating at 50, 70 and 90°C, respectively. The score plots can be used to explain patterns and similarities of the samples, such plots show up groupings of similar spectra and consider samples to be similar if they lie closer to each other and dissimilar if they are apart from each other. PCA score plots were able to display discrimination of the native BSFL protein and BSFL-Glu conjugates.

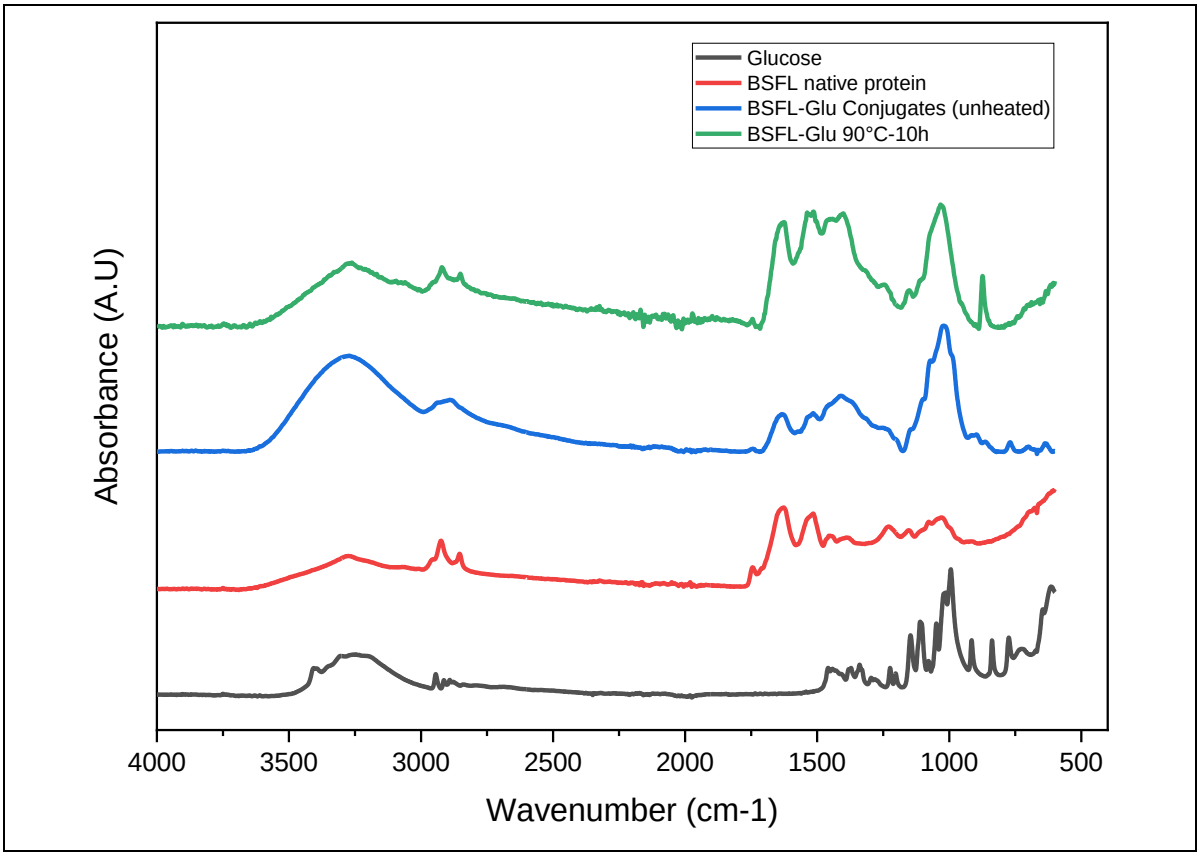


Figure 5.5 Attenuated total reflectance Fourier transform mid-infrared spectroscopy of glucose, native BSFL protein, BSFL-Glu conjugates (unheated) and BSFL-Glu 90°C – 10 h.

In all cases, the variance explained by the PC1 and PC2 was above 80% (Figure 5.6). At 50°C PC1 (70%) and PC2 (21%) had an accumulated total variance of 91%, at 70°C PC1 (83%) and PC2 (13%) had an accumulated total variance of 96% and at 90°C PC1 (87%) and PC2 (8%) had an accumulated total variance of 95% and these indicate an acceptable amount of the sample's variability explained by the respective PCAs. With reference to Figure 5.6 A – C, it can be observed that native BSFL protein and BSFL-Glu samples are diametrically opposed through PC1. Thus the greater distinction between the samples was achieved due to the higher reaction rate.

The loadings plot (Figure 5.6 D – E), show the coefficient of the linear combinations associated with each PC. One can make correlations between the wavelengths and the loading regarding the principal components. It is vital to note that variables of large loadings lie away from the origin and variables of little importance lie near the origin. The main absorbance peaks which contribute to the discrimination between the samples are those related to the protein content, such as the amide I, II, and also the carbohydrate related bands at 1230 cm^{-1} and between 1160 and 1000 cm^{-1}. Table 5.2 displays the loadings for the first two components from PCA, indicating which of the frequencies had more influence on the discrimination of the native BSFL and BSFL-Glu conjugates. With reference to PC1, the influence was mainly due to vibrations in the amide I and amide II bands. Moreover, PC2 is influenced by C=O stretch at 1626 – 1576 cm^{-1} and CH_3 bending vibrations. This confirmed that conformational/structural changes occurred due to the Maillard reaction.

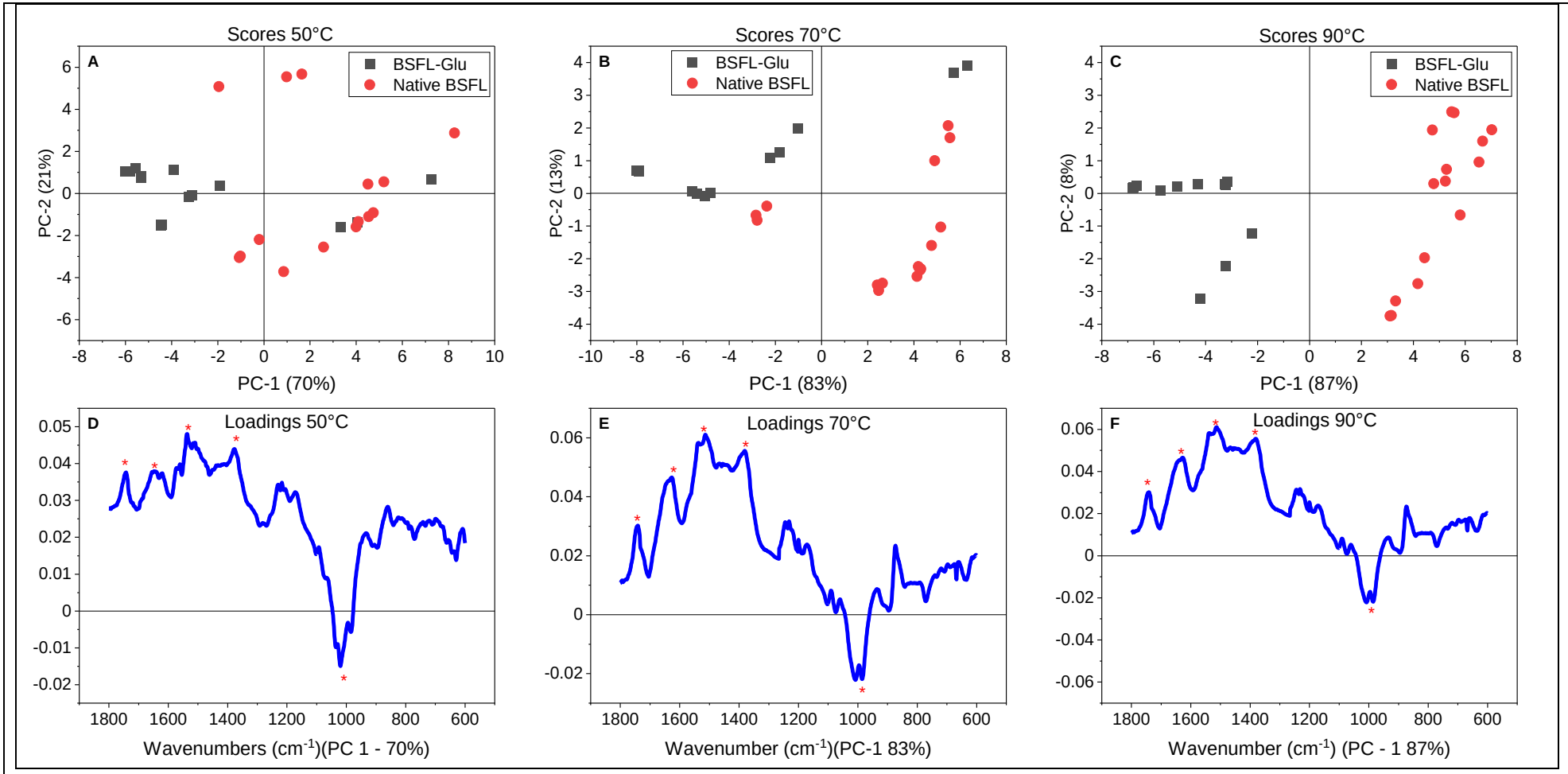


Figure 5.6 PCA score plots (A, B and C) and loadings (D, E and F) of native BSFL (red circles) and BSFL-Glu conjugates (grey squares) heated at 50, 70 and 90°C.

Table 5.2 Original variables (wavenumbers) from PCA with more impact on the first two principal components and the vibrational modes associated with.

Principal component	Order of relevance	Band (from - to)	Max band	Associated to
PC_1 – 50°C	1	1542 – 1521	1537	Amide II region of proteins
	2	1663 – 1612	1648	Amide I region of proteins
	3	1792 – 1701	1743	C=O stretch
	4	1399 – 1336	1377	Amide III region of proteins
	5	1041 – 1000	1018	C–O stretching vibration of carbohydrate
PC_1 – 70°C	1	1526 - 1501	1537	Amide II region of proteins
	2	1671 – 1699	1627	Amide I region of proteins
	3	1423 – 1370	1381	Amide III region of proteins
	4	1794 –1706	1741	C=O stretch
	5	1021 – 994	1007	C–O stretching vibration of carbohydrate
PC_1 – 90°C	1	1670 - 1590	1627	Amide I region of proteins
	2	1526 - 1501	1513	Amide II region of proteins
	3	1774 – 1704	1741	C=O stretch
	4	1021 – 994	1006	C–O stretching vibration of carbohydrate
	5	994 – 979	984	C–O stretching vibration of carbohydrate

Principal component	Order of relevance	Band (from - to)	Max band	Associated to
PC_2 – 50°C	1	1789 – 1704	1729	C=O stretch
	2	1626 – 1576	1596	N-H bend, C-N stretch
	4	1370 – 1341	1353	CH_3 bending vibrations
	3	1241 – 1180	1217	Stretching vibration of C-O group (1228 and 1155 cm^{-1})
PC_2 – 70°C	1	1789 – 1704	1738	C=O stretch
	3	1681 – 1575	1591	N-H bend, C-N stretch
	2	1375 – 1335	1365	CH_3 bending vibrations
	4	1241 – 1180	1217	Stretching vibration of C-O group (1228 and 1155 cm^{-1})
PC_2 – 90°C	1	1780 – 1703	1739	C=O stretch
	2	1393 – 1340	1365	CH_3 bending vibrations
	3	1238 – 1194	1218	Stretching vibration of C-O group (1228 and 1155 cm^{-1})
	4	1044 - 978	1021	C–O stretching vibration of carbohydrate

5.4.6. SIMCA native and glycated BSFL protein classification

Soft independent modelling of class analogy (SIMCA), a supervised chemometric classification tool, was used to establish the best classification model for discrimination between native BSFL protein and BSFL-Glu conjugates based on PCA. The PCA analysis revealed that the largest differences between native BSFL protein and BSFL-Glu conjugates were due to the alterations in the amide I and amide II region 1800 – 1400 cm^{-1}. Therefore, subsequent PCA analysis focused only on the 1800 – 600 cm^{-1} region. The training data set (60 samples) was able to classify native and BSFL-Glu samples and achieved 100% sample classification efficiency. This can be visualised using the Coomans plot (Figure 5.10). The Coomans plot is useful to assign whether a set of unknown value samples is similar to a group of known measured samples. The model was then applied to the test (prediction) samples (30 samples). From the Coomans plot in Figure 5.11, it can be seen that there were no samples in the lower left quadrant. This indicates that in this model there were no samples that were classified as inconclusive by the model. As can be seen in Table 5.3 only 6 samples could not be allocated to the correct class (two from class 1 and four from class 2). Thus, the proposed approach was able to achieve an accuracy of 91.2% specificity of 100% was obtained. Table 5.4 exhibits the class and global performance parameters for the prediction stage given by SIMCA. An excellent classification task was performed obtaining SEN, SPEC and PREC values of 100% for the two classes. Moreover, NER and ACC values of 100% confirm the observations given by class parameters and Coomans' plot (Figure 5.8). Therefore, the applied methodology provides a rapid and powerful tool for the successful discrimination of native and glycated BSFL samples. This study describes for the first time the application of SIMCA in heated native BSFL protein and BSFL-Glu conjugates and therefore paving way for further understanding of conjugation with edible insect protein with glucose.

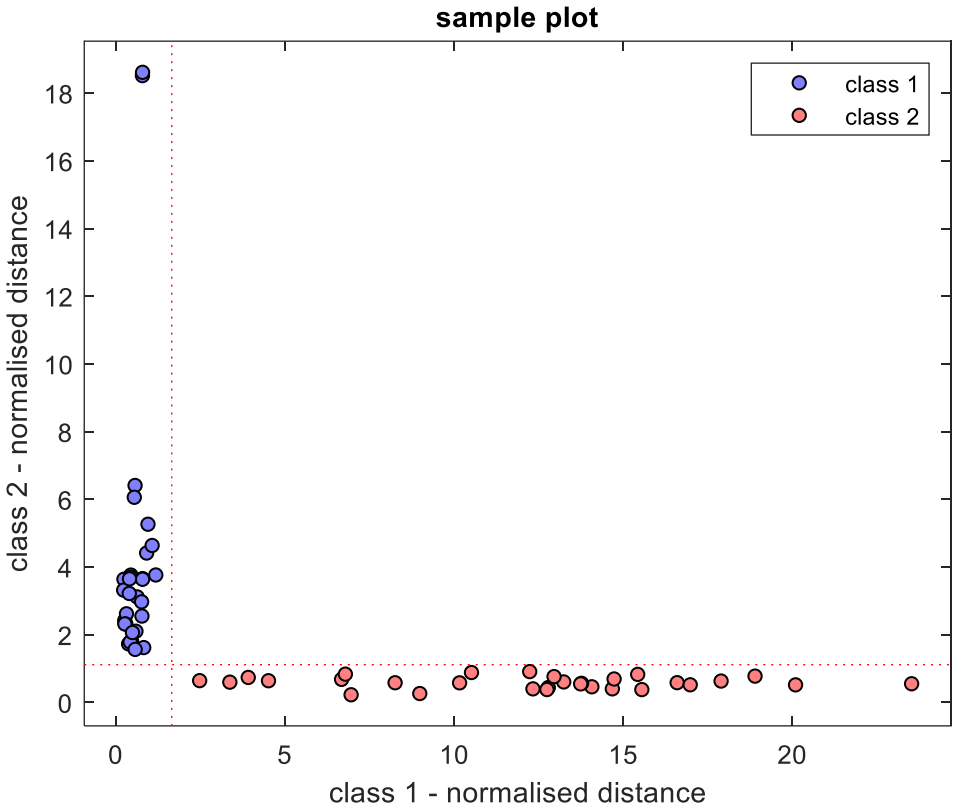


Figure 5.7 Coomans plot training step for classification of heated native BSFL protein (class 2) and BSFL-Glu conjugates (class 1) in the spectral region 1800 – 600 cm^{-1} (95% confidence interval).

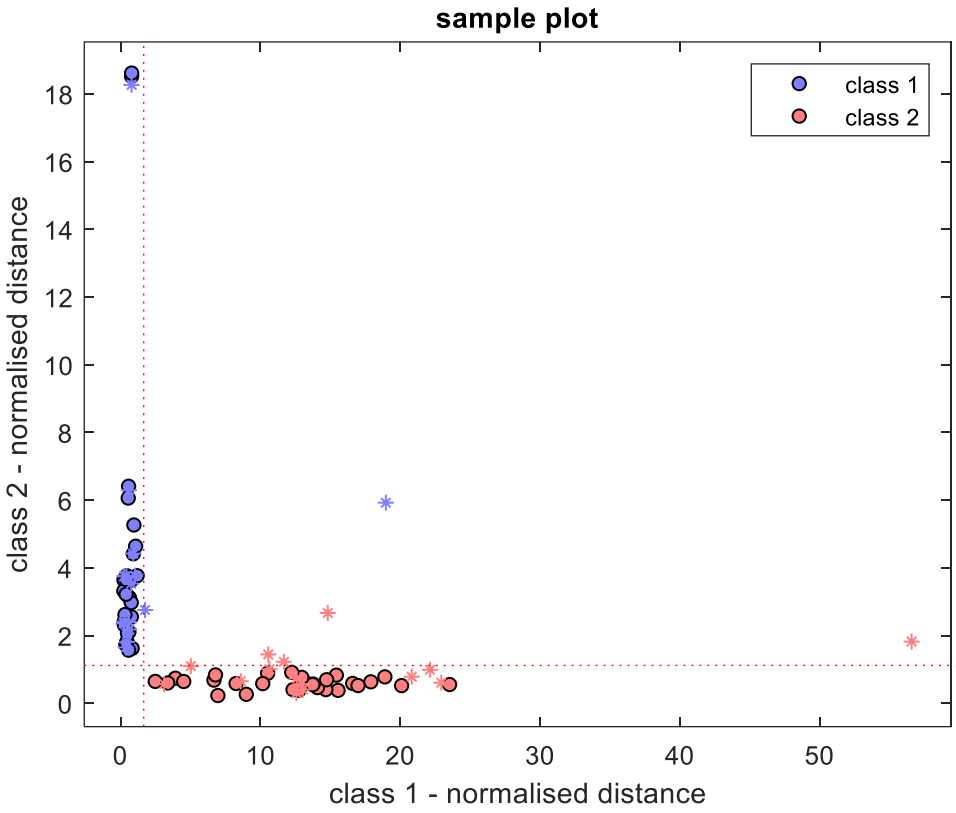


Figure 5.8 Coomans plot training prediction step for classification of heated native BSFL protein (class 2) and BSFL-Glu conjugates (class 1) in the spectral region 1800 – 600 cm^{-1}(95% confidence interval).

Table 5.3 Confusion matrix from the prediction stage using SIMCA.

Real/prediction	**Class 1 (C1)**	**Class 2 (C2)**	**Not assigned**
Class 1	13	0	2P2 = (BSFL-Glu50-2h) P3 = (BSFL-Glu 50-4h)
Class 2	0	11	P21 = (BSFL 90-10h) P26 = (BSFL 50-6h) P28 = (BSFL 50-2h) P30 = (BSFL 90-2h)

Table 5.4 Class and global performance parameters from the prediction stage by SIMCA.

	Class Performance Parameters		**Global Perf Parameters**	
	Class 1	**Class 2**		
Sensitivity	100	1.00	Non error rate	1.0
Specificity	1.00	1.00	Accuracy	1.0
Precision	1.00	1.00	Error	0.0
Non assigned	0.15	0.36		

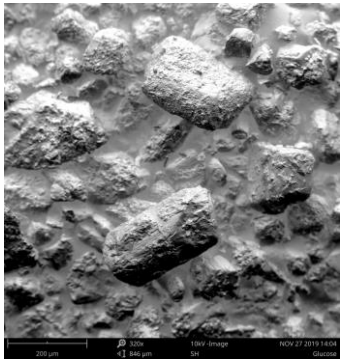
5.4.7. Microstructure analysis of native and glycated BSFL protein

The microscopic structure of food ingredients largely influences the techno-functional properties of food systems. Hence, knowledge of the protein surface morphology is vital to understanding changes taking place in heat-treated native and conjugated BSFL protein. The surface morphology of the unheated (glucose and native BSFL protein) and heated (native BSFL protein and BSFL-Glu conjugates) samples was examined using scanning electron microscopy at 320 X magnification. In the case of unheated glucose, large particles which exhibit blocks with slightly roughened surfaces were observed (Figure 5.2). In contrast, native BSFL protein showed non-homogeneous flaky, thinner sheets with partially dented surfaces. In this study, the non-purified BSFL protein obtained using alkaline and acid precipitation had a protein content of 73.5% (see discussion in chapter 3, section 3.4.1) and thus could contain other non-protein components. The application of SEM was useful in clearly distinguishing the surface morphology of the initial reactants (glucose and BSFL protein). Casein and whey protein concentrate were analysed with the view to compare with native BSFL. Casein exhibited a structure with wrinkled surfaces while whey protein concentrate appeared spherical with dimpled surfaces. Both these animal (dairy-based) protein structures which are commonly used in food products showed distinct surface characteristics compared to the native BSFL. These observations or differences in surface morphology could further be used to understand the functionalities of these proteins to develop tailor-made food ingredients with novel techno-functional properties to be incorporated in food products such as emulsions, sausages or bakery products.

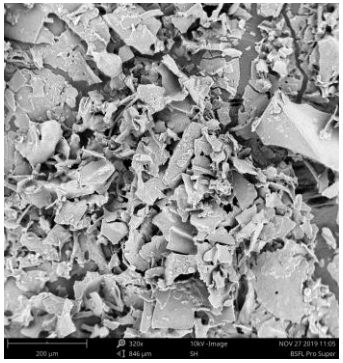
In the case of native proteins heated at 50°C, a rough, uneven, compact and continuous surface was observed between 2 – 6 h. At longer heating times, especially at 8 and 10 h, respectively, large spherical particles were observed (Figure 5.3). The appearance of these structures could be attributed to the unfolding or denaturation of the native BSFL protein at prolonged heating times. BSFL-Glu conjugates had considerable roughness, less compact (more spread) compared to native BSFL protein. This loosened (less compact) surface structure with cavities is due to the wet-heat treatment which resulted in attached glucose on the surface of the BSFL protein resulting in conformational change. BSFL-Glu conjugates heated at 10 h also exhibited spherical particles similar to those observed in the native BSFL protein indicating

unfolding of the structure at prolonged heating times and these results are consistent with the Zeta potential and FT-IR analyses which confirmed alteration of the protein structure through particle charge and functional groups, respectively. This phenomenon was similar to the findings reported by Wang *et al.* (2020) in whey protein isolate conjugated with inulin under wet-heat conditions. In general, a similar pattern was observed for samples incubated at higher reaction temperatures (Appendix E and F). Therefore, it can be said that the Maillard reaction results in protein unfolding and reduces the protein molecular aggregation.

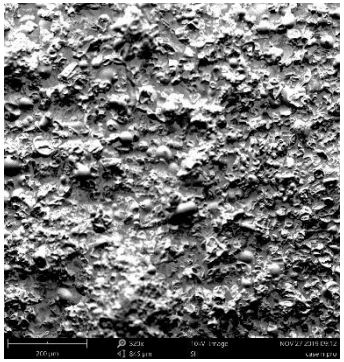
Native Glucose



Native BSFL protein



Native Casein protein



Native whey protein isolate

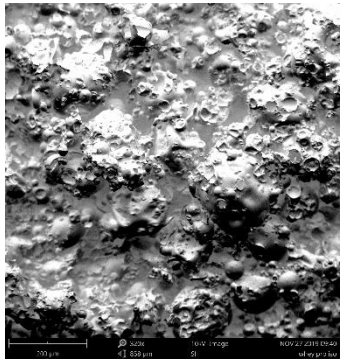


Figure 5.9 Scanning electron micrographs of native (unheated) glucose (left) and native BSFL protein (right). Images are displayed at 320 X magnification (scale bars correspond to 200 µm).

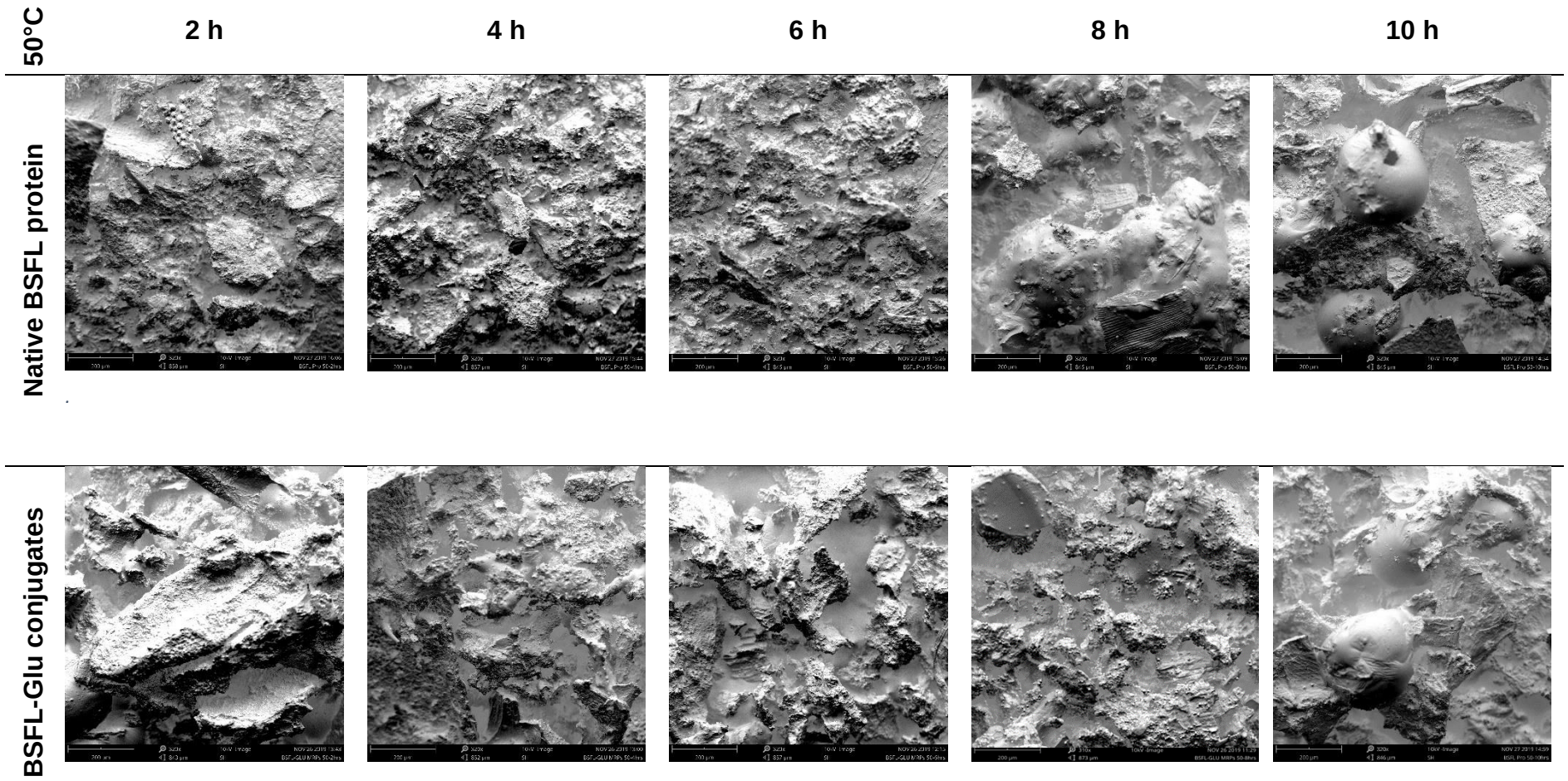


Figure 5.10 Scanning electron micrographs of heated native BSFL protein (top) and BSFL-Glu conjugates reacted at 50°C as a function of time. Images are displayed at 320X magnification (scale bars correspond to 200 µm).

5.5. Conclusions

In this study, the feasibility of using ATR FT-IR combined with chemometric methods (PCA and SIMCA) to study the structural modification in native BSFL and BSFL-Glu conjugates were investigated. SIMCA proved to be a powerful, rapid and reliable tool for the classification of native and glycated BSFL proteins. Moreover, the apparent changes in the protein charge via the zeta potential, surface modifications revealed by the SEM and thermal properties indicate that glucose was successfully conjugated with the black soldier fly larvae protein. SEM also showed that surface structure became looser and more porous after glycation with glucose. Compared with the native BSFL protein, TGA analysis revealed that BSFL-Glu conjugates showed greater thermal stability at 90°C. These structural modifications are vital in influencing the changes in protein techno-functionality in food applications. The techniques used were complementary and provided different levels of useful information to probe the structural modifications due to glycation. Taken collectively, the findings of this study clearly illustrate that the degree of glycation results in structural changes to the native insect protein and thus conjugation hold a possibility for delivering novel food structures with enhanced functionalities and expand the application of BSFL-Glu conjugates in food applications.

5.6. References

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CHAPTER 6: Summary and Conclusions

Black soldier fly (*Hermetia illucens*), an insect rich in protein and lipids is one of the most promising edible insect species for incorporation in food products due to the environmental benefits, coupled with available technology for their rearing. The need to increase the global consumption of edible insects is paramount due to the high demand for protein sources for human consumption. To meet the increasing protein demand of the growing world population to achieve the 2030 Agenda for SDGs, notably SDG-2 Zero Hunger, insects have emerged as promising alternative protein sources. Very little information from a food science and technology point of view is available on the characteristics and functionality of extracted insect proteins and structural modifications due to conjugation via Maillard reaction. This study aimed to provide an in-depth insight into the techno-functional, antioxidant and structural properties of native and glycated BSFL protein with the view to find alternative novel food ingredients.

The first research objective (chapter 3) was the preparation, isolation or extraction and characterisation of BSFL protein concentrates using alkali and isoelectric precipitation extraction and alkaline extraction. The proximate composition, colour, bulk density and amino acid profile of BSFL flours and protein concentrates were established. The hypothesis that extraction of BSFL proteins with alkaline and acid extraction will have high crude protein content was accepted. Crude protein content and amino acid composition were determined and compared to well-known protein sources (cow's milk and egg).

The second research objective (Chapter three)was to establish the techno-functional properties of BSFL flours and protein concentrates. The hydration properties (oil and water binding capacity), protein solubility, emulsification and foam properties demonstrated that BSFL flour and protein concentrates can be useful in multiple food applications in formulated food products. Therefore, BSFL flours and protein concentrates are expected to compete successfully with other traditional sources or insect-derived ingredients in the market.

The third research objective (Chapter 3) was to describe the structural modifications in BSFL flours and protein concentrates. The zeta potential of BSFL protein concentrates was pH-dependent and the isoelectric point was established at

pH 4.5. This is of critical essence in food systems since the isoelectric point tends to coincide with minimum solubility. The FT-IR spectra for BSFL protein concentrates revealed the presence of amide I (1600 – 1700 cm^{-1}) and amide II (1500 – 1600 cm^{-1}) bands with lower intensities compared to the BSFL flours indicating that the methods of protein extraction induced structural changes which consequently influences the techno-functional properties.

The second research objective (Chapter 4) aimed to investigate the effect of conjugation temperature (50, 70 and 90°C) and time (2 – 10 h) on the antioxidant activity and techno-functional properties of BSFL protein reacted with glucose. In general, BSFL protein conjugates synthesised at higher temperature and time combinations (90°C – 10 h) exhibited superior antioxidant activities. Therefore, the hypothesis that BSFL-Glu conjugates will exhibit higher antioxidant properties compared to the native BSFL protein is accepted. In addition, BSFL-Glu conjugated at 90°C showed significantly higher emulsion stability and capacity due to the Maillard reaction. This suggests that these conjugates have the potential to be used as novel functional food ingredients in food processing.

The aim of research chapter five was to characterise the native BSFL protein and BSFL-Glu conjugates prepared via the Maillard reaction using novel analytical techniques, ATR coupled with a chemometric approach (PCA and SIMCA). The complementary techniques such as DSC, TGA, SEM and zeta potential were applied to further probe the structural modifications due to the Maillard reaction. The zeta potential of BSFL-Glu conjugates heated at 90°C (greater than 30 mV) revealed that they can be potentially used as a stabilizer, preventing flocculation in food applications. Moreover, BSFL- Glu conjugates prepared at 90°C (6 and 10 h) exhibited greater thermal stability compared to other studies when subjected to TGA analysis. Soft independent modelling of class analogy (SIMCA), a supervised chemometric classification tool, was used to establish the best classification model for discrimination between native BSFL protein and BSFL-Glu conjugates based on PCA. The applied model was able to distinguish between native BSFL-Glu and BSFL conjugates with an accuracy of 91%. Although much more research on insect proteins and the effect of glycation is needed, the present study gives a first insight into its potential for food use. The following conclusions can therefore be drawn from this study:

1. Alkali solution and acid precipitation is a suitable method to extract edible proteins from BSFL.

2. BSFL flours and protein concentrates have beneficial physico-chemical and functional properties which are comparable or in some instances superior to commercial alternatives.
3. BSFL protein concentrates have desirable physico-chemical and techno-functional properties and can be promoted as novel functional ingredients for use in food applications.
4. BSFL-Glu conjugates synthesised at 90°C have superior antioxidant activity and improved functionality.
5. Emulsions stabilised with BSFL-Glu are more stabilised compared to native BSFL protein.
6. The surface charge, functional groups and thermal stability of native BSFL protein were altered due to glycation with glucose and resulted in conjugates with improved thermal stability which can be used as novel functional ingredients.
7. SICMA can be used as a classification tool to distinguish between native BSFL and BSFL-Glu conjugates.

CHAPTER 7: Recommendations

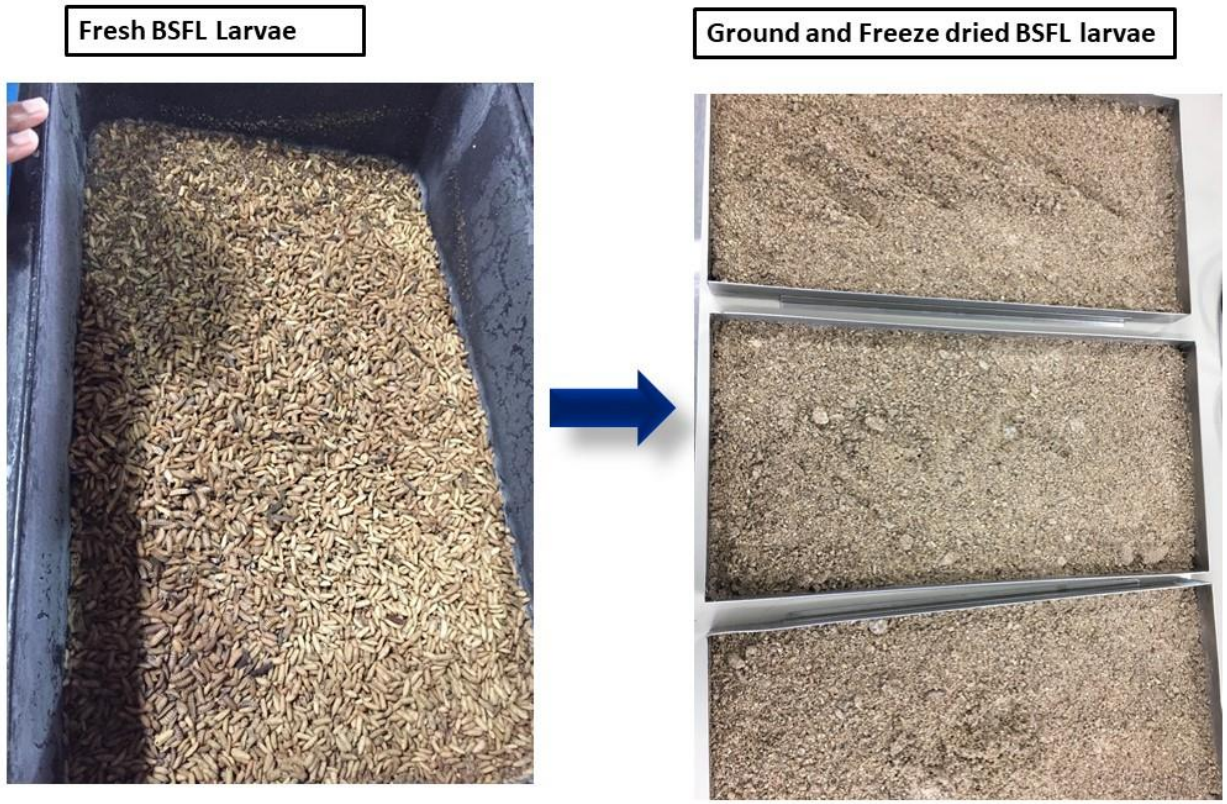
In this dissertation, BSFL flours and protein concentrates were characterised and established. The physico-chemical, techno-functional, antioxidant and structural properties of native BSFL protein and BSFL-conjugates were established with the view to illustrate the potential use as novel functional ingredients. However, some challenges for food technologists and researchers in the food industry remain namely:

1. The performance of BSFL flours in bakery goods such as biscuits and rusks should be further investigated and consumer acceptance be established.
2. Investigate how to obtain higher purity protein fractions using novel techniques such as pulsed electric field (PEF) and to study the functional properties of these protein fractions.
3. Design an insect protein production process on an industrial scale using sustainable procedures, e.g. in relation to water/energy use and feasibility.
4. Consumer acceptance of edible insect-derived food products or foods incorporating BSFL-Glu conjugates.

It is recommended that further work needs to be done to fully characterise the BSFL protein in terms of potential allergenicity, molecular weight (using Sodium dodecyl sulphate–polyacrylamide gel electrophoresis), tertiary and quaternary structure (using circular dichroism) and rheological properties. In terms of the conjugates, the use of longer temperature-time combinations should be explored. Further work should also focus on the elucidation of the Maillard reaction pathways with insect proteins using analytical techniques such as UPLC (ultra-performance liquid chromatography), NMR (Nuclear Magnetic Resonance) spectroscopy and SEM (Scanning electron microscopy) at higher magnification. Furthermore, regarding the food regulations in South Africa, regulations should be explored and developed towards the utilisation of edible insects or insect-derived components in food products.

Appendix A

Fresh BSFL Larvae (Left) and ground and freeze-dried BSFL larvae

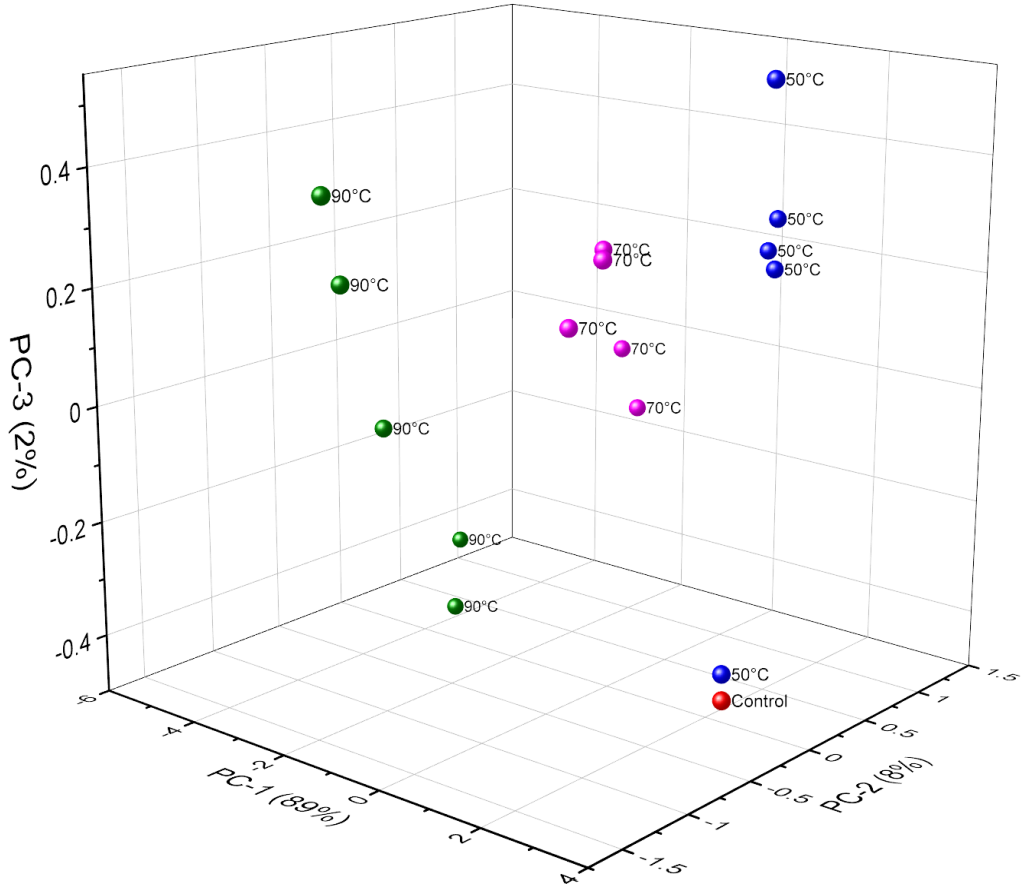


Appendix B

Freeze-dried BSFL flour –FD, defatted BSFL flour – DF, alkaline and acid precipitation extraction BSFL protein concentrate – PC1, alkaline extraction BSFL protein concentrate – PC2.

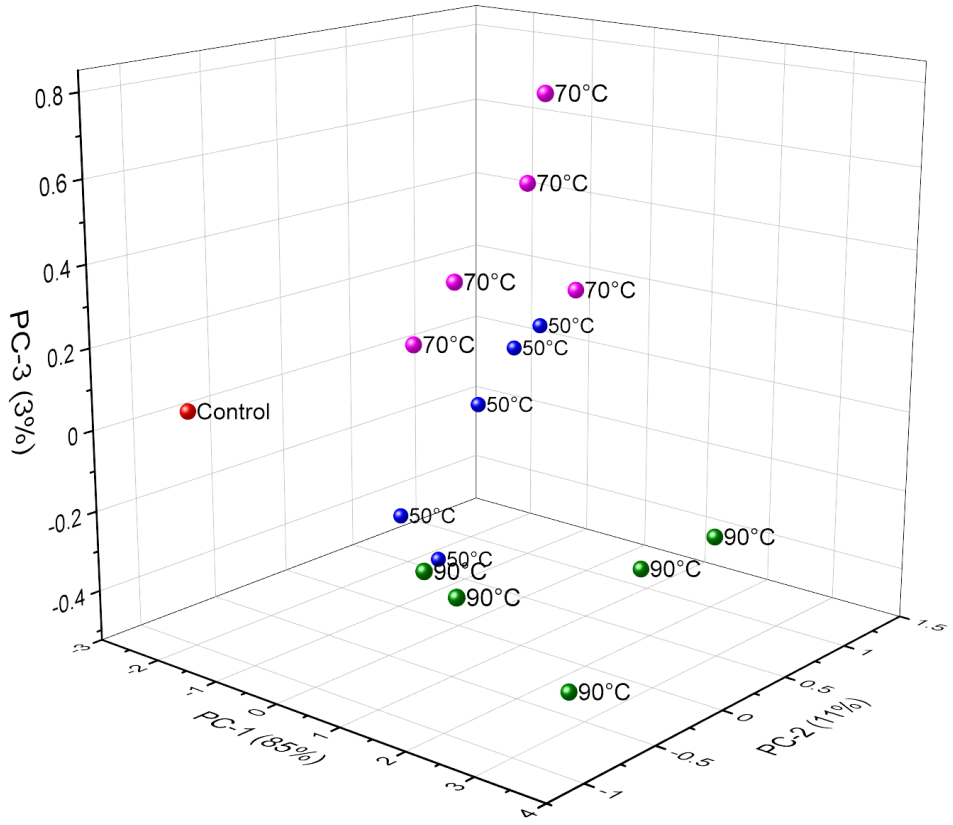
Appendix C

Three dimensional (3D) PCA score plots of antioxidant properties of BSFL-Glu conjugates heated at 50, 70 and 90°C showing PC1 vs. PC2 vs. PC3.



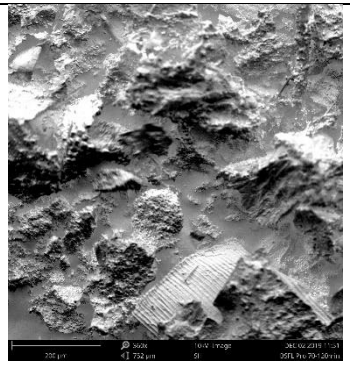
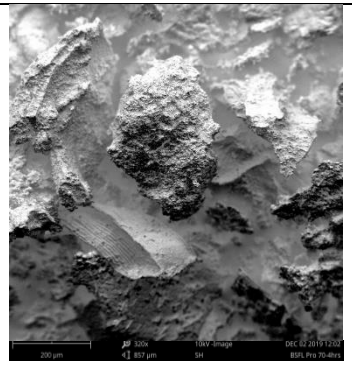
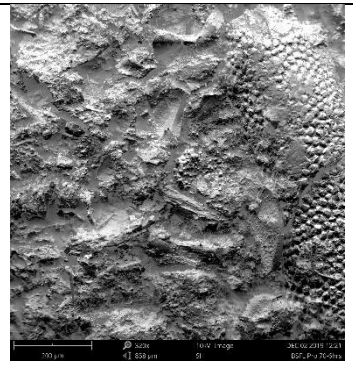
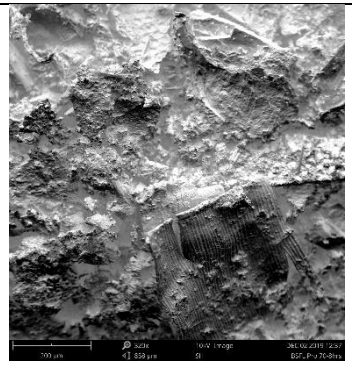
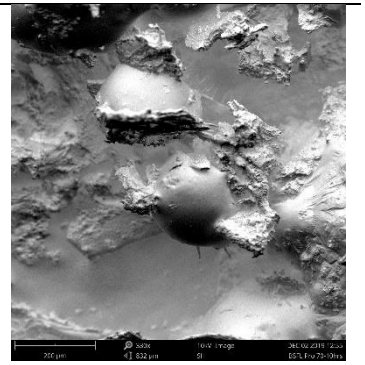
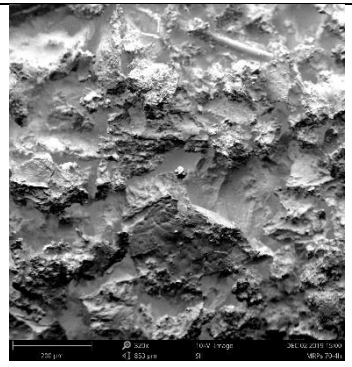
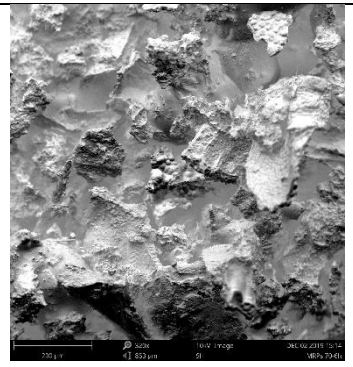
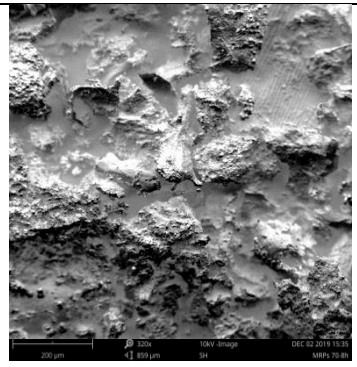
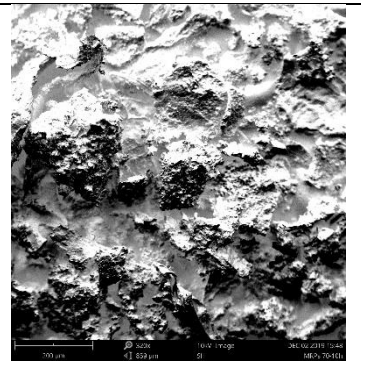
Appendix D

Three dimensional (3D) PCA score plots of antioxidant properties of BSFL-Glu conjugates heated at 50, 70 and 90°C showing PC1 vs. PC2 vs. PC3.



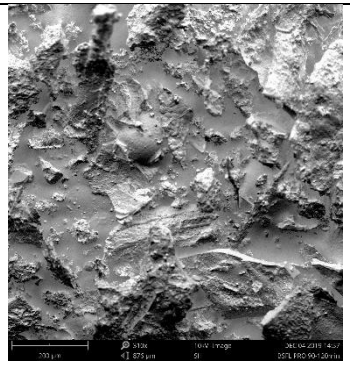
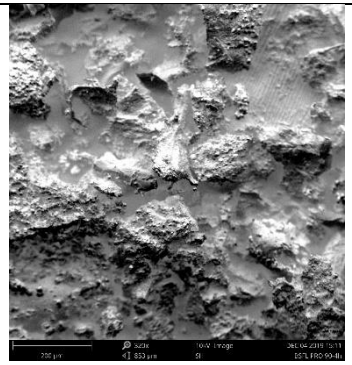
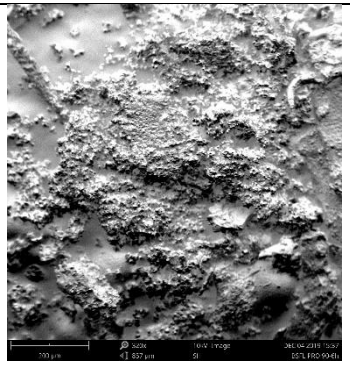
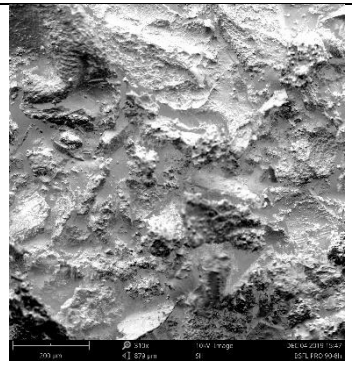
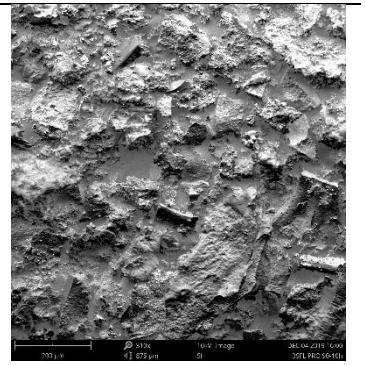
Appendix E

Scanning electron micrographs of heated native BSFL protein (top) and BSFL-Glu conjugates reacted at 70°C as a function of time. Images are displayed at 320X magnification (scale bars correspond to 200 µm).

70°	2 h	4 h	6 h	8 h	10 h
Native BSFL protein					
BSFL-Glu conjugates					

Appendix F

Scanning electron micrographs of heated native BSFL protein (top) and BSFL-Glu conjugates reacted at 90°C as a function of time. Images are displayed at 320X magnification (scale bars correspond to 200 µm).

90°	2 h	4 h	6 h	8 h	10 h
Native BSFL protein					
BSFL-Glu conjugates