



PURIFICATION AND CHARACTERISATION OF THE NATIVE BLACK SOLDIER FLY LARVA PROTEIN

by

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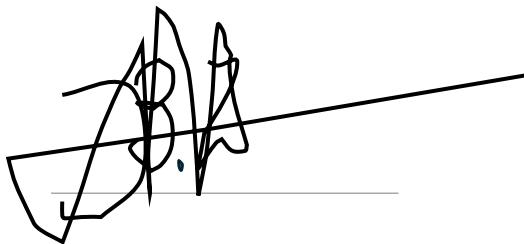
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DECLARATION BY AUTHOR

I, **Bongisiwe Zozo**, declare that the contents of this thesis are composed of my original work, and contain no material published or written by another person except where due reference has been made in the text. Furthermore, the thesis has not previously been submitted for academic examination towards any qualification and it represents my opinions and not necessarily those of the Cape Peninsula University of Technology.



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ABSTRACT

The larvae of the black soldier fly (BSF) are one of the promising edible insect species for industrial food production, showing great potential as a sustainable alternative to conventional protein sources. To fully utilize the potential of insect protein as food, it is important to refine and enhance its functional characteristics. This study aimed to investigate the effects of various purification and characterization techniques, such as salting in/out, ultrafiltration, and ultrasound treatments, on native BSF larva proteins. The goal was to enhance their functional and nutritional properties for potential food-related applications.

Protein isolates were extracted using alkaline extraction and acid precipitation (AAF), salting-in (AAFSI), salting-out (AAFSO), and salting-in-out (AAFSISO) coupled with ultrafiltration. Additionally, BSF larval proteins were subjected to ultrasound treatment at varying power levels (150 W, 200 W, 250 W, and 300 W) and treatment durations (10, 20, 30, and 40 min). The physicochemical properties, such as colour change, amino acids, zeta potential and sulfhydryl group levels, were measured. Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), x-ray diffraction and circular dichroism (CD) were used to analyze protein structures, while functional properties like solubility, surface hydrophobicity, water binding capacity, oil binding capacity, foaming capacity, and foam stability were evaluated.

The extraction method had a significant impact ($p < 0.05$) on the crude protein content of the isolates. For example, the AAF extraction yielded 71.25% protein, which increased to 85.02% with the addition of salting-out-assisted treatment. Furthermore, the total essential amino acid content was 320.48 mg/g for AAF, 300.40 mg/g for AAFSI, 286.15 mg/g for AAFSO, and 296.88 mg/g for AAFSISO. These values all exceed the World Health Organization's recommended minimum of 277 mg/g for adult daily requirements. The zeta potentials of all samples were found to be significantly negative ($p < 0.05$), with values ranging from -9.81 to -26.86 mV, with no significant differences ($p > 0.05$) observed in the levels of exposed sulfhydryl groups between AAF and AAFSI proteins. FTIR of protein extracts derived from AAFSO and AAFSISO revealed significant N-H free stretching vibrations, indicating structural transformations confirmed by CD analysis. Furthermore, this study thoroughly examined the influence of alkaline extraction and isoelectric precipitation methods assisted by salting-in (1% NaCl) and salting-out (80% $(\text{NH}_4)_2\text{SO}_4$) techniques on the functional properties of BSF larvae proteins. The addition of $(\text{NH}_4)_2\text{SO}_4$ significantly ($p <$

0.05) improved protein solubility, particularly at pH 2, suggesting its potential use in acidic food systems. Correlation analysis demonstrated a significant positive relationship ($p < 0.05$) between various functional properties, including foaming capacity, foam stability, emulsification properties, solubility, and surface hydrophobicity, emphasizing their interconnected nature and potential applications in food formulations. Foaming capacity and foam stability showed a positive relationship with protein solubility at pH 4 ($r = 0.88$, $p < 0.05$; $r = 0.74$, $p < 0.01$, respectively).

Additionally, the impact of ultrasound treatment on enhancing the functional and nutritional attributes of BSF larval proteins was investigated. Ultrasound, a promising technique for modifying protein characteristics, was employed at different power levels (150 W, 200 W, 250 W, and 300 W) and treatment durations (10, 20, 30, and 40 min), inducing structural modifications and enhancing functional properties. Ultrasound treatment at varying power levels and durations significantly increased the lightness (L^*), solubility, surface hydrophobicity, water-binding capacity, oil-binding capacity, foaming capacity, and foam stability of BSF larval proteins. These improvements were most pronounced at higher ultrasound power and longer treatment times. P200T10 exhibited higher lightness ($L^* = 74.99$, $p > 0.05$) and reduced redness ($a^* = 2.55$, $p < 0.05$) compared to the other isolates. Optimal conditions, such as 200 W for 40 min (foaming) and 250 W for 10-20 min (emulsification), maximized these properties. Excessive treatment, however, could lead to protein aggregation and reduce functionality. FS showed a positive correlation with solubility ($r = 0.70$, $p < 0.001$), supporting the idea that ultrasound disrupts disulfide bonds and exposes sulfhydryl groups. However, FS and exposed SH exhibited weak or negative correlations with WBC ($r = -0.29$, $p < 0.05$) and OBC ($r = 0.35$, $p < 0.05$), suggesting that while these structural changes improve solubility, they may decrease binding capacities.

The integration of salting-out extractions, ultrafiltration, and ultrasound treatments effectively enhanced the functional and nutritional properties of BSF larval proteins. These techniques produced insect proteins with excellent nutritional quality and functional properties, making them suitable as sustainable functional ingredients for various food-related applications. Future research should focus on optimizing these extraction and treatment techniques to ensure scalable and commercially viable production of BSF larval proteins. This includes fine-tuning ultrasound parameters and exploring additional treatment combinations to maximize the potential of BSF larvae as a sustainable and functional protein source in the global protein market.

DEDICATION

I dedicate this thesis to a lineage of women who came before me: my late great-grandmother, uNombhayana Gqobho (MaNgethe); my grandmother, uNokruxeshe Zozo; and my mother, uNomsa Zozo.

Mom, you birthed a force to be reckoned with. Thank you for imparting the values of hard work and resilience.

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CAMAGU.

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RESEARCH OUTPUTS DURING CANDIDATURE

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LIST OF ACRONYMS

Abbreviation	Description
BSF	Black soldier fly
FTIR	Fourier transform infrared.
SEM	Scanning electron microscopy.
TGA	Thermogravimetric analysis
XRD	X-ray diffraction
SDS	Sodium dodecyl-sulphate
PAGE	Polyacrylamide gel electrophoresis
CD	Circular Dichroism
UV	Ultraviolet
ANS	1-anilino-8-naphthalenesulfonate
DSC	Differential scanning calorimetry
FAO	Food and Agriculture Organization
¹ H NMR	Proton nuclear magnetic resonance
OPA	Orthophthalaldehyde
Fmoc	9-fluorenylmethyloxycarbonyl
COVID	Coronavirus disease
DESA	Department of Economic and Social Affairs
SDGs	Sustainable Development Goals
UF	Ultrafiltration
DM	Dry Matter
MW	Molecular Weight
EFSA	European Food Safety Authority
EDTA	Ethylenediaminetetraacetic acid
EAA	Essential amino acids

LIST OF SYMBOLS

Symbol	Meaning	Unit
H_0	Surface hydrophobicity	relative value
kDa	Kilodalton	KDa
t	Time	min
T	Temperature	°C
W	Working solution volume	L

CHAPTER ONE

INTRODUCTION

*"Insects are the future of food, a sustainable solution to global hunger and malnutrition."
(Arnold van Huis, 2013)*

CHAPTER 1: INTRODUCTION

1.1. Background

According to the United Nations Food and Agriculture Organization (FAO), the global population is projected to reach nine billion people by 2050, leading to a substantial increase in food demand. The literature agrees that traditional protein sources cannot meet the estimated 260 million tons of protein required by 2050. Therefore, there is a need to investigate alternative sustainable and nutritious protein sources (FAO, 2013; McClements, 2019). In addition, the COVID-19 pandemic has highlighted the importance of food security, as many food processors and food supply chain stakeholders have been shut down, resulting in meat shortages and increased concerns about food insecurity.

Owing to their high protein content and the presence of all amino acids essential for human consumption, insects have received significant attention from the FAO as an underutilized sustainable protein source (van Huis, 2013a). Despite the fact that entomophagy, or the consumption of insects as food, is practiced by over two billion people worldwide, most developed countries and some urban areas in developing countries have severe objections to consuming edible insects because of the expectation of distaste and disgust about their nature and appearance. Studies have found that eating edible insects as milled powders and/or pastes in food products, as well as using their different functional fractions, such as proteins, fatty acids, and chitin, is more acceptable when they are disguised or invisible in food. People are increasingly experimenting with edible insects, not just eating them whole, but grinding them into powders or pastes, or using specific components like proteins, fatty acids, and chitin (Yi et al., 2013). Among these, the black soldier fly (*Hermetia illucens*) stands out as one of the most promising candidates for commercial protein production, and there's already a clear roadmap for introducing it into the human diet (Mintah et al., 2020). Depending on the source, BSF contains roughly 37-69% protein and 27-39% fat by dry mass (Zozo et al., 2022). Factors such as the type and quality of feed, larval density, and protein extraction can significantly influence the larvae's nutritional profile (Barragan-Fonseca et al., 2017). Processing BSF proteins into functional food additives is proving to be a smart strategy for boosting consumer acceptance. Various methods can be employed to extract high amounts of protein, leading to specific modifications in the protein structure or composition that can influence

its final functional characteristics, thermal stability, and potential applications, thermal stability, and use of the protein (Mintah et al., 2020; Bußler et al., 2016; Yi et al., 2013). Some researchers have shown enhancement on BSF protein extraction using conventional alkaline extraction followed by acid precipitation methods (Azagoh et al., 2016; Mintah et al., 2020; Purschke et al., 2018; Zozo et al., 2022), however, there is no research available on salt-assisted and/or ultrasonication techniques for BSF protein extraction. Defatting BSF meals has also been shown to enhance protein content, as highlighted by Mshayisa and Van Wyk (2021) and Zozo et al. (2022). Furthermore, Bußler et al. (2016) noted that the choice of protein extraction method can significantly influence techno-functional properties through alterations in protein composition.

Therefore, understanding the protein profile, referring to the composition and distribution of different types of proteins within a sample, along with protein functionality and overall quality, can be a crucial strategy to enhance consumer acceptance of insect-based ingredients in food formulation development. Although previous investigations have focused on the properties of insect proteins, information on the extraction of BSF larval proteins using alternative methods remains limited (Bose et al., 2023). We still have a lot to learn about BSF protein extracts and how they work, but the more we understand them, the easier it will be to use them in food products.

1.2. Statement of the research problem

With the global population projected to hit 9 billion by 2050 (DESA, 2009), the demand for food-grade proteins will only intensify. This means we need to look beyond conventional livestock and explore alternative sources. Edible insects, such as proteins from BSF larvae, are a promising option; not only do they offer high nutritional value, but they also convert feed more efficiently than traditional protein sources (Lu et al., 2022). In response to the growing global demand, many countries and international bodies, including FAO, have actively promoted the development of the insect industry. While a considerable amount of research has focused on extracting protein from BSF larvae through alkaline extraction followed by acid precipitation (Bußler et al., 2016; Mintah et al., 2020), far less is known about the purification of insect proteins, particularly those from BSF larvae, using techniques such as salting-in, salting-out, and ultrasound. So, for food applications, it's important to learn more about the chemical composition, structure, and thermal properties of purified native proteins from BSF larvae using these extraction methods. This

information is very important for solving malnutrition caused by a lack of protein and making sure that the world's growing population has access to more sustainable and healthy food.

1.3. Broad objective

This study aimed to purify the native BSF larvae protein using different extraction methods and characterize the extracts using chemical analyses, structural analyses, thermal analyses, and techno-functional techniques to obtain more information on BSF larvae proteins to assist with future food applications.

1.4. Specific objectives of the research

The specific objectives of this study were as follows:

- a) To examine the effect of salting-in and salting-out coupled with ultrafiltration extraction on the structural, physicochemical, and thermal properties of BSF larval proteins.
- b) Compare the purity (protein content) and quality (amino acid composition), molecular weight distribution, and an overview of the secondary structure of the native protein extracts with those of traditional known proteins (controls).
- c) To determine the effect of alkaline-acid precipitation and salting-in/out-assisted protein extraction on the physicochemical and techno-functional properties of BSF larval isolates.
- d) To investigate how ultrasound and salt-assisted alkaline isoelectric precipitation influence the structural and functional properties of BSF larvae proteins and to identify the mechanisms behind these effects.

1.5. Research hypotheses and questions.

The hypotheses and questions posed in this study are as follows:

Hypothesis 1: The structure, purity, and yield of insect proteins are influenced by numerous factors, among which the method of extraction is a critical one.

- o What is the effect of the salt-assisted extraction method on the structure, purity, yield, and functionality of BSF larvae proteins?

Hypothesis 2: The application of ultrasound significantly increases the protein yield, enhances extraction efficiency, promotes the recovery of targeted compounds, and improves their functionality, which is well established for various protein extraction methods, including those involving insect proteins.

- o How does the use of ultrasound impact the purity, yield, functionality, and structure of BSF larvae proteins?

1.6. Significance of the study

The primary objective of this research is to address the issue of declining protein sources for the growing population and to develop alternative protein options. It is crucial to establish a safe and sustainable food system, as outlined in the Sustainable Development Goals (SDGs) for 2030, which aim to eliminate hunger, achieve food security and improved nutrition, and promote sustainable agriculture. Proteins are the second most important macronutrient, after calories, that are required for human survival. Traditional animal protein sources are resource-intensive, making protein production a significant challenge (Calicioglu et al., 2019). Thus, there is an urgent need for alternative protein sources that are nutritionally similar or superior to those derived from conventional sources. As a result, the search for sustainable proteins to support global food security has led to increasing research into the use of edible insects as an alternative food source for human consumption (Meyer-rochow, 1975; van Huis, 2013a). Insects are a good source of protein because they have all the amino acids that humans need (Bosch et al., 2014; Chakravorty et al., 2014; Zozo et al., 2022).

Eating whole insects isn't common in many developed countries and some cities in developing countries. Extracting insect proteins for human food could help people accept eating insects more, and these proteins could then be used to make a variety of food products. This is especially important in countries where people don't usually eat insects (Bußler et al., 2016; Yi et al., 2013). It has been shown that established methods like salt and ultrasound extractions can improve the efficiency of protein extraction and keep the proteins' functions (Okur et al., 2017; Jiang et al.,

2021). But no one has looked into using these methods to get proteins from BSF larvae. It is important to look into different ways to extract proteins from BSF larvae and to explain the structural and functional properties of the proteins that are obtained in order to make sure that they are safe and effective as a protein source. This will enable the development of highly purified protein products for human consumption.

1.7. Delineation of the study

In this study, we explored the impact of various extraction methodologies on the purification of proteins derived from BSF larvae. It is pertinent to emphasize that the investigation focused solely on BSF larvae and did not extend to alternative insect species. Our work expanded on prior research by employing ultrasound extraction with time and power as the main factors (Huang et al., 2020; Mishyna et al., 2019).

1.8. Thesis outline

This thesis is divided into seven chapters, with three of them dedicated to the study objectives (Chapters 4 to 6).

Chapter 1 (the current chapter) presents the fundamental background, motivation, and objectives to provide context for the study.

Chapter 2 offers a literature review of the relevant knowledge regarding edible insect protein extraction. The impact of protein extraction on insect proteins is discussed, and the characteristics of some insect proteins (structural and functional) are highlighted. Additionally, the factors influencing protein extraction, such as the larval stage, feeding diet, and killing method, are explored. The chapter concludes by identifying gaps and research problems that this study aims to address.

Chapter 3 describes the procedures and methods employed in this thesis, including the typical set-up of the experimental rig, description of materials, mathematical tools, analytical solution techniques, and general characterization methods used for the analysis of products throughout the thesis.

Chapter 4 examines the influence of salting-in and salting-out extraction coupled with ultrafiltration on the structural, physicochemical, and thermal characteristics of BSF larvae protein isolates. Purity and yield were determined, and physicochemical analysis, such as colour and thermal stability, surface charge, and protein size using sodium dodecyl-sulphate polyacrylamide gel electrophoresis, was employed. Structural analysis was conducted using amino acids, circular dichroism, X-ray diffraction, and Fourier Transform Infrared Spectroscopy.

Chapter 5 investigates the consequences of salt treatment on the functional properties of BSF larvae proteins. The study provides a basis for further examination of these isolates for applications in food and nutraceuticals, as well as an analysis of the differences between the measured components of the isolates. The study evaluates functional properties such as protein solubility, surface hydrophobicity, bulk density, water/oil binding capacity, foam properties, and emulsion properties.

Chapter 6 assesses the effects of ultrasound/salt-assisted alkaline isoelectric precipitation on the protein extraction yield, amino acid composition, secondary structures, solubility, emulsifying properties, water holding capacity, oil binding capacity, sodium dodecyl sulphate polyacrylamide gel electrophoresis, sulfhydryl content, surface hydrophobicity, tertiary structure, and thermal stability of BSF larvae proteins.

Chapter 7 summarizes the main findings of this research and provides recommendations for purification processes and future studies to expand on the knowledge gained through this research.

A schematic illustration of the thesis structure is depicted in Figure 1.1.

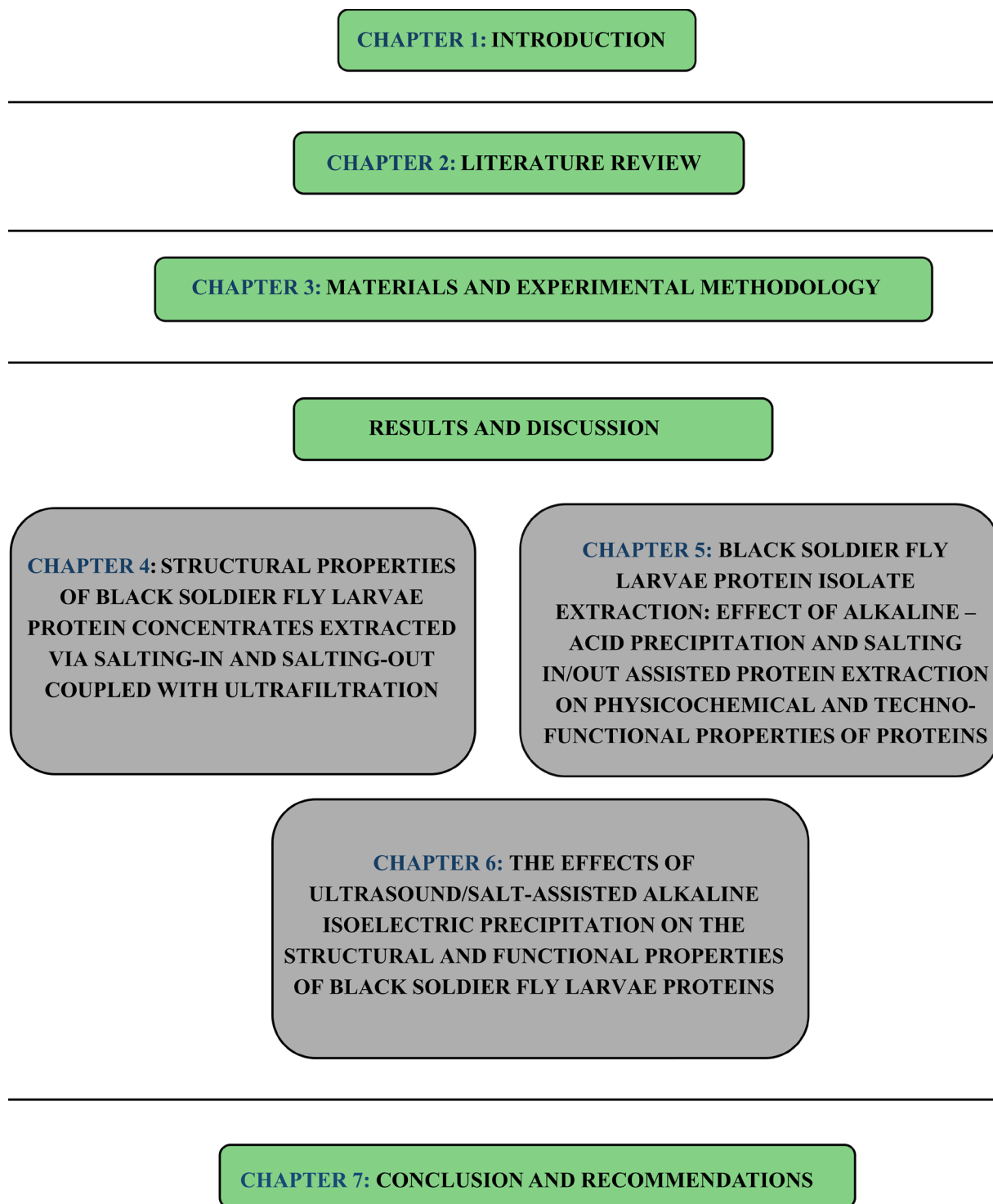


Figure 1.1. The thesis road map

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CHAPTER TWO

LITERATURE REVIEW

This chapter reviews the relevant literature that underpins this thesis and highlights important research gaps that are addressed in this work. Most importantly, this chapter is targeted at developing a background into the nature and properties of black soldier fly larvae protein as related to its purification and characterization.

“Edible insects offer a promising solution to the global challenges of food security, environmental sustainability, and human health.”
(Food and Agriculture Organization, 2013)

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

According to the United States Census Bureau's global population clock, the world's population reached 8 billion in November 2022. On average, approximately 140 individuals are added to the population every minute, with births exceeding deaths. By 2030, the population is expected to reach 8.5 billion and grow to approximately 9.7 billion by 2050. According to the Food and Agriculture Organisation (FAO), a 70% increase in food production is required to satisfy food supply demands. The challenge of securing an adequate food supply is complicated by global conflicts in regions that were previously regarded as significant. The need to create and sustain a safe, sustainable food system is highlighted by the Sustainable Development Goals (SDGs) for 2030. This is done to eradicate hunger, attain food security, enhance nutrition, and foster sustainable agriculture.

Edible insects have been targeted as a new sustainable source of protein by researchers to help with global food security (Meyer-Rochow, 1975; van Huis, 2013). Around 2,000 different kinds of insects are consumed by people all throughout the world, mostly in non-Western countries. Consumption of insects is done as part of the practice of entomophagy (Jongema, 2017). Insects have a lot of protein in them, meaning they are proteinaceous, similar to animal proteins. Additionally, insects have all the amino acids that are required by humans (Bosch et al., 2014; Chakravorty et al., 2014; Zozo et al., 2022). Also, their feed conversion efficiencies are excellent (Barragan-Fonseca et al., 2017; Oonincx et al., 2015; Driemeyer, 2016). Some species, like BSF larvae, can recycle organic matter that would otherwise be garbage (EFSA, 2015; Woods et al., 2019). This combination of being abundant in nutrients and having a small environmental impact could help relieve some of the stress on high-impact industries like meat and dairy. Edible insects could be a great source of food, but people in different parts of the world have very varied opinions about them (Hartmann & Siegrist, 2017; Tan et al., 2015; Verbeke, 2015).

In several African countries, certain species, such as stink bugs (*Hemiptera: tessaratomidae*), are consumed and have cultural significance. For instance, stink bugs are considered a delicacy among the VhaVenda people in South Africa's Limpopo Province

(Hlongwane et al., 2020; Vanqa et al., 2022). Caterpillars (*Imbrasia epimethea*) are currently popular culinary items among various populations in the northern part of the Republic of Congo (Mabossy-mobouna & Ombeni, 2021), whereas termites in Nigeria are used to increase protein intake (Bukkens, 2005). Entomophagy used to be a common food source in developing countries, but now that Western ideas have spread around the world, it is less common (Chakravorty et al., 2014; Premalatha et al., 2011).

Food neophobia is the fear of trying or eating foods that are new to you or that come from other cultures. This is one reason why people in the West are willing to eat insects (Verbeke, 2015). When people see insects, they are often disgusted or, even worse, scared of them. Such behaviors have made it difficult to sell edible insects in the markets (Hartmann & Siegrist, 2017; Tan et al., 2015; Verbeke, 2015). In such cases, there is less chance of consumption of insects in the future as they are disgusted by them. Looking at it, food neophobia is a weaker reason than disgust (La Narnera et al., 2018). Besides disgust and food neophobia, people's feelings are based on other things. The role in how they feel about new foods is played by their age, gender, lack of knowledge, and experience with foods. Also, convenience and environmental attitudes play a role in choosing food (La Barbera et al., 2018).

Whole insects are less preferred by Western customers than processed and hidden insects, as reported by researchers (Gmuer et al., 2016; Hartmann et al., 2015; Schösler et al., 2012). Some products have been produced from insects, such as burger patties out of crickets (*Acheta domestica*) and mealworms (*Tenebrio molitor*) with different amounts of each (Caparros Megido et al., 2016; Schouteten et al., 2016). Throughout Europe, these products can be found. Insects such as crickets (*Gryllidae*), grasshoppers (*Locusta migratoria*), and silkworms (*Bombyx mori*), are found in the Western market (EFSA, 2015). Somehow, *Hermetia illucens*, known as the black soldier fly (BSF), has been ignored for the consumption of insects by people. There is discussion on whether they can still be in different types of cattle feed as an environmentally friendly protein source (Mertenant et al., 2019; Woods et al., 2019). Even so, in the Western markets, they are not accepted as a food source for human consumption.

People may accept consuming insects if they come in a form of a known food product, and in places where insects are not consumed, this will be of great significance. Other methods for

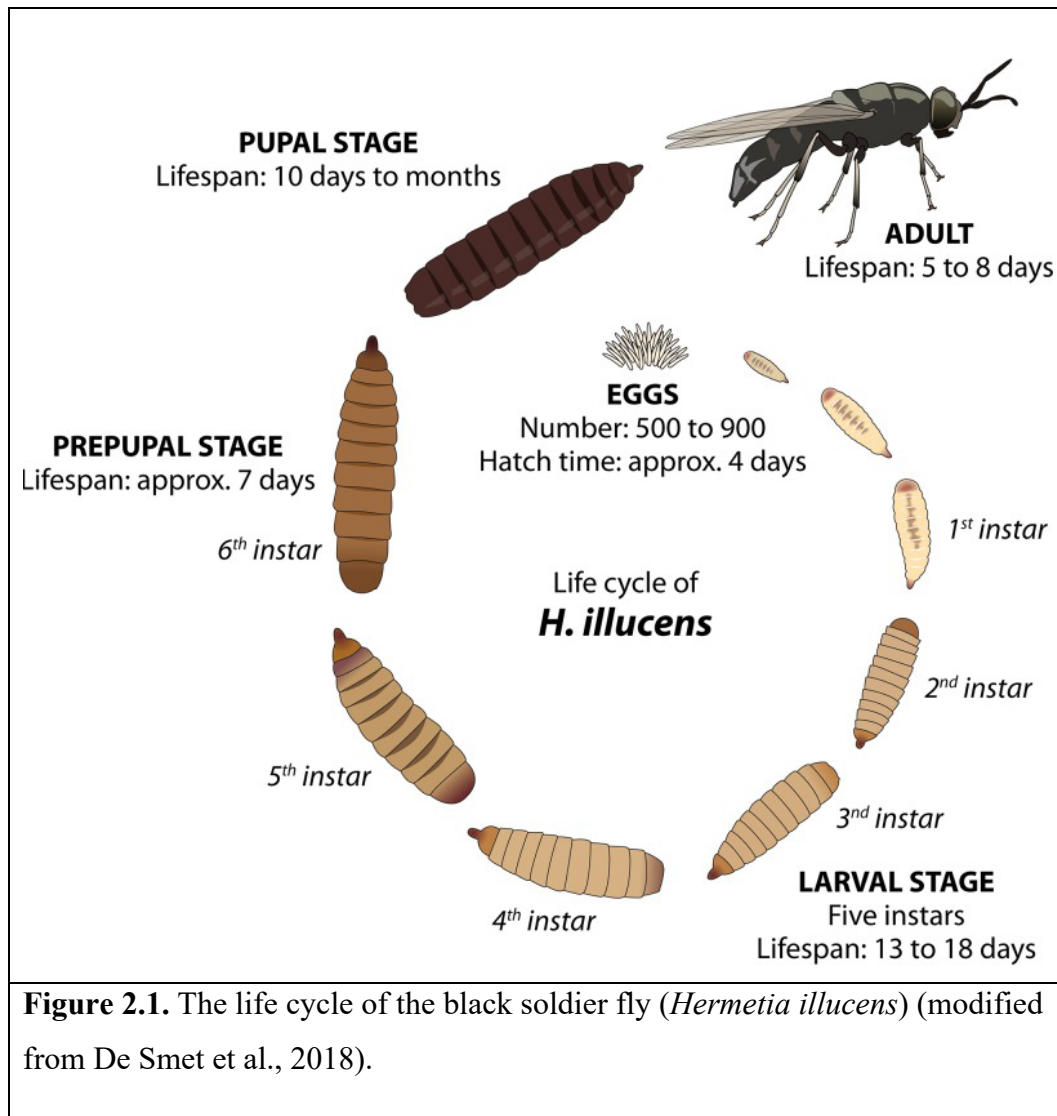
getting protein from insects include alkaline extraction and acid precipitation. But this method has a big flaw: it doesn't have enough protein. Salt extraction is a well-known method that can help with protein extraction and keep the proteins' function (Jiang et al., 2021). We don't know yet if using salt and ultrasonics to get proteins out of BSF larvae works. To make a BSF larval protein product that is very pure and safe for people to eat, scientists should look into different ways to get proteins out of the larvae.

The goal of this literature review is to sum up what scientists already know about the biological traits of BSF larvae, with a focus on studies that have been published about nutritional value. The review talks about the protein extraction methods needed to make pure BSF larvae protein and how instrumental analysis can be used to help make insect protein products. This review's goal is to introduce new ideas and encourage creative thinking in the field of insect protein extraction, not to repeat what has already been done.

2.1.1. Black soldier fly biology

The black soldier fly (*Hermetia illucens*) is a kind of fly that is part of the Diptera order and the Stratiomyidae family. In warm, tropical, and subtropical areas, this is where insects grow in America. From there, the insects were transported to different regions such as Australia, India, Africa, and Europe. They were transported by people's movement, and that how they how they got there (Gayatri et al., 2013; Martínez-Sánchez et al., 2011). However, the BSF larvae can withstand rough seasons such as lack of food, lack of water, or not having enough oxygen (Diener et al., 2011).

Figure 2.1 shows that the adult black soldier fly is black, looks like a wasp, and is 15 to 20 mm long (Hardouin & Mahoux, 2004). The larvae of this species can grow up to 27 mm in length and 6 mm in width and weigh up to 220 mg in their final larval stage. They have a dull, whitish colour (Diclaro & Kaufman, 2009). Larvae are known to consume food quickly, ranging from 25 to 500 mg of fresh matter per larva per day, and can feed on a wide variety of decaying organic materials, such as rotting fruits and vegetables, coffee bean pulp, distillers' grains, fish offal, animal manure, and human excreta (Hardouin & Mahoux, 2004; Diener et al., 2011; van Huis, 2013).



The larval maturation phase is about two months in the best conditions, but it can go up to four months because of things like temperature, food quality, and amount (Hardouin & Mahoux, 2004). When the larva reaches the end of its larval stage (prepupa), it stops eating and moving around and empties its digestive tract (Hardouin & Mahoux, 2004). After that, the prepupae look for dry, protected places to pupate (Diener et al. 2011). The pupal stage lasts about 14 days, but it can last a lot longer, even up to five months (Hardouin & Mahoux, 2004). Two days after they come out, adult females mate and lay their eggs in dry places and cracks near a food source (Diener et al. 2011; van Huis, 2013). In the late 1990s, BSF was promoted to get rid of waste material through turning organic materials into protein- and fat-rich biomass. Such waste can be used as a kind of

livestock feed. It is also used to manufacture biodiesel and chitin (Diener et al. 2011; van Huis, 2013). For example, this insect can turn garbage into a useful product; this is a grown BSF larvae that is. As such, they can be used as a long-term source of food for humans (Wang et al., 2017).

The projected future scarcity of sufficient nutritious and healthy food will be addressed by this research. Their variety of micronutrients and macronutrients makes BSF larvae noticeable from the perspective of nutrition. Moreover, livestock such as chickens, fish, and pigs use BSF larvae as a form of animal feed (Pieterse et al., 2019; Huyben et al., 2019; Zarantoniello et al., 2021; Veldkamp & Bosch, 2015). BSF larvae contain the same nutrients that are good for animal health. The food industry can use these nutrients as meat substitutes (Bessa et al., 2019), butter/margarine alternatives (Delicato et al., 2020; Smetana et al., 2020), bioactive compounds (Müller et al., 2017), and possible functional ingredients (Caligiani et al., 2018).

2.2. Challenges for the use of insect proteins

As mentioned, insects are a good source of macronutrients such as proteins, vitamins, and minerals. Therefore, global food security and environmental sustainability can be improved by the use of consuming insects (van Huis, 2013). Although these insects can help with the food industry, they are still not accepted because of some challenges.

a) Cultural Acceptance

Culturally, some people have a big problem with using insect proteins for human consumption. Some people say they are gross and do not taste good, especially in the West (Deroy et al., 2015). Therefore, the cultural beliefs and how they feel about eating insects remain two major challenges. To then get people to eat more insect-based food, new and exciting beliefs need to be looked at, running of educational campaigns, other other ways to promote the consumption of insects.

b) Regulatory Frameworks

Globally, there are clear regulations as to how to sell insect-based food, even in Africa (Halloran et al., 2015; Niassy et al., 2022). Even so, these regulations that are current are not sufficient to ensure that insect proteins are safe and labelled correctly. There needs to be clear rules that make sure insect-based foods are safe, of good quality, and properly labelled so that people can trust them and the market can grow.

c) Allergen Concerns

Certain insects or parts of insect proteins may cause allergies in some people, which could be harmful to their health (Fukutomi & Kawakami, 2021). It is especially worrying that this could cross-react with known allergies like dust mites or shellfish. Allergen testing and labelling rules are needed if we are to find and reduce rising concerns about insect-based food items.

d) Economic Viability

The economic viability in insect farming and processing is the most profound issue (Rumpold & Schlüter, 2013). Due to elevated production costs, insufficient infrastructure, and ambiguous market demand, there is limited investment in insect protein production manufacturing. A comparison indicates that plant-based proteins exert minimal environmental impact and incur the lowest production costs. Plant-based proteins show less environmental impact. Additionally, they incur the lowest production costs. This is when in comparison to the insect-based protein. For example, 1kg of beef protein will need 151400 litres, 1kg of soy protein will need 1,500 liters of water (Mekonnen & Hoekstra, 2012). Lower levels of greenhouse gas emissions are generated by plant proteins. For example, soy protein generates approximately 2 kg of CO₂ equivalent per kilogram, whereas beef produces 27 kilograms. For example, bovine protein production is sustainable and less efficient than the production of insect proteins. On the other hand, to produce 1 kg of protein from crickets, you need about 1.7 kg of feed. In other cases (van Huis et al., 2013). As mentioned before, less greenhouse gases and ammonia are emitted when farming insects compared to when farming known animal livestock. To produce insect protein, currently, you will need between \$3 - \$5 per kg. Although this is higher than plant proteins, which cost between \$0.50 - \$2 per kg, it is way less than premium animal proteins such as beef. In the form of grants and subsidies, the government is willing to fund insect farmers. In such innovation and investment, an increasing producer's profitability would be recognized.

e) Supply Chain Logistics

The supply chain would try to make insect-based food products easier to work easier. This is usually the logistical problems that come up (Halloran et al., 2015). This is because special care and processing are needed for insects as compared to regular cattle needs. While the quality and safety of the products are preserved, insect needs to be addressed in time for the customers. For this to be successful, a set of clear and strong supply chain infrastructure must be created. This

would include looking into the storage, transportation, processing facilities, and others. All different insect-based feed products will need to be included in the supply chain. For example, different processing and shipping would be needed for the dried pupae and protein extracts that could be used as animal feed. Dried pupae, which are often less processed, cost less to make. Additionally, dried pupae need to be stored properly to keep them from going bad (van Huis, 2013). Although it is easier to move protein extract, its extraction method requires a lot of time and energy. In this case, then, protein extracts are expensive to make. Another example of dried pupae, they do not cost much to process, and they don't need a lot of processing, but their storage costs a lot, since they need to be stored properly in order to retain their freshness (van Huis, 2013). Therefore, you need to compare the costs, such as on storage and transportation, to be able to choose the best product or improve the supply chain.

f) Consumer Education and Awareness

There is a lack of information on insect proteins' beneficial health and environmental benefits (Deroy et al., 2015). This can help insect-based food to be bought more, and the growth of market can grow. Insect proteins can be promoted effectively by cooking, public awareness campaigns, and collaborations with retailers. It would also help to incorporate it known diets to enhance environmental sustainability.

Insect proteins represent a viable alternative to conventional animal protein sources; however, several challenges must be addressed to realise their full potential. The insect protein sector encounters several significant challenges, including consumer acceptance, ensuring safety for individuals with allergies, establishing economic viability, determining distribution methods, and educating the public. Collaborative efforts among industry leaders, legislators, researchers, and consumers can address the barriers to insect protein utilization, thereby maximizing their benefits for a sustainable food future.

2.3. Insect processing methods applied for protein extraction.

2.3.1. Insect feed and fasting time

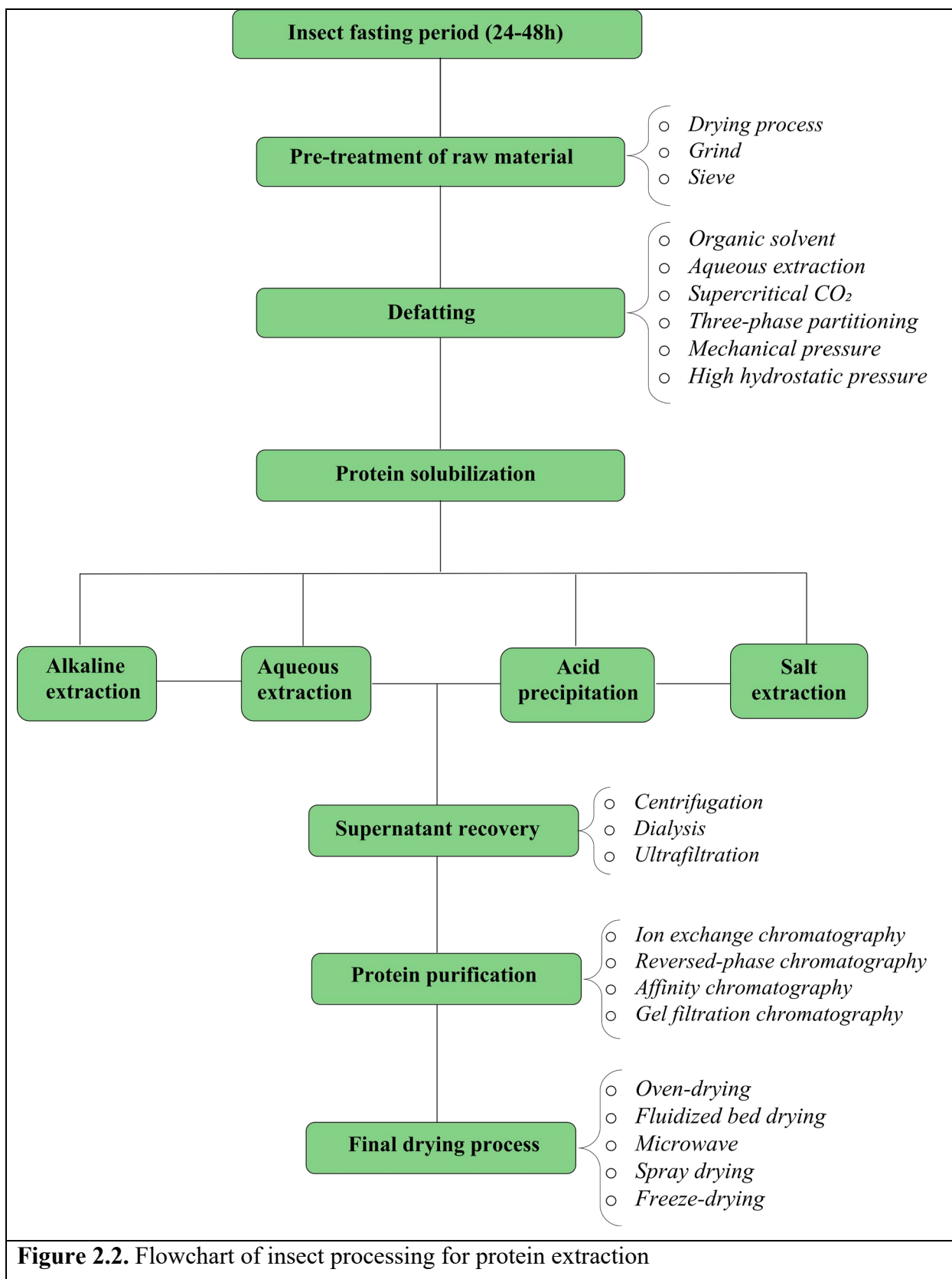
Many people know that BSF are good at turning organic waste into edible biomass. The type of substrate employed affects the makeup of the BSF larvae. In 2017, Spranghers et al. did an experiment where they raised BSF larvae on four different types of food: chicken feed, vegetable

waste, biogas digestate, and restaurant garbage. The researchers saw big differences in the total biomass of the prepupae they collected from the four substrates. The chicken feed had the most biomass, while the digested biogas had the least. The protein concentration of the four substrates was very different, ranging from 399 to 431 g/kg of dry matter. The amino acid profile didn't vary notably; meanwhile, the ash content and fatty acid profile (such as lauric acid) were very different.

Fuso et al. (2021) looked at the nutritional composition of BSF larvae grown on vegetable combinations. The researchers divided the 49 different substrates into three groups according to seasonal availability. The amount of protein in BSF prepupae ranged from 35% to 49% of dry matter. The lowest amount was in insects that ate fruit by-products, while the highest amount was in insects that ate leftovers. Before processing insects, it is very important to think about how long they have been fasting (**Figure 2.2**). It is important to keep pollutants to a very low level so that they don't affect later tests, such as protein analysis (Zielińska et al., 2018). Mishyna et al. (2019) raised adult grasshoppers (*S. gregaria*), larvae, and honeybee pupae (*Apis mellifera*) in a controlled environment to keep pollutants to a minimum. Before the protein extraction, the insects had to be defatted for at least 24 hours. This method mirrored that employed by Khampakool et al. (2020), who subjected *Protaetia brevitarsis* larvae to a 24-hour fasting period to ensure the gut was free of faeces or other waste that might alter the nutritional value. According to reports, a time frame of 24 hours is the most effective approach to achieving this goal.

2.3.2. Pre-treatment

The input material must be pre-treated to minimize the moisture content and make the powder finer before the protein can be extracted (**Figure 2.2**). Most of the time, these first steps make the succeeding steps in the process go more smoothly. For instance, this first process could improve the protein level in beans by removing most of the fiber-rich parts (Gravel & Doyen, 2020). Insects are also high in fibre, just like legumes, so you may directly compare the two. Drying foods can help them survive longer since it lowers their water content, which means there are fewer ways for them to break down (Melgar-Lalanne et al., 2019). The first step is normally to dry the bugs, grind them up, and then sift them to get a fine powder with greater surface area for the solvents to operate on (Gravel & Doyen, 2020; Zielińska et al., 2018; Bußler et al., 2016; Zhao et al., 2016).



The drying stages could happen at the start or end of the process of separating or cleaning proteins. Melgar-Lalanne et al. (2019) looked at several ways to dry insects that were used as raw materials. The study looked at both old and new ways to dry samples, such as freeze-drying and solar drying.

When drying insects in the lab, freeze-drying is a frequently used procedure. This procedure removes water from frozen samples by sublimation in a vacuum, and the samples' colour, flavour, smell, and physical shape don't change much (Shadung, 2012). Researchers used different drying methods on mealworms (*Tenebrio molitor*) to see how they affected the stability of nutrients and the likely oxidation of lipids. The study in question found that different drying processes, like microwave, fluidized bed, and hoover drying, could change the nutritional value of mealworms. For example, they might make protein less soluble and lipids more likely to oxidise (Sogari et al., 2019).

2.3.3. Insect powder defatting step

People usually think of defatting as a step before extracting protein (**Figure 2.2**) since it cleans the flour and makes the derived protein more useful. Varying ways of defatting can provide you with protein extracts with varying technofunctional qualities. Hexane has been a common solvent for defatting, but it is no longer used since it is bad for the environment, unsafe, and affects the function of proteins (L'Hocine et al., 2006). Kim et al. (2022) looked at how removing water-soluble fats and defatting with organic solvents, including hexane, methanol, and ethanol, affected samples of the white-spotted flower chafer (*Protaetia brevitarsis*). Hexane was the best way to defat protein since it changed their amino acid makeup, made them more soluble in water, and improved their functional capabilities, like their ability to foam and emulsify. Kim et al. (2022), on the other hand, looked at how to defat *Acheta domesticus*. They said that the aqueous extraction worked better when the goal was technofunctionality. The effectiveness of aqueous extraction for defatting was compared to a number of organic solvent-based extraction methods for different insects. As expected, water extraction produced the least amount of oil of all the methods that have been published (Tzompa-Sosa et al., 2014)

These studies show that different defatting methods work better and have better results, but we also need to think about how long these methods will last. Metrics like E-factor (environmental factor), Process Mass Intensity (PMI), or other life cycle assessment tools can help us figure out

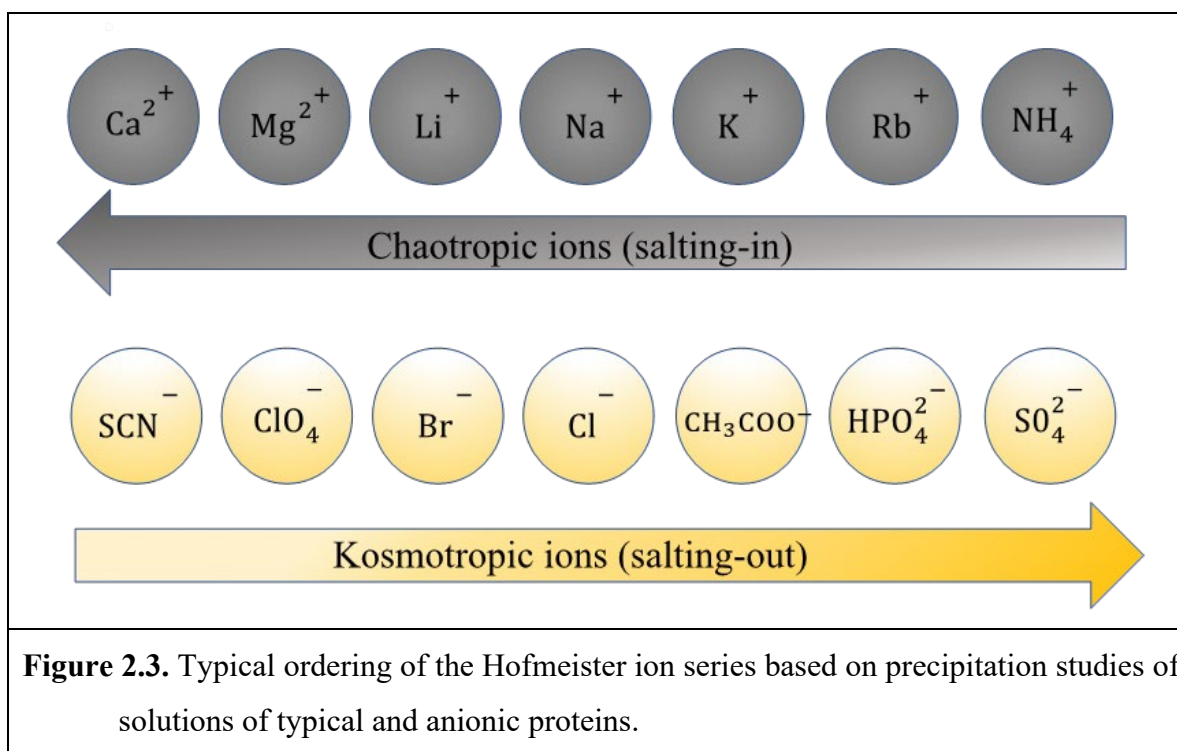
how each approach affects the environment. Hexane-based extraction, for example, has a high E-factor because it creates dangerous waste, even if it is good at getting oil and protein to work together. Although aqueous extraction doesn't get as much oil, it's better for the environment because it has a lower PMI and less chemical waste (L'Hocine et al., 2006; Kim et al., 2022). Finding ways to balance efficiency and environmental responsibility can be easier if you can measure and compare the sustainability index of these processes.

2.3.4. Supernatant recovery and extraction

There are several ways to extract protein, and the methods used to do so can affect the process. The features of the raw materials also affect how well the extraction works. The kind of insect, its stage of life, and how it eats are all important. When trying to get the most protein out of something, you should also think about the sample concentration, pH, salt addition, temperature, and extraction time. A common way to separate proteins from other things is to change the medium's pH and ionic strength. This makes proteins easier to dissolve. Most proteins, like those in insects, have an isoelectric point (pI) between pH 4 and pH 5. This makes proteins repel each other more strongly and makes them interact with water more, which makes them more soluble (Lam et al., 2018; Singhal et al., 2016). According to Hall et al. (2017), the pH must reach a certain level, which is the isoelectric point of the target proteins, to get a certain group of proteins out. Hall et al. (2017) demonstrated the successful separation of these proteins from a mixture through meticulous pH regulation to achieve the isoelectric point of the targeted protein group. This method is also very useful in biochemistry, where it is important to get pure and specific proteins for more research or use.

You can also change the ionic strength to change how soluble proteins are during extraction. In cases where there is a low amount of salt (NaCl), the salt ions will behave as a “shield” on the surface of the protein. This also happens when the ionic strength is between 0.5-1 M. When it comes to a time when a protein needs to interact with water and dissolve, this makes things easier. One way to do this is by salting in: low concentration, namely, 0.15M. The salting-in method helps protein to dissolve more easily by giving the protein a surface charge and preventing it from clumping together (Okur et al., 2017). Another way is salting out, this one makes proteins clump together and they would fall out of the solution. This is done by raising the

ionic strength (Duong-Ly & Gabelli, 2014). How different ions can be salty or not salty is shown by the Hofmeister series in **Figure 2.3**. (Okur et al., 2017), which shows how different ions can make salty or not salty.



Ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ is commonly utilised for salting out due to the efficacy of both its cation and anion in reducing protein solubility, as demonstrated by the Hofmeister series (Duong-Ly & Gabelli, 2014). Ammonium sulphate also dissolves very well in water and can be used in amounts up to 90%, which makes it easier to precipitate different types of proteins (Rangel et al., 2003; Doung-Ly & Gabelli, 2014). According to reports, there is a low risk of protein denaturation during salt extraction (Novák & Havlíček, 2016), which mainly keeps the proteins' original structure. Because of this, salt extraction produces a protein isolate with a high salt content, which needs to be desalinated. Dialysis usually removes salts while keeping proteins at a lab scale, while diafiltration is done on a larger scale (Sun & Arntfield, 2021). Membrane filtration is another way to lower the amount of salt. We don't know much about insect proteins yet, so we can't use membrane filtration to study them. We need to know more about the size and properties of proteins

first. However, further research is needed on the size and properties of insect proteins because these methods have not been thoroughly described in the literature.

2.4. Characterization of insect-based protein.

2.4.1. Protein composition

As mentioned earlier and supported by several researchers in recent years, BSF can consume and break down various types of waste, converting it into valuable products rich in nutrients, such as protein (Newton et al., 2004; Sheppard et al., 2002; Li et al., 2011). Several earlier studies have looked into black soldier fly larvae meal as a source of protein and compared it to other sources of protein, such as beef and chicken (**Table 2.1**). BSF larvae often contain a high amount of protein, ranging from 30 to 67.7g per 100g of dry matter (DM), which is comparable to that of beef and chicken. During the short larval stage, the nutritional makeup changes, with the amount of protein decreasing as the larvae get older. According to Rachmawati et al. (2015), the protein content of *Hermetia illucens* larvae dropped from 61% (DM) at five days to 44% at 15 days and 42% at 20 days. Bußler et al. (2016) found that the non-defatted *Hermetia illucens* flour had 30% less protein. This information is useful because it lets you collect larvae at the right stages of growth to get the right nutritonal profiles for certain uses.

Table 2.1. Nutrient content as crude protein of beef (ground, raw), chicken (ground, raw), black soldier fly larvae, mealworm, cricket, and mopani worm (g/100 g, Dry Matter Basis).

Species	Latin name	Crude protein	Reference
Beef	<i>Not applicable</i>	50.8 to 65.4	U.S. Department of Agriculture, Agricultural Research Service (2019)
Chicken	<i>Not applicable</i>	66.8 to 86.2	U.S. Department of Agriculture, Agricultural Research Service (2019)
Mealworm	<i>Tenebrio molitor</i>	50.1 to 76.2	Ghaly (2009; Yi et al. (2013)
Black soldier fly	<i>Hermetia illucens</i>	30.0 to 67.7	Bußler et al. (2016); Caligiani et al. (2018); Zozo et. al (2022);
Cricket	<i>Acheta domesticus</i>	64.4 to 73.6	Barker et al. (1998); Osimani et al. (2018))
Mopani worm	<i>Gonimbrasia belina</i>	52.7 to 55.4	Kwiri et al. (2014; Vanqa et al. (2022)

Still, harvesting before pupation would make it hard to get the larvae out of the leftover feed before they turn into prepupae, which is when they start to move away from their food source on their own (Wang & Shelomi, 2017). Studies have indicated that the substrate has a big effect on the nutritional makeup of BSF larvae. However, these effects are not always linear and need more research (Liland et al., 2017; Liu et al., 2008; Meneguz et al., 2018). In general, BSF larvae have a protein composition that is similar to that of other well-known edible insects like mealworms, crickets, and mopani worms (**Table 2.1**), making them viable options for culinary use.

2.4.2. Amino acid profile of proteins

Amino acids are essential for all organisms as they are the fundamental components of proteins. Proteins constitute every organ and confer its physiological function (McDonald et al., 2001). Diverse animal species possess distinct amino acid requirements, which may also fluctuate within the same species based on the animal's physiological stage and seasonal variations (McDonald et al., 2001). The quantity of amino acids is influenced by food processing methods, including the larvae's developmental stage, their nutritional intake, and the method of euthanasia. The amino acid profile of BSF larvae aligns closely with the reference requirements established by the World Health Organisation (Joint WHO/FAO/UNU Expert Consultation, 2007) (**Table 2.2**).

These indicated that they could serve as an appropriate protein supply for individuals and may represent a more environmentally sustainable alternative to conventional protein sources (Zozo et al., 2022). Lysine is particularly crucial in developing countries as it is a limiting amino acid in numerous cereals, which are inexpensive and constitute a significant portion of the diet for many individuals globally (Joint WHO/FAO/UNU Expert Consultation, 2007). Incorporating BSF larval protein into the diet facilitates the fulfilment of the daily lysine requirement of 4.8g per 100g of protein (Liu et al., 2017). The amino acid composition of the larvae is less influenced by external stimuli compared to certain other nutrients. BSF larvae's amino acid composition changes throughout their life cycle, as indicated by the research. The amino acid composition increases as they grow older; it is noted from younger larvae at the age of 4 up to 6 days (Liu et al., 2017). These indicated that they could serve as an appropriate protein supply for individuals and may represent a more environmentally sustainable alternative to conventional protein sources (Zozo et al., 2022).

Table 2.2. Total amino acid profile of black soldier fly larvae (expressed as g/100g) reared on different feeds compared to FAO (Food and Agriculture Organization) recommendation for adults.

Author	Liland et al. 2017	Mshayisa et al. 2022	Cappellozza et al. 2019	Lalander et al. 2019	FAO, 2013
Stage	Larva	Larva	Larva	Larva	
Feed	Wheat	Organic feed	Vegetables	Mix food	
Killing	Freezing	Blanching	Freezing	Blanching	
Histidine	2.70	1.82	1.24	2.91	1.60
Isoleucine	4.00	2.49	1.59	4.13	3.00
Leucine	7.70	4.02	2.70	6.79	6.10
Lysine	5.60	3.73	1.98	6.17	4.80
Methionine	1.70	2.53	1.76	1.84	2.30
Phenylalanine	4.60	4.14	1.88	4.03	4.10
Threonine	4.30	2.87	2.15	3.86	2.50
Valine	5.90	3.39	2.50	5.84	4.00
Alanine	6.30	3.58	3.89	5.90	-
Arginine	6.00	3.38	4.83	4.90	-
Aspartic acid	8.70	5.89	-	9.10	-
Glutamic acid	19.10	6.89	6.49	9.84	-
Glycine	6.40	3.08	2.29	5.27	-
Tyrosine	3.90	6.89	2.00	6.02	-
Proline	7.60	2.57	1.46	5.12	-
Serine	5.50	2.13	1.79	4.13	-

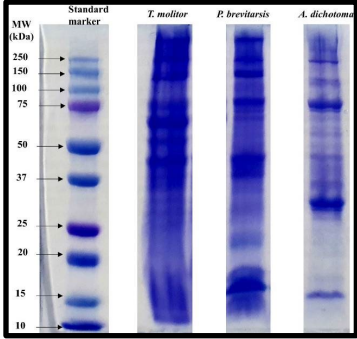
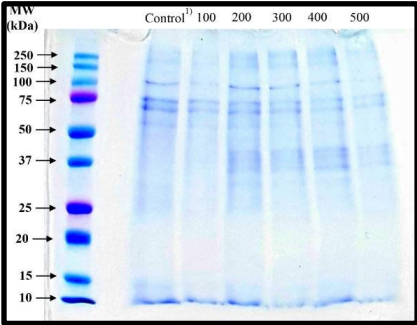
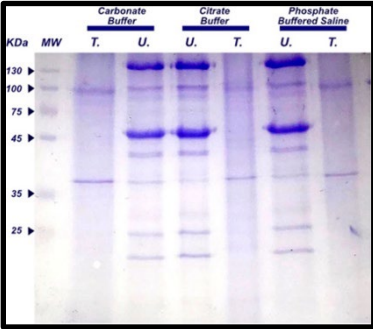
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2.4.3. Molecular weight of the protein/polypeptide

The molecular weight determination is a way to elucidate the makeup of proteins, and this is done by PAGE, or Polyacrylamide Gel Electrophoresis. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), 2D-gel electrophoresis, and native PAGE are tools that are also used to determine the molecular weight. A technique that is well known for molecular weight determination of proteins is called SDS-PAGE. This can be looked into by using different dyes to be able to see them. The preparation of the stacking gel and gel separation is the most difficult part when running SDS-PAGE analysis. Other parts, such as preparation of the sample, loading of the sample on the gel, staining, and also destaining, can also be difficult to prepare (Holzmüller & Kulozik, 2016). To prepare samples for SDS-PAGE, they are boiled with SDS and β -mercaptoethanol. β -mercaptoethanol cleaves disulfide bonds in proteins, while SDS is an anionic detergent that binds to proteins, facilitating further denaturation. The denatured protein unfolds and assumes a rod-like conformation, with negatively charged SDS permeating the peptide chains. The negative charges on this denatured rod-shaped protein maintain its conformation, hence preserving its denatured structure. Coomassie Brilliant Blue and Bromophenol Blue are ionizable tracking dyes that enable visualization of protein migration within the gel (Gallagher, 2012). Upon activation of the electric field, the negatively charged SDS-protein commences its migration towards the anode. The gel's distinctive molecular sieving characteristics subsequently result in the separation of proteins. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. The molecular weight of the protein influences the efficacy of gel separation of biomolecules.

Table 2.3. Various techniques of structural characterization of insect proteins.

Type of insects	Analysis technique	Extraction method used	Concluding remarks	Image	Reference
<i>Tenebrio molitor</i> <i>Allomyrina dichotoma</i> <i>Protaetia brevitarsis seulensis</i>	SDS - PAGE	Protein extracted with 0.15% phosphate buffer	The three insect species evaluated in the present study, the <i>Tenebrio molitor</i> emulsion was found to have the best gelling properties.		Kim et al. (2020)
<i>Protaetia brevitarsis seulensis</i>	SDS PAGE	High hydrostatic pressure	Edible insect protein subjected to high pressure treatment is reduced in molecular weight.		Kim et al. (2020)
<i>Ulomoides dermestoides</i> <i>Tenebrio molitor</i>	SDS PAGE	Three buffering systems assisted by sonication	The protein profile revealed by electrophoresis of both insects' crude extracts showed very different patterns and molecular weight bands.		Flores et al. (2020)

A study by Kim et al. (2020) identified protein bands exceeding 75 kDa in all edible insects in their ground and defatted forms; however, *Tenebrio molitor* and Japanese rhinoceros beetle (*Allomyrina dichotoma*) displayed only faint protein bands above 75 kDa following extraction with 0.02% ascorbic acid. The protein bands of *Tenebrio molitor* at 75 kDa or greater diminished following defatting with n-hexane, and no bands were observed post-centrifugation. The absence of protein bands over 75 kDa may adversely impact the technical activity of proteins, given that associated proteins and enzymes can be identified within the range of 30 to 95 kDa. In another study by Flores et al. (2020). *Ulomoides dermestoides* displayed most of its bands around 45 kDa, but there were also low molecular weight bands between 15 and 25 kDa, and a strong band around 130 kDa. The protein samples from *Tenebrio molitor*, *Allomyrina dichotoma*, *Protaetia brevitarsis seulensis*, and *Ulomoides dermestoides* were examined using these techniques (**Table 2.3**).

2.5. Functional properties of the protein

It is important to have a full grasp of the proteins' functional properties to include insect protein to be modern food chain. In this case, "functionality" means any property of food or its ingredients, other than nutritional ones, that impacts how it can be used. This includes viscosity, the ability to absorb water and lipids, solubility, emulsification, gelling, and foaming (Hall et al., 2017). Protein functionality is about the physical and chemical properties of proteins that affect how they work in a food matrix during processing and when they're in the body (Kinsella, 1979). Understanding how insect proteins work will help in making and keeping food products that contain insect proteins. Also, it can show clearly what the best uses for it as a food element are, and it could even replace some of the functional food components that are now out there. Proteins are helpful depending on their profile and structural integrity, which are greatly affected by the conditions used to extract and treat them (**Table 2.4**).

2.5.1. Protein solubility

The quality of a protein is directly connected to how well it dissolves in water. This can be measured at a pH level between 2 and 12. People think that the most important techno-functional property of proteins is how soluble they are, because it has a big effect on other functional properties.

Table 2.4. Summary of the effect of extraction methods on protein functionalities.

Type of insects	Species	Extraction technique	Functional properties studied	Concluding remarks	Reference
Cricket	<i>Gryllidae</i>	Enzymatic hydrolysis	Emulsion capacity index. Foamability and foam stability.	Enzymatic hydrolysis of whole crickets successfully creates protein hydrolysates with improved protein functionality.	Hall et al. (2017)
Black soldier fly	<i>Hermetia illucens</i>	Water-soluble extraction	Protein solubility. Water and oil binding capacity. Emulsifying capacity.	The techno-functional properties can be effectively manipulated. The protein composition of the samples may influence the functional properties.	Bußler et al. (2016)
Grasshopper	<i>Caelifera</i>	Alkaline and sonication-assisted extractions.	Foaming properties. Emulsifying properties.	Grasshopper demonstrated high emulsifying properties, comparable to whey proteins; there were differences in functional properties between proteins.	Mishyna et al. (2019)
Bombay locusts	<i>Nomadacris succincta</i>	Ultrasound-assisted extraction	Protein solubility. Foaming properties. Emulsifying properties.	Ultrasound significantly improved foam capacity while it had no adverse effect on emulsion activity index. Ultrasound has the potential for enhancement of the protein solubility of Bombay locust.	Kingwascharapong et al. (2021)
Cricket	<i>Gryllidae</i>	Alkaline extraction–acid precipitation and including ammonium sulphate	Protein solubility. Emulsifying properties. Foaming properties. water-holding capacity.	The addition of high concentrations of salt ions led to decreased solubility. These techniques decrease the water-holding capacity and emulsifying properties of the cricket.	Edward et al. (2023)
Mealworms	<i>Tenebrio molitor</i>	Salting-in/out-assisted extractions	Emulsion stability index. Foam properties.	The treated sample displayed the greatest emulsion stability index and foam properties.	Jiang et al. (2021)
Superworm	<i>Zophobas morio</i>	Aqueous extraction	Foamability and foam stability.	The supernatant fractions obtained contained some amount of oil in a concentration of around 0.1%, which may also influence foamability.	Yi et al. (2013))
Antarctic krill	<i>Euphausia superba</i>	Ultrasound-assisted deionized water	Foaming capacity, foam stability, and emulsifying capacity.	All these properties contributed to the improved foaming capacity, foam stability, and emulsifying capacity of the krill proteins.	Lin et al. (2020)

Including emulsifying, foaming, and gelation (Purschke et al., 2018). The protein solubility of migratory locust ranged from 10% to 22%, with the lowest solubility at pH 5 and the maximum at pH 9. It was found that the isoelectric point of most proteins is about pH 4. These results are in line with other studies that show that insect protein flours from mealworm larvae (3-45%; pH 2-9), black soldier fly larvae (10-35%; pH 2-9), house crickets (12-23%; pH 3-9), and tropical banded crickets (5-25%; pH 3-10) do not dissolve well in weakly acid environments (Bußler et al., 2016; Hall et al., 2017). Enzymatic hydrolysis of locust flour increased the protein solubility of the hydrolysates throughout a wide pH range, from 10-22% to as high as 55% in alkaline conditions (Purschke et al., 2018). The results are in line with what Sweers et al. (2024) found: that pre-treatment methods heat treatment and strong hydrostatic pressure, can greatly increase protein solubility. Zhao et al. (2016) found that the mealworm extract's protein solubility in distilled water was lowest between pH 4 and 5, and then it rose outside of the pH range. The solubility reached its highest point at pH 9. Adding NaCl to the solution made it less soluble.

2.5.2. Water and oil absorption

In culinary contexts, the term “water absorption capacity” refers to the ability to hold water against gravity. This includes bonded water, hydrodynamic water, capillary water, and physically trapped water. Protein-water interactions can change a number of functional properties, such as solubility, foaming, emulsification, viscosity, gelation, and coagulation. The fact that proteins can absorb oil because they physically trap it and have a lot of non-polar side chains that interact with the fatty acids in the oil (Zhao et al., 2016). Being able to hold on to water and oil is an important functional quality. The ability to hold water affects the texture, juiciness, flavour, and most importantly, the shelf life of baked goods (Hall et al., 2017). The way food feels and tastes is affected by how well it can absorb and hold onto fat, as well as how well it can mix with fat in emulsions. Son et al. (2019) found that oil concentration in mealworm powders makes it harder for them to absorb water and oil. Defatted powder with less fat has better emulsion or foam formation properties and good stability. This observation is confirmed by Borremans et al. (2020).

2.5.3. Foaming capacity and foam stability

Foaming capacity (FC) and foam stability (FS) are the two most used ways to measure foaming qualities. Proteins unfold to form an interfacial skin that holds are bubbles in suspension and stops

them from collapsing. This is how foams develop. Stone et al. (2019) looked at how well cricket and mealworm powder froth compared to protein concentrates from yellow pea and faba bean. The pulse proteins made a foam than the cricket protein foam, but the cricket protein foam was more stable after 30 min. The mealworm protein did not foam; hence, it is not good for uses that need foam. The insect powders included a lot more lipids than the pulse proteins. This could have made several of the insect proteins' functional qualities worse.

Kim et al. (2020) looked into the amino acid composition and functional features of proteins from three different types of edible insects, one of which was *Tenebrio molitor*. Grinding, defatting, and extraction were the three stages used to get the protein out. The extraction technique made *Tenebrio molitor* froth more and made it more stable. Haber et al. (2019) said that removing fat made the protein foam better, and that alkaline conditions made the protein foam even better.

2.5.4. Emulsion activity and stability

Colloidal dispersions are emulsions that are made up of at least two liquids that don't mix. You can tell them apart by how the two liquids are spread out in space (Dickinson, 2015). An oil-in-water emulsion is made when water droplets are spread out in a continuous phase of oil. When an emulsion forms, the area between the two liquids gets bigger, which raises the energy level. Even though the energy has gone up, smaller droplets can fit into more places in the system, which makes the configurational entropy higher. Proteins can make and keep emulsions stable by lowering the surface tension at the oil-water interface. This is because they are amphiphilic. Proteins can make a coating that stops coalescence, flocculation, creaming, sedimentation, and Oswald ripening (Gravel & Doyen, 2020). As a result, proteins are very important as emulsifiers and surfactants in many natural and processed foods, like egg yolk, soy milk, butter, and mayonnaise. For instance, casein micelles and whey proteins in homogenized milk replace the lipoprotein membrane with a stronger protein film that is less likely to cream than natural milk (Gould & Wolf, 2018; Zielińska et al., 2018).

Insects flour and protein extracts made using different solvent extraction procedures are only a few of the materials that have been examined for their ability to form emulsions and stay stable. These tests are all affected by outside variables. The emulsion capacity tells you how much oil can be mixed with one gram of protein, and the emulsion stability tells you how well the

emulsion can stay together over time (Lam & Nickerson, 2013). A new study looked at the emulsifying qualities of BSF larvae and how stable they are physically and chemically. The functionality of the protein can be improved by the use of sustainable techniques, such as ultrasound and ohmic heating (Queiroz et al., 2023). To increase the use of BSF larvae in the food industry, it is essential to try out different methods and see which one works.

2.5.5. Surface hydrophobicity and –SH groups

Examining the individual physicochemical properties of proteins can elucidate their functions. The presence of free sulfhydryl groups (SH) is one such factor. Heating proteins that have cysteine and cystine residues can cause polymerization, which leads to a thiol-disulfide exchange process and the creation of a strong polymer structure. When proteins denature, they may unfold and expose their thiol groups, which then interact with other proteins (Van Der Plancken et al., 2005). In globular tertiary protein structures, hydrophilic amino acid side groups are mostly found on the surface, while hydrophobic amino acid residues like leucine, isoleucine, valine, phenylalanine, and tryptophan are found in the core of the structure. The folding of proteins during food processing exposes most of the hydrophobic residues. This usually makes things less soluble because nonpolar groups cause hydrophobic interactions between molecules, which makes proteins stick together even more (Mintah et al., 2020; Trevino et al., 2006).

Santiago et al. (2021) investigated the effects of heat treatment and NaCl concentration on the secondary structures of black cricket proteins. This study primarily examined hydrophobic surface exposure and sulfhydryl groups. The authors observed that elevating the temperature to 85 °C for 15 minutes resulted in an increasingly hydrophobic patches precipitated this occurrence. Mishyna et al. (2019) obtained analogous results while examining proteins from honeybee brood. Santiago et al. (2012) also found that hydrophobicity dropped at temperatures above 90°C for 15 min and dropped much more after NaCl was added. This shows that heat and higher NaCl concentrations made black cricket proteins stick together more easily. Under varied conditions of heat and salt addition (salting-in/out). Jiang et al. (2021) looked into the physicochemical and technological usefulness of a *Tenebrio molitor* protein extract. We looked at the levels of SH in the salting-in group and compared them to the untreated group. There were no big differences in

the levels of exposed SH patches. But when the protein samples were treated with salting-out, they had more disulfide bonds than the untreated and salting-in groups.

2.6. Thermal characterization of proteins

There are different ways to thermally characterise proteins that look at how their physical properties change with temperature in a controlled temperature program. Because each protein has a different structure and folding pattern, they all react differently to heat. This causes protein to fold or unfold because of the thermodynamic barrier of transition. Higher temperatures change the shape of polypeptides at the secondary, tertiary, and quaternary structural levels. This causes proteins to unfold and stick together. Thermodynamic parameters are important for modelling how stable something is over time and how stable it is at different temperatures. Changes in proteins caused by temperature include physicochemical changes, including melting, crystallisation, exothermic or endothermic processes, and degradation. There are a number of ways to look at how proteins change physically, including spectroscopy (FTIR, NMR, UV-visible, Raman, far- and near-UV, circular dichroism, and fluorescence) and scattering methods (light, x-ray, and neutron). These methods are employed alongside thermal techniques to forecast alterations in protein structures contingent upon their configuration and thermal properties (Zhou et al., 2014; Zölls et al., 2012).

2.7. Structural morphology and characterization of protein

2.7.1. SEM

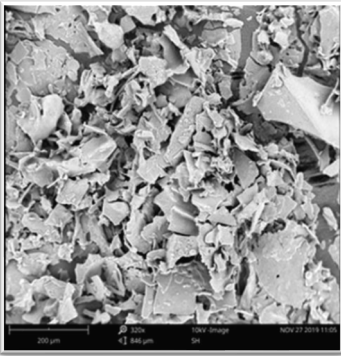
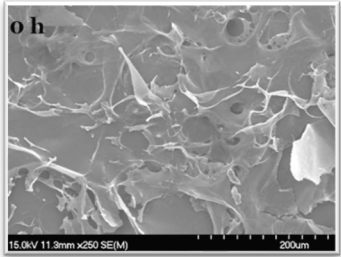
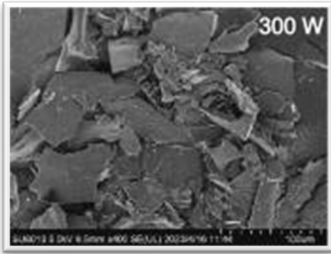
Scanning Electron Microscopy (SEM) is a very useful way to get clear pictures of surfaces. It can help you understand the shape better. The shape and size of plant proteins. A lot of people use this method to learn about plant proteins' structure, shape, size, surface morphology, and changes. These trains tell us about the protein samples' surface topography and how uniform they are. Depending on how uniform they are and other surface properties, insect proteins can also be used for different things. For example, plant-based proteins work well in packaging films when the protein particles are smaller and more even. You can use SEM to see how proteins are put together. This means using a focused electron beam to look at the surface of a sample. The series of signals can tell the shape the composition of the surface. Unstained insect proteins with high contrast can be viewed using SEM imaging. Also, other substances such as proteins, viruses, and many organic

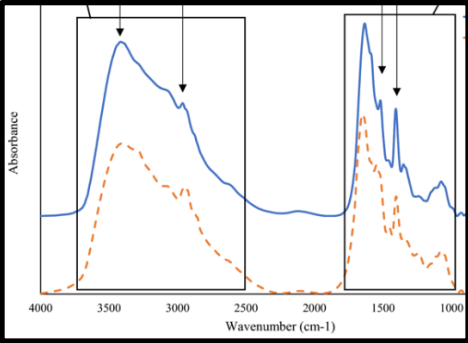
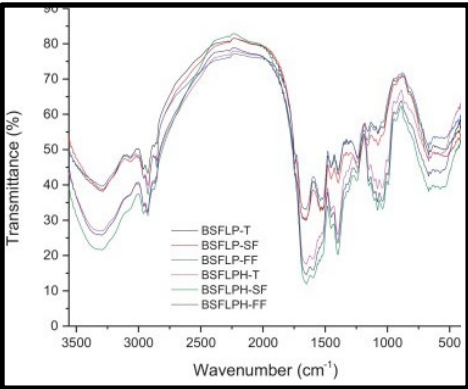
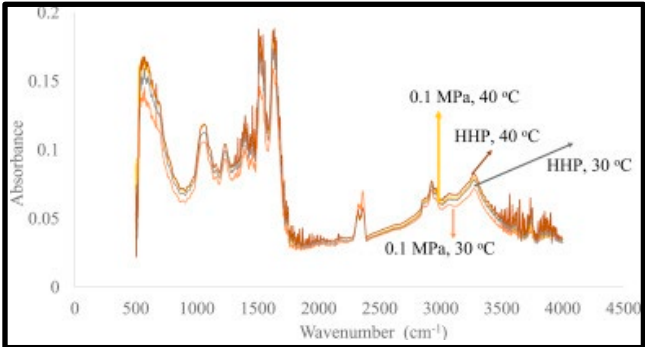
compounds can be analyzed with SEM analysis. The effect of vacuum ultrasound-assisted extraction has been studied by many researchers. Additionally, the vacuum ultrasound-assisted enzymatic extraction on the microstructure of sesame bran has been studied in many proteins. The extraction by vacuum ultrasound-assisted enzyme was reported to have the highest mortality of cells. The formation of cavitation bubbles during ultrasonication was indicated by the fragmentation, hollowness, and crimping of cells. As a result, this leads to the degradation of the cell walls (Görgüç et al., 2020). The evaluation of the quality of yields can be done by SEM, as this will come from the quantitative analytical techniques. The different insect protein surface morphological features are shown in **Table 2.5**. To look at the overall shape and structural changes, at 1,000x to 10,000x magnification range, the general observation can be made. Insect protein analysis can be found thoroughly in this range. This would provide information on their surface topography, consistency, and possible uses in different fields (Görgüç et al., 2020).

2.7.2. FTIR

Fourier Transform Infrared (FTIR) spectroscopy is a technique that uses infrared light to make a unique spectrum. This is done by measuring how radiation interacts with molecular vibrations in a sample. Vibrational transitions are caused when molecules absorb infrared light, between different energy states. In this case, absorption bands are created specifically for certain functional groups in the molecule. Useful information comes from measuring the intensity and the absorption bands. This will detail the macromolecules' biology, such as proteins and the changes in shape. To study insect protein functional groups, in recent years, FTIR has proven to be useful, other applications as well. With very little sample preparation, the FTIR would timely check the secondary structure, content, and thermal stability of the samples. Researchers have found the infrared range between $4000\text{--}400\text{ cm}^{-1}$ to be linked to the functional groups of proteins. Bands that are specific for proteins are amide I (C=O stretching), amide II (N-H bending coupled with C-N stretching), and amide III (C-N stretching coupled with N-H bending) (Yaman & Durakli Velioglu, 2019; Thummajitsakul et al., 2023; Zozo et al., 2022). When looking at the secondary structure of proteins, the important marker band is amide I.

Table 2.5. Various techniques of structural characterization of insect proteins.

Type of insects	Analysis technique	Extraction method used	Concluding remarks	Image	Reference
Black Soldier Fly	SEM	Alkaline and Isoelectric Precipitation	These findings suggest that the protein extraction methods changed or modified the microstructure of the BSF larvae proteins.		Mshayisa et al. (2022)
Silver carp	SEM	Buffer extraction	The particle of myosin protein was large, uneven and irregular structure (0 h).		Liu et al. (2016)
Housefly larvae	SEM	Alkaline/acid precipitation with ultrasonic treatment (300W)	The surface area of protein molecules is also increased, which improves their hydration, thus enhancing surface hydrophobicity and solubility.		Huang et al. (2023)

Bombay locusts <i>Legends abbreviations</i> ultrasound-assisted process at 60% amplitude for 20 min (UAP-60/20, orange dotted line) and typical extraction process (TP, blue line).	FTIR	Aqueous extraction with ultrasound treatment (60% amplitude for 20 min)	The selected treatment of 60% amplitude for 20 min had a clear impact on the molecular structure and functional groups which could provide high extraction yield with good functional and antioxidative properties.		Kingwascharapong et al. (2021)
Back soldier fly larvae <i>Legends abbreviations</i> BSFLP-T (control/traditional), BSFLP-FF (sonic treatment at a fixed frequency), and BSFLP-SF (sonic treatment by sweep frequency) and BSFL protein hydrolysate (BSFPH).	FTIR	Alkaline extraction with different treatment	Ultrasonication could be useful in altering the structure and improving functional and physical attributes.		Mintah et al. (2020)
Cricket powder <i>Legends abbreviations</i> High Hydrostatic Pressure (HHP) extraction	FTIR	High hydrostatic pressure extraction at different temperatures	It was observed that temperature and pressure interaction caused a significant difference in some absorption peaks in the case of cricket powder.		Bolat et al. (2021)

The protein secondary structure in its different forms correlates with different peaks in the wavelength range. Namely, these bands are α -helices, β -sheets, twists, and random coils. Therefore, studies can look into how proteins combine together, change form, and fold, physiologically. Additionally, researchers can look into the shape, position, and intensity of the amide I band (Oliver et al., 2009).

2.7.3. Circular Dichroism

A technique that is very sensitive for the reason that it uses protein, rather than chiral molecules, to absorb left- and right-circularly polarized light differently is called Circular Dichroism (CD) spectroscopy. This technique is also used to get information on the secondary structures and tertiary conformations of proteins. When circularly polarized light goes through a protein sample, it absorbs differently depending on how the protein is shaped. This is called circular dichroism (Kelly et al., 2005) (**Figure 2.4**). This variation in absorption creates unique CD spectra that show the secondary structure of the protein, such as α -helices, β -sheets, turns, and random coils. Most of the time, CD spectra are taken in the far-ultraviolet (UV) range, which is between 190 and 250 nm (**Figure 2.4**).

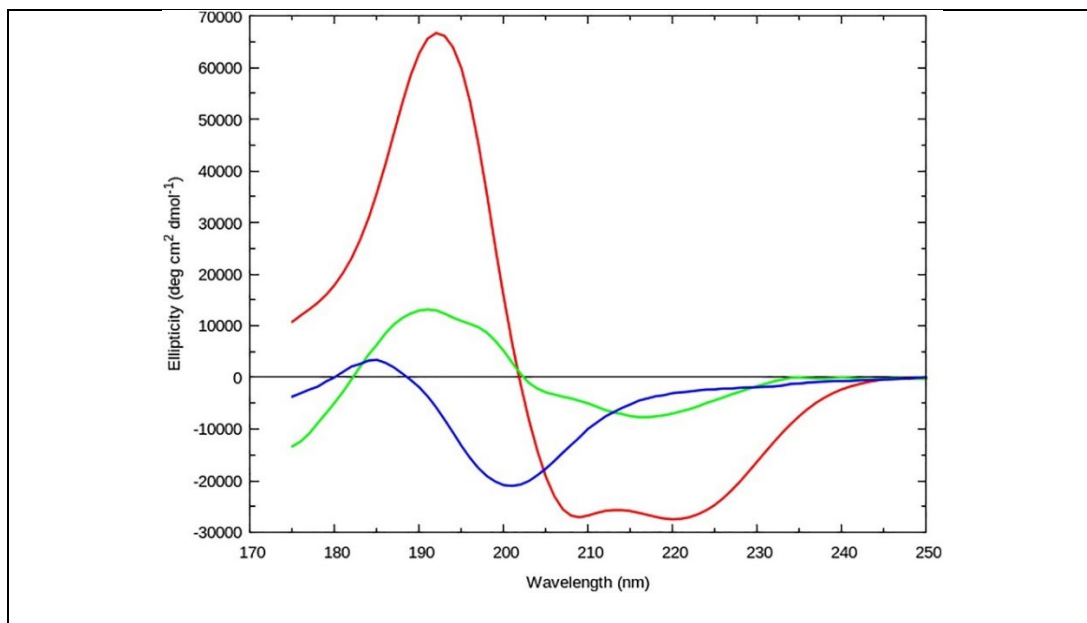
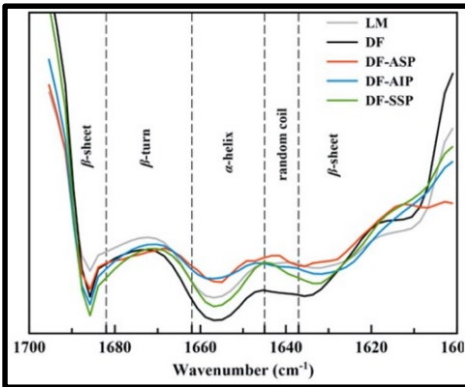
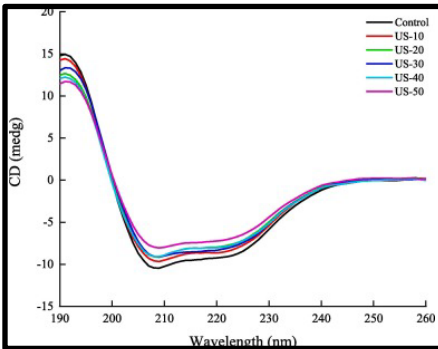
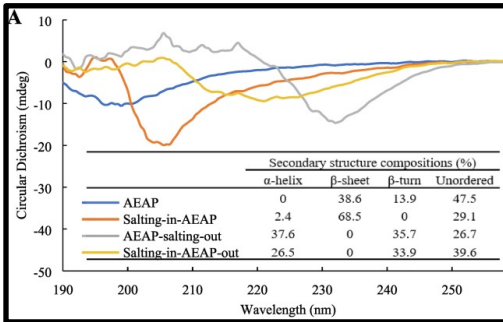


Figure 2.4. Illustrative CD spectra showing the characteristic patterns for three different secondary structures: α -helical protein (red line), β -sheet protein (green line), and random coil protein (blue line).

In the domains of taxonomy, structural biology, molecular biophysics, and drug development, CD spectroscopy can be used in many different ways to study insect proteins. Comparative analysis of CD spectra across various insect groups can enhance understanding of differences in protein folding and conformation. This aids in species identification and phylogenetic analysis (**Table 2.6**). In structural biology, CD spectroscopy is crucial for understanding the folding and stability of insect proteins under both normal and stress conditions (Miles et al., 2021). Investigating the alterations in CD spectra of insect proteins in relation to temperature, pH, denaturants, and ligand binding enables researchers to gain insights into the thermodynamic and kinetic processes governing protein folding, unfolding, and aggregation. This provides additional insights into the functional roles and molecular interactions of the proteins. Researchers can determine the biochemical mechanisms of insect proteins by examining the effects of substrate binding, cofactor binding, and allosteric modulation on their CD spectra (Micsonai et al., 2021)

Table 2.6. Circular Dichroism characterization of insect proteins.

Mealworm	CD	Isoelectric precipitation after alkaline extraction Salting extraction	The application of isoelectric precipitation led to the recovery of a protein fraction that was dominated by β -structures.		Gkinali et al. (2022)																													
Mealworm	CD	Ultrasound-assisted alkaline extraction	US treatment times increased, indicating that the α -helix content of the protein decreased, and the β -sheet content increased.		Zhang et al. (2023)																													
Mealworm	CD	Salting-in/out-assisted extractions	After salting-out assisted treatments, the β -sheet and random coil were changed to α -helix and β -turns suggesting a more compact and ordered conformation.	 <table><tr><th rowspan="2"></th><th colspan="4">Secondary structure compositions (%)</th></tr><tr><th>α-helix</th><th>β-sheet</th><th>β-turn</th><th>Unordered</th></tr><tr><td>AEAP</td><td>0</td><td>38.6</td><td>13.9</td><td>47.5</td></tr><tr><td>Salting-in-AEAP</td><td>2.4</td><td>68.5</td><td>0</td><td>29.1</td></tr><tr><td>AEAP-salting-out</td><td>37.6</td><td>0</td><td>35.7</td><td>26.7</td></tr><tr><td>Salting-in-AEAP-out</td><td>26.5</td><td>0</td><td>33.9</td><td>39.6</td></tr></table>		Secondary structure compositions (%)				α -helix	β -sheet	β -turn	Unordered	AEAP	0	38.6	13.9	47.5	Salting-in-AEAP	2.4	68.5	0	29.1	AEAP-salting-out	37.6	0	35.7	26.7	Salting-in-AEAP-out	26.5	0	33.9	39.6	Jiang et al. (2021)
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2.8. Gap analysis and research novelty

For centuries, numerous African and Asian nations have consumed and utilised insects as a food source. Recent years have seen scientists beginning to understand the production, utilisation, and purification of insect protein. Entomophagy has been recognised as a forward-looking direction for solving the problem of hunger and malnutrition in countries with a growing population and low income, and also as a substitute for traditional sources of protein (Halloran et al., 2015; van Huis, 2013). Most insects are rich nutrient sources of protein, fat, and vitamins (Janssen et al., 2017; Zielińska et al., 2018). However, in most Western countries, the direct consumption of whole insects is unacceptable (Gmuer et al., 2016; Hartmann & Siegrist, 2017; Schösler et al., 2012). Nutrient extraction represents a more effective method for producing food products.

Various methods have been employed for protein extraction and purification, including the acid-alkaline extraction method. However, these extraction methods frequently present challenges in usability, require significant time investment, incur high costs, and/or exhibit suboptimal effectiveness. Salting-in and salting-out of proteins are established techniques that enhance the efficiency of protein extraction and purification while preserving protein functionality (Yi et al., 2016). Extraction through salting-in and salting-out offers several advantages compared to conventional methods, including time and energy efficiency, environmental friendliness, cost-effectiveness, high polarity, low viscosity, high yield, and ease of scalability (Su & Chiang, 2006). Another method that has gotten a lot of attention is ultrasound extraction. This method is good for the environment and lets you make a lot of things with very little solvent. Many scientists have used this technology to get physiologically active molecules from crops, such as plant proteins (Ojha et al., 2015) and polysaccharides (Ventura et al., 2017). The food industry often needs more than one way to process food in order to meet its needs. Purification and extraction can work better by combining different methods.

To date, no research has looked at using both ultrasound technology and salting techniques to get, clean, and identify BSF larvae proteins. This study utilised salting-in and salting-out extraction methods for the extraction and purification of BSF larvae protein. Ultrasound made it easier to do the salting extractions. Different methods of analysis were used to make the purified proteins clearer. We looked at how salting-in and salting-out affected the properties and functions of BSF larvae protein to find a good way to use it in food and nutraceuticals. We posited that the ultrasound-assisted salt extraction method might substantially improve the characteristics of BSF larvae protein.

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CHAPTER THREE

MATERIALS AND EXPERIMENTAL METHODOLOGY

This chapter details the general description of materials and experimental methods used to achieve the objectives of the research on the purification and characterization of black soldier fly larvae protein. The specific experimental conditions, methodology, and mathematical tools are presented in the relevant chapters.

“The idea of eating insects may seem unconventional to some, but it's a logical and practical solution to the challenges of feeding a growing population in a resource-constrained world.”
(Marcel Dicke, 2010)

CHAPTER 3: MATERIALS AND EXPERIMENTAL METHODOLOGY

3.1. Materials

3.1.1. Chemicals

Unless stated otherwise, all chemicals used in the study were of analytical grade. The following chemicals were obtained from Sigma-Aldrich (South Africa, Johannesburg): hexane, isopropanol (88%), petroleum ether, o-phthalaldehyde (OPA), fluorenyl-methylchloroformate (Fmoc), nitric acid (HNO₃, 55%), ethylenediaminetetraacetic acid (EDTA), 1-anilino-8-naphthalenesulfonate (ANS), and potassium hydroxide. Sodium chloride and ammonium sulphate were acquired from Science World (Cape Town, South Africa). The prepared solutions and reagents were stored under appropriate conditions to prevent contamination or deterioration, and all solutions were prepared with ultrapure water (Milli-Q system, Millipore, Gradient model), which has a resistivity of 18.2 MΩ.cm.

3.1.2. Preparation of the insect flour

The larval stage of black soldier flies (BSF), aged ~14 days, was obtained from MaltEnto in Epping Cape Town, South Africa. The BSF larvae were reared on spent brewer's grain as their food source. The BSF flour was prepared according to the method of Mshayisa et al. (2021) and Zozo et al. (2022) with slight modifications. The larvae were cleaned and then blanched at 85°C for 2 min. The blanched larvae were frozen at -80°C in a blast freezer (NuAire Ultra-Low Freezer NU-9483E, NuAire Limited, United States) and stored until further processing. To obtain a stable product, the frozen larvae were ground using a laboratory blender and then freeze-dried under a vacuum of 750 mTorr for 3 days. This was followed by defatting with a 3:2 (v/v) mixture of hexane and isopropanol. The defatting process involved stirring one part of the ground BSF larvae sample (g) with five parts of the solvent mixture (mL) on a magnetic stirrer for 2 hours, decanting the solvent-fat mixture after sedimentation of the solids, and repeating the procedure twice. The residual solvent was removed by evaporation overnight in a fume hood. The resultant defatted sample was finely ground using a mortar and pestle, and the integument (the outer covering or skin of the BSF larvae) was removed by sieving through a stainless-steel sieve with an aperture size of 500 µm to produce defatted BSF larvae flour.

3.2. Characterization of insect proteins

3.2.1. Preparation of the protein isolates

Four different methods were used to prepare BSF larvae protein (see **Table 3.1**). The salt extraction isolates were prepared according to the method of Jiang et al. (2021) with modifications. The alkaline-acid extraction (AAF) was conducted as previously described by Azagoh et al. (2016) with slight modifications. The defatted BSFL flour was mixed with Milli-Q water in a ratio of 1:10 (w/v) and the pH was adjusted to 10 by using 1 M NaOH solution. The slurry was stirred for 30 min ($60^{\circ}\text{C} \pm 1.0$) and then cooled to room temperature before centrifugation (Z-326 K, Centrifuge, Hermle Labortechnik GmbH, German) at 25°C , 10,000 rpm for 30 min. The pH of the supernatant was adjusted to 4.5 with 1 M HCl, and the suspension was left at 4°C overnight for protein precipitation. The precipitate was recovered by centrifugation and then resuspended in 250 mL of Milli-Q water and pH adjusted to 7.0 with 1 M NaOH.

The water-soluble isolate was obtained after centrifugation, and a 200 mL solution was subjected to dialysis through ultrafiltration using a 3 kDa nominal molecular mass cut-off membrane in an Amicon ultrafiltration cell (8400, Amicon, Beverly, MA, USA). The ultrafiltration step, using the specified 3 kDa disc, was employed to remove salt from the samples. This disc effectively eliminates all impurities, including salt, from the samples. The process continued until no liquid was observed passing through the disc, with the disc being replaced every three hours. The retained solution containing the proteins ($>3\text{kDa}$) was freeze-dried and stored at -18°C for further analysis.

The salting-in extraction (AAFSI) was similar to the AAF method, except for the addition of 1% NaCl (w/v) to the NaOH solution to promote protein solubility (Yi et al., 2017).

The salting-out extraction (AAFSO) followed the AAF process, except for the addition of 80% $(\text{NH}_4)_2\text{SO}_4$ (w/v) to the supernatant to enhance protein precipitation (Duong-Ly & Gabelli, 2014).

The salting-in/out extraction (AAFSISO) involved both salting-in and salting-out methods, wherein 1% NaCl (w/v) was added to the NaOH solution to facilitate protein solubility and 80% $(\text{NH}_4)_2\text{SO}_4$ (w/v) was added to the supernatant to promote protein precipitation.

Table 3.1. Preparation of the protein isolates

Protein isolation details		
	Abbreviation	Salt addition
Alkaline - acid extraction + filtration	AAF	None (control sample)
AAF + salting-in	AAFSI	1% NaCl
AAF + salting-out	AAFSO	80% (NH ₄) ₂ SO ₄
AAF + salting in & salting out	AAFSISO	1% NaCl + 80% (NH ₄) ₂ SO ₄

3.2.2. Protein yield

The crude protein content was determined by using the Dumas combustion method (TruSpec-N Leco, St. Joseph, MI, USA) and adopting a protein-to-nitrogen conversion coefficient of 5.60, as advised by Janssen et al. (2017) for edible insects. EDTA was used as the standard reference in accordance with the laboratory's established procedure. The extraction purity was expressed as the final protein concentration in the protein concentrates on a dry basis (%). The extraction yield (% w/w) was calculated as follows:

$$\text{Extraction yield (\%)} = \frac{\text{mass of extracted protein (g)}}{\text{dry mass of the initial sample (g)}} \times 100$$

3.2.3. Physicochemical properties

3.2.3.1. Colour change.

The insect flours and protein concentrates were evaluated for their colour using a spectrophotometer (Konica Minolta Sensing Americas Inc., Tokyo, Japan) based on the CIE L* a* b* colour space system. The instrument was calibrated using the white calibration plate, followed by zero calibration. The powdered samples were evenly spread in the cuvette (diameter 30 mm) for reflectance measurement. The measurements were performed in triplicate at three different positions in the samples, and the results were recorded in terms of L* (lightness/brightness), a* (redness/greenness), and b* (yellowness/blueness). The overall colour difference (ΔE) was calculated using the values of ΔL^* , Δa^* , and Δb^* , where ΔE is a quantitative representation of the difference between two colours.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

3.2.3.2. Thermal properties

The thermal properties that include differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were evaluated on the freeze-dried protein concentrates. In both these 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 protein concentrates was evaluated using a TGA system (TGA-7, Perkin Elmer, Singapore). The analysis was performed from 30 to 650°C at a heating rate of 20°C min⁻¹ with nitrogen as carrier gas at a flow rate of 50 mL min⁻¹.

3.2.3.3. Surface Charge (Zeta Potential)

The zeta potential of protein isolates was measured based on the method of Deng et al. (2017) and Mshayisa et al. (2021). The protein isolate solutions (1%, w/v) were prepared by dispersing the protein samples in Milli-Q water. The solutions were filtered through Whatman No. 1 filter paper. The pH of these diluted solutions was adjusted to pH 8 with 0.1 M HCl or 0.1 NaOH. The zeta potential was determined using a Zetasizer Nano Series (Malvern Instruments, Malvern, Worcestershire, UK), following the methodology of Mshayisa et al. (2021).

3.2.3.4. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The soluble proteins from the BSF larvae protein isolates were analysed with SDS-PAGE to determine the molecular weight (MW) distribution of the proteins. This was performed using a mini Protean Tetra Electrophoresis Cell System (Bio-Rad, United States) and 4 – 20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, United States). A 50 mg sample was dissolved in 1 mL of Laemmli buffer (62.5mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.01% bromophenol blue and 20% glycerol). After 2 min of agitation and 10 min in an ultrasonic bath, the samples were heated at 100°C for 10 min, and then centrifuged for 2 min at 10,000 rpm. 10 µL of the solution was applied to the gel and the electrophoresis was run at 180 V. The gels were stained with Coomassie Brilliant blue G250 (Bio-Rad, United States). The Sigma Pre-Stained Protein Marker was employed to identify different protein molecular weight fractions.

3.2.4. Techno-functional properties

3.2.4.1. Protein Solubility

The solubility of the protein concentrates was evaluated using a modified version of the method described by Hall et al. (2017). Briefly, 400 mg of each sample was dispersed in 20 mL of phosphate buffers ranging from pH 2 to 10. The mixture was stirred using a magnetic stirrer at room temperature for 30 min, centrifuged at 7500×g for 20 min at 4°C and then filtered using Whatman No. 1 filter paper. The protein content of the supernatant and the total protein in the sample were measured using the bicinchoninic acid protein assay (BCA) method, with BSA serving as a standard, following the manufacturer's protocol (Sigma, St. Louis, MO, USA). Protein solubility was expressed as a percentage and calculated as follows:

$$\text{Protein solubility (\%)} = \frac{\text{Protein in the supernatant}}{\text{Total protein content in the sample}} \times 100$$

3.2.4.2. Surface hydrophobicity

The determination of surface hydrophobicity followed a previously established method, as outlined by Chen et al. (2018). In summary, protein solutions ranging from 0.04 to 0.20 mg/mL were prepared in 0.1 M phosphate buffer at pH 7.0. Subsequently, 20 µL of an 8 mM solution of ANS (1-anilino-8-naphthalenesulfonate) in phosphate buffer was added to 4 mL of the protein solution, followed by vortex-mixing for 10 sec. The resultant mixture was then kept in the dark for 15 min, and fluorescence intensity values (FI) were measured at excitation and emission wavelengths of 390 and 470 nm, respectively, using a F2500 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA, USA). To obtain accurate measurements, FI values of ANS-blank and diluted protein solutions without ANS were also determined and subtracted from the FI of the protein solutions containing ANS. The initial slope of the plot depicting the corrected FI against protein concentration was computed using linear regression analysis and utilized as an indicator of protein hydrophobicity.

3.2.4.3. Free sulfhydryl group (SH) and disulfide bond (SS) content

Free SH values were determined as previously described by Deng et al. (2019). Briefly, 30 mg BSF larvae was dissolved in 10 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M Gly, 4 mM EDTA,

pH 8 containing 8 M urea) to which 50 μ L of 4 mg/ml Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid) in Tris-Gly buffer was added. The mixture was incubated at 25°C for 0 min followed by centrifugation at 8000 $\times$ g for 5 min. Absorbance of the supernatant was measured at 412 nm using buffer containing Ellman's reagent as blank. Total SH was determined with 15 mg of BSF larvae protein that was dispersed in 10 mL Tris-Gly buffer and 100 μ L of 2-mercaptoethanol. These dispersions were incubated at 25°C for 60 min and 10 mL of 12% trichloroacetic acid (TCA) was added followed by centrifugation at 8000 $\times$ g for 10 min. The precipitates were washed with 10 mL of 12% TCA three times and then centrifuged. The protein precipitate was dissolved in 10 mL Tris-Gly buffer and 50 μ L Ellman's reagent was added for a colour reaction as per above. The SH content was calculated as follows:

$$\text{SH}(\frac{\text{uM}}{\text{g}}) = \frac{73.53 \times A_{412} \times D}{C}$$

where A₄₁₂ was the absorbance at 412 nm, D was the dilution factor; C was the protein concentration (mg/mL). The SS content = (Total SH value - Free SH value)/2.

3.2.4.4. Bulk density

The method described by Mintah et al. (2020) was followed with some modifications. First, a 50 mL measuring cylinder, pre-weighed (W_1 - g), to determine its empty weight, was used. Then, 5 g of the sample was added to the cylinder and compacted through tapping until its volume remained constant. Afterward, the cylinder with the sample was weighed again (W_2 - g), the final volume (V_1 - ml) was recorded, and the density (g/mL) was calculated using the provided formula:

$$\text{Bulk density} = \frac{W_2 - W_1}{V_1}$$

3.2.4.5. Water and oil binding capacity

The water-binding capacity (WBC) of the protein concentrates was measured using a modified version of the methods reported by Purschke et al. (2018) and Deng et al. (2019). In brief, 0.5 g of the sample was mixed with 2.5 mL Milli-Q water at 25°C in a pre-weighed centrifuge tube. The centrifuge tube was then vortexed for 2 min (using Vortex-Genie 2, Scientific Industry Inc., Bohemia, NY, USA), and centrifuged at 3000 $\times$ g for 10 min. The water that was not bound was

decanted, and the tube was inverted onto Whatman No. 1 filter paper for an hour before re-weighing. The weight of the remaining material + centrifuge tube was determined. WBC of the samples was calculated as:

$$\text{WBC}(\frac{\text{g}_{\text{water}}}{\text{g}_{\text{DM}}}) = \frac{W_2 - W_1}{W_{o,DM}}$$

Where W_2 (g) was the weight of centrifuge tube and protein with bound water in g; W_1 (g) was the weight of the centrifuge tube and protein without water in mg; W_0 (g) was the weight of the protein sample based on dry matter in g. The oil binding capacity (OBC) was assessed by substituting Milli-Q water with commercial sunflower oil. The experimental procedure was identical to the WBC assay, except for the mixing step, which was performed for 2 min. OBC was calculated as follows:

$$\text{OBC}(\frac{\text{g}_{\text{oil}}}{\text{g}_{\text{DM}}}) = \frac{W_2 - W_1}{W_{o,DM}}$$

3.2.4.6. Foaming capacity and stability

The foaming characteristics of the protein concentrates were assessed using a method adapted from Mshayisa and Van Wyk (2021). Edible insect and protein concentrates were reconstituted in Milli-Q water (5% w/v) at pH 7 and centrifuged at 10,000 rpm for 15 min. Next, 20 mL of the supernatant was subjected to high shear homogenization (Polytron PT 2500E) at 16,000 rpm for 3 min. The homogenized mixture was transferred to a 50 mL graduated cylinder, and the total volume was recorded at both 0 and 30 min after homogenization to evaluate the stability of the foam. The foaming capacity and foam stability were calculated from the formula:

$$\text{Foaming capacity (FC)} = \frac{V_0}{V} \times 100$$

$$\text{Foam stability (FS)} = \frac{V_1}{V_0} \times 100$$

where: V_0 (ml), V_1 (ml), V (ml) were the foam volumes at 0 min, 30 min and initial volume, respectively.

3.2.4.7. Emulsifying properties

The emulsifying properties of the protein concentrates were evaluated by adapting the procedures described by Coelho and Salas-Mellado (2018) and Zielińska et al. (2018). Specifically, the sample was dispersed in distilled water (5% w/v) at pH 7 and subjected to centrifugation (Thermo Electron Corporation Jouan MR1812, Waltham, MA USA) at a speed of 9,000 rpm for 15 min. The resultant supernatant was then combined with sunflower oil (1:1 v/v) and homogenized (Polytron PT 2500 E, Thermofisher, Cape Town, South Africa) at 18,000 rpm for 1 min. Aliquots of 15 mL of the emulsion samples were centrifuged at 3,000 rpm for 5 min, and the volume of each layer was determined using a 50 mL graduated tube. The emulsifying activity was calculated as:

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

The emulsion was heated in a water bath (80°C for 30 min) to determine the stability values. Emulsion stability was calculated as:

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

where: V (mL) - total volume of tube contents, V_{el} (mL) - volume of the emulsified layer, V_{30} (mL) - volume of the emulsified layer after heating.

3.2.5. Structural property determination

3.2.5.1. Amino acid composition

A 0.5 g fine powder sample was weighed and placed into a screw-capped test tube. Then, 7 mL of 6 M HCl was added, and the tube was capped and heated for 6 hours at 110°C to carry out hydrolysis. After cooling to room temperature, the sample was dried using a Reacti-Therm™ heating/stirring module at 70°C under a stream of nitrogen. The remaining residue was quantitatively transferred and made up to volume in 100 mL volumetric flasks with 0.1 M HCl. The resultant solutions were filtered using 0.22 µm pore size cellulose membrane syringe filters

(Sigma-Aldrich, Johannesburg, South Africa) and transferred into high-performance liquid chromatography (HPLC) vials.

The analysis was performed on an Agilent 1260 Infinity II liquid chromatography system (Chemetrix, Midrand, South Africa), equipped with a μ -degasser (G7111A), 1260 binary pump (G7111B), 1260 standard auto-sampler (G7129B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G7117C), and a Zorbax Eclipse-AAA column (150 mm $\times$ 4.6 mm, i.d., particle size 5 μ m; Chemetrix). The chromatographic conditions employed were as follows: mobile phase A was acetonitrile: methanol: water (45:45:10, vol.%), the mobile phase B consisted of 5.5 g of Na₂HPO₄ per 1L of distilled water, adjusted to pH 7.8 using 10 M NaOH solution. The hydrolysed samples and solutions of the standard amino acid mixture were derivatised with OPA and FMOC using the autosampler programme for solution mixing, and 0.5 μ l of each sample was injected into a Zorbax Eclipse-AAA column at 40°C, with detection at λ_1 =338 nm and λ_2 =262 nm. The separation was performed at a flow rate of 1 ml/min employing a solvent gradient (vol.%).

3.2.5.2. Circular Dichroism (CD)

Protein solutions were formulated with a final concentration of 0.5 mg/mL in 100 mM phosphate buffer with pH 7.0. A Jasco J-600 Spectropolarimeter was utilized to collect data at 25°C with a 0.1 cm path length quartz cell, a 10 nm/min scan rate, and a 0.2 nm band width. Spectra were recorded as an average of 3 scans between 190 to 260 nm. The spectrum of 100 mM phosphate buffer served as a reference and was subtracted from the average of three spectra to derive the corrected spectrum for each sample. Duplicate sample spectra were recorded. The CONTIN software was used to deconvolute the spectra for the identification of secondary structures. CONTIN, a software developed by Provencher and Glöckner (DICHROWEB website), makes use of a large database of proteins with known conformations to fit the CD of unknown proteins by a linear combination of the spectra. This method yields reliable estimates of α -helix (right-handed coiled structure in proteins formed by a twisting polypeptide chain), β -turns (protein structural motifs that cause the chain to reverse direction, typically composed of a four-amino-acid sequence) and β -sheet (secondary protein structures where neighbouring segments of the polypeptide chain

align side by side in a sheet-like arrangement, stabilized by hydrogen bonds between adjacent strands) in a protein.

3.2.5.3. Fourier Transform Infrared Spectroscopy (FTIR)

Protein concentrate samples were subjected to analysis via FT-IR using a Perkin Elmer instrument equipped with a universal attenuated total reflectance (UATR) polarisation accessory, as described by Zozo et al. (2021). Before data collection for each sample, a background spectrum was collected. The samples, which had been ground to powder form using a mortar and pestle, were placed directly onto the surface of the ATR crystal. Spectra were collected by co-adding 32 scans with a resolution of 4 cm⁻¹ in the range of 400 – 4000 cm⁻¹. To eliminate any residual contribution from previous samples, the UATR crystal was cleaned with acetone before each scan. After baseline correction and smoothing were performed using the Savitzky-Golay function on Origin Pro 8.6 software for Windows® (Dell Technologies, Cape Town, South Africa), the spectrum data were imported to Unscrambler 11 software for Windows to standardise the normal variations.

3.2.5.4. X-Ray Diffraction (XRD)

The XRD pattern of the protein isolates was determined using a diffractometer, Panalytical-X'pert PRO (Almelo, Netherlands) with CuK (Å) = 1.54056 radiation. Samples were scanned at the rate of 3.2° /min from 5° to 40° (2θ) with a step size of 0.013°. Crystallinity measurement was carried out by X'pert HighScore plus software. Crystalline index (CrI) values of the proteins were calculated using the formula below.

$$\text{CrI}_{110} = \frac{I_{110} - I_{am}}{I_{am}} \times 100$$

I_{110} is the maximum intensity at $2\theta = 19^\circ$.

I_{am} is the intensity of amorphous diffraction at $2\theta = 13^\circ$.

3.3. Statistical multivariate analysis: principal component analysis (PCA)

This projection method uses only the most important principal components of the PCA, which are factors relating to sensor signals. PCA calculation is based on a singular value decomposition of

the data array of the fingerprint region of the FTIR spectrum. The first two scores of the PCA results were used to make a projection plot that provided a visual determination of the similarity among the fingerprints. Unscrambler 11 for Windows was used for the PCA in this work.

3.4. Data analysis

All experiments were analysed in triplicate and the results were presented as means $\pm$ standard deviation. All data were subjected to multivariate analysis of variance (MAN-OVA) 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 mean inter-actions. SPSS 29.0 for Windows® (Dell Technologies, Cape Town, South Africa) was used for the statistical analyses and the level of confidence required for significance was selected at $p < 0.05$. Graphs and figures were generated using Origin software, version 9.60 (Origin labs, Northampton, MA, USA). Pearson correlation coefficients (r) were used to assess the relationships among functional properties. The strength and direction of the correlations were categorized as follows: low correlation ($r = 0.1$ to 0.3), moderate correlation ($r = 0.3$ to 0.5), and high correlation ($r = 0.5$ to 1.0). A correlation of $r = 1.0$ indicates a perfect positive relationship, while $r = -1.0$ represents a perfect negative relationship. A correlation of $r = 0$ indicates no linear relationship between the variables.

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CHAPTER FOUR

STRUCTURAL PROPERTIES OF BLACK SOLDIER FLY LARVAE PROTEIN CONCENTRATES EXTRACTED VIA SALTING-IN AND SALTING-OUT COUPLED WITH ULTRAFILTRATION

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“The consumption of insects has been a traditional practice in many cultures for centuries. Now, it's time to embrace this ancient wisdom on a global scale to ensure food security for future generations.”
(Afton Halloran, 2016)

4.1. Abstract

For industrial food production, the black soldier fly (BSF) larvae are one of the promising edible insect species. Therefore, the aim of this study was to investigate the effect of salting-in/out extraction coupled with ultrafiltration techniques on BSF larvae protein. Protein extraction was performed with alkaline and acid precipitation coupled with ultrafiltration. The alkaline extraction and acid precipitation (AAF), salting-in (AAFSI), salting-out (AAFSO), and salting-in-out (AAFSISO) were used for the extraction of protein isolates. The extraction method had an influence on the crude protein content, significantly ($p < 0.05$). For example, in the use of salting-out assisted treatment, the AAF extraction resulted in a protein yield of 71.25% which went up to 85.02%. The essential amino acids total sum was 320.48 mg/g for AAF, 300.40 mg/g for AAFSI, 286.15 mg/g for AAFSO, and 296.88 mg/g for AAFSISO. All these adult daily requirements are found by the World Health Organization (minimum of 277 mg/g). All samples exhibited significantly ($p < 0.05$) negative zeta potentials, ranging from -9.81 to -26.86 mV. Moreover, no significant differences ($p > 0.05$) were observed in the levels of exposed sulfhydryl groups between AAF and AAFSI proteins (6.75 and 6.90 $\mu\text{M/g}$, respectively). Fourier transform infrared spectroscopy (FTIR) of protein extracts derived from AAFSO and AAFSISO revealed a significant N–H free stretching vibration in the spectral range of 3500–3200 cm^{-1} . The β -turns and random coil structures were transformed into α -helix and β -sheet by salting-out treatments, as demonstrated by circular dichroism, indicating a more compact and organized conformation. AAFSO and AAFSISO had the highest content of ordered protein structures and showed the largest ΔH values, measured at 141 J/g and 137 J/g, respectively. This research demonstrates that the integration of salting-out extractions and ultrafiltration techniques holds significant potential for protein extraction, especially as an innovative approach to producing insect proteins for food-related applications.

4.2. Introduction

The growing interest in eating more sustainable proteins has led to the use of edible insect proteins in human cuisine. It is conceivable that the adverse perceptions associated with insects may be alleviated by incorporating them in a manner that is not perceived as a functional component. This method would involve adding bug powders or flours to everyday foods to make them more appealing to customers. (Gmuer et al., 2016; Hartmann & Siegrist, 2017; Schösler et al., 2012; Tan et al., 2015) these are some of the studies that have been done on this subject. The black soldier fly (BSF) larvae has gained attention in recent years because of their known for their low environmental impact, as they are reared on a different organic waste materials (van Huis, 2013). In the aspect of nutrition, this insect is very rich in (30.0 to 67.7%) and lipids (20 to 39%) (Bußler et al., 2016b; Mshayisa et al., 2021; Queiroz et al., 2021; Vanqa et al., 2022). Non-striated muscle and structural proteins were found in BSF larvae in significant amounts. This then supports the substitution of traditional meat and soy protein sources.(Altmann et al., 2018).

It is normally not acceptable for most people to eat whole insects, but other ways of eating insects are growing more common. Therefore, getting insect derivatives is a better way to make ingredients that can be used in processed foods. BSF larvae can be used in different applications as previous research has stated. Animal feed, aquaculture, and human consumption are applications that can be used (Nguyen et al., 2015; Ghaly, 2009; Sheppard et al., 2002; Wang et al., 2017). It is important to note that the extraction process that is utilised has a significant impact on the profile, quality, and yield of BSF larva protein isolates. The physicochemical and structural features of proteins are directly affected by the extraction methods that are used.

Pretreatment, defatting, protein extraction, and protein purification are the four basic processes that are commonly involved in the process of isolating proteins from living organisms. Blanching and freeze-drying the insect larvae is often the initial process, which is followed by grinding and screening the larvae to generate a fine powder. The powder enhances surface area, improving protein-solvent interaction during extraction. (Zozo et al., 2022; Nguyen et al., 2015; Ghaly, 2009; Choi et al., 2017) these are some of the researchers who have successfully implemented a defatting phase in order to increase the amount of protein that can be extracted from insects in a laboratory setting. When their respective flours were defatted with hexane, both in

Tenebrio molitor and *Hermetia illucens* observed a significant increase in protein content. Specifically, the protein content of *Tenebrio molitor* increased from $58 \pm 1.2\%$ to $65 \pm 0.3\%$, while that of *Hermetia illucens* increased from $35 \pm 0.2\%$ to $45 \pm 1.4\%$ (Bußler et al., 2016a).

The insect protein extraction has employed numerous techniques. Yi et al. (2013) used acidic solubilisation to extract protein isolates from the supernatants of five insect species (*Tenebrio molitor*, *Zophobas morio*, *Alphitobius diaperinus*, *Acheta domesticus*, and *Blaptica dubia*) with a protein yield of 50 to 61%. Chatsuwan et al. (2018) extracted *Patanga succincta* and *Chondracris rosea* at 49% and 43%, respectively. A protein yield of 51.7% and purity of 82.3% were reached by using either 1M or 4M NaOH to increase the solubility of protein in a protein concentrate prepared from *Locusta migratoria*, as stated by Purschke et al. (2018). This was accomplished by increasing the concentration of NaOH from 1 M to 4 M. These findings show the feasibility of employing both aqueous and alkaline solubilisation techniques for the manufacture of insect protein isolate. However, Makker et al. (2014) say that the high pH and temperature needed for these approaches can cause proteins to denature and lose their ability to work.

Ultrafiltration (UF) is a common way to clean proteins from many different sources. In a study, tangential ultrafiltration was employed for protein fractionation utilising a membrane molecular weight cut-off of < 300 kDa on algal proteins solubilised at pH 7 and 12 (Ursu et al., 2014). Most of the proteins, on the other hand, were in the retentate. This was because the macromolecular aggregates of algal proteins were too big to fit through the molecular weight cut-off. There has been little research on using membrane filtration for insect proteins because we need to know a lot about their size and other characteristics. Not much study has been done in this field yet; however, Sweers et al. (2023) looked into ultrafiltration and stressed how important protein hydrolysis, aggregation, and pH are for getting proteins back. This work seeks to further examine these facets to enhance the utilisation of membrane filtration techniques for insect proteins.

The food business needs more than one method for extracting proteins because they need to be functional and useful in products. So, we need to look into more methods, including salting-out extractions, ultrafiltration, and microfiltration, to go along with the ones we already have and get high extraction efficiency and purity. Salt-based extraction is a well-known method in the food industry. To get biologically active compounds out of crops, like pea protein (Sun & Arntfield,

2011), oleosin (Sun et al., 2022), soy protein (Jiang et al., 2010), and *Tenebrio molitor* protein (Jiang et al., 2021) have been used. Other traditional methods are not better than salt-based extraction because they make it easy to scale up, it is good for the environment, are cheap, produce high yields, have a high polarity, and low viscosity. Salting-in extraction or salting-out extraction are the two ways to do this. To make it more soluble and encourage protein precipitation, the salting-in method adds salt to the protein solution, while the salting-out method reduces protein solubility and facilitates protein precipitation and aggregation by the addition of salt to the protein solution (Duong-Ly & Gabelli, 2014). According to A property explained in the Hofmeister series (Okur et al., 2017), the ionic strength of the salts would differ in their capacity to salt in/out proteins.

As a result, NaCl is often used as to salt in proteins, and $(\text{NH}_4)_2\text{SO}_4$ is employed to speed up the process of salting out proteins. These methods that use salting agents have been demonstrated to work for separating different proteins from water solutions, like ovalbumin, bovine serum albumin, myoglobin, and cytochrome (Okur et al., 2017; Gadalkar et al., 2017; Mune et al., 2014). Some researchers have reported that an effective increase in the solubility of albumin and globulin was present for plant proteins (during the process of alkaline extraction), and enhanced protein extraction efficiency can be achieved using NaCl. Thus, the quantity and quality of native proteins can be simultaneously improved (De Chirico et al., 2020; Mune et al., 2014; Gadalkar et al., 2017).

However, to date, there is no evidence of research conducted on the effect of the salting-in/out extraction techniques coupled with ultrafiltration on BSF larvae protein. It is essential to evaluate the efficiency of these methods and their impact on the quality and yield of BSF larvae protein isolates. Therefore, the aim of this study was to elucidate the effect of salting-in and salting-out extraction coupled with ultrafiltration on the physicochemical, structural, and thermal characteristics of BSF larvae protein isolates.

4.3. Methodology

The experimental design, quantification, and analysis of data were described in detail in Sections 3.1, 3.2, and 3.4 of Chapter 3. Refer to the schematic diagram below for a summary of the steps and methods involved (**Figure 4.1**).

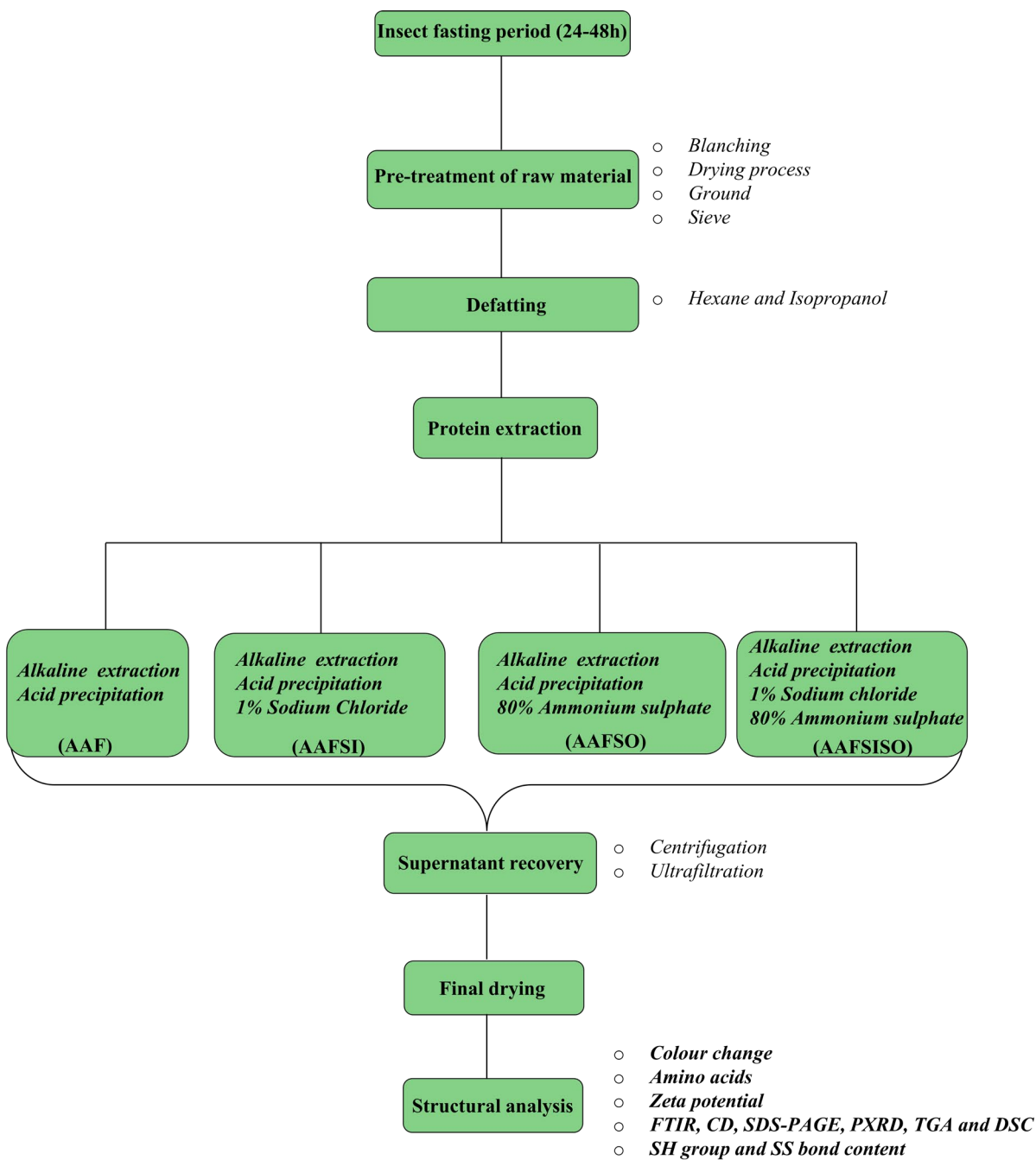


Figure 4.1. Summary of the methodology schematic, as presented in Chapter 3.

4.4. Results and discussion.

4.4.1. Colour change

Determination of colour in food or novel ingredients is useful due to the association between colour, taste, and flavour perceptions in humans. The colour measurements for the proteins extracted from the BSF larvae using different techniques are displayed in **Table 4.1**. There is a significant difference ($p < 0.05$) in the colour attributes in terms of lightness (L^*), redness (a^*), yellowness (b^*), and the colour difference (ΔE). AAFSISO isolates were characterised by significantly ($p < 0.05$) higher lightness (higher L^* value), lower redness (a^*), and yellowness (b^*) than the AAF isolates. The L^* value of AAF was significantly ($p < 0.05$) lower when compared to other samples; these findings are similar to those reported by Bußler et al. (2016). The significantly higher L^* value observed for the AAFSISO isolates may be attributed to size effects, as smaller particle sizes typically scatter lighter uniformly, resulting in increased lightness (Wu et al., 2022).

Generally, BSF larvae flour is darker in appearance, and the resultant protein is much lighter, especially when using AAFSISO extraction. In this study, ΔE denotes the overall colour variation between the control sample and samples containing added salt. The parameter is used as a colour quality indicator, and a value below 1.5 is considered not visible to consumers. In contrast, a value above 6.0 is found to be visible for consumers (Wibowo et al., 2014). This study found that the range of ΔE was between 11.63 and 21.35, indicating that colour differences were perceptible at a glance, meaning that the changes in colour could be easily distinguished without the need for a closer examination. Due to the addition of salts, the redness (a^*) and yellowness (b^*) were significantly ($p < 0.05$) decreased; similar results were obtained by Wu et al. (2022), who studied the effect of salt addition on textural and quality properties in the chicken-surimi products.

The use of alkaline conditions during the extraction of AAF proteins can result in the co-extraction of phenolics from BSF larvae protein, producing protein isolates with a dark green to brown colour. As explained by Janssen et al. (2019), this occurs since phenolic compounds, when exposed to alkaline conditions, undergo enzymatic and non-enzymatic oxidation to form quinones, which can then bind to protein and contribute to the darker colour (Xu & Diosady, 2002). Salts, on the other hand, can slow down phenolic oxidation and ionic interactions, which makes the protein powder lighter in colour (Hadnacev et al., 2018).

Table 4.1. Effect of extraction on the colour attributes of black soldier fly larvae protein isolates.

				
Isolation	AAF	AAFSI	AAFSO	AAFSISO
L[*]	60.94 ± 0.09 ^a	72.67 ± 0.47 ^b	75.34 ± 0.46 ^c	81.65 ± 0.46 ^d
a[*]	3.50 ± 0.71 ^c	2.00 ± 0.00 ^b	1.93 ± 0.09 ^b	0.85 ± 0.09 ^a
b[*]	13.76 ± 0.35 ^b	15.60 ± 0.57 ^c	15.34 ± 0.46 ^c	9.10 ± 0.46 ^a
ΔE	Control	11.82 ± 0.26 ^a	14.38 ± 0.16 ^b	21.35 ± 0.16 ^c

Results are reported as mean ± standard deviation. Different superscripts in the rows indicate significant differences between treatments ($p \leq 0.05$). AAF (alkaline-acid extraction); AAFSI (salting in technique); AAFSO (salting out technique) and AAFSISO (salting-in and out technique).

Treatments that remove phenolic compounds lead to lighter-coloured protein isolates (Xu & Diosady, 2002). The results of this study indicate that AAFSISO produces protein powders that are lighter in colour and may be more desirable for food products where a lighter appearance is preferred, such as dairy alternatives or foams and emulsions. Furthermore, the lighter colour could be linked to higher protein purity or differences in protein band intensity, as these factors can influence the light-scattering properties and overall visual appearance of the powders.

4.4.2. Amino acid composition, protein quality analysis, and zeta potential

The essential and non-essential amino acid content of BSF larvae extracted with various methods and FAO (Food and Agriculture Organisation) protein intake recommendations for adults are displayed in **Table 4.2**. The method of extraction had a significant ($p < 0.05$) influence on the crude protein yields of the isolates. For instance, the basic AAF extraction resulted in a protein yield of 15.90%, which increased to 19.47% when the salting-in-assisted treatment was used. When alkali solutions were employed, protein solubility was directly related to the ionic strength at lower ion concentrations, resulting in more protein salting-in. Salting-out-assisted treatments also notably increased the crude protein yields, consistent with previous findings that higher concentrations of $(\text{NH}_4)_2\text{SO}_4$ lead to increased protein yields (Duong-Ly & Gabelli, 2014). However, no significant ($p > 0.05$) differences in crude protein yields were observed between AAFSI and AAFSISO extractions, which yielded 36.10% and 44.45%, respectively (**Table 4.2**).

Moreover, these protein yields were in line with those previously reported for BSF larvae (ranging from 26.40% to 59.9%), the variation being attributed to any one or more of: variations in process parameters such as pH, temperature, heating duration, NaCl, and $(\text{NH}_4)_2\text{SO}_4$ concentrations, and the liquid-to-solid ratio (Lam et al., 2018). The amino acid composition of a protein not only plays a role in determining its structure but also has broader implications. The protein extracts in this study showed the presence of all essential amino acids (EAA) except for tryptophan and cysteine, which were not measured. Tryptophan is susceptible to degradation under acidic hydrolysis conditions, and severe conditions result in its destruction, which can lead to inaccurate measurements. Similarly, cysteine's reactivity and sensitivity to oxidation are attributed to its thiol (-SH) group. During standard acidic hydrolysis, cysteine may undergo oxidative changes, resulting in inaccurate measurements.

Table 4.2. Amino acid (AA) composition (mg/g of crude protein) of the black soldier fly larvae protein extracts, compared with the adult daily requirements, long with additional data on protein extraction yield (%), protein content (%), and zeta potential (mV).

Essential amino acids	AAF	AAFSI	AAFSO	AAFSISO	Adult daily requirements 1985 FAO/WHO/UNU
Histidine (His)	34.54 ± 0.66 ^d	38.71 ± 0.41 ^c	26.97 ± 0.04 ^b	16.09 ± 0.12 ^a	15
Isoleucine (Ile)	32.04 ± 0.05 ^b	32.89 ± 0.16 ^c	27.20 ± 0.28 ^a	38.28 ± 0.39 ^d	30
Leucine (Leu)	63.29 ± 0.41 ^d	59.26 ± 0.36 ^c	49.91 ± 0.13 ^a	53.35 ± 0.49 ^b	59
Lysine (Lys)	38.34 ± 0.48 ^b	28.60 ± 0.57 ^a	37.60 ± 0.57 ^b	45.24 ± 0.33 ^c	45
Methionine (Met)	25.28 ± 0.40 ^b	16.48 ± 0.67 ^a	26.78 ± 0.32 ^c	25.98 ± 0.04 ^{bc}	22
Phenylalanine (Phe)	42.21 ± 0.30 ^b	44.35 ± 0.49 ^c	46.23 ± 0.32 ^d	39.13 ± 0.18 ^a	38
Threonine (Thr)	41.43 ± 0.60 ^d	35.17 ± 0.24 ^c	28.12 ± 0.17 ^b	22.20 ± 0.28 ^a	23
Valine (Val)	43.36 ± 0.51 ^b	45.05 ± 0.07 ^c	43.36 ± 0.50 ^b	40.16 ± 0.23 ^a	39
Tryptophan (Trp)	-	-	-	-	6
Sum of EAA	320.48 ± 2.09 ^d	300.4 ± 0.69 ^c	286.15 ± 0.21 ^b	296.88 ± 16.57 ^a	277
Non-essential amino acids					
Alanine (Ala)	47.35 ± 0.49 ^c	52.95 ± 0.08 ^d	28.26 ± 0.37 ^a	35.28 ± 0.39 ^b	
Arginine (Arg)	45.24 ± 0.34 ^c	48.04 ± 0.06 ^d	22.85 ± 0.21 ^a	28.23 ± 0.33 ^b	
Aspartic acid (Asp)	75.83 ± 0.24 ^c	92.08 ± 0.11 ^d	37.0 ± 0.04 ^a	46.21 ± 0.30 ^b	
Glutamic acid (Glu)	101.11 ± 0.16 ^c	130.38 ± 0.53 ^d	53.69 ± 0.98 ^a	72.62 ± 0.54 ^b	
Glycine (Gly)	51.15 ± 0.21 ^c	51.11 ± 0.15 ^c	24.66 ± 0.49 ^a	32.84 ± 0.23 ^b	
Proline (Pro)	33.92 ± 0.11 ^c	35.06 ± 0.08 ^c	22.64 ± 0.51 ^a	27.86 ± 0.20 ^b	
Serine (Ser)	36.66 ± 0.49 ^c	37.93 ± 0.04 ^d	21.37 ± 0.52 ^a	26.42 ± 0.59 ^b	
Tyrosine (Tyr)	69.69 ± 0.44 ^c	70.98 ± 0.03 ^d	39.02 ± 0.02 ^a	49.61 ± 0.55 ^b	
Sum of total amino acids	782.83 ± 2.00 ^c	818.99 ± 1.54 ^d	535.65 ± 0.92 ^a	599.47 ± 2.07 ^b	
Crude protein content (%)	71.24 ± 0.03 ^a	77.61 ± 0.15 ^c	85.02 ± 0.27 ^d	75.34 ± 0.45 ^b	
Yield (%)	15.90 ± 0.22 ^a	19.47 ± 0.12 ^b	36.10 ± 0.89 ^c	44.45 ± 0.50 ^d	
Zeta potential (mV)	-9.81 ± 0.73 ^d	-14.96 ± 0.17 ^c	-24.10 ± 0.34 ^b	-26.86 ± 0.56 ^a	

Results are reported as mean ± standard deviation. Different superscripts in the row indicate significant differences between treatments ($p \leq 0.05$). AAF (alkaline-acid extraction); AAFSI (salting in technique); AAFSO (salting out technique) and AAFSISO (salting-in and out technique).

Table 4.2 provides a breakdown of the amino acid composition, including the essential amino acids: threonine, methionine, valine, histidine, lysine, leucine, phenylalanine, and isoleucine. There were significant differences in amino acid compositions among the protein isolates, indicating that the methods used for extraction and isolation influenced the resultant amino acid profiles. In quantitative terms, the total sum of all the essential amino acids was 321.95 mg/g for AAF, 300.98 mg/g for AAFSI, 286.31 mg/g for AAFSO, and 281.84 mg/g for AAFSISO. These values meet the adult daily requirements established by the World Health Organization (WHO), which specify a minimum of 277 mg/g (WHO/FAO/UNU, 2007). However, when compared directly to the Food and Agriculture Organization (FAO) recommendations, which are higher than those of the WHO (e.g., 343 mg/g for adults; FAO, 2013), only the AAFSI isolate meets the required threshold. The AAF method likely preserves the native structure and composition of proteins, resulting in a moderate yield and a balanced amino acid profile. The AAFSISO isolate displayed lower values for essential amino acids like histidine, threonine, methionine, and valine when compared to other extracts. Therefore, the amino acid profile of the protein is of great importance for BSF larvae protein to be considered as a supplement in food products or a novel functional ingredient. The salting-out treatments use high concentrations of salts to precipitate proteins, which significantly alter the amino acid composition by preferentially isolating certain proteins. The different ionic strengths and pH levels used can also impact the solubility and stability of specific amino acids.

Except for lysine and methionine, the AAFSI isolate exhibited higher levels of all essential amino acids in comparison to the daily requirements of the FAO for adults, which makes the extraction method noteworthy from a nutritional perspective. It's worth noting that Spranghers et al. (2017) have previously demonstrated that the concentration of each amino acid may vary depending on the diet of BSF larvae. Here, we highlight that amino acid content can also differ after protein extraction and the subsequent processing steps, such as the method used for killing the larvae (Bessa et al., 2020). AAFSI made proteins more soluble at lower ion concentrations, which led to increased protein yields. The way the ions interact with each other can change the amino acid profile in some way.

Different ways of extracting proteins could cause changes in the amino acid content of the BSF larvae; this was reported by Mintah et al. (2020). BSF larvae protein extract is a sustainable

alternative to other protein sources like meat and chicken since it has higher levels of several essential amino acids (EAAs). BSF larvae protein extraction can be a simple way to get the right amount of lysine (45 mg/g) for food fortification. In populations where lysine is a dietary concern, especially in developing countries where it is often found in low amounts (Bessa et al., 2020),

Moreover, the decrease in S-S bonds or total SH groups of the AAFSI protein extracted was attributed to the diminishing amounts of methionine (Chen et al., 2018). The effect of the extraction of methionine on the total amount of essential amino acids is shown in **Table 4.2**. AAFSI had more tyrosine, serine, alanine, arginine, and proline than the other groups, while AAF has the greatest value, followed by AAFSI and AAFSO. To cause proteins to selectively precipitate, NaCl and $(\text{NH}_4)_2\text{SO}_4$ must be used; this can also affect the amino acid composition of the final extract. For comparison, a protein from milk, namely casein, has about 377 mg/g of essential amino acids (Kamal et al., 2018), while a frequently utilised as a reference protein in biochemical studies, bovine serum albumin (BSA), comprises approximately 358 mg/g of essential amino acids (Jung et al., 2008). These values are far higher than those of the BSF protein isolates. This shows that BSF-derived proteins need to be further refined or supplemented in order to have nutritional profiles that are similar to those of well-known protein sources. Moreover, the BSF proteins could be a good alternative protein source in food because they can meet the WHO's basic nutritional needs. Amino acid profile and composition of BSF larvae proteins can be enhanced by salting-in and salting-out extraction techniques, as indicated by the results. This is rendering them desirable as nutritional components in diverse food processing applications.

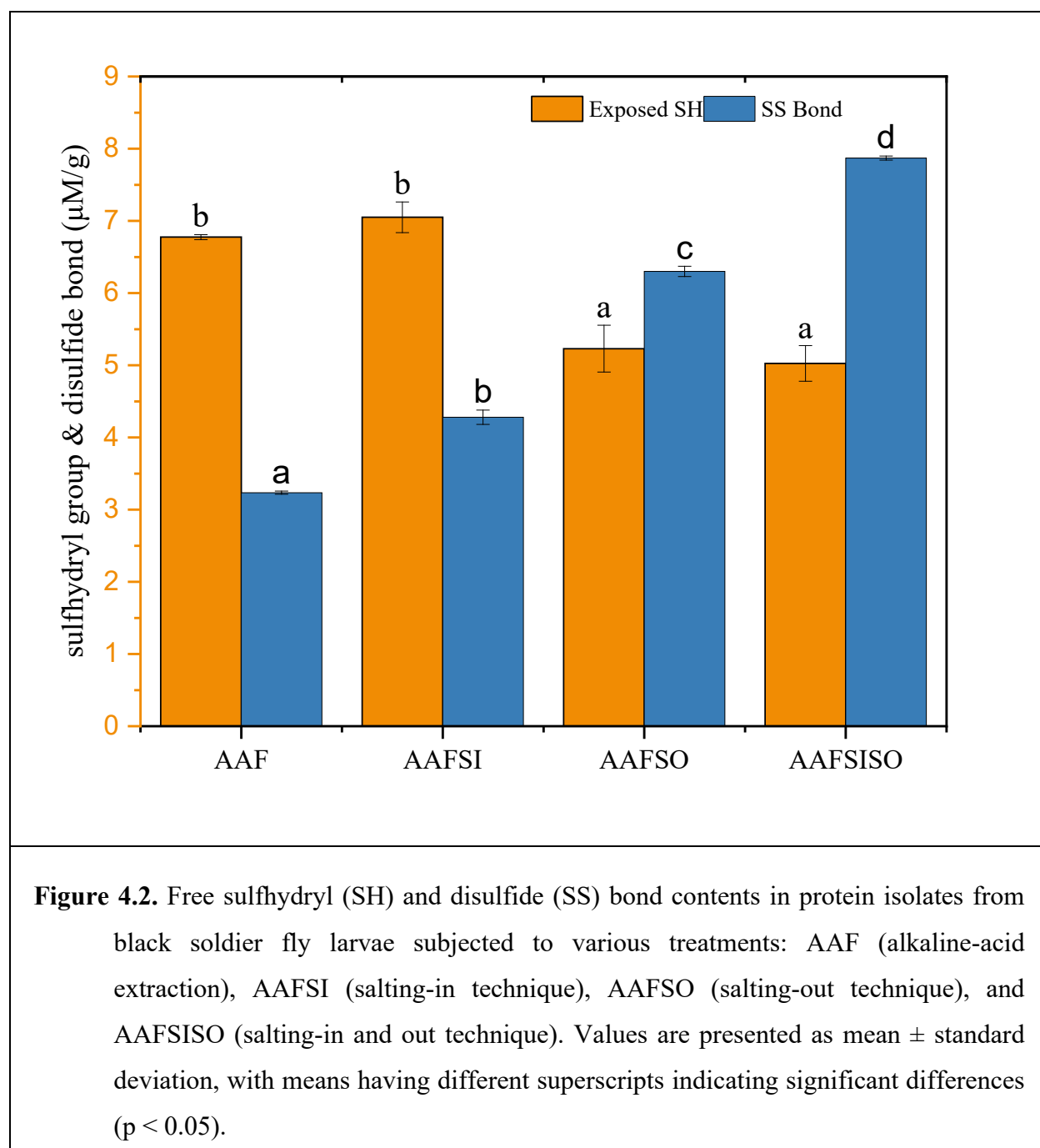
The zeta potential of a dietary protein macromolecule shows how its structure, content, and function have changed (Barba et al., 2019). A stable zeta potential value is usually between -30 mV and -60 mV or between $+30$ mV and $+60$ mV. For a colloidal system to stay stable, the zeta potential needs to be in these ranges. When it is, the particles are more likely to push each other apart, which stops them from coming together (Subaşı et al., 2020; Casanova et al., 2020). A higher absolute zeta potential results in stronger electrostatic repulsion and a greater separation distance between particles in a suspension, reducing aggregation and flocculation driven by Van der Waals

interactions (Thaiphanit et al., 2016). The zeta potential of proteins can also be affected by outside influences like temperature, ionic strength, and pH (Deng et al., 2019).

In this investigation, all BSF larvae isolates at pH 8 displayed negative zeta potentials, varying from -9.81 to -26.86 mV. The zeta potential values for AAF and AAFSI (-9.81 mV and -14.96 mV, respectively) were substantially ($p < 0.05$) greater than those for the salting-out procedures, with AAFSO at -24.10 mV and AAFSISO at -26.86 mV, as shown in **Table 4.2**. The AAFSO extraction, which has a more negative zeta potential (-24.10 mV) than AAFSI (-14.96 mV), shows that proteins extracted this way keep their natural electrostatic properties with little ionic influence, which makes them moderately stable and increases electrostatic repulsion. The combined salting-in and salting-out method (AAFSISO), which gives a zeta potential of -26.86 mV, suggests a synergistic effect that may lead to a net decrease in the electrostatic repulsion between protein particles. This could be because of a balance between ionic strength and protein interactions. A suspension's zeta potential is higher, which means that particles are more likely to push each other away. This makes them less likely to clump together (Azagoh et al., 2016). These results indicate that the proteins subjected to the salting-out approach exhibited enhanced electrostatic repulsion, possibly facilitating their higher solubility. When looking at these zeta potential data in light of using the protein extract as a new food additive, the anionic charge suggests that it might be used in recipes where colloidal stability is very important. The negative zeta potential's repulsive forces may help with problems like particle aggregation, flocculation, and sedimentation in food matrices like drinks, emulsions, and dairy substitutes

4.4.3. Free sulfhydryl group (SH) and disulfide bond (SS) content

Proteins have sulfhydryl (SH) groups and disulfide bonds (SS), which help them stay stable and strong (Deng et al., 2019; Hu et al., 2021). But heating and high pressure, which are examples of processing procedures, can destroy sulfhydryl content, which can make proteins less useful in culinary applications (Lin et al., 2020). **Figure 4.2** shows the amounts of free SH and disulfide bonds. In this study, no significant differences ($p > 0.05$) were observed in the levels of exposed SH groups between AAF and AAFSI proteins (6.75 and 6.90 $\mu\text{M/g}$, respectively), although these levels exceeded those of the proteins extracted by salting-out extraction (**Figure 4.2**).



Proteins with more exposed SH groups, such as those in AAF and AAFSI extracts, are more likely to stick together or clump together, which makes them less soluble (Deng et al., 2019). These results corroborate the study of zeta potential delineated in section 4.4.2. The disulfide bonds (SS) content in the salting-out protein samples (AAFSO and AAFSISO) was considerably ($p > 0.05$)

elevated compared to the AAF and AAFSI proteins. The SS content in the salting-out samples was 6.30 and 7.87 $\mu\text{M/g}$, whereas the AAF and AAFSI proteins displayed SS amounts of 3.24 and 4.28 $\mu\text{M/g}$, respectively. The findings of this study correspond with those presented by Jiang et al. (2021) about the extraction of proteins from *Tenebrio molitor*.

There are a lot of SS bonds because there is a lot of salt in the water. This can cause proteins to unfold because of the salting-out effect. This unfolding reveals more cysteine residues, which are the building blocks of SS bonds, in the protein. Consequently, this further demonstrates that the existence of SS bonds in a protein improves its thermal stability by stabilising its folded conformations and diminishing conformational entropy (Du et al., 2014). Furthermore, the molecular weight and SS content of a protein can influence emulsion stabilisation, with proteins derived by salting-out exhibiting greater thermal stability, according to the research conducted by Zhang et al. (2019). Disulphide bonds, a type of covalent bond, can make peptide chains in protein structures more stable. This means that proteins with more disulfide bonds are more stable in general. Du et al. (2014) showed that disulfide bonds are very important for proteins because they help keep their folded shapes stable, lower conformational entropy, and make them more stable at high temperatures.

In general, this work shows that salting-out extraction methods improve the structural stability and thermal characteristics of BSF larvae proteins by increasing the amount of disulfide bonds. These insights are essential for refining protein extraction methods to yield BSF larvae protein isolates with specific functional attributes for incorporation as innovative components in diverse food applications.

4.4.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to check the changes in the secondary structure that happened because of the extraction methods used (Figure 4.3). This analytical method gives us useful information about proteins' molecular structure, functional groups, and secondary structure. Infrared spectra of the BSF larvae isolates are categorized into five distinct regions, each corresponding to the Amide group, which originates from the presence of peptide bonds between amino acids (as shown in Table 4.3).

Table 4.3. Characteristic infrared band frequencies and their corresponding functional groups in black soldier fly larvae protein isolates.

Classification	Approximate frequency (cm ⁻¹)	Functional group and vibration type
Amine <i>A</i>	3274	N–H stretching
Amine <i>B</i>	2927	C–H stretching
amide <i>I</i>	1665	C=O stretching
amide <i>II</i>	1558	N–H bending, C–N stretching
amide <i>III</i>	1399	N–H bending, C–N stretching

In the FTIR spectra of protein extracts obtained from AAFSO and AAFSISO, a clear N–H free stretching vibration was evident within the range of 3500 – 3200 cm⁻¹. However, it's worth noting that when it comes to insect powders, some researchers have associated the peaks in the same range with vibrations of the O–H group stretching (as seen in studies by Lozano-Vazquez et al. (2015) and Ozel et al. (2018)). The Amide B group, which encompasses the 3100 – 2900 cm⁻¹ range, appears to manifest as a split of two peaks with the alkaline and acid precipitation and salting-in extraction (AAF and AAFSI) showing high intensities. This wavelength result indicates the asymmetric and symmetric stretching of C–H bonds.

Subsequently, the Amide I and II bands stand out as the most prominent and characteristic vibrational bands originating from the protein backbone in FTIR spectra, as discussed by Kong & Yu (2007). The Amide I band is typically found in the range of 1700 – 1600 cm⁻¹, attributed to the stretching vibrations of the carbonyl group C=O within the peptide linkage. It is pertinent to note that the frequency of the Amide I band is intricately linked to the secondary structural characteristics of proteins (Lozano-Vazquez et al., 2015). **Figure 4.3** shows that AAF and AAFSI have a strong peak at 1634 cm⁻¹, but the salting-out extraction has a weaker peak. These results are in line with the amino acid content data in section 4.4.2, which clearly show how different extraction methods affect protein structure. The impact on protein structure is expected in the form of altered secondary structural content, specifically in the balance between α -helices, β -sheets, and random coil structures. Kong & Yu (2007) say that the range from 1640 to 1620 cm⁻¹ is linked to the presence of β -sheet structures.

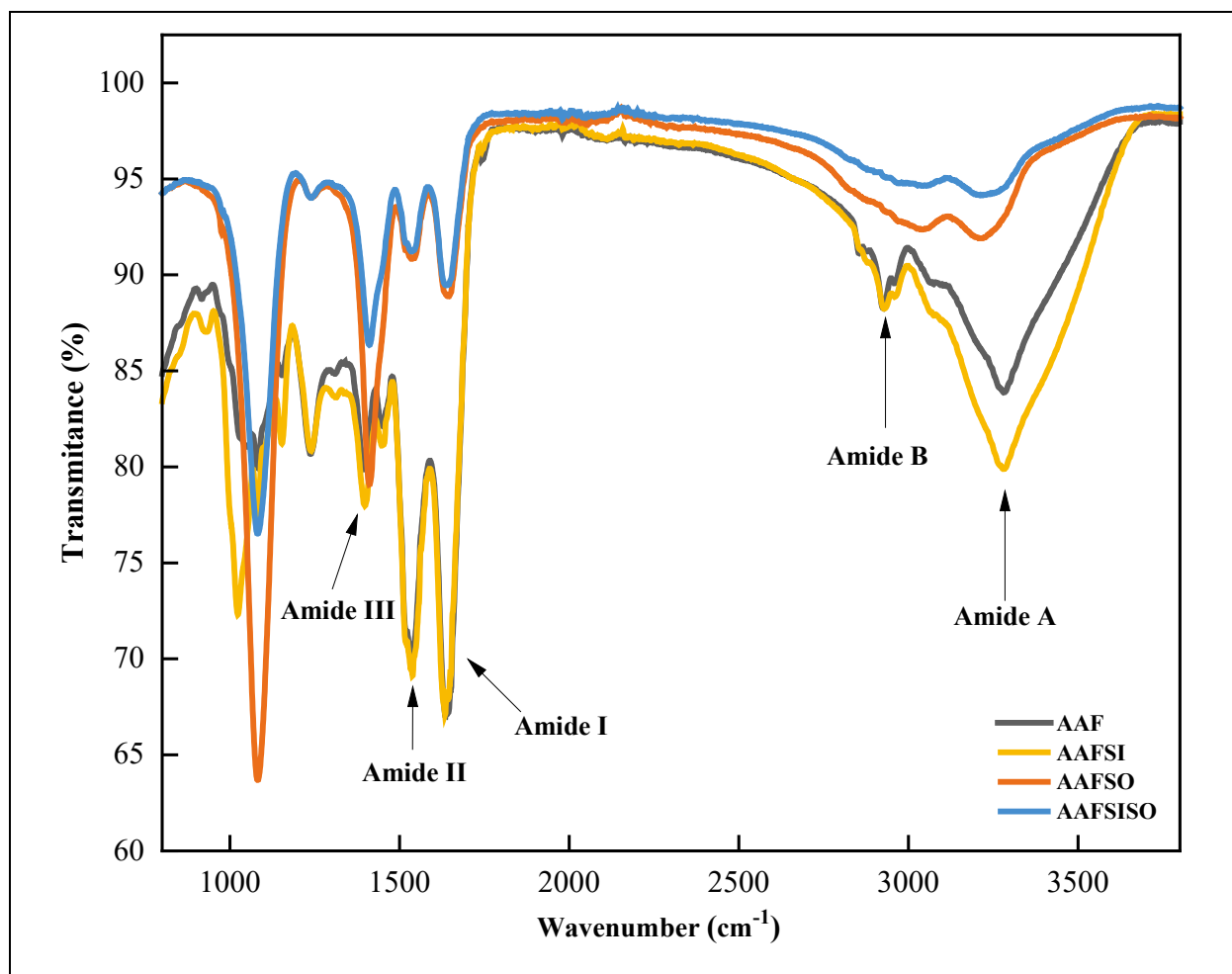


Figure 4.3. Fourier Transform Infrared (FTIR) spectroscopy spectrum of black soldier fly larvae protein isolates obtained through different treatments: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) - blue.

For the AAF and AAFSI samples, the stronger peak at 1634 cm^{-1} shows that there is more β -sheet content, which usually means that the structure is more stable and organised. The FTIR spectra of the salting-out (AAFSO and AAFSISO) protein concentrates were mostly the same. However, variations were observed in a few characteristic peaks and intensities (**Figure 4.3**), suggesting minor differences in structure, amino acids, and protein function groups. The FTIR analysis suggests that the salting-out extraction methods (AAFSO and AAFSISO) might disrupt the protein's secondary structure, leading to lower peak intensities in the Amide I and II regions. The

preservation of structural integrity, as indicated by the FTIR analysis, is better with AAF and AAFSI methods, making them more suitable for applications where maintaining protein structure is critical.

4.4.5. Circular Dichroism (CD) analysis

When analysing the structure of proteins and estimating the proportions of secondary structural components, CD offers useful information that is complementary to FTIR spectroscopy. The secondary structure of the proteins in phosphate buffer, as analysed by CD spectroscopy, is shown in **Figure 4.4**. The presence of α -helical structures is indicated by negative peaks at 204 and 234 nm, while the presence of β -sheets is signalled by a negative peak at 218 nm and a positive peak at 198 nm.

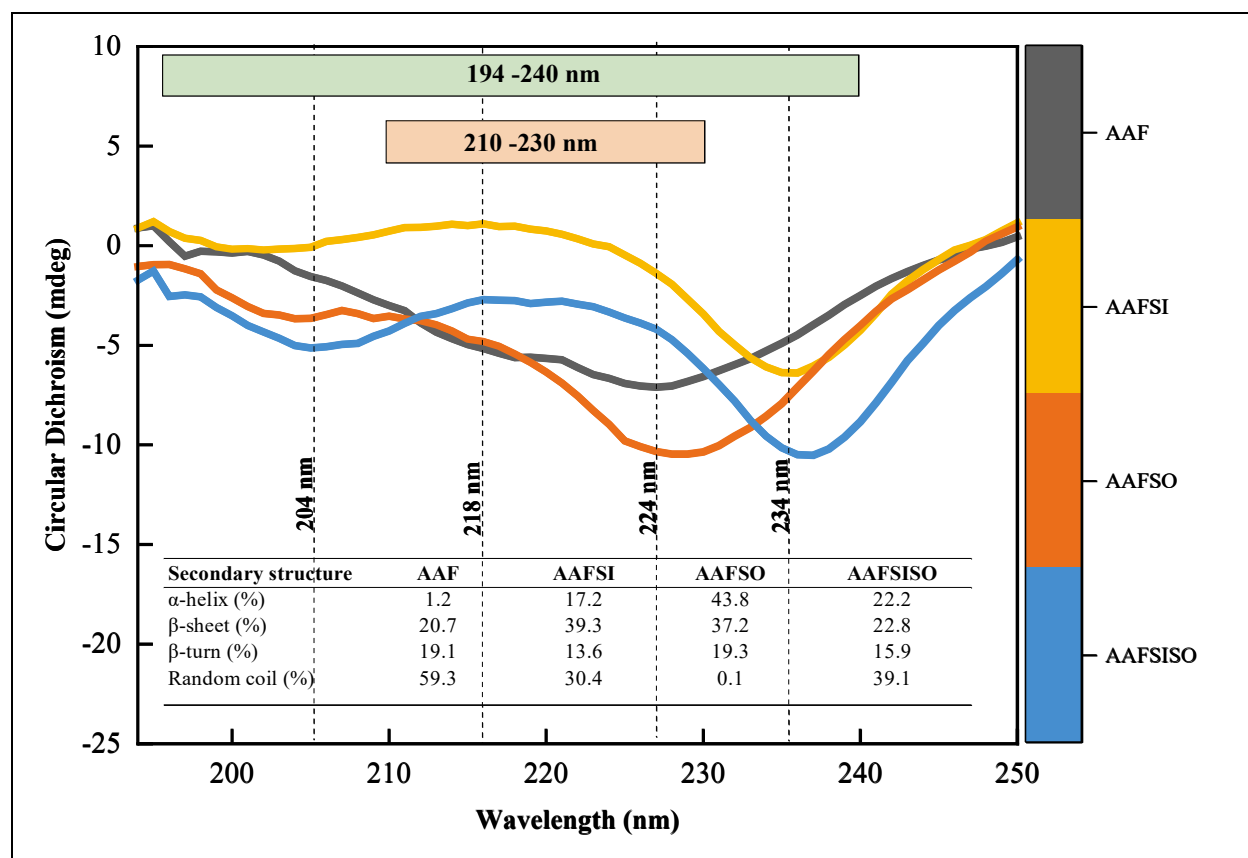


Figure 4.4. Effect of different extraction methods on the structural properties of black soldier fly larvae proteins: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) - blue.

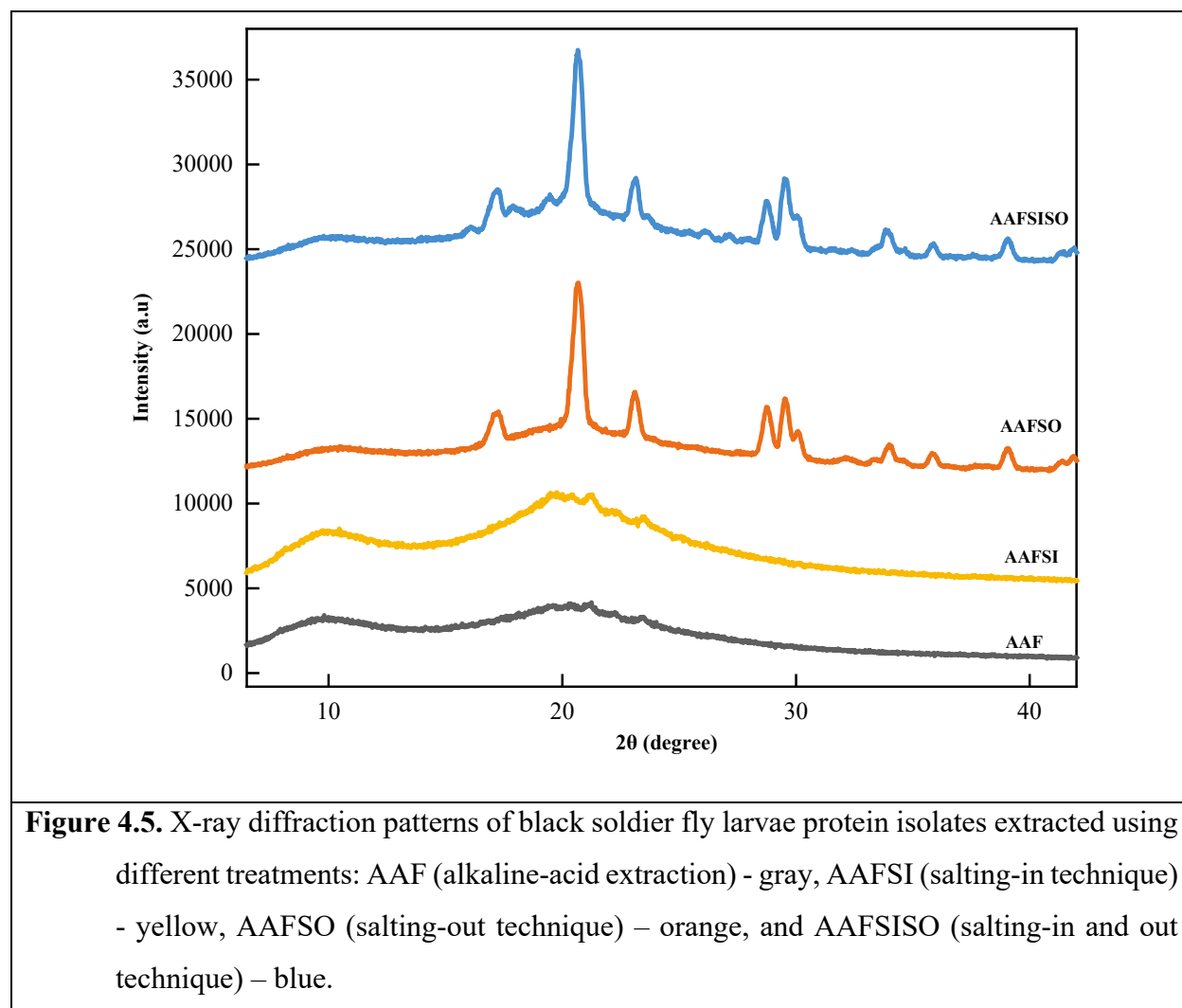
A positive peak at 206 nm suggests the presence of β -turns, whereas a negative peak at 224 nm and a positive peak within the 210 – 230 nm range indicate the existence of unordered structural elements (Malik & Saini, 2018). The CD spectra of the AAF proteins were systematically compared with those acquired after salting-assisted treatments. The employment of salt treatments exerted a discernible influence on the secondary structures, eliciting alterations within the 194 – 240 nm spectral range. At first, the basic AAF protein had 20.7% β -sheet, 19.0% β -turn, and 59.3% unordered structures. Salting-out treatments have demonstrated the conversion of β -sheet and random coil structures into α -helices and β -turns from AAF, indicating a more compact and organised conformation. Salt has a big effect on how protein-folding intermediates are described. For example, sulphate ions (SO_4^{2-}) usually help keep native protein shapes stable (Crevenna et al., 2012).

Figure 4.4 shows that the AAFSI technique led to a higher β -sheet composition than the basic AAF protein. This observation suggested a more folded shape within this group (Ijarotimi et al., 2017), as the β -sheet structure is typically linked to a more compact and stable protein conformation. An increase in β -sheet content usually means that the protein has kept or even improved its ordered structure under the AAFSI extraction conditions, which is better than the less organised forms seen in other techniques. The AAFSISO protein had 22.2% α -helix and 22.8% β -sheet composition. This finding, along with FTIR data, indicates that AAFSISO maintains a less compact and less ordered conformation in comparison to AAFSI and AAFSO. The greater percentage of unordered structures (39.1%) corresponds with the FTIR finding of reduced β -sheet intensity at 1634 cm^{-1} , which suggests that the extraction procedure partially disrupted the ordered protein structures. These results show how sensitive secondary structures are to the conditions of extraction. Jiang et al. (2021) also said that random coil structures change into α -helix and β -structure following salting-out treatment. This means that *Tenebrio molitor* has a more compact and ordered shape. Their work emphasises the importance of salt effects in delineating protein folding phases and elucidates how salt ions can stabilise the natural structure of proteins.

4.4.6. Powder X-ray Diffraction

X-ray diffraction analysis was conducted to investigate the variations in the crystal structure of the protein extracts from BSF larvae (**Figure 4.5**). Weak peaks were detected for AAF and AAFSI at

2 θ positions of approximately 9° and 20°. In contrast, AAFSO and AAFSISO displayed several broad and intense peaks at around 16.5°, 19.0°, 23.0°, 28.8°, 29.6°, 34.1°, 35.9°, and 39.3°. The calculation of the crystallinity index, by comparing the counts at 19° to 13°, yielded average crystallinity values of 81.0% for AAFSO and 82.2% for AAFSISO.



This is comparable to the crystallinity level identified in commercial protein (casein), recorded at 87.6 (Tan et al., 2021). These findings indicate that the BSF larvae samples exhibit a harder crystalline structure, possibly enhancing their stability (Sagheer et al., 2009). The amino acid study in section 4.4.2 showed that the BSF larvae protein isolates have a lot of hydrophobic residues, which are known to help create stable and organised structures like β -sheets.

These organised structures may help explain the level of crystallinity that was seen (Sagheer et al., 2009). Adding salt had a big effect on the protein isolates' crystal structure. Adding ammonium sulphate made the diffraction intensity go up more than adding other salts did. This means that using ammonium salt made the protein isolate crystals bigger. Salt ions, especially sulphate ions (SO_4^{2-}), are known to help proteins stay stable by increasing hydrophobic interactions and lowering the electrostatic repulsion between charged groups (Crevenna et al., 2012). This stabilising effect probably helped the protein chains line up in a more orderly way, which led to higher crystallinity in the salt-treated samples, as shown by methods like AAFSO and AAFSI. Joshi et al. (2011) found that lentil protein isolates with a higher percentage of crystallinity had a higher diffraction intensity and vice versa. This shows that a more crystalline structure leads to a stronger diffraction intensity.

These results suggest that BSF larvae protein typically possesses an organised structure; nonetheless, it remains more amorphous in comparison to protein derived from prawns (Kaya et al., 2015). This study is the inaugural investigation into the impact of salting-in/out extraction techniques combined with ultrafiltration on BSF larvae protein. Consequently, it contributes to the scarce body of information in this domain by providing a comprehensive elucidation of the crystalline structure of the BSF larvae protein via X-ray diffraction research.

4.4.7. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The alterations in the distribution of molar masses in AAF, AAFSI, AAFSO, and AAFSISO were confirmed using SDS-PAGE with protein staining. In **Figure 4.6**, the marker was included alongside the protein extract line to facilitate band identification. The SDS-PAGE profiles in **Figure 4.6** revealed noticeable differences in the width of electrophoretic bands, indicating distinct molecular weight distributions in BSF larvae extracted using different methods. Ravi et al. (2020) previously reported a similar protein distribution across various product processing techniques, such as conventional drying, freezing methods, microwave drying, scalding, and blanching. The lower molecular weight protein bands observed in **Figure 4.6** may be attributed to disulfide bond cleavage under reducing conditions, destabilizing protein quaternary and tertiary structures, and producing smaller protein fragments. This disruption reduces the overall stability of the protein and alters its molar mass distribution.

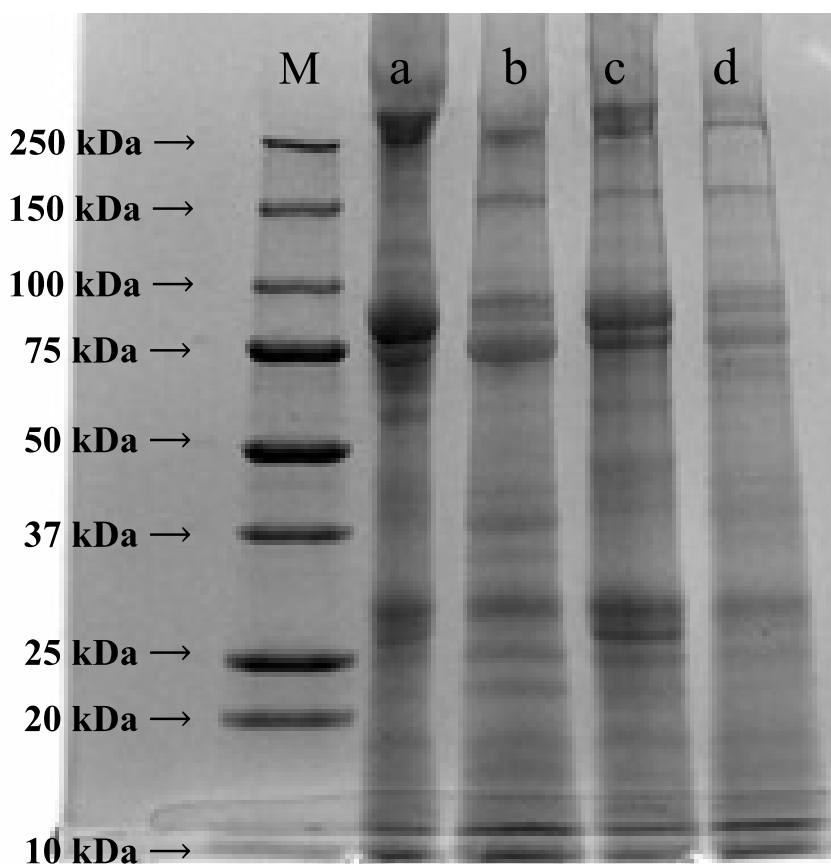


Figure 4.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of black soldier fly larvae proteins obtained through different treatments: (a) AAF (alkaline-acid extraction), (b) AAFSI (salting-in technique), (c) AAFSO (salting-out technique), and (d) AAFSISO (salting-in and out technique). M represents the molecular weight marker.

Structural and muscular proteins were found to be highly prevalent, as reported by Rabani et al. (2019). This suggests stronger bands in the molecular weight range of 75 to 50 kDa in insect samples. This range corresponds to key structural proteins like actin and myosin, which are commonly found in insect muscle tissues. Additionally, it appears more pronounced compared to the lower molecular weight bands observed in the current study. To preserve the structural integrity and molar mass distribution of proteins, the differences highlighted are the significant role in the extraction methods (Andersen, 2011).

The enzymes, muscle proteins, and exoskeleton proteins in studies on *H. illucens* and *Tenebrio molitor* reported the same range of results (Yi et al., 2013; Ravi et al., 2020). According to Leni et al. (2019) the proteins with a size of 31 kDa could correspond to glutelin proteins from the actin family, with troponin C being the most abundant. Chen et al. (2018) and Leni et al. (2019) identifies Hexamerin F as a common protein in insect larvae, also as the primary protein between bands of 116 kDa and 66 kDa. Protein extraction, defatting, drying, and killing are methods applied to BSF larvae samples, which result in different protein profiles. Moreover, this emphasizes the need to consider different elucidation methods (Ravi et al., 2020; Ravi et al., 2019). The increase in size is caused by certain proteins not migrating fully through gels due to aggregation, which can be indicated by the presence of a darker region at the top of the gel in the protein extract area. The separation and identification of such proteins can be assisted by using gels with larger pores in the future.

SDS-PAGE analysis showed that AAFSI had only a few low molecular weight proteins (less than 15 kDa) and more prominent bands in the range of 80 to 20 kDa. Vinayashree & Vasu (2021) reported a different outcome for salt-extracted (0.5 mol/L NaCl) pumpkin protein, showing a lighter band at 110–20 kDa and a darker band at less than 10 kDa. Conversely, AAFSI exhibited a stronger band near 30 kDa, suggesting potential variations in raw materials or protein extraction methods. AAF exhibited a broader band, while AAFSO showed a more intense and darker staining in the 75 kDa range, suggesting a higher concentration of high molecular weight proteins exceeding 20 kDa. It is noteworthy that the SDS-PAGE intensities for AAFSISO differed significantly from the control and AAFSI. The molar mass distribution of proteins showed a visibly stained broadband near the top of the SDS-PAGE gel during electrophoresis (the same amount of protein was applied in each lane). This conjugation observed in SDS-PAGE was further supported by DSC analysis, as discussed below. Consequently, the concentration of AAF and AAFSO extraction favoured the presence of high molecular weight proteins in BSF larvae.

4.4.8. Thermal properties (DSC and TGA)

The alterations in the tertiary and quaternary structures of food proteins may result in changes to their functional properties. It is crucial to explore such structural modifications because they are closely associated with the functional properties of the protein in food. DSC can directly measure

the heat changes in the sample at different temperatures and provide valuable information about the thermal stability of the sample. **Figures 4.7A** and **4.7B** display the TGA and DSC curves for the BSF larvae isolates, respectively. Both TGA and DSC were employed to evaluate the impact of salting-assisted extraction processes on the thermal stability of the isolates. The TGA tests showed that there were three different heat processes: (I) an initial dehydration phase occurring between 50 and 100°C, (II) a rapid decomposition phase in the range of 200 – 300°C, involving the breakdown of non-covalent bonds, including intermolecular and intramolecular hydrogen bonds, electrostatic bonds, and hydrophobic interactions, (III) A slower degradation phase spanning 300 – 600°C. Qu et al. (2018) say that the lower degradation phase (300–600°C) also includes the breakdown of covalent bonds at higher temperatures.

The groups have more water association because of the use of salting-in therapy (AAFSI), which led to a bigger drop in mass in the first stage. Also, the degradation temperatures for these groups were higher during the second thermal stage, which means that there were more thermally stable structures present. Nonetheless, the residual weights for the salting-out group were markedly lower than those of the non-salting-out group after the second stage, corroborating the findings of Jiang et al. (2021). In the third stage, the weight losses were more noticeable, which showed that the salting-out treatments made the proteins more thermally stable overall (as shown in **Figure 4.7A**).

One probable reason for this could be that the salting-out group had more disulphide (SS) bonds, which aided keep folded shapes and lowered conformational entropy, making the material more stable at high temperatures. This implies that the salting-out effect may further augment the stability of BSF larvae proteins in comparison to salting-in. DSC analysis gives a lot of information on the qualitative and quantitative thermal properties of solid materials. This includes things like melting and degradation temperatures and, in particular, the heat-induced denaturation of proteins. The thermal denaturation temperature (T_d) is often used to tell how stable a protein is at high temperatures, and the enthalpy (ΔH) shows how organised the structure of a protein is. The DSC thermogram showed two important endothermic transitions: one for AAFSO between 40 and 60°C and another for AAF and AAFSI at 70°C (**Figure 4.7B**).

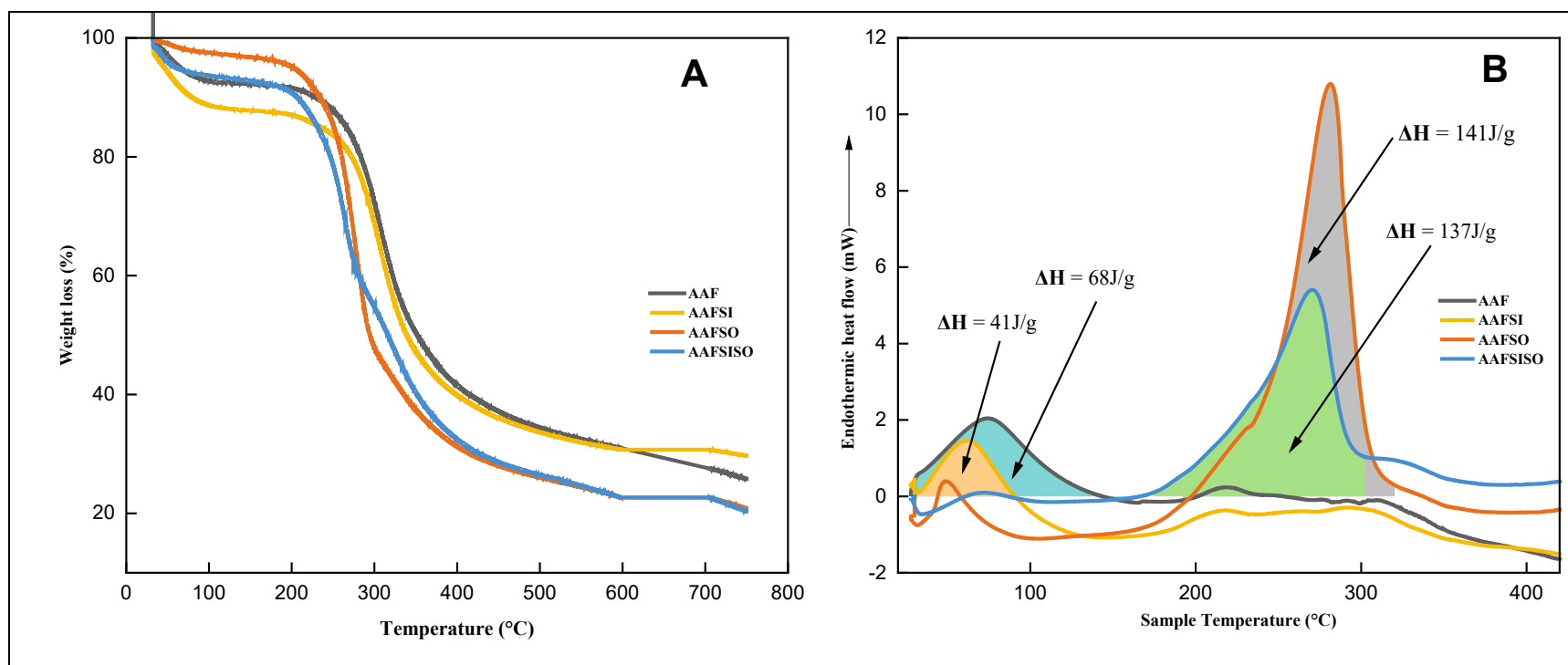


Figure 4.7. Effects of salting-in/out on (A) thermogravimetric analysis (TGA) and (B) differential scanning calorimetry (DSC) of black soldier fly larvae protein isolates obtained through different treatments: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) – blue.

AAFSO exhibited an endothermic peak at approximately 35°C, which may be attributed to the formation of new intermolecular bonds during the initial heating process, as discussed by Fitzsimons et al. (2007). The peaks below 100°C represent the denaturation of proteins with lower thermal stability, likely indicating proteins that unfold at lower temperatures. The peaks between 200–300°C are indicative of more thermally stable proteins undergoing denaturation, reflecting the breakdown of stronger intramolecular and intermolecular bonds such as hydrogen bonds and disulfide bridges.

As depicted in **Figure 4.7B**, AAFSO exhibited a higher enthalpy value compared to the other insect protein extracts, and this elevated enthalpy value is indicative of a greater content of ordered protein structures, as noted by Chandrapala et al. (2011). Conversely, AAF displayed the lowest ΔH value at 68 J/g, suggesting that AAF had the poorest thermal stability, which aligns with the results regarding its ordered structure. AAFSO and AAFSISO, on the other hand, had the highest content of ordered protein structures and exhibited the largest ΔH values, measured at 141 J/g and 137 J/g, respectively. Additionally, these elevated ΔH values can be ascribed to the breakdown of hydrogen and covalent bonds within the protein structure, including processes such as deamidation, desulfuration, or decarboxylation, as elaborated by Sobral et al. (2011).

4.5. Conclusions

To study the diverse attributes of BSF larvae proteins, this study used an extensive array of analytical methodologies. Looking into their composition, structural features, and thermal stability. The big changes in characteristics of BSF larvae proteins were due to the procedure used to extract proteins, especially salting-out-aided treatments. The increased amount of protein content and changed amino acid content were due to the procedures, making them an alternative possibility for food uses. TGA and DSC studies demonstrated an enhanced thermal stability through salting-out extraction. The shapes and lower conformational entropy can be kept folded with this better heat stability, due to more disulfide (SS) bonding. By looking at amide stretches and their functional groups, FTIR analysis revealed information about the proteins' secondary structure, especially the existence of β -sheet structures. The salted-out treated samples showed more stability. In confirmation of the zeta potential tests, the electrostatic repulsion was stronger.

In general, this study showed that in places where lysine deficiency is a problem, BSF larvae proteins could be a long-lasting and nutritious source of protein. As a way to solve

nutritional problems in different groups of people, this research can assist in creating new ways to process food and functional ingredients. The results also show how important extraction procedures are for changing the characteristics of proteins from insects, which will make them even more useful in the food business and other fields. It will be important to test the functional qualities of these proteins in different types of food, along with sensory tests, to see how useful they are and how well they are liked by consumers.

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CHAPTER FIVE

EFFECT OF ALKALINE EXTRACTION- ISOELECTRIC PRECIPITATION AND SALT-ASSISTED EXTRACTION ON PHYSICOCHEMICAL AND TECHNOFUNCTIONAL PROPERTIES OF BLACK SOLDIER FLY LARVAE PROTEINS

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“Insects are not just pests; they are valuable food resources. By incorporating insects into our diets, we can create a more sustainable and resilient food system for future generations.”

(Daniella Martin, 2023)

5.1. Abstract

Black soldier fly (BSF) larvae are becoming a more popular source of protein in the food industry, because of their long-lasting replacement for traditional protein sources. However, insect proteins' functional properties need to be improved to use their benefits. The objective of this work was to comprehensively investigate the impact of alkaline extraction and isoelectric precipitation procedures, supplemented by salting-in (NaCl) and salting-out ($(\text{NH}_4)_2\text{SO}_4$) techniques, on the functional characteristics of BSF larvae proteins. We found out the physicochemical and techno-functional properties of the proteins. The bulk density of untreated proteins (AAF) was 1.23 g/mL, whereas the bulk density of salt-treated isolates was 0.99 g/mL (AAFSI), 1.15 g/mL (AAFSO), and 1.52 g/mL (AAFSISO). The AAFSISO isolate had the highest water binding capacity (WBC) at 4.45 g/g, which was much higher than the WBC values for other insect protein fractions. The isolates had very different oil binding capacities ($p > 0.05$): AAF at 1.58 g/g, AAFSI at 2.17 g/g, AAFSO at 1.04 g/g, and AAFSISO at 1.36 g/g. The addition of $(\text{NH}_4)_2\text{SO}_4$ considerably ($p < 0.05$) affected how soluble the protein was, especially at pH 2. This suggests that it could be useful in acidic food systems. Salt treatment changed the surface hydrophobicity, with salting-out approaches lowering hydrophobicity and changing how proteins work. There was a good association between the surface hydrophobicity and the oil binding capacity ($r = 0.74$, $p < 0.05$) and the emulsion stability index ($r = 0.83$, $p < 0.05$). Correlation analysis revealed a substantial positive link ($p < 0.05$) among the various functional aspects, underscoring their interconnectedness. Foaming capacity and foam stability exhibited significant correlations with protein solubility at pH 4 ($r = 0.88$, $p < 0.05$; $r = 0.74$, $p < 0.01$, respectively). These results underscore the possible application of BSF larvae proteins in food formulations, particularly in light of the positive connections identified among emulsification characteristics, solubility, and surface hydrophobicity. The revealed correlations and functional qualities establish a foundation for the continued investigation of BSF larvae proteins as sustainable functional ingredients in food applications, corresponding with the increasing interest in alternative protein sources within the food industry.

5.2. Introduction

Due to environmental issues and a growing global population, it is important to find other ways to provide a steady supply of protein (Choi et al., 2017; Kwak et al., 2020). Edible insects have become a prominent protein substitute, exhibiting a remarkable protein conversion ratio (the effectiveness of transforming ingested feed into body protein) among available options (Post, 2012). A lot of studies have revealed that people eat insects because they are good for them (Khampakool et al., 2020). Improving knowledge and increasing exposure may facilitate the acceptability of edible insects; however, their influence is limited by food neophobia (Kim et al., 2020; Piha et al., 2018). Besides eating whole insects, adding edible insects to traditional foods might be a way to make them more nutritious (Bessa et al., 2019; Haber et al., 2019). Insects are hard to promote as a food source in Western society since they don't look good and people don't like them, which is a big problem that needs to be solved (Kim et al., 2022). In order to remove the negative ideas people have about eating insects, some researchers have studied ways to alter the appearance (Yi et al., 2013). The function and benefits of insect-derived proteins need to be enhanced to promote their use. The functional properties include foaming capacity, foam stability, emulsifying capacity, emulsion stability, water-oil binding capacities, and surface characteristics (Kim et al., 2022; Yi et al., 2013; Yi et al., 2017).

Additionally, to improve the proteins' functional properties, studies looked into ways to best extract the proteins from different methods. Most studies available focus on mealworms; therefore, there is still a lack of information, especially on edible insects (Yi et al., 2013; Preece et al., 2017). The black soldier fly (BSF), among other edible insects, appears to be a highly promising insect for the commercial production of proteins. This is because it offers numerous advantages both for humans and the environment. There are many studies on how insect proteins can be extracted in the literature (Jiang et al., 2021; Yi et al., 2013); however, the extraction method substantially influences the value of the extracts, as reported by the study by Yi et al. (2017)

Extraction of proteins can be affected by many things. For example, a good way to adjust the ionic strength of proteins is by the addition of salts (Bußler et al., 2016). Testing the functional qualities and efficiency of the extraction is important. This is done to make sure that, instead of using regular protein sources or as a component in food formulation, insect protein is used. Protein isolates can be improved by the protein extraction technique, which will make them have many advantages when used in the food industry.

Good ways to extract proteins are alkaline extraction with isoelectric/acid precipitation, since it is easy and cheap (Yi et al., 2017). This can be done by modifying the pH to their isoelectric point; such a method solubilizes proteins in a high pH environment and subsequently precipitates them (Preece et al., 2017). Alkaline extraction and acid precipitation are assisted by salt, and the extraction yield will be increased, and also clean the proteins. Good effects on food production processes and good effects on the physicochemical qualities are caused by salt-assisted extraction (Yi et al., 2013; Zhao et al., 2016). To improve protein extraction, recent studies have started using salt. To our knowledge, no studies have been conducted on how salt-assisted extraction affects the functional characteristics of BSF larvae proteins. Therefore, to enhance alkaline extraction and isoelectric precipitation, this study used sodium chloride (NaCl) and ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$, respectively.

This study aimed to examine the impact of salt treatment on the functional properties of BSF larvae isolates, emphasising critical parameters including bulk density, water binding capacity, oil binding capacity, solubility, surface hydrophobicity, foaming capacity, and emulsification characteristics. These measured components provide a foundation for additional investigation of BSF isolates for food and nutraceutical uses. This research offers a basis for the development of BSF larvae protein extracts with specific physicochemical and techno-functional properties, serving as a guide for their potential application as nutritional supplements or functional ingredients.

5.3. Methodology

The experimental design, quantification, and analysis of data were described in detail in Sections 3.1, 3.2, and 3.4 of Chapter 3. Refer to the schematic diagram below for a summary of the steps and methods involved (Figure 5.1).

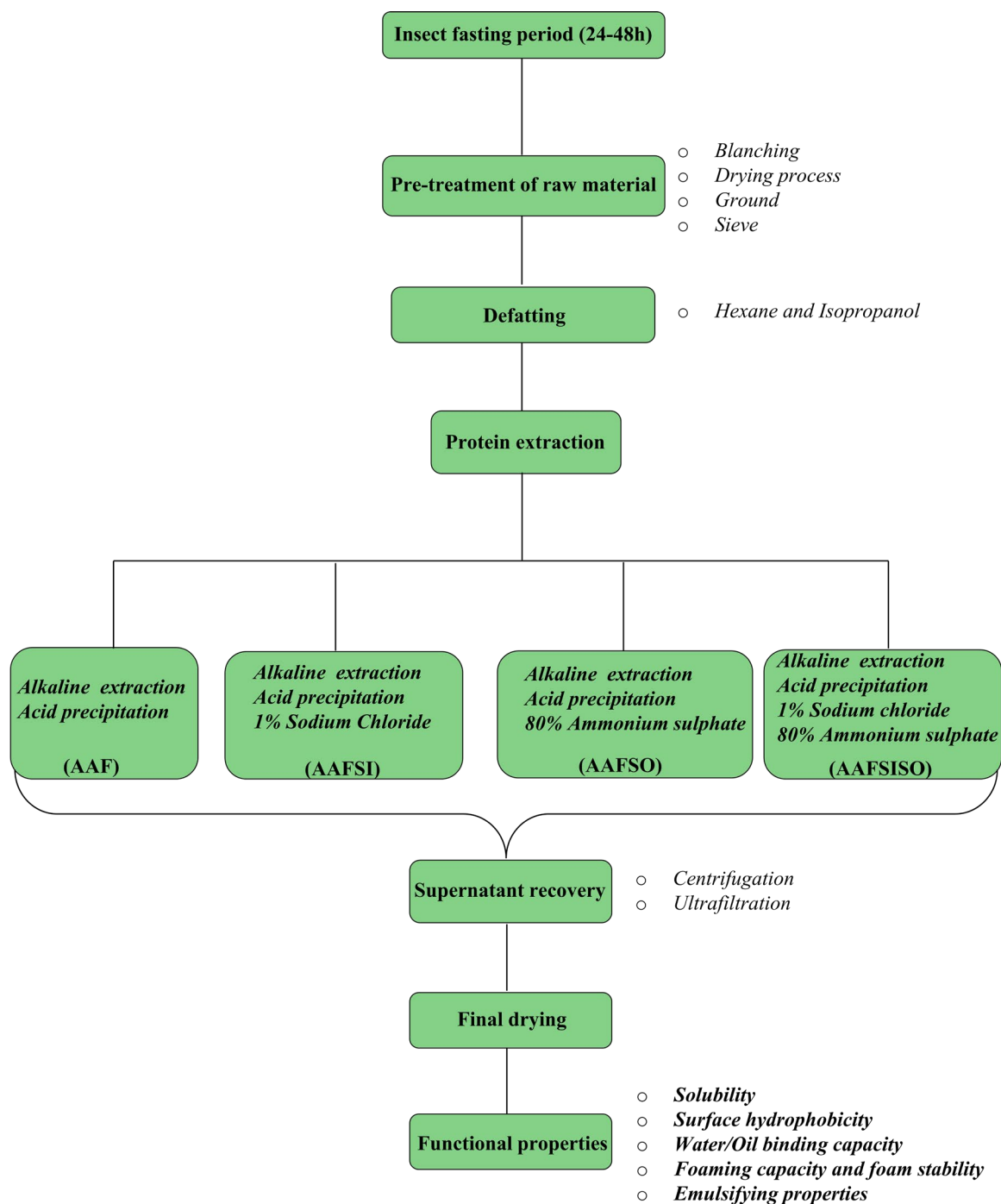


Figure 5.1. Summary of the methodology schematic, as presented in Chapter 3.

5.4. Results and discussions

5.4.1. Solubility of proteins

Protein solubility is a crucial factor that influences various functional properties, making it a critical determinant of protein functionality in the food processing industry. The impact of pH and ionic strength on the protein isolates derived from BSF larvae was investigated, and the

findings are illustrated in **Figure 5.2**. The solubility of the BSF larvae proteins followed a characteristic U-shaped curve within the examined pH range 2–10, indicating a substantial dependence on pH. The solubility of the AAFSO and AAFSISO proteins was significantly ($p < 0.05$) greater than that of the AAF and AAFSI proteins at pH 4–8 (**Figure 5.2**).

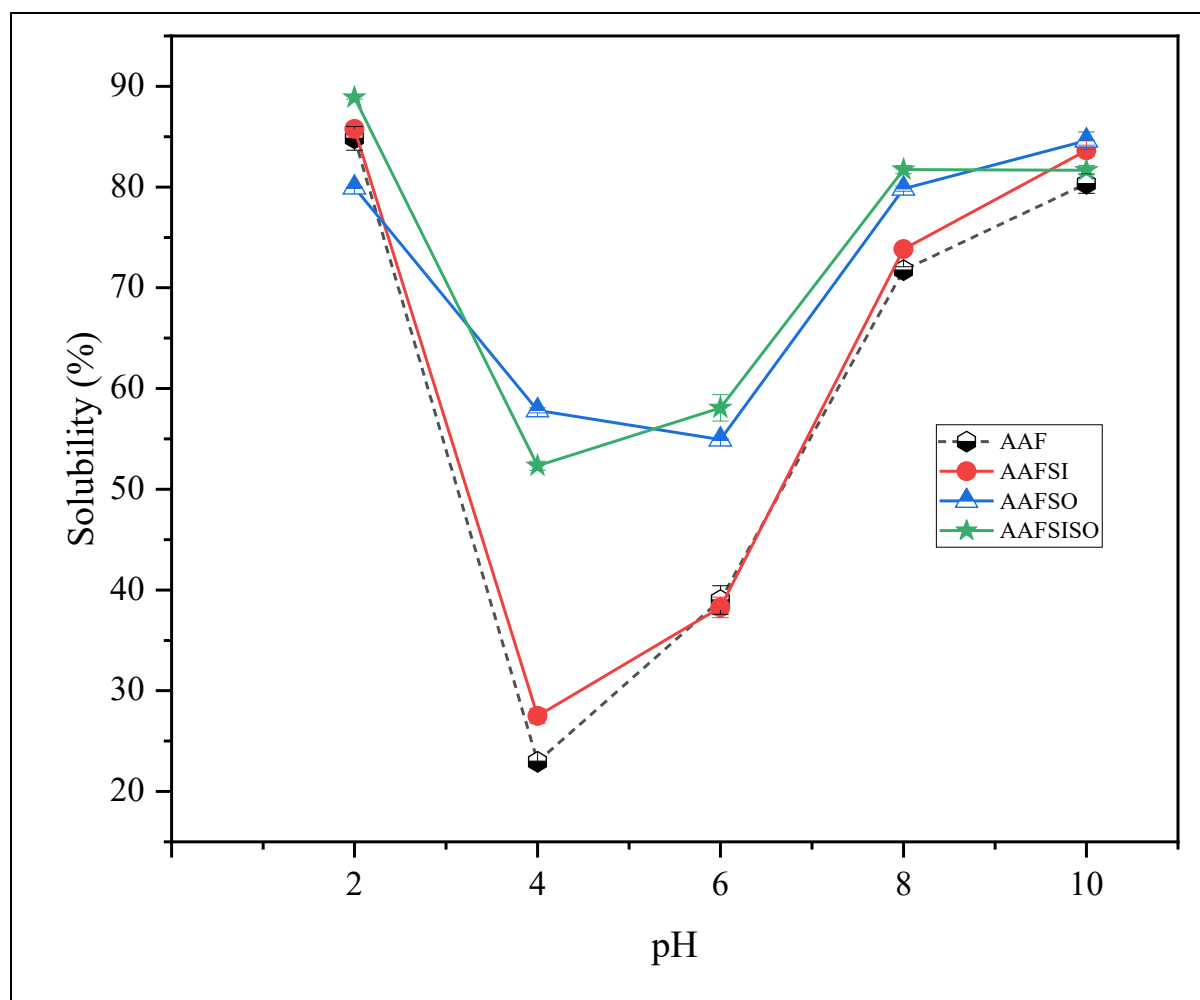


Figure 5.2. Impact of extraction methods on solubility of black soldier fly larvae isolates obtained by: AAF (alkaline-acid extraction – black pentagon); AAFSI (salting in technique – red circle); AAFSO (salting out technique – blue triangle) and AAFSISO (salting-in and out technique – green stars). Error bars show standard deviation.

The greatest levels of solubility were recorded at pH 2 (89% for AAFSISO) and pH 10 (85.21% for AAFSO), which were likely due to the attainment of the isoelectric point. Previous studies reported similar patterns of solubility (Jiang et al., 2021; Yuliana et al., 2014; Azagoh et al., 2016), consistent with our results. With the exception of the AAFSO protein group, BSF larvae isolates show remarkably enhanced solubility at pH 2 compared to pH 8 and 10. Mintah et al.

(2020) reported similar findings with a study on *Tenebrio molitor* protein solubility at pH 2 compared to pH 10. For the industrial applications, neutral pH levels are a crucial aspect, and based on these research findings, protein solubility is weakly acidic and alkaline. For nutrition and sports drinks as well as acidified sauces, our BSF larvae meet these criteria, therefore can be used in acidic food environments, including nutrition and sports drinks, as well as acidified sauces.

Some proteins may aggregate and precipitate, while others may remain soluble based on the varying solubility of insect proteins and their characteristics. AAFSISO had the highest solubility at pH 2, while both AAF and AAFSI had the lowest solubility at pH 4 in our study. This may be related to surface hydrophobicity, and solubility is a factor that significantly affects it (**Table 5.1**). Protein solubility can be affected by the addition of salt, as demonstrated by the salt-assisted extraction method. Therefore, this serves as an extrinsic factor that impacts protein solubility (Purschke et al., 2018). The protein structure can be preserved by the addition of $(\text{NH}_4)_2\text{SO}_4$ as discovered in this study. This would lead to improved solubility (Deng et al., 2019). When NaCl was added to the solution at pH 2, there was no significant difference ($p > 0.05$) in solubility for the AAFSI samples,

The presence of salt counterions surrounding the proteins may explain this phenomenon. Among protein molecules, this neutralizes surface charges and modulates electrostatic interactions (Mshayisa & Van Wyk, 2021). However, there is no information in the literature reporting on the protein solubility profile of BSF larvae protein extracted by salt extraction. Salting in and salting out showed that they are suitable for the extraction of insect proteins. However, the method's effectiveness is dependent on the optimized method to fit the extraction process of that particular species; additionally, how well the extraction method fits the unique characteristics of the protein being studied. This is done to maintain the integrity and function of the protein while the isolate is extracted efficiently and effectively. To make suitable proteins, these methods are very important, and in the field of alternative protein research, they are essential. Future studies would look into how protein surfaces are hydrophobic and how the overall structure of a protein affects how the packaging is put together.

5.4.2. Surface hydrophobicity and bulk density

The foaming and interactions with lipids and other proteins are influenced by the surface hydrophobicity because it affects protein surface (Zozo et al., 2022; Duong-Ly & Gabelli,

2014; Coelho & Salas-Mellado, 2018). Table 5.1 displays the alterations in the surface hydrophobicity of the different BSF larvae protein isolates. It is evident that salting out treatment has a substantial influence ($p < 0.05$) on the surface hydrophobicity when compared with the untreated sample (AAF). Moreover, there were significant differences between AAF and AAFSI (1056.41 and 1021.37, respectively), which were significantly ($p < 0.05$) higher than those of salting-out proteins (**Table 5.1**).

Table 5.1. Effect of extraction methods on surface hydrophobicity (H_o relative value) and bulk density of black soldier fly larvae protein isolates.

Extraction method	Surface hydrophobicity	Bulk density (g/mL)
AAF	1056.41 ± 0.93^d	1.23 ± 0.02^b
AAFSI	1021.37 ± 4.08^c	0.99 ± 0.01^a
AAFSO	594.59 ± 8.47^b	1.15 ± 0.06^b
AAFSISO	540.02 ± 6.45^a	1.52 ± 0.03^c

The values are expressed as means $\pm$ standard deviations ($n = 3$). The letters above each data point indicate that the values are statistically significantly different ($p < 0.05$). AAF (alkaline-acid extraction); AAFSI (salting-in technique); AAFSO (salting-out technique), and AAFSISO (salting-in and out technique).

During alkaline extraction and acid precipitation, proteins may partially unfold, exposing more hydrophobic residues on the protein surface and increasing total hydrophobicity (Zielińska et al., 2018). The surface hydrophobicity of AAFSO and AAFSISO was discovered to be closer to the reported values for whey protein (468.0), with both isolates having a higher value than whey protein (**Table 5.1**) (Zielińska et al., 2018). This is attributed to partial denaturation and subsequent aggregation of hydrophobic groups, which is consistent with previous observations (Munialo et al., 2022). The higher surface hydrophobicity may result in enhanced protein functionality due to changes in interfacial or surface tension. According to Preece et al. (2017) the protein hydrophobicity increases as a result of the exposure of hydrophobic groups and regions enclosed within the molecules to the more polar surrounding environment, which is due to protein denaturation to a certain extent. On the other hand, a high concentration of salt can enhance surface hydrophobicity owing to the salting-out mechanism, which breaks the hydration layer on the surface and disrupts the charge balance of protein molecules.

Zhang et al. (2017) discovered that surface hydrophobicity augmented with solubility in the context of soy protein isolates. This indicates that proteins that form fewer or more

loosely connected aggregates are likely to have more hydrophobic areas on their surface, which makes them more hydrophobic and soluble. When proteins stick together more tightly, on the other hand, their hydrophobic regions get buried inside the protein complexes. This makes the surface less hydrophobic and makes the proteins less soluble. However, these findings may be influenced by the concentration of salt ions, which can reduce the competition between protein molecules and water. This encourages protein–protein interactions and aggregation, which can, ironically, make the surface more hydrophobic if aggregation causes partial unfolding. These methods may elucidate the enhanced solubility of salted-out proteins, as illustrated in **Figure 5.2**.

Bulk density is an important parameter used to describe protein products. It is very important for both economic and functional reasons (Muranyi et al., 2016). Table 5.1 shows that the BSF larval isolates had different bulk densities, except for AAF and AAFSO. The bulk density of AAF was 1.23 g/mL. The bulk density of the salt-treated isolates was 0.99 g/mL (AAFSI), 1.15 g/mL (AAFSO), and 1.52 g/mL (AAFSISO). Among the concentrates, AAFSI exhibited a considerably ($p < 0.05$) reduced bulk density compared to the salt-extracted concentrate. Also, the bulk density of AAFSISO was much larger than that of AAFSO ($p < 0.05$). This could be because the extraction process changed the structure of the protein instead of the salt, as the salt would have been eliminated during ultrafiltration.

What is interesting is that extraction in the presence of NaCl significantly decreased the bulk density of the BSF larvae protein, dropping from 1.23 g/mL to 0.99 g/mL, while the addition of ammonium sulfate during extraction resulted in a significant increase in bulk density, rising to 1.52 g/mL (Table 5.1). One possible explanation could be that NaCl enhanced the solubility of the BSF larvae proteins, leading to a more dispersed or diluted protein solution with a lower bulk density. Conversely, the ammonium sulfate extraction likely caused protein precipitation, resulting in denser, more compact protein particles that increased the bulk density. The reduction in protein bulk density is attributed to structural modifications of the protein during extraction (Timilsena et al., 2016). Proteins with low bulk density are beneficial because they are simpler to move and share (Siow et al., 2017). Mishyna et al. (2019) reported results that are consistent with our findings, of which *Hermetia illucens*, *Macrotermes subhyllanus*, and *Gonimbrasia belina* varied between 0.51 and 0.64 g/mL of bulk densities of the protein concentrates. The NaCl extraction concentrate showed a bulk density comparable to that of most species in our study. Whereas a higher bulk density of 1.52 g/mL for the

ammonium sulfate extraction concentrate, higher than 0.57 g/mL reported by Vanqa et al. (2022) for BSF larvae protein extract.

There are various reasons why bulk density can be different, such as differences in the composition of the raw materials. This results in the size distribution of the particles, the way proteins clump together, and the way things are processed, such as drying and extraction procedures. For example, extracting ammonium sulphate may make the particles denser by causing proteins to precipitate, whereas extracting NaCl may make proteins more soluble, making them lighter and more spread out. Different ways of drying, such as spray-drying and freeze-drying, could also have an effect on the ultimate bulk density of the protein products.

5.4.3. Water binding capacity and oil binding capacity

The water binding capacity (WBC) of a protein is closely related to the texture, mouthfeel, and viscosity of food products (Malik & Saini, 2018). The oil-binding capacity (OBC) of a protein, which is the amount of oil it can hold, is also affected by the kind of protein, the pH, and the ionic strength (Mintah et al., 2020; Wagner et al., 2000). **Figure 5.3** shows the WBC and OBC values for the proteins in BSF larvae.

It is worth noting that the AAFSI did not generate a statistically significant change in the WBC of the protein, as evidenced by the data ($p > 0.05$). On the other hand, the highest WBC was reported for the AAFSISO than for other proteins, and this was statistically significant ($p < 0.05$). To date, no research has studied the effect of WBC of BSF larvae proteins using the extraction of varied salt-assisted techniques. Additionally, our results for WBC are higher than the values reported by Jian et al. (2021) for BSF larval flour fractions (0.4–0.8 g/g). Again, *Tenebrio molitor* (1.87 g/g) (Zielińska et al., 2018) and *Spodoptera gregaria* (2.18 g/g) (Mintah et al., 2020). The flour of *Galleria sigillatus* (2.34 g/g) and *Spodoptera gregaria* (2.18 g/g) had lower values when compared to AAFSISO isolate for WBC (4.45 g/g). However, the flour of *Galleria sigillatus* (2.34 g/g) and *Spodoptera gregaria* (2.18 g/g) reported the highest WBC values. This could be due to the high level of hydrophilic amino acids (390.6 and 371.4 mg/g, respectively) (Zielińska et al., 2018).

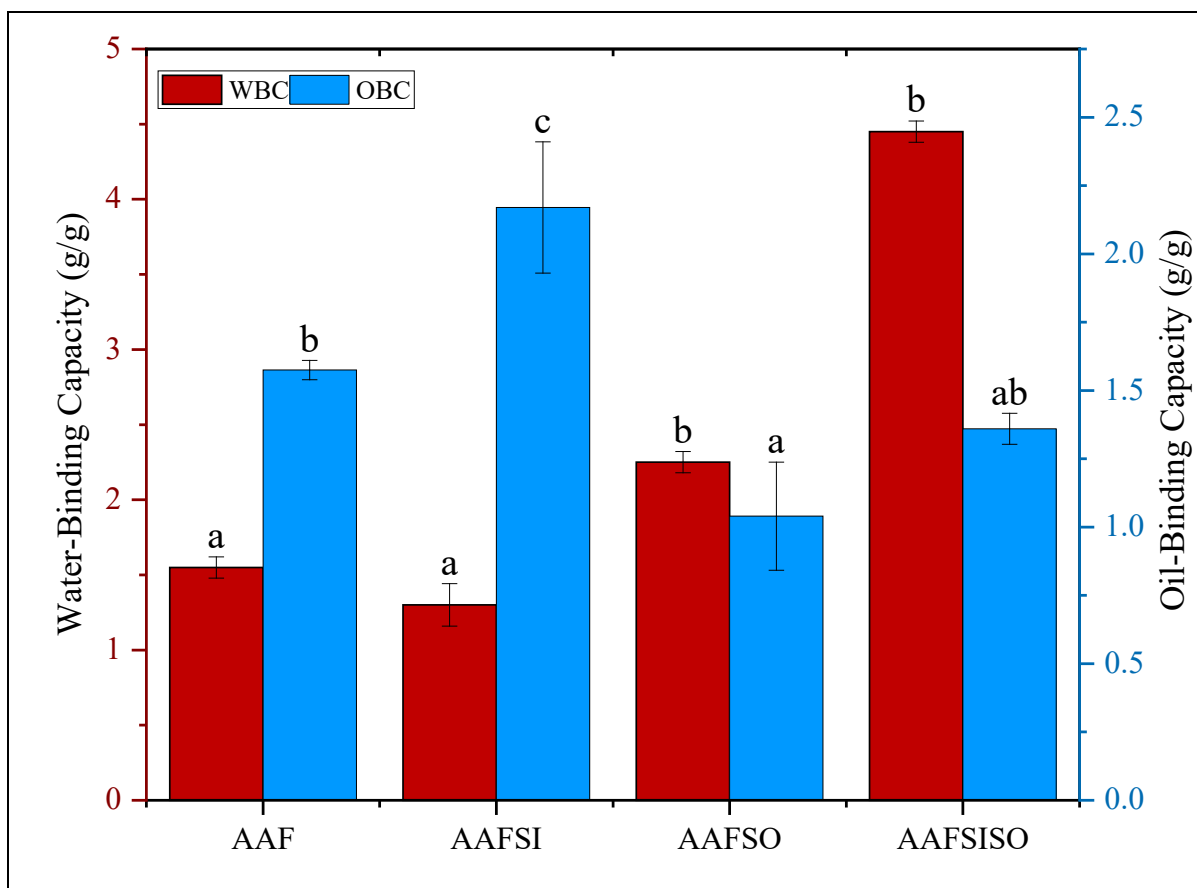


Figure 5.3. Effect of extraction methods on water and oil binding capacity of black soldier fly larvae isolate obtained by: AAF (alkaline-acid extraction); AAFSI (salting in technique); AAFSO (salting-out technique), and AAFSISO (salting-in and out technique). Various letters suggest notable contrasts with statistical significance at $p < 0.05$. The error bars depict the standard deviation.

These data also indicate that BSF larvae could serve as a significant component in the food industry, as proteins with WBC ranging from 1.49 to 4.72 g/g are advised for incorporation in viscous meals (Akpossan et al., 2015). The OBC of proteins is frequently a sign of how well they can interact with lipids. This will affect how well they emulsify, how long they last, and how well they keep their flavour (Akpossan et al., 2015). Type of protein, oil used, and hydrophobicity are other things that can affect this ability. The OBC for the BSF larvae protein isolate for salt-treated samples was enhanced significantly ($p < 0.05$). The high level of oil binding may be linked to the protein's high hydrophobicity. Therefore, in our study, oil binding capacity (OBC) was reported to be 2.17 g/g, while a surface hydrophobicity value was 1021.37.

The increased surface hydrophobicity is associated with increased oil binding capacity, as shown by AAFSI results. This then shows that the hydrophobic interactions are crucial in oil binding. The formulation of meat alternatives and extenders, alongside sausages, emulsions, and cake batters, is important for the protein binding ability for oils or fat samples. (Akpossan et al., 2015). AAFSI had the highest ($p < 0.05$) OBC (**Figure 5.3**) when compared to other proteins. The results of AAFSI at pH 10 are in agreement with other studies with regard to the solubility of the hydrophobic interaction thereof. However, AAF was reported to be 1.58 g/g, AAFSI to be 2.17 g/g, AAFSO to be 1.04 g/g, and AAFSISO to be 1.36 g/g, showing a lot of significant differences ($p > 0.05$)

The differences in hydrophobic or hydrophilic protein surfaces cause differences in OBC, and also the extraction process utilised could have an effect on this. For example, we reported significant differences between the protein isolates of AAFSI and AAFSO. This is because precipitates made by salting-out may have a different structure or shape than those made by salting-in, which can affect how well they can bind to oil. Salting-out may precipitate a broader spectrum of proteins, including those with diminished affinity for oil binding, resulting in a reduced overall oil binding capacity. The differences seen in both WBC and OBC across BSF larval proteins can help us make protein concentrates better and more beneficial in a variety of insect-based meal recipes.

5.4.4. Foaming capacity and foam stability

Foaming capacity (FC) refers to the ability of protein molecules to quickly dissolve and spread out, creating a solid layer surrounding a gas bubble. Foam stability (FS), on the other hand, refers to the ability of intermolecular polymerisation to create a stable foam that traps air (Preece et al., 2017). **Figure 5.4** shows the FS and FC of BSF larvae isolates. In our study, all four isolates displayed remarkable FC values, surpassing those documented for *Tenebrio molitor* and *Schistocerca gregaria* (34.67% and 19.33%, respectively), as indicated by Mintah et al. (2020). AAF and AAFSI showed differences in foaming capacity (FC), and they were markedly greater ($p < 0.05$) than those between AAFSO and AAFSISO protein extracts, exhibiting FC values of 11.00% and 14.79%, respectively. While the difference between AAFSO and AAFSISO was statistically significant, the magnitude of this difference (11% vs. 15%) is relatively small. The main observation here is that the SO-treated extracts consistently exhibited higher FC values, indicating that the salting-out method contributes to improved foaming properties.

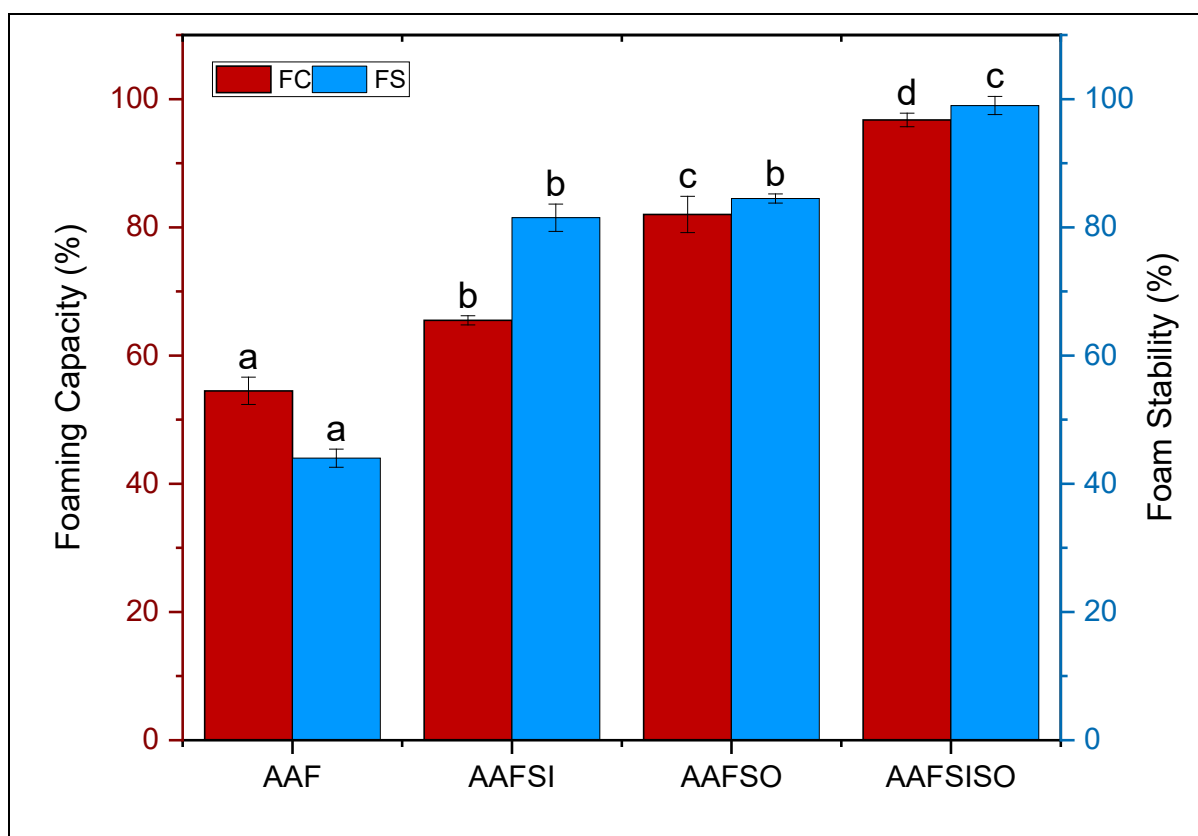


Figure 5.4. Effect of extraction methods on foaming capacity and foam stability of black soldier fly larvae isolates obtained by: AAF (alkaline-acid extraction); AAFSI (salting in technique); AAFSO (salting out technique) and AAFSISO (salting-in and out technique). Various letters (a, b, c, d) point to significant variations at $p < 0.05$. Error bars show standard deviation.

Correspondingly, the FS results revealed that AAFSISO proteins exhibited the highest stability at 99.99%, followed by AAFSO at 84.50% ($p < 0.05$, **Figure 5.4**). The salting out extraction may enhance both FC and FS by exposing hydrophilic residues and improving protein solubility during the isolation step (Preece et al., 2017). This result aligns with the findings of a study conducted by Yuliana et al. (2014), where the FS of cashew nutshell protein demonstrated an increase from 56.03% to 76.91% at the 60-min mark following salt addition. This increase in FS was attributed to the enhanced surface activity and solubility of the protein.

On the contrary, the low foaming capacity of AAFSI can be attributed to the globular proteins that are highly ordered and resist surface denaturation. For proteins to function effectively as foaming agents, they must possess the ability to (i) undergo rapid conformational changes and rearrangements at the interface and (ii) rapidly adsorb at the air-water interface

during bubbling. In a study by Gnana et al. (2022), the comparison was made between the foaming properties of mealworm and cricket powders and those of protein concentrates derived from yellow pea and faba bean. In a comparison between pulse proteins and cricket proteins, the cricket proteins produced foam that was more stable over time, while the pulse proteins generated more foam overall. These differences in FC could arise from the distinct conformational characteristics of the proteins. It has been established that the FC of proteins can be influenced by factors such as the balance of hydrophilic/hydrophobic groups and solubility, as well as the protein's composition and structural properties (Gnana et al., 2022).

Globular proteins, such as those found at the air-water interface, possess a reduced capacity to unfold, which in turn restricts their ability to capture air bubbles (Akpossan et al., 2015). The scientific community is currently exploring alternatives to eggs, a widely used foaming agent in food products (Akpossan et al., 2015). Presently, the food industry utilizes protein concentrates and isolates derived from wheat, soy, and dairy as ingredients. Nevertheless, both consumers and food processors are seeking alternative sources of protein to mitigate the allergenic concerns associated with the eight priority allergens, which comprise egg, soy, wheat, fish, peanut, crustaceans, dairy, and tree nuts. The findings of this study indicate that the AAFSISO protein preparation exhibits promising foaming properties and could be considered as a potential foaming agent for use in food applications.

5.4.5. Emulsifying properties

Emulsification is the process by which proteins enable and maintain the interface between oil and water. Generally, soluble, amphiphilic, and versatile proteins serve as effective emulsifiers (Hojilla-Evangelista & Evangelista, 2009). The emulsifying properties of BSF larvae were evaluated using the emulsifying activity index (EAI) and emulsion stability index (ESI), as shown in **Figure 5.5**. Our protein isolates exhibited a clear ($p < 0.05$) variation in EAI values, spanning from 32.50 to 87.78 m^2/g (**Figure 5.5**). These values corresponded to, or surpassed, those reported for other edible insects, including *Patanga succincta* (23.23 m^2/g) (Mishyna et al., 2021), *Gryllobates sigillatus* (32 m^2/g) (Hall et al., 2017), and *Chondracris roseaapbrunner* (36.96 m^2/g) (Chatsuwan et al., 2018). The salting-out proteins demonstrated the most promising results, making them suitable candidates for emulsifying agents. The process of emulsification is mainly caused by proteins sticking to the surface of oil droplets and the water around them in emulsions (Akpossan et al., 2015). The higher EAI in the salting-out group could be because there were more tiny, soluble proteins at the oil-water interface since proteins

are more soluble (Aletor et al., 2002). The variations in EAI are believed to be associated with disparities in surface hydrophobicity and protein-protein/oil interactions (Gravel & Doyen, 2020).

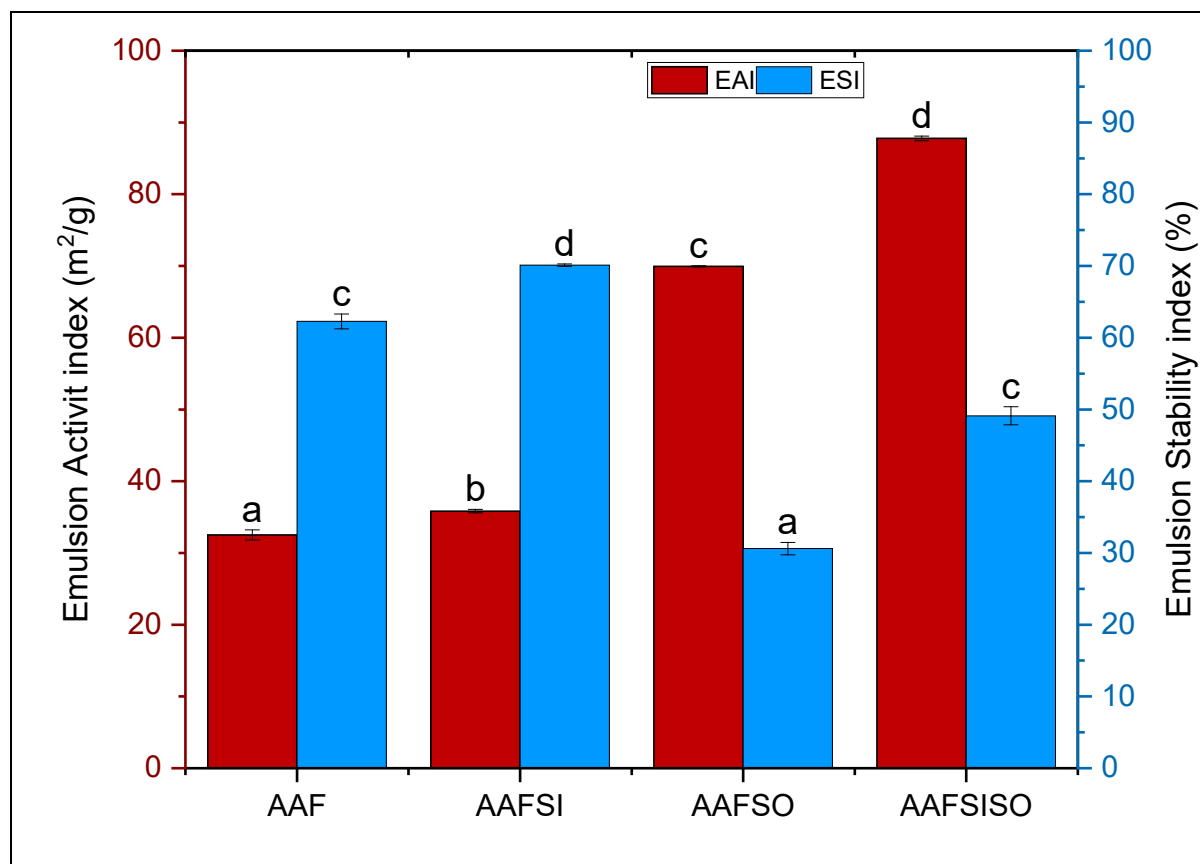


Figure 5.5. Effect of extraction methods on emulsion stability index and emulsion activity index of black soldier fly larvae isolate obtained by: AAF (alkaline-acid extraction); AAFSI (salting in technique); AAFSO (salting out technique) and AAFSISO (salting-in and out technique). Various letters suggest notable contrasts with statistical significance at $p < 0.05$. The error bars depict the standard deviation.

Gravel and Doyen (2020) posited that proteins exhibiting elevated surface hydrophobicity are inclined to adsorb at the oil-water interface, hence augmenting the emulsification effect. Gravel and Doyen (2020) assert a strong association between elevated surface hydrophobicity and a high emulsion stability index, aligning with our findings. Mshayisa et al. (2022) observed that elevating the ionic strength of the extraction solution can augment the shielding of surface charge, resulting in heightened protein-protein interactions and diminished protein-oil

interactions. The stability of an emulsion is determined by the balance between repulsive and intramolecular forces at the water/oil interface, which can be impacted by numerous parameters such as ionic strength, pH, amino acid polarity, and protein composition (Stone et al., 2019b).

Our findings, as demonstrated by the ESI values, indicated that the AAF and AAFSI proteins had superior values (62.28% and 70.12%, respectively) in comparison to the *Tenebrio molitor* proteins (51.31%), corroborating the earlier research by Zielińska et al. (2018) (**Figure 5.5**). The AAFSO proteins also caused the emulsions to break down faster than the AAFSI proteins, which was more than twice as fast (**Figure 5.5**).

The outcomes from salt-out-assisted treatments indicated a reduction in ESI, aligning with prior research that showed proteins isolated using isoelectric precipitation exhibited elevated ESI levels compared to those isolated through ammonium sulphate precipitation (Deng et al., 2019). The elevated hydrophobicity of BSF larvae isolates may facilitate protein adsorption at the oil/water interface, leading to a reduction in interfacial tension and an augmentation of ESI levels (Jiang et al., 2021). Casein and whey proteins are the most prevalent emulsifiers used in the food business right now. Researchers are still looking for other sources of protein that could replace these traditional ones, though. The BSF larval extracts examined in this study yielded comparable outcomes and may serve as alternative protein emulsifiers in both current food products and novel formulations.

5.4.6. Pearson's correlation analysis

To investigate the possible relationship between the functional parameters of BSF larvae proteins and the variables listed earlier, the correlation coefficients were calculated between these variables. Pearson's correlation matrix is shown in **Figure 5.6**. To our knowledge, there are no comparable studies on BSF larvae protein available currently. A strong, positive correlation was observed between the emulsion activity index and foam capacity ($r = 0.98$, $p < 0.001$). The emulsion activity index showed a strong positive correlation with solubility at pH 4 ($r = 0.92$, $p < 0.01$), pH 6 ($r = 0.98$, $p < 0.001$), and pH 8 ($r = 0.98$, $p < 0.001$).

Additionally, a strong, positive correlation was noted between solubility at pH 8 and foam stability ($r = 0.85$, $p < 0.01$), as well as with foaming capacity ($r = 0.98$, $p < 0.001$), water binding capacity ($r = 0.84$, $p < 0.01$). A negative correlation was observed with oil-binding capacity ($r = -0.64$, $p < 0.05$) and emulsion stability index ($r = -0.75$, $p < 0.05$). Furthermore, a moderate, positive correlation was observed between surface hydrophobicity and oil binding capacity ($r = 0.74$, $p < 0.05$) as well as emulsion stability index ($r = 0.83$, $p < 0.05$).

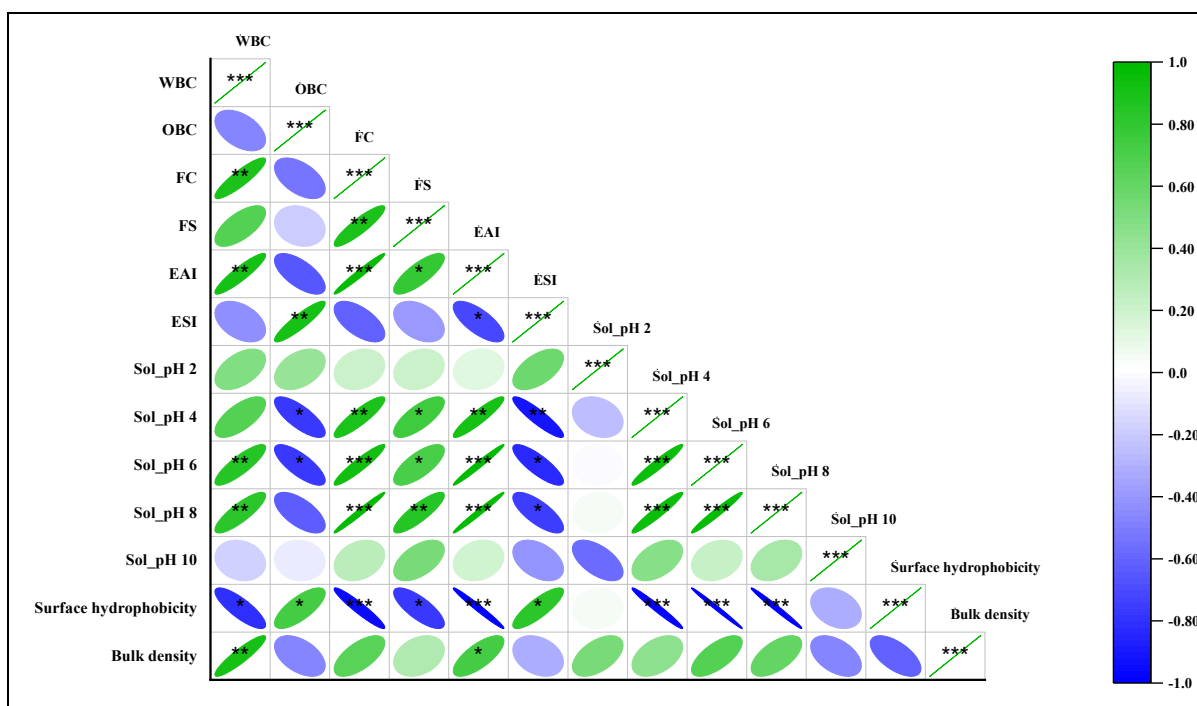


Figure 5.6. Correlation matrix between the black soldier fly larvae protein isolates functional properties via Pearson's linear correlation analysis; the colour gradation and the size of the circle represent the correlation coefficient (r value). Significance levels: * $p < 0.05$ (moderate significance), ** $p < 0.01$ (higher significance), *** $p < 0.001$ (highest significance), blue (represents negative correlation) green (represents positive correlation).

The data depicted in Figures 5.2 and 5.4 suggest that the enhanced oil-binding capacity and emulsion stability index observed in BSF larvae proteins after salt treatment with NaCl and $(\text{NH}_4)_2\text{SO}_4$ is attributable to the increased surface hydrophobicity. This increase enhances the ability to bind water and convert it into immobilized water, as well as strengthens the interactions among the exposed hydrophobic groups. Lastly, a weak, positive correlation was observed between bulk density and foam stability ($r = 0.65$, $p < 0.005$) and foam capacity ($r = 0.31$, $p < 0.005$).

Water binding capacity and surface hydrophobicity had a statistically significant negative association ($r = -0.82$, $p < 0.01$). This study is the core investigation into the effects of salt on BSF larval proteins. Also, for making food items with different, long-lasting food ingredients for populations that are expanding, these results are important. Strong interrelations ($p < 0.05$) among the functional features of BSF larvae proteins were reported on our findings.

Such findings suggest that BSF larvae protein may be an appropriate candidate for incorporation into food formulations.

5.5. Conclusion

In conclusion, techno-functional and physicochemical attributes of BSF larvae proteins were extensively investigated. Protein solubility, surface hydrophobicity, bulk density, foaming capacity and stability, emulsifying characteristics, and water and oil binding capacity were studied. Within the food industry, such findings will provide significant insights into their prospective applications. Salting out extraction using $(\text{NH}_4)_2\text{SO}_4$, making it more soluble at pH 2, and suggesting that it could be used in acidic food systems. In addition, the hydrophobicity of the surface was changed by the salt treatment. Making hydrophobicity values much lower when treated by salting out, changing the functionality of the proteins. Salting-in (NaCl) lowered bulk density, while salting-out $(\text{NH}_4)_2\text{SO}_4$ raised it. This shows how important salt treatment methods are for getting the most protein based on the properties you want to focus on. The best result will rely on the protein product or ingredient's individual needs and how it will be used. Depending on the desired qualities and functions of the protein extract, both the decrease and increase in bulk density could be useful.

Moreover, salt treatment had a big effect on the ability to bind water and oil, make foam, and emulsify, with salting-out proteins showing better functional capabilities. The study showed that the AAFSISO protein obtained through salting-out had the potential to have good foaming capabilities and might be used as a foaming agent in food. Correlation analyses demonstrated robust positive associations among various functional properties, including emulsion activity index and foam capacity, solubility at pH 4 and foam capacity, solubility at pH 8 and foam stability, foam capacity and water binding capacity, as well as surface hydrophobicity and oil binding capacity, underscoring the interrelatedness of these attributes in BSF larval proteins. These results indicate the possible application of BSF larvae proteins in food formulations, especially in light of the favourable relationships identified among emulsification characteristics, solubility, and surface hydrophobicity. The revealed correlations establish a basis for the continued investigation of BSF larvae proteins as sustainable functional ingredients in food applications, corresponding with the increasing interest in alternative protein sources within the food industry.

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CHAPTER SIX

THE EFFECT OF ULTRASOUND AND SALT-ASSISTED ALKALINE ISOELECTRIC PRECIPITATION ON THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF BLACK SOLDIER FLY LARVAE PROTEINS

“Entomophagy, or the consumption of insects, presents an opportunity to address food security and environmental challenges simultaneously. It’s time to embrace insects as a valuable food source.”

(Angela Garb, 1992)

6.1. Abstract

This study investigated the mechanisms by which ultrasound and salt-assisted alkaline isoelectric precipitation enhance structural and functional properties of black soldier fly (BSF) larvae proteins. These properties are essential for diverse applications in the food, feed, and biotechnology industries. Additionally, the proteins were isolated using salt extraction techniques, including salting-in (NaCl) and salting-out ((NH₄)₂SO₄), followed by ultrasound treatment to modify their properties further. Ultrasound, a promising technology for altering protein characteristics. This was applied to facilitate structural changes and improve functional properties. The treatments incorporated varying power levels (150 W, 200 W, 250 W, and 300 W) and durations (10, 20, 30, and 40 min). Compared to the control ($L^* = 60.94 \pm 0.07$), ultrasound treatment significantly ($p < 0.05$) influenced the colour features of BSF larvae protein isolates, with increased lightness (L^*) observed under specific conditions such as P200T10 ($L^* = 75.02 \pm 0.03$). Solubility of BSF larval proteins improved markedly with ultrasound treatment, reaching a maximum of 89.18% after treatment at 200 W for 30 min, indicating effective breakdown of protein aggregates and unfolding of protein structures. Longer treatment times generally increase surface hydrophobicity for P150 and P200. However, at higher power levels, such as P250 and P300, excessive treatment reduces surface hydrophobicity. Enhanced WBC (6.40 g/g) was observed in isolate P250T40, while the maximum OBC (6.30 g/g) was observed in isolate P300T10, demonstrating improved moisture retention and oil absorption capacities. Our results demonstrate that P200T40 achieved the highest foaming capacity of 98.03% and foaming stability of 99.33%. The DSC results demonstrate that ultrasound treatment significantly affects the thermal stability and structural properties of BSF larvae proteins. The technique of ultrasound-assisted extraction has emerged as promising for the improvement of these functional properties. BSF larval proteins appear to be well-suited for diverse industrial applications. To unlock its full potential, future research should aim to optimize ultrasound parameters, ensuring scalable and economically viable production for the global protein market.

6.2. Introduction

By 2050, the global population is anticipated to reach approximately 9.7 billion (Borges et al., 2022). Substantial growth in the demand for affordable, nutritious dietary protein sources for human consumption will be one of the outcomes (Zhang et al., 2022; Ordoñez-Araque & Egas-Montenegro, 2021). The primary source of high-quality proteins is considered animal-derived proteins, particularly those from meat, dairy, and eggs. This is due to their superior nutritional value (e.g., high amounts of essential amino acids and micronutrients) and functional properties (e.g., gelling, emulsifying, foaming, and water/fat holding capacities). These properties contribute to the development of high-quality profiles for animal-derived food products. According to van Huis. (2013) and the Food and Agriculture Organisation (FAO), an increase of approximately 70% in the demand for animal-derived proteins in response to global population growth, particularly in developing countries. However, significant environmental problems, such as greenhouse gas and ammonia emissions and pollution (Kim et al., 2020), have resulted from the continuous expansion of conventional livestock production. Therefore, sustainable and alternative protein sources need to be developed and applied as suitable alternatives to conventional animal-derived proteins.

Insect proteins have gathered substantial attention from the FAO due to their advantages over traditional protein sources, for example, insects such as BSF larvae emit less greenhouse gases and ammonia, resulting in less pollution. They require less space and water and possess a high protein content. In addition, they also contain essential amino acids required for human consumption (Lamsal et al., 2019; Mshayisa et al., 2022; Leni et al., 2018; Huang et al., 2023). Consequently, insect proteins have emerged as promising alternative protein sources alongside algae and plant-based sources, and recent studies have shown that edible insects contain high levels of nutrients, including proteins (15–82% dry basis) and fat (4–77% dry basis) (Köhler et al., 2019; Paul et al., 2017). It has been endorsed that the content of essential amino acids (Lamsal et al., 2019; Mshayisa et al., 2022; Leni et al., 2018; Huang et al., 2023) is found in acceptably high levels in insects. Lipids with a higher proportion of unsaturated fatty acids, valuable vitamins (e.g., vitamin A, vitamin C, vitamin E, and niacin), and minerals (e.g., potassium, calcium, iron, magnesium, and zinc) (Zhang et al., 2017; Rumpold & Schlüter, 2013) as well. However, factors such as insect species, developmental stage of the insect, season, and insect feed can result in variations in the nutrient content of insect proteins (Rumpold & Schlüter, 2013; van Huis, 2013; Makkar et al., 2014; Sogari et al., 2019). Additionally, the extraction processes used, such as the

extraction techniques applied, process flow, and conditions, as well as the powdering and drying methods used, can affect the nutrient content of insect protein extracts (Köhler et al., 2019; Mshayisa et al., 2022; Purschke et al., 2018; Zozo et al., 2022).

Protein extraction is an essential process in the food industry; however, traditional extraction methods, such as solvent extraction, acid precipitation, and enzymatic hydrolysis, often have low yields and may introduce interfering compounds, resulting in complex purification steps (Ge et al., 2010). Chemical and mechanical extraction methods using alkaline solution, salt, and ethanol as solvents, with stirring the mixture at a specific temperature, are commonly used. However, these techniques are labor-intensive, time-consuming, and require large amounts of samples. For example, the denaturation of proteins, decrease in bioactivity, and reduction of the nutritive value may be caused by some extraction solvents (Zhu et al., 2009; Jodayree et al., 2012). Recent developments in non-thermal processing methods have generated interest in the food industry (Ashokkumar et al., 2008) and ultrasound-assisted extraction, microwave-assisted extraction, ionic liquid solution extraction, and high-density steam flash explosions have been reported as new technologies for protein extraction (Ge et al., 2010; Zhang et al., 2013). These advanced methods offer higher yields, faster extraction times, and lower sample requirements. The use of high-intensity ultrasound has accumulated substantial attention owing to its potential to develop novel, milder, and selected processes to enhance the quality and safety of processed foods.

The possibility of improving existing processes is offered by this technology. The most efficient, based on the final protein yield, is alkaline extraction; others are salt precipitation and solvent precipitation (Liu et al., 2020). Wu et al. (2021) attributed the higher protein yield of ultrasound-assisted alkaline extraction to ultrasound's ability to significantly enhance extraction efficiency. Acoustic cavitation induces cell wall rupture and the promotion of mass transport. Kadam et al. (2015) also noted that ultrasound vibration could effectively promote the recovery of proteins or bioactive peptides from solid raw materials into the aqueous phase. However, the disadvantage of excessive ultrasound treatment can lead to the formation of reactive free radicals and acute lipid and protein oxidation (Li et al., 2021). Ultrasound-assisted alkaline extraction can destroy hydrogen bonds or electrostatic forces via the cavitation effect, thus altering the molecular structures of the intended protein (Li et al., 2020).

Moreover, the turbulence, shock waves, and microjets generated during sonication can result in structural and physical changes in protein isolates. These are described as partial unfolding of protein structures, improved dispersion of protein molecules, and disruption of large protein aggregates (Ashokkumar, 2015; Bhargava et al., 2021). The cavitation phenomenon, wherein rapid formation and collapse of bubbles produce localized high pressure and temperature. They are primarily responsible for these effects, promoting conformational changes in proteins (Preece et al., 2017). During cavitation, microjets and shear forces enhance surface exposure of hydrophobic residues, and in such cases, an increase in the solubility and functional properties of proteins, such as emulsification and foaming, is perceived (Kang et al., 2022).

Gelling properties, foaming capacities, emulsifying characteristics, and protein solubility are enhanced by ultrasound-assisted treatment, as indicated by Xiong et al. (2018), additionally Zhang et al. (2023) indicated that ultrasound-assisted alkaline extraction could be applied as a potential and effective extraction method to extract protein from *Tenebrio molitor* larvae. The objective of increasing the yield and functionality of targeted proteins by ultrasound-assisted alkaline extraction has been widely studied from conventional plant- or animal-based sources (Alavi et al., 2021; Naik et al., 2022; Wang et al., 2021). However, there are no studies on the use of ultrasound treatment on the properties of BSF larvae protein extract. There is a lack of information on how the combination of sonication and salt treatment can affect the structural and functional properties of BSF larvae protein extract. Thus, the objective of this study was to scrutinize the effects of ultrasound-assisted alkaline extraction and isoelectric precipitation extraction at five different power levels (0, 150, 200, 250, and 300W) and extraction times (0, 10, 20, 30, and 40 min). The impact on the techno-functional, structural, and nutritional content, tertiary structure, and thermal stability of black soldier fly (BSF; *Hermetia illucens*) larvae will emerge from this study. The results will establish a novel ultrasound-assisted extraction method with higher extraction efficiency to produce BSF larvae protein, potentially promoting the development of a novel and sustainable protein with superior quality profiles.

6.3. Materials and Methods

6.3.1. Chemicals and materials

The chemicals and materials used in this chapter are the same as those described in the experiments in Chapters 3 and 4. Detailed information about the chemicals, their suppliers, the flour preparation, and the protein analysis methods is provided in Section 3.3 of Chapter 3. The structural analysis from Chapter 4 and the physicochemical and techno-functional property analysis from Chapter 5 were also applied. A distinct procedure for ultrasound protein extraction is presented below (**Table 6.1**).

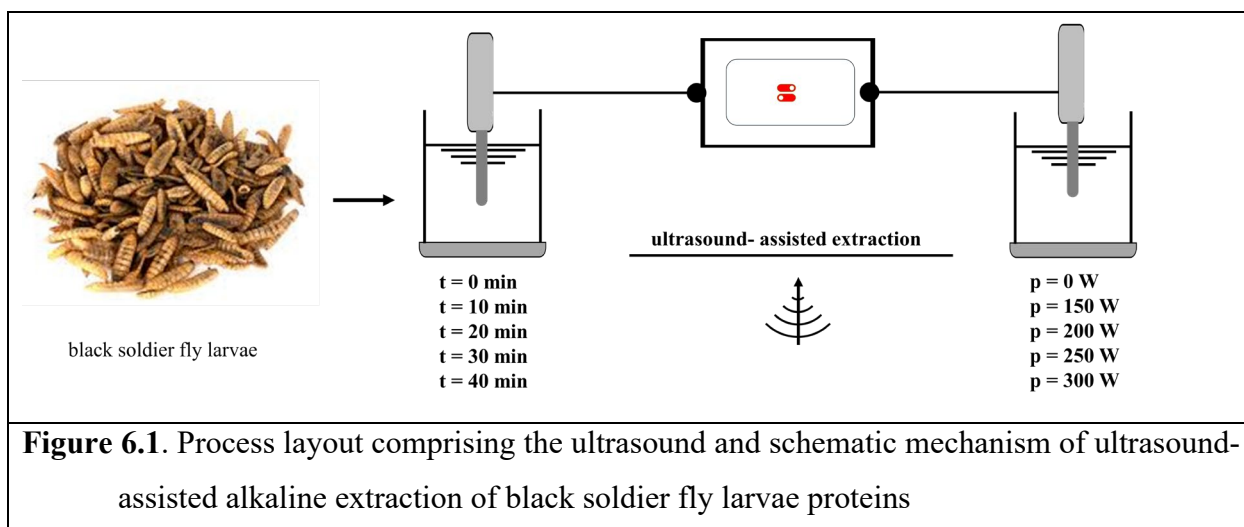
6.3.2. Protein extraction based on alkaline extraction with isoelectric precipitation method and ultrasound extraction.

The salt-extracted isolates were prepared according to the method described by Jiang et al. (2021), with minor modifications. Alkaline extraction with isoelectric precipitation was conducted as previously described by Azagoh et al. (2016), with slight modifications.

Table 6.1. Ultrasound-assisted extraction of the black soldier fly larvae protein isolates.

Extraction power (W)	Extraction time (min)	Abbreviation	Sonication Energy (J)
0	0	P0T0 (control)	0
150	10	P150T10	89.74 ± 0.40
	20	P150T20	135.06 ± 0.36
	30	P150T30	202.54 ± 0.05
	40	P150T40	283.60 ± 0.02
200	10	P200T10	91.84 ± 0.02
	20	P200T20	183.73 ± 0.03
	30	P200T30	275.59 ± 0.00
	40	P200T40	367.43 ± 0.06
250	10	P250T10	113.41 ± 0.05
	20	P250T20	226.43 ± 0.34
	30	P250T30	303.56 ± 0.02
	40	P250T40	453.28 ± 0.09
300	10	P300T10	135.09 ± 0.02
	20	P300T20	270.04 ± 0.01
	30	P300T30	405.14 ± 0.05
	40	P300T40	540,15 ± 0,02

The defatted BSF larvae flour was mixed with Milli-Q water at a ratio of 1:10 (w/v), and the pH was adjusted to 10 using a 1 M NaOH solution and a 1% NaCl (w/v) in NaOH solution to improve protein solubility (Yi et al., 2017). Ultrasonicated treatments were applied instead of mixing (see **Figure 6.1**). The protein dispersions were placed in an ice bath to control the increase in temperature during the ultrasound treatment. The probe was inserted into the solution and subjected to ultrasound treatment (Branson Sonifier, Emerson, St. Louis, MO, USA) fixed at 20 kHz, pulse ON and OFF for 15 s at exposure times of 0, 10, 20, 30, and 40 min, and the output power was varied at 0, 150, 200, 250, and 300 W.



The solution was centrifuged (Z-326 K, Hermle Labortechnik GmbH, Germany) at 25°C and $10,000 \times g$ for 30 min. The pH of the supernatant was adjusted to 4.5, and 1 M HCl and 80% $(\text{NH}_4)_2\text{SO}_4$ (w/v) were added to the supernatant to enhance protein precipitation (Duong-Ly & Gabelli, 2014). The suspension was then incubated overnight at 4°C for protein precipitation. The precipitate was recovered by centrifugation and then resuspended in 250 mL of water, and the pH was adjusted to 7.0 with 1 M NaOH. The water-soluble isolate was obtained after centrifugation, and a 200 mL solution was subjected to dialysis through ultrafiltration using a three kDa nominal molecular mass cut-off membrane in an Amicon ultrafiltration cell (8400, Amicon, Beverly, MA, USA). The dialysate was subsequently lyophilized and preserved at -18°C for subsequent analysis. The samples in the control group were not subjected to the ultrasonic treatment (**Table 6.1**).

6.4. Results and discussion

6.4.1 Influence of ultrasound treatment on colour change, protein content, and yield

Colour is a critical sensory parameter that significantly impacts consumer acceptance of food products. Colour is an essential determinant of marketability (Nidhina & Muthukumar, 2015). In this study, the influence of sonication on extraction efficiency and associated biochemical modifications is examined. The relationship between protein extraction yield and changes in the colour characteristics of BSF larvae proteins under varying ultrasound conditions was analyzed. The effects of ultrasound power and time on the colour of BSF larvae proteins are illustrated in **Table 6.2**. Also displayed are the protein content and protein recovery yield. It is important to note that L^* represents the lightness of the sample, hence higher L^* values indicate lighter colours and lower values darker colours. A^* describes the redness-greenness continuum, with positive values indicating redness and negative values indicating greenness. B^* describes the yellowness-blueness continuum, with positive values indicating yellowness and negative values indicating blueness.

The colour of the proteins changed following ultrasonic treatment when compared with the control proteins (P0T0). The results of the ultrasonication treatment on the L^* values showed significant improvements. The most significant differences were observed in P200T10 compared to the control sample's L^* values. P200T10 had L^* values of 74.99 ± 0.03 , while P0T0 had values of 60.87 ± 0.07 . The major change in L^* values for the 250W samples after treatment was observed at 20 min compared to the control sample. The 300W samples showed significant changes in L^* values at all run times compared to the control, indicating a transition from black to white colour. P0T0 exhibited significantly lower brightness values compared to other isolates ($L^* = 60.87$, $p < 0.05$). Furthermore, P200T10 exhibited higher lightness ($L^* = 74.99$, $p > 0.05$) and reduced redness ($a^* = 2.55$, $p < 0.05$) compared to the other isolates. These results, together with other samples treated at 300W, suggest that the turbulence, shock waves, and shear forces generated by sonication may enhance sample lightness under a power of 300W. In contrast, no substantial differences in lightness were observed between samples treated at 150W and 200W ($p > 0.05$).

Ultrasound treatment has been widely explored in various fields due to its ability to induce physical and biochemical changes. In the context of BSF larvae, ultrasound treatment may affect the biochemical composition of proteins, which could subsequently influence colour attributes.

Table 6.2 Colour measurement of colour values (L*, a*, and b*), change in colour (ΔE), protein content and protein recovery yields of seventeen fractions from black soldier fly larvae proteins.

Sample	L*	a*	b*	ΔE	Protein content (%)	Protein recovery yield (%)
P0T0	60.87 $\pm$ 0.07 ^a	3.00 $\pm$ 0.50 ^{ef}	13.51 $\pm$ 0.25 ^a	-	89.43 $\pm$ 1.72 ^f	62.74 $\pm$ 1.25 ^a
P150T10	69.14 $\pm$ 0.02 ^e	3.16 $\pm$ 0.00 ^{bcde}	16.03 $\pm$ 0.03 ^h	14.40 $\pm$ 0.08 ^f	75.63 $\pm$ 0.57 ^{bcde}	74.19 $\pm$ 1.32 ^{bcd}
P150T20	71.07 $\pm$ 0.00 ⁱ	2.82 $\pm$ 0.00 ^{ab}	15.45 $\pm$ 0.00 ^d	13.63 $\pm$ 0.05 ^e	78.54 $\pm$ 1.03 ^e	71.44 $\pm$ 0.92 ^d
P150T30	70.55 $\pm$ 0.02 ^g	2.87 $\pm$ 0.01 ^{bcd}	15.27 $\pm$ 0.02 ^c	13.68 $\pm$ 0.01 ^e	77.14 $\pm$ 1.81 ^{abcd}	72.74 $\pm$ 1.79 ^{bcd}
P150T40	70.68 $\pm$ 0.07 ^{gh}	2.90 $\pm$ 0.01 ^{bcd}	15.21 $\pm$ 0.01 ^c	13.60 $\pm$ 0.00 ^e	72.63 $\pm$ 1.50 ^{ab}	77.26 $\pm$ 1.53 ^{bcd}
P200T10	74.99 $\pm$ 0.03 ^k	2.55 $\pm$ 0.01 ^a	14.11 $\pm$ 0.02 ^b	12.35 $\pm$ 0.15 ^b	77.75 $\pm$ 0.79 ^{de}	72.17 $\pm$ 0.72 ^{cd}
P200T20	71.74 $\pm$ 0.01 ^j	3.18 $\pm$ 0.00 ^{bcde}	15.84 $\pm$ 0.00 ^{fg}	12.89 $\pm$ 0.05 ^{cd}	75.20 $\pm$ 0.53 ^{ab}	72.07 $\pm$ 0.53 ^{bcd}
P200T30	69.94 $\pm$ 0.01 ^f	3.22 $\pm$ 0.01 ^{bcde}	15.59 $\pm$ 0.01 ^{de}	14.35 $\pm$ 0.15 ^f	74.00 $\pm$ 0.01 ^{ab}	75.83 $\pm$ 0.01 ^{bcd}
P200T40	70.20 $\pm$ 0.00 ^f	3.40 $\pm$ 0.00 ^{def}	15.52 $\pm$ 0.01 ^{de}	13.17 $\pm$ 0.03 ^{de}	78.38 $\pm$ 0.12 ^{cde}	71.82 $\pm$ 0.18 ^{cd}
P250T10	70.74 $\pm$ 0.01 ^g	3.23 $\pm$ 0.00 ^{bcde}	15.85 $\pm$ 0.02 ^g	13.14 $\pm$ 0.04 ^{de}	72.73 $\pm$ 0.68 ^a	71.15 $\pm$ 0.73 ^b
P250T20	71.10 $\pm$ 0.07 ^h	3.21 $\pm$ 0.02 ^{bcde}	16.04 $\pm$ 0.04 ^h	13.43 $\pm$ 0.02 ^{de}	72.80 $\pm$ 0.03 ^a	77.07 $\pm$ 0.03 ^{bc}
P250T30	68.51 $\pm$ 0.03 ^d	3.29 $\pm$ 0.01 ^{cdef}	15.70 $\pm$ 0.01 ^{efg}	15.03 $\pm$ 0.02 ^{gh}	73.56 $\pm$ 0.92 ^a	76.24 $\pm$ 0.93 ^{bc}
P250T40	66.05 $\pm$ 0.02 ^b	3.67 $\pm$ 0.01 ^f	16.48 $\pm$ 0.01 ⁱ	18.07 $\pm$ 0.02 ⁱ	72.31 $\pm$ 0.25 ^a	77.56 $\pm$ 0.27 ^b
P300T10	71.10 $\pm$ 0.14 ⁱ	3.10 $\pm$ 0.01 ^{bcde}	15.65 $\pm$ 0.01 ^{def}	12.54 $\pm$ 0.07 ^{bc}	72.93 $\pm$ 0.34 ^{ab}	74.88 $\pm$ 0.35 ^{bcd}
P300T20	70.88 $\pm$ 0.35 ^g	3.15 $\pm$ 0.05 ^{bcde}	15.52 $\pm$ 0.01 ^{de}	13.12 $\pm$ 0.07 ^{cd}	72.60 $\pm$ 1.27 ^{ab}	77.07 $\pm$ 1.30 ^{bcd}
P300T30	68.35 $\pm$ 0.02 ^{cd}	3.06 $\pm$ 0.00 ^{bcd}	15.57 $\pm$ 0.01 ^{de}	15.12 $\pm$ 0.39 ^h	76.03 $\pm$ 1.23 ^{abc}	73.29 $\pm$ 1.23 ^{bcd}
P300T40	68.18 $\pm$ 0.01 ^c	3.21 $\pm$ 0.00 ^{bcde}	15.48 $\pm$ 0.01 ^{de}	14.56 $\pm$ 0.34 ^{fg}	73.64 $\pm$ 1.97 ^{abcd}	76.20 $\pm$ 1.93 ^{bcd}

Values are means $\pm$ standard deviations (n = 3). Different superscript letters above each of the data points in a column indicate statistically significantly different values (p < 0.05).

Ultrasound treatment can enhance protein extraction by disrupting cell walls and improving extraction efficiency. A study by Mintah et al. (2020), indicated that the sonic treatment aided the enzyme action from the substrate, in a way that resulted in a product (hydrolysate) with decreased luminosity, giving a slightly less light appearance than the non-treated black soldier fly larvae sample.

Following the application of power ultrasound, the b^* values of BSF protein isolates increased, resulting in significant differences ($p < 0.05$) between the b^* values of ultrasound-treated BSF larvae protein isolates and those of the control (P0T0) protein isolates. The a^* values of all ultrasound-treated BSF larvae protein isolates, including P150T20, P150T30, P150T40, P200T10, and P300T30, showed no significant differences compared to the control isolate (P0T0), as indicated by shared significance letters ('e' or 'f') ($p > 0.05$). Furthermore, statistically significant differences were observed in the colour characteristics of the ultrasound-treated BSF larvae protein isolates across various treatment durations compared to the control group. The whiteness index values of BSF larvae-treated proteins increase with treatment compared to the control, and there is no consistent trend of decline with increasing power. The effects of ultrasound on the colour of food depend on the intrinsic characteristics of the food and the ultrasound conditions. Bi et al. (2015) reported that ultrasound treatment on egg white protein decreased the L^* value and increased the values of a^* and b^* . This was attributed to an increase in the number of hydrophobic groups exposed on the protein surface, resulting in the release and exposure of riboflavin. Riboflavin is a bright yellow pigment, and its exposure plays a significant role in altering the colour of treated proteins.

In the study by Adekunle et al. (2010), sonication of tomato juice led to a decrease in its L^* , a^* , and b^* values due to the degradation of lycopene. The degradation of lycopene, which imparts a red colour to tomato juice, results in a loss of redness (decreased a^*), diminished yellowness (decreased b^*), and reduced lightness (decreased L^*), leading to a paler appearance of the juice. On the other hand, Valero et al. (2007) found no significant effect of ultrasound treatments on the colour characteristics of an orange juice. Nevertheless, Pingret et al. (2013) reported the degradation or loss of other compounds, including pigments and antioxidants such as anthocyanins, carotenoids, ascorbic acid, and total phenolic content, by ultrasound pre-treatments, particularly when a liquid medium was used during sonication. The authors suggested mechanisms such as pyrolysis and oxidation by OH radicals formed by cavitation. The combination of

ultrasound treatment with salt-assisted acid-alkaline protein extraction method offers a systematic approach to optimizing protein extraction from BSF larvae, which is crucial for various applications in the food, feed, and biotechnology industries.

Lastly, ultrasound also enhanced protein recovery yields significantly, with treated samples achieving yields up to 77.89% compared to 62.74% for the control. A similar increase in protein yield (from 3.1 to 4.2 mg/mL) after ultrasonic treatment has been described previously for rapeseed meal proteins (Yagoub et al., 2017). It may occur due to induced cavitation, which facilitates the rupture of cells (Vinatoru, 2001) and increases protein extractability. However, no consistent trend of increased yield with higher ultrasound power and longer treatment time was observed (**Table 6.2**). Ultrasound-assisted extractions resulted in lower protein content in BSF larvae isolates compared to the control ($87.71 \pm 1.72\%$), with treated samples ranging between $72.05 \pm 0.25\%$ and $79.58 \pm 1.03\%$. The decrease in protein content could be attributed to protein denaturation, hydrolysis, or aggregation due to the formation of disulfide bridges through the oxidation of sulphur-containing amino acids in the protein induced by hydroxyl-free radicals generated at high ultrasound power (Wang et al., 2021). It is well-documented that alkaline conditions promote enzymatic and non-enzymatic oxidation of phenolic compounds into quinones, which can react with proteins to produce green and brown colours that cannot be washed from protein isolates. This phenomenon has also been observed in insect protein isolates, as described by Janssen et al. (2019).

The variation in the colour characteristics of ultrasound-treated protein isolates observed in this study does not show a consistent trend with sonication time. In contrast, literature such as Zou & Jiang (2016) reports a clear relationship between sonication time and colour changes, attributed to the alteration of chromophores and protein oxidation. The discrepancies between the findings in this study and those reported in the literature warrant further investigation to understand the specific factors influencing the colour characteristics of black soldier fly protein isolates. Sonication can have a positive or negative effect on the pigments present in food constituents. The sonication may facilitate the release of pigments from proteins and potentially disrupt the sites containing pigments, resulting in the absorption of light. The improvement of colour for BSF larvae protein isolates by ultrasound in our study may be helpful to enhance their aesthetic perception and will make them more attractive and desirable for consumers.

6.4.2 Amino acid composition

Amino acids play a pivotal role in protein and nitrogenous compound production, as well as in regulating metabolic pathways. Although they constitute a small percentage of the total, certain amino acids act as crucial precursors that significantly impact the quality of food products (Jobgen et al., 2006; Meijer, 2003). Proteins are composed of a variety of amino acids, and the content, proportion, and availability of these amino acids greatly influence their nutritional value (Chronakis & Madsen, 2011). The total amino acid composition of the control sample and the protein obtained through ultrasound-assisted extractions are presented in **Table 6.3**. The total amino acid content of the control and various ultrasound-treated samples ranged from 724.18 to 791.06 mg/g (**Table 6.3**). All samples contained essential amino acids surpassing FAO/WHO recommendations for adults, except for lysine, which was slightly below the recommended level (**Table 6.3**).

“Available lysine content” refers to the portion of protein-bound lysine with a free amino group that can be efficiently absorbed during digestion. Ultrasound treatment caused significant variations ($p < 0.05$) in lysine content across samples P200T40 (41.41 ± 1.51 ; 8% higher), P250T10 (42.14 ± 0.22 ; 10% higher), P250T20 (41.95 ± 0.65 ; 9% higher), and P250T30 (41.37 ± 0.67 ; 8% higher) when compared with the untreated sample P0T0 (38.34 ± 0.34), these findings indicate a notable increase in lysine content in the treated samples. These results suggest that P250T10 and other ultrasound-treated samples with significantly higher lysine content could serve as suitable alternatives to low-lysine insect-based foods, as demonstrated by Bradford Harris et al. (2013). The rest of the samples showed no significant differences in lysine content compared to the untreated sample (P0T0). This stability may be due to structural alterations caused by ultrasonication, exposing the ϵ -amino groups of lysine and interacting with other protein functional groups, resulting in a slight reduction in lysine content (Zhang et al., 2021). The maximum reduction was observed at 50% amplitude for 30 min, likely due to lysine’s role in protein aggregation during ultrasonic treatment (Ma et al., 2022). This trend was also seen in sunflower protein after ultrasound treatment (Malik et al., 2017). Ultrasound-assisted extracts generally exhibited higher concentrations of essential amino acids (EAAs) compared to the control sample P0T0 (320.48 ± 1.48 mg/g), with significant increases observed in P200T30 (338.71 ± 9.31 mg/g; 6% higher), P250T10 (348.88 ± 1.32 mg/g; 9% higher), P250T20 (347.41 ± 2.01 mg/g; 8% higher), P250T30 (328.85 ± 2.25 mg/g; 3% higher), and P250T40 (338.31 ± 2.51 mg/g; 6% higher).

Table 6.3. Amino acid (AA) composition (mg/g of crude protein) of the black soldier fly larvae protein extracts, compared with the adult daily requirements. Values are means ± standard deviation (n = 3). Different letters above each of the data points indicate statistical significantly different values (p < 0.05).

Essential Aas	P0T0	P150T10	P150T20	P150T30	P150T40	P200T10	P200T20	P200T30	P200T40	FAO/WHO /UNU
His	34.54 ± 0.47 ^{abc}	33.74 ± 0.47 ^a	34.74 ± 0.47 ^{abc}	33.84 ± 0.47 ^a	34.39 ± 0.52 ^{abc}	34.94 ± 0.57 ^{abcd}	36.04 ± 0.33 ^{cdef}	36.59 ± 0.28 ^{defg}	37.14 ± 0.23 ^{efg}	15
Ile	32.04 ± 0.04 ^{ab}	31.24 ± 0.04 ^a	32.24 ± 0.03 ^{abc}	31.34 ± 0.03 ^a	31.89 ± 0.02 ^{ab}	32.44 ± 0.07 ^{abc}	33.04 ± 0.33 ^{bcd}	33.59 ± 0.28 ^{cde}	34.14 ± 0.23 ^{de}	30
Leu	63.29 ± 0.29 ^{ab}	62.49 ± 0.29 ^a	63.64 ± 0.44 ^{abc}	62.71 ± 0.41 ^a	63.30 ± 0.40 ^{ab}	63.90 ± 0.39 ^{abc}	65.00 ± 1.29 ^{bcd}	65.55 ± 1.25 ^{cde}	66.11 ± 1.21 ^{de}	59
Lys	38.34 ± 0.34 ^{ab}	37.54 ± 0.34 ^a	38.69 ± 0.49 ^{abc}	37.76 ± 0.46 ^a	38.35 ± 0.44 ^{ab}	38.94 ± 0.44 ^{abc}	40.29 ± 1.59 ^{abcd}	40.85 ± 1.55 ^{bcd}	41.41 ± 1.51 ^{cd}	45
Met	25.28 ± 0.28 ^{ab}	24.48 ± 0.28 ^a	25.63 ± 0.43 ^{abc}	24.70 ± 0.40 ^a	25.28 ± 0.38 ^{ab}	25.88 ± 0.38 ^{abc}	27.03 ± 1.33 ^{bcd}	27.59 ± 1.29 ^{bcd}	28.15 ± 1.25 ^{de}	22
Phe	42.21 ± 0.21 ^{abc}	41.41 ± 0.21 ^a	42.56 ± 0.36 ^{abcd}	41.63 ± 0.33 ^{ab}	42.21 ± 0.31 ^{abc}	42.81 ± 0.31 ^{abcde}	44.16 ± 1.46 ^{cdefg}	44.72 ± 1.42 ^{deg}	45.28 ± 1.38 ^{efg}	38
Thr	41.43 ± 0.43 ^{abc}	40.63 ± 0.43 ^a	41.78 ± 0.58 ^{abc}	40.85 ± 0.55 ^{ab}	41.43 ± 0.53 ^{abc}	42.03 ± 0.53 ^{abcd}	43.38 ± 1.68 ^{abcde}	43.94 ± 1.64 ^{bcd}	44.49 ± 1.59 ^{efg}	23
Val	43.36 ± 0.36 ^{ab}	42.56 ± 0.36 ^a	43.71 ± 0.51 ^{abc}	42.78 ± 0.48 ^a	43.36 ± 0.46 ^{ab}	43.96 ± 0.46 ^{abc}	45.31 ± 1.61 ^{abcde}	45.87 ± 1.57 ^{bcd}	46.43 ± 1.53 ^{cde}	39
Sum of EAAs	320.48 ± 1.48 ^{ab}	314.08 ± 1.48 ^a	322.98 ± 2.38 ^{ab}	315.60 ± 2.20 ^a	320.20 ± 2.00 ^{ab}	324.90 ± 1.90 ^{abc}	334.20 ± 9.64 ^{bcd}	338.71 ± 9.31 ^{cdef}	343.12 ± 8.92 ^{def}	277
Non-essential										
Ala	47.35 ± 0.35 ^{ab}	46.55 ± 0.35 ^a	47.70 ± 0.50 ^{abcd}	46.77 ± 0.47 ^a	47.35 ± 0.45 ^{ab}	47.95 ± 0.45 ^{abcd}	49.30 ± 1.60 ^{abcde}	49.86 ± 1.60 ^{bcd}	50.41 ± 1.51 ^{de}	
Arg	45.24 ± 0.24 ^{ab}	44.44 ± 0.24 ^a	45.59 ± 0.39 ^{abcd}	44.66 ± 0.36 ^a	45.24 ± 0.34 ^{ab}	45.84 ± 0.34 ^{abcd}	47.19 ± 1.49 ^{abcde}	47.75 ± 1.45 ^{bcd}	48.31 ± 1.41 ^{de}	
Asp	75.83 ± 0.17 ^{ab}	75.03 ± 0.17 ^a	76.18 ± 0.02 ^{abc}	75.25 ± 0.05 ^a	75.83 ± 0.07 ^{ab}	76.43 ± 0.07 ^{abcd}	77.78 ± 1.08 ^{defg}	78.34 ± 1.04 ^{efg}	78.90 ± 1.00 ^{fg}	
Glu	101.11 ± 0.11 ^{ab}	100.31 ± 0.11 ^a	101.46 ± 0.26 ^{abcd}	100.53 ± 0.23 ^a	101.11 ± 0.22 ^{ab}	101.71 ± 0.21 ^{abcd}	103.06 ± 1.36 ^{abcde}	103.62 ± 1.32 ^{bcd}	104.18 ± 1.28 ^{de}	
Gly	51.15 ± 0.15 ^{ab}	50.35 ± 0.15 ^a	51.50 ± 0.30 ^{abcd}	50.57 ± 0.27 ^a	51.15 ± 0.25 ^{ab}	51.75 ± 0.25 ^{abcd}	53.10 ± 1.40 ^{abcde}	53.66 ± 1.36 ^{efgh}	54.22 ± 1.32 ^{de}	
Pro	33.92 ± 0.08 ^{ab}	33.12 ± 0.08 ^a	34.27 ± 0.07 ^{abcd}	33.34 ± 0.04 ^a	33.92 ± 0.02 ^{ab}	34.52 ± 0.02 ^{abcd}	35.87 ± 1.17 ^{defg}	36.43 ± 1.13 ^{efg}	36.99 ± 1.09 ^{fgh}	
Ser	36.66 ± 0.35 ^{abc}	35.86 ± 0.35 ^a	37.01 ± 0.20 ^{abc}	36.08 ± 0.22 ^{ab}	36.66 ± 0.24 ^{abc}	37.26 ± 0.24 ^{bcd}	38.61 ± 0.91 ^{def}	39.17 ± 0.87 ^{efg}	39.72 ± 0.82 ^{fg}	
Tyr	69.69 ± 0.31 ^{abc}	68.89 ± 0.31 ^a	70.04 ± 0.16 ^{abc}	69.11 ± 0.19 ^{ab}	69.69 ± 0.21 ^{abc}	70.29 ± 0.21 ^{abcd}	71.64 ± 0.94 ^{def}	72.20 ± 0.90 ^{abcd}	72.76 ± 0.86 ^{fg}	
Sum of total AA's	736.18 ± 1.18 ^{abc}	724.18 ± 1.18 ^a	741.13 ± 3.13 ^{abc}	727.25 ± 2.75 ^{ab}	735.93 ± 2.43 ^{abc}	744.83 ± 2.33 ^{abcd}	763.63 ± 18.13 ^{cdef}	772.01 ± 17.51 ^{defg}	780.28 ± 16.78 ^{efg}	
EAAI	4.64	4.52	4.69	4.55	4.64	4.70	4.92	5.00	5.09	

Table 6.3 continued

Essential Aas	P0T0	P250T10	P250T20	P250T30	P250T40	P300T10	P300T20	P300T30	P300T40	FAO/WHO /UNU
His	34.54 ± 0.47 ^{abc}	37.89 ± 1.02 ^g	37.69 ± 0.62 ^{fg}	37.14 ± 0.57 ^{efg}	36.59 ± 0.52 ^{defg}	36.04 ± 0.47 ^{cdef}	35.49 ± 0.41 ^{bcd}	34.94 ± 0.36 ^{abcd}	34.39 ± 0.31 ^{abc}	15
Ile	32.04 ± 0.04 ^{ab}	34.84 ± 1.06 ^e	34.64 ± 0.66 ^e	34.09 ± 0.61 ^{de}	33.54 ± 0.56 ^{cde}	32.99 ± 0.51 ^{bcd}	32.44 ± 0.46 ^{abc}	31.89 ± 0.41 ^{ab}	31.34 ± 0.36 ^a	30
Leu	63.29 ± 0.29 ^{ab}	66.82 ± 0.08 ^e	66.65 ± 0.35 ^e	66.07 ± 0.37 ^{de}	65.50 ± 0.40 ^{cde}	64.90 ± 0.40 ^{bcd}	64.34 ± 0.44 ^{abcd}	63.76 ± 0.46 ^{abc}	63.19 ± 0.49 ^{ab}	59
Lys	38.34 ± 0.34 ^{ab}	42.12 ± 0.22 ^d	41.95 ± 0.65 ^d	41.37 ± 0.67 ^{cd}	40.80 ± 0.70 ^{bcd}	40.20 ± 0.70 ^{abcd}	39.64 ± 0.74 ^{abcd}	39.06 ± 0.76 ^{abcd}	38.49 ± 0.79 ^{ab}	45
Met	25.28 ± 0.28 ^{ab}	28.86 ± 0.04 ^e	28.69 ± 0.39 ^e	28.11 ± 0.41 ^{de}	27.54 ± 0.44 ^{cde}	26.94 ± 0.44 ^{bcd}	26.38 ± 0.46 ^{abcd}	25.80 ± 0.50 ^{abc}	25.23 ± 0.53 ^{ab}	22
Phe	42.21 ± 0.21 ^{abc}	45.99 ± 0.09 ^g	45.82 ± 0.52 ^{fg}	45.24 ± 0.54 ^{efg}	44.67 ± 0.57 ^{cdefg}	44.07 ± 0.57 ^{bcd}	43.51 ± 0.61 ^{abc}	42.93 ± 0.63 ^{abc}	42.36 ± 0.66 ^{abcd}	38
Thr	41.43 ± 0.43 ^{abc}	45.21 ± 0.31 ^e	45.03 ± 0.73 ^{de}	44.45 ± 0.75 ^{cde}	43.88 ± 0.78 ^{bcd}	43.29 ± 0.79 ^{abc}	42.72 ± 0.82 ^{abc}	42.15 ± 0.85 ^{abc}	41.58 ± 0.88 ^{abc}	23
Val	43.36 ± 0.36 ^{ab}	47.14 ± 0.24 ^e	46.97 ± 0.67 ^{de}	46.39 ± 0.67 ^{cde}	45.82 ± 0.72 ^{bcd}	45.22 ± 0.72 ^{abc}	44.66 ± 0.76 ^{abc}	44.08 ± 0.78 ^{abcd}	43.51 ± 0.81 ^{abc}	39
Sum of EAA	320.48 ± 1.48 ^{ab}	348.88 ± 1.32 ^f	347.41 ± 2.01 ^{ef}	342.85 ± 2.25 ^{def}	338.31 ± 2.51 ^{cdef}	333.65 ± 2.65 ^{bcd}	329.15 ± 2.95 ^{abcd}	329.15 ± 3.22 ^{abc}	329.15 ± 3.48 ^{ab}	277
Non-essential AA										
Ala	47.35 ± 0.35 ^{ab}	51.13 ± 0.23 ^e	50.95 ± 0.65 ^e	50.37 ± 0.67 ^{cde}	49.80 ± 0.70 ^{bcd}	49.21 ± 0.71 ^{abc}	48.64 ± 0.74 ^{abc}	48.07 ± 0.77 ^{abcd}	47.50 ± 0.89 ^{abc}	
Arg	45.24 ± 0.24 ^{ab}	49.02 ± 0.12 ^e	48.85 ± 0.55 ^e	48.27 ± 0.57 ^{cde}	47.70 ± 0.60 ^{bcd}	47.10 ± 0.60 ^{abc}	46.54 ± 0.64 ^{abc}	45.96 ± 0.66 ^{abcd}	45.39 ± 0.69 ^{abc}	
Asp	75.83 ± 0.17 ^{ab}	79.61 ± 0.29 ^g	79.44 ± 0.14 ^g	78.86 ± 0.16 ^{fg}	78.29 ± 0.19 ^{efg}	77.69 ± 0.19 ^{cdef}	77.13 ± 0.23 ^{bcd}	76.55 ± 0.25 ^{bac}	75.98 ± 0.28 ^{ab}	
Glu	101.11 ± 0.11 ^{ab}	104.89 ± 0.01 ^e	104.72 ± 0.42 ^e	104.14 ± 0.44 ^{cde}	103.57 ± 0.47 ^{bcd}	102.97 ± 0.47 ^{abcd}	102.41 ± 0.51 ^{abc}	101.83 ± 0.53 ^{abcd}	101.26 ± 0.56 ^{abc}	
Gly	51.15 ± 0.15 ^{ab}	54.93 ± 0.03 ^e	54.76 ± 0.46 ^e	54.19 ± 0.48 ^{cde}	53.61 ± 0.51 ^{bcd}	53.01 ± 0.51 ^{abc}	52.45 ± 0.55 ^{abc}	51.87 ± 0.57 ^{abcd}	51.30 ± 0.60 ^{abc}	
Pro	33.92 ± 0.08 ^{ab}	37.70 ± 0.20 ^{gh}	37.53 ± 0.23 ^{gh}	36.95 ± 0.25 ^{fgh}	36.38 ± 0.28 ^{efgh}	35.78 ± 0.28 ^{cdef}	35.22 ± 0.32 ^{bcd}	34.64 ± 0.34 ^{abcd}	34.07 ± 0.37 ^{abc}	
Ser	36.66 ± 0.35 ^{abc}	40.44 ± 0.46 ^g	40.26 ± 0.04 ^g	39.68 ± 0.02 ^{fg}	39.11 ± 0.01 ^{efg}	38.52 ± 0.02 ^{def}	37.95 ± 0.05 ^{cdefg}	37.38 ± 0.08 ^{bcd}	36.81 ± 0.11 ^{abc}	
Tyr	69.69 ± 0.31 ^{abc}	73.47 ± 0.43 ^g	73.30 ± 0.00 ^g	72.72 ± 0.02 ^{fg}	72.15 ± 0.05 ^{efg}	71.55 ± 0.05 ^{def}	70.99 ± 0.09 ^{cdef}	70.41 ± 0.11 ^{bcd}	69.84 ± 0.14 ^{abc}	
Sum of total Aas	736.18 ± 1.18 ^{abc}	791.06 ± 2.44 ^g	788.35 ± 3.85 ^{fg}	779.75 ± 4.25 ^{efg}	771.20 ± 4.71 ^{defg}	762.39 ± 4.89 ^{cdef}	753.92 ± 5.42 ^{bcd}	745.38 ± 5.88 ^{abcd}	736.84 ± 6.38 ^{abc}	
EAAI	4.64	5.20	5.17	5.08	5.00	4.91	4.82	4.73	4.65	

However, samples P150T10, P150T20, and P150T40 showed no significant differences in EAA content compared to P0T0 ($p > 0.05$), indicating that these ultrasound conditions maintained essential amino acid levels. The amino acid composition of BSF larvae proteins revealed high levels of aspartic acid and glutamic acid for P200 and P250 values, suggesting acidic characteristics (Zhao et al., 2021). The P250T10 group had the highest levels of both essential and non-essential amino acids compared to the control, although this value was not significantly different from the other P250 samples, indicating modifications in amino acid content due to ultrasound treatment characteristics (Zhao et al., 2021). Cross all groups, the essential amino acid index (EAAI) values ranged from 4.52 to 5.20. These minor variations suggested that the overall protein quality was maintained across treatments. These findings indicate that the quality of the BSF larvae protein was not adversely affected by the ultrasound treatment. Ma et al. (2022) reported similar findings, who studied the impact of ultrasound treatment on the protein quality of housefly larvae (*Musca domestica*), to demonstrate that ultrasound-induced structural modifications maintained essential amino acid content. In addition, Malik et al. (2017) reported that dephenolized sunflower meal highlights the potential of ultrasound to preserve protein quality, and together, these findings value the usefulness of ultrasound-assisted extraction as a means of maintaining protein quality across different protein sources, including insects.

Similar studies on *Tenebrio molitor* larvae protein (Zhang et al., 2022) found that ultrasound did not negatively affect amino acid content and composition. Factors such as temperature, exposure time, and power can alter amino acid levels in BSF larvae proteins. A noticeable trend in methionine content for higher-power-treated samples (P250 and P300) shows a slight decrease as processing time increases. Although most time intervals showed no statistically significant changes, P250 exhibited a significant difference, as indicated by its distinct superscript letters compared to other samples. The observed reduction might be partially attributed to oxidation during extraction, consistent with findings from Chambal et al. (2012) and Le et al. (2009). Among the essential amino acids, leucine, followed by histidine, valine, phenylalanine, and threonine, were the most abundant (**Table 6.3**). For non-essential amino acids, glutamic acid followed by aspartic acid were the primary contributors, then serine and alanine (**Table 6.3**). These isolates contained many hydrophobic residues, with amino acids such as leucine, isoleucine, valine, phenylalanine, and alanine being prominent, which contribute to the savory flavor of the proteins (Mintah et al., 2019).

For P250-treated samples, histidine, aspartic acid, proline, leucine, isoleucine, tyrosine, and serine levels increased compared to the control. However, only histidine, leucine, isoleucine, and tyrosine showed statistically significant increases (>5% difference) compared to the control (**Table 6.3**). Proline, detected in all samples, holds significant metabolic and physiological functions such as antioxidant role and protein structure, and stability (Li & Wu, 2018). In grasshoppers, the application of ultrasound enhances the sensory experience, such as rancid smell and taste, while increasing the concentration of specific amino acids in meat products (Cruz-López et al., 2022). Similarly, Das et al. (2020) found increased amino acids in bottled gourd juice after ultrasound treatment compared to pasteurization. The increased levels can be attributed to more molecular regions being exposed during ultrasound treatment.

For this study, the total amount of amino acids, the essential building blocks of protein molecules, increased significantly with higher ultrasound power ($p < 0.05$), rising from 736.18 ± 1.18 mg/100g in the control (P0T0) to 785.35 ± 3.85 mg/100g in P250T40 and 762.39 ± 4.89 mg/100g in P300T10. This suggests that ultrasound's cavitation effect and mechanical force expose previously buried amino acids by decomposing protein aggregates or unfolding proteins. Ultrasound treatment increased the levels of hydrophobic amino acids compared to the P0T0 sample, suggesting that ultrasound can induce conformational changes in proteins. Emulsifying, gelling, and foaming, these functional properties of proteins were heavily influenced by their structural domains, and ionic regions and ionized amino acids contribute to the stability of emulsions. In contrast, methionine can promote gel formation through protein denaturation due to its sulfur-containing side chain. Studies by Jafari et al. (2016), Makri & Doxastakis (2006), and Yang et al. (2018) demonstrated that proteins from ultrasound-treated samples are expected to display enhanced gelling and emulsifying properties. In summary, the ultrasound is a feasible, quick, and simple auxiliary method to extract high-quality BSF larvae proteins with potential to find multiple techno-functional applications in the food industries.

6.4.3 Free sulfhydryl group (SH) and disulfide bond (SS) content

The disulfide bond, a covalent bond formed between the sulfhydryl groups of two cysteine residues through oxidation, is crucial for stabilizing protein conformation (O'Sullivan et al., 2016a). Compared to other forces maintaining protein structure, such as electrostatic interactions and hydrogen bonding, disulfide bonds have relatively high bond energies and short action distances (Zhang et al., 2014a). In our study, we investigated the effect of ultrasound-assisted extraction of

BSF larvae protein as a function of power (150 W, 200 W, 250 W, and 300 W) and time (10, 20, 30, and 40 min). As shown in **Figure 6.2**, there were significant differences ($p < 0.05$) in the content of exposed sulfhydryl (SH) and disulfide (SS) bonds among the treated samples. However, at 250 and 300 W (**Figure 6.2C** and **Figure 6.2D**), the SH content significantly decreased ($p < 0.05$), as indicated by the subscript letters. A similar trend was observed at 150 and 200 W, though to a lesser extent. The energy generated by ultrasonic cavitation can homolytically dissociate water molecules into hydrogen atoms and highly reactive hydroxyl radicals, which expose SH groups in protein molecules, subsequently oxidizing them into more stable disulfide bonds (Li et al., 2013). This decrease in exposed SH group content results from the expansion of the protein molecular space structure caused by ultrasound and the oxidation by hydroxyl radicals. Exposed SH groups are critical active groups in protein molecules; their interaction with disulfide bonds can enhance protein interactions and facilitate the irreversible adsorption of proteins at the oil-water interface, resulting in a highly viscoelastic membrane that resists protein aggregation. Consequently, exposed SH content significantly impacts the functional characteristics of proteins. Disintegration of disulfide bonds via sonication can also improve protein digestibility. For instance, Figueroa-González et al. (2022) found that amaranth protein isolates treated with a combination of pH shift and ultrasound had lower SH contents and higher SS bonds compared to those obtained using a control method. This difference is attributed to the oxidation of SH groups, the formation of intra- or intermolecular disulfide bridges, SH/SS interchange reactions, and the formation of mercaptide ion species under alkaline conditions (Figueroa-González et al., 2022).

In **Figure 6.2**, the disulfide (SS) content of BSF larvae proteins was notably higher in the ultrasound-treated group compared to the control sample, likely due to the exposure of buried groups during protein unfolding (Wang et al., 2015). At all tested time and power points, including P300T10, P300T20, and P300T30, the SS bond content of ultrasound-treated samples was consistently higher than that of the control samples. However, the SS content showed a declining trend from P300T10 to P300T40 ($p < 0.05$), despite remaining above the control levels. This difference could be explained by the production of hydrogen peroxide after prolonged ultrasound exposure, which oxidizes some sulfhydryl bonds and reduces sulfhydryl content (Gülseren et al., 2007).

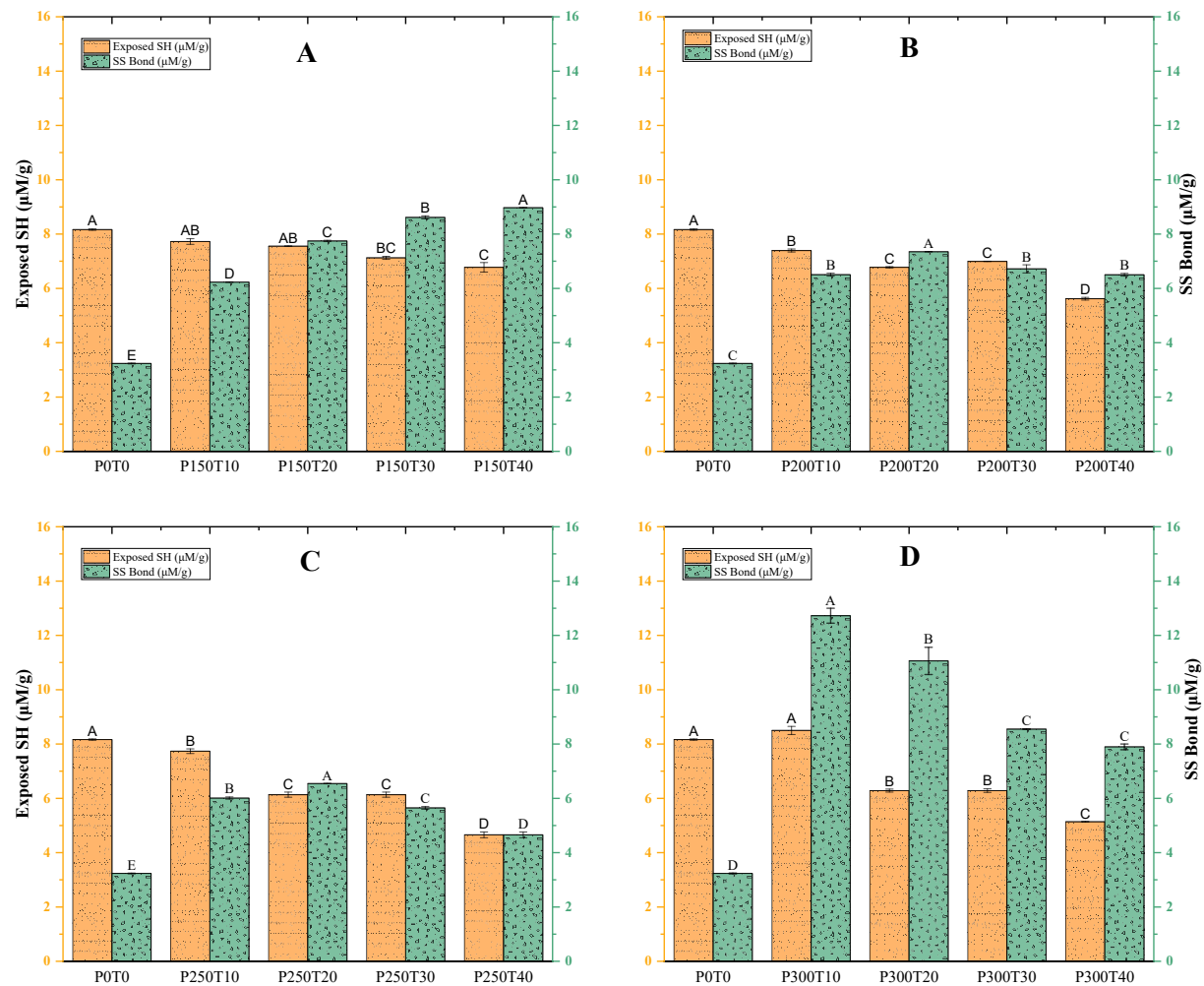


Figure 6.2. Free sulphydryl contents (Exposed SH (uM/g), disulfide bond (SS Bond (uM/g) contents of protein isolates extracted from black soldier fly larvae with different treatments (A – P150, B – P200, C – P250, and D – P300). Values are mean $\pm$ standard deviation; means with different superscript are significantly different ($p < 0.05$).

Increased exposure to air during the ultrasound process can further exacerbate this oxidation. Oxygen, being a reactive species, accelerates the oxidative modification of proteins, particularly the thiol groups (-SH) in cysteine residues. Prolonged exposure to air can increase the formation of reactive oxygen species (ROS), such as hydrogen peroxide, thereby enhancing the extent of protein oxidation. The SS bond content in BSF larvae showed an initial increase followed by a decrease as ultrasound treatment time increased at P200, P250, and P300, although the values consistently remained higher than those of the control sample. This may be due to sonication time exposing internal sulfhydryl groups to the surface, promoting disulfide bond generation in BSF larvae protein. During ultrasonication, active sulfhydryl groups in proteins tend to form disulfide bonds (Zhang et al., 2017). In agreement with our study, Zhang et al. (2023) found that the sulfhydryl group proportion decreases after a long-term ultrasound treatment (40–50 min). Alavi et al. (2021) hypothesized that the decrease in the sulfhydryl group content resulted from the oxidation of susceptible sulfhydryl groups by cavitation-generated hydrogen peroxide.

A reduction in SH group concentration has been observed in defatted wheat germ protein after high ultrasound treatment times (70 min or more) (Yang et al., 2017) and in walnut proteins treated with high ultrasound power (≥ 600 W for 15 min) (Zhu et al., 2018a). In contrast, an increase in SH group concentration has been reported in proteins treated with ultrasound, compared to untreated or control samples, from multiple alternative protein sources, including soy proteins (Hu, et al., 2013), brewer's spent grain (Li et al., 2021), pecan protein (Wang et al., 2021), and rapeseed and napin proteins (Pan et al., 2020). Gülseren et al., (2007b) observed a decrease in free thiol quantity in proteins based on sonication duration, proposing that hydrogen peroxide generated by cavitation might oxidize free SH groups, decreasing free SH content of BSA.

During sonication, protein oxidation causes molecular structure unfolding. Conformational changes from the exposure of embedded sulfhydryl groups and an increase in active sulfhydryl group content are observed. Nonpolar amino acid residues inside the protein are exposed on the surface, increasing surface hydrophobicity (Segat et al., 2014). Higher sulfhydryl content correlates with higher surface hydrophobicity and greater protein denaturation (Jiang et al., 2021). Our speculation whether ultrasound-induced protein treatment increases sulfhydryl content is thwarted by Wang et al. (2020, who state that some exposed sulfhydryl groups are easily oxidized to disulfide bonds, decreasing exposed SH sulfhydryl content. Overall, the study highlights the

potential of ultrasound-assisted extraction in modifying protein structure. The need for precise control over treatment parameters to optimize desirable outcomes is emphasized.

6.4.4 Effect of ultrasound on protein solubility

Protein solubility is a critical factor in assessing the functional properties of proteins. It is closely related to their emulsification and foaming capabilities. Protein denaturation and aggregation, key determinants of protein functionality and potential applications, influence solubility (Pongsawatmanit et al., 2006). As good solubility is a prerequisite for the effective use of novel proteins for food application by the industry, the effect of ultrasound on the solubility of BSF larval proteins is illustrated in **Figure 6.3**.

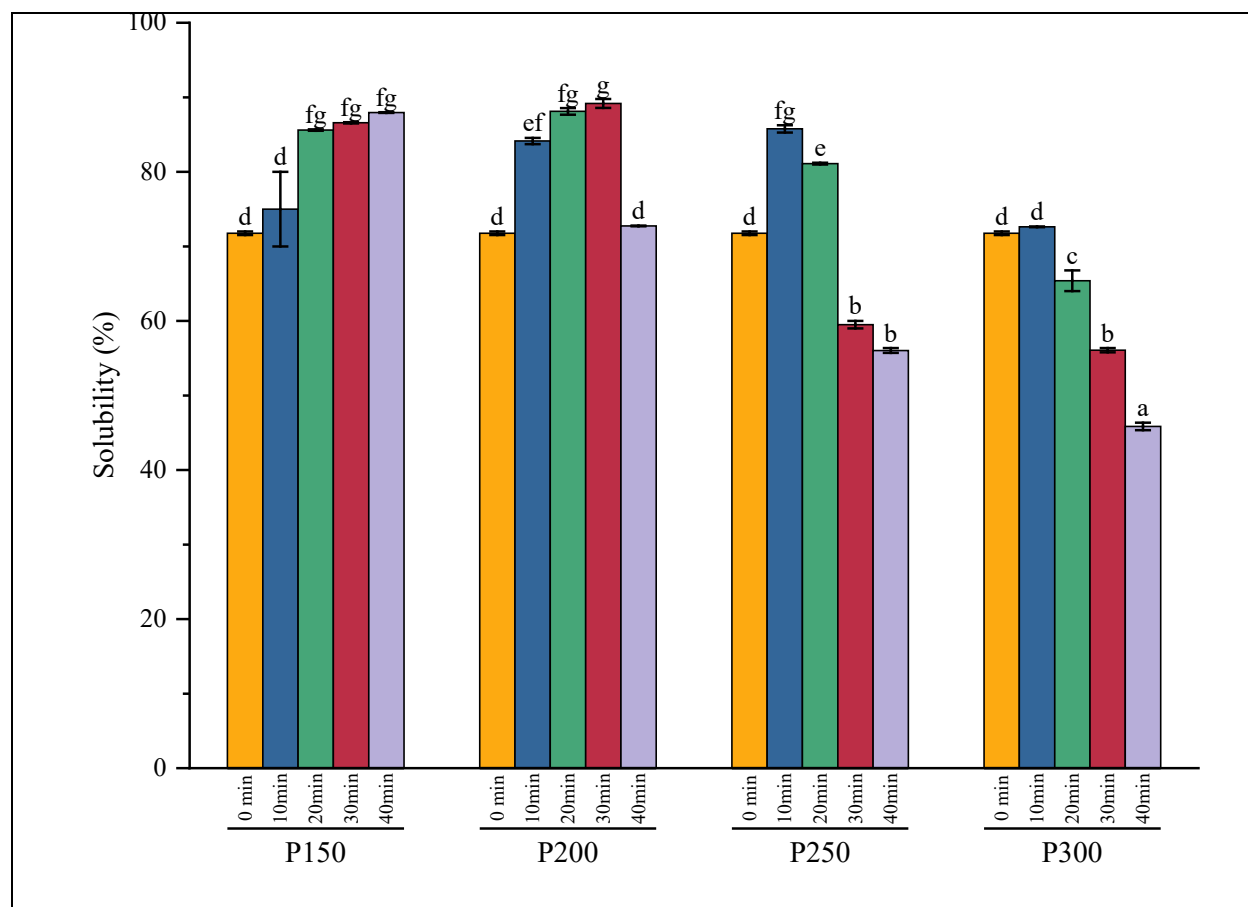


Figure 6.3. Effect of ultrasound on the solubility properties of black soldier fly larvae protein isolates. Values are presented as mean $\pm$ standard deviation, with error bars representing the variability within the data. Means with different superscript letters are significantly different ($p < 0.05$).

Ultrasonic treatment significantly enhanced the solubility of BSF larval proteins under conditions P150 (20, 30, and 40 min), P200 (10, 20, and 30 min), and P250 (10 and 20 min), transforming initially less soluble proteins into more soluble forms. Generally, in the cases referred to above, the solubility increased with longer processing times, reaching a maximum of 89.18% after treatment at 200 W for 30 min. This trend was observed for treatments at P150T40, P200T30, and P250T10, where solubility consistently improved with increased processing times. This increase can be attributed to two main factors: (1) the breakdown of protein aggregates, ultrasonic cavitation effects break down larger protein aggregates into smaller particles. This reduction in size increases the interaction between protein and water, enhancing water solubility (Zhu et al., 2018); (2) unfolding of protein molecules: Ultrasonic treatment partially unfolds protein molecules, exposing hydrophilic groups to water, which enhances protein-water interactions and increases solubility (Gharibzahedi & Smith, 2020; Mir et al., 2019; Tang et al., 2009; Wen et al., 2019).

Initially, as the ultrasound intensity increased (moving from 150 W to 200 W and 250 W), protein solubility also increased. However, beyond a certain threshold, further increases in ultrasound power of 300 W and extended treatment times led to a significant decrease in solubility ($p < 0.05$, **Figure 6.3**). This is because ultrasound-generated cavitation and shear forces depolymerize protein aggregates, due to improved solvation (Yan et al., 2021). However, excessive sonication causes proteins to aggregate, thereby reducing their solubility. This can be confirmed using SDS-PAGE (**Section 6.4.14**), where the appearance of higher-molecular-weight bands indicates protein aggregation, as aggregated proteins tend to migrate more slowly or form multiple bands. Ultrasound treatment may cause protein denaturation and increased hydration owing to high pressure, shear, cavitation, turbulence, surface electrostatic, and thermodynamic effects. This promotes hydration and enhances the solubility of BSF larval proteins. Specifically, the ultrasound-assisted extraction method improved the solubility of the BSF proteins by 16.65% (**Appendix B1**). The solubility of oleosin also showed a similar trend, increasing by 8.5% and 10.6% using ultrasonic and ultrasonic-assisted salt extraction methods, respectively. This study indicates that ultrasound can increase the solubility of BSF larvae proteins under less severe conditions (e.g., lower intensities and shorter treatment times), likely due to the disruption of protein aggregates. The increased solubility may also be influenced by the extraction conditions and the use of salts in the preparation of all protein isolates. Ultrasound-assisted treatment under selected conditions of power (particularly 150–200 W) and treatment times between 20 and 40

min significantly improved the solubility of BSF larval proteins. In contrast, higher power levels or treatment times approaching 40 min showed a less pronounced or inconsistent effect. Therefore, the ultrasound-assisted salt treatment method can positively impact the extraction of BSF larval proteins.

6.4.5 Effect of surface hydrophobicity

Protein surface hydrophobicity is a characteristic that describes the number of hydrophobic groups protruding from the surface of a protein molecule. This feature is frequently used to assess protein conformational changes and is closely associated with functional properties (Chandrapala et al., 2011). As depicted in **Figure 6.4**, hydrophobic interactions play a significant role in influencing protein behaviour in solution, as reflected by the surface hydrophobicity measurements, which in turn affect the stability of native protein structures. Our results indicated that ultrasonic treatment of BSF resulted in increased surface hydrophobicity compared to the untreated group ($p < 0.05$) under the following conditions: P150 for 30 and 40 min, P200 for 20, 30, and 40 min, P250 for 20 min, and P300 for 20 min. In the control samples, most hydrophobic regions were likely concealed within the protein molecules. However, after ultrasonic treatment, these regions were exposed, increasing surface hydrophobicity (Zhang et al., 2017). When the duration of ultrasonic treatment was kept constant, the hydrophobicity of the BSF surface exhibited an upward trend. This was before reaching a peak and then decreasing as the ultrasonic power increased. This shows that shorter treatment times or lower power levels cannot disrupt the compact three-dimensional structure of the protein; therefore, within the protein molecule, most hydrophobic amino acid residues remain. Longer treatment times or higher power levels of ultrasound enhanced the surface hydrophobicity of the proteins.

The cavitation effect and mechanical stress generated by ultrasound can explain the increase followed by the decrease in surface hydrophobicity by ultrasound. Firstly, in surface hydrophobicity, an increase is caused by the exposure of hydrophobic amino acids buried within the protein's interior. However, with prolonged ultrasound treatment, excessive disruption of the protein structure may result in denaturation or aggregation. This causes a decrease in surface hydrophobicity after reaching a peak. This pattern highlights the optimal treatment time where the balance between protein exposure and structural stability is achieved (Jin et al., 2021). Sonication of P300T40 led to a significant reduction ($p < 0.05$) in surface hydrophobicity by 5% compared with the control (**Figure 6.4**). This decrease in surface hydrophobicity in the sonicated BSF protein

could be attributed to a decrease in the number of exposed nonpolar amino acid residues due to changes in the protein structure. A reduction in surface hydrophobicity due to protein aggregation has been previously reported (Tang et al., 2009). Ultrasound-assisted alkali treatment showed similar trends to alkali treatment but improved the solubility of rice proteins.

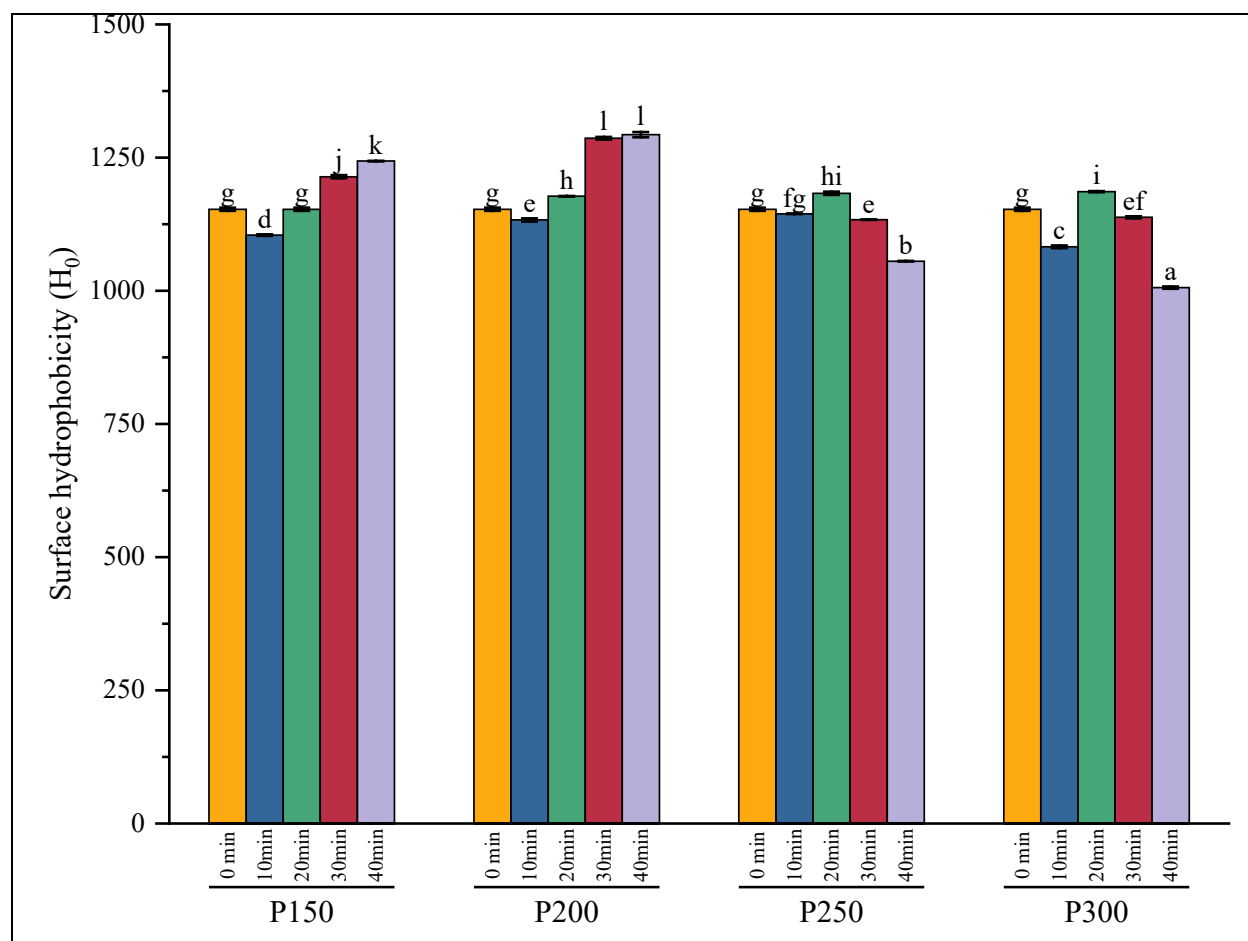


Figure 6.4. Effect of ultrasound on the surface hydrophobicity properties of black soldier fly larvae protein isolates. Values are presented as mean $\pm$ standard deviation, with error bars representing the variability within the data. Means with different superscript letters are significantly different ($p < 0.05$).

This improvement might be attributed to the fact that violent agitation and acoustic cavitation caused by ultrasound can damage the unfolding protein hydrophobicity (Zhang et al., 2017). The chemical and physical changes resulted in a viscous medium through the cyclic generation and collapse of cavities. Therefore, in the vicinity of these cavities, increased pressure is the basis for physical damage, contributing to increased protein solubility. Insoluble proteins are transformed

into soluble proteins, enhancing the impact of alkali treatment (Yildiz et al., 2017). Surface hydrophobicity of various proteins has been shown to be significantly enhanced by ultrasonic treatments, as demonstrated in several studies. Surface hydrophobicity of oleosin was increased when treated at intensities ranging from 200 to 600 W, as reported by Sun et al. (2020). This was likely due to the disruption of its protein structure, which exposed hydrophobic regions previously hidden within the protein. The black soldier fly larvae protein resulted in increased surface hydrophobicity when treated for 30 minutes at 500W, similar results reported by Kumar et al. (2020). Additionally, Zhang (2018) reported similar results for peanut protein isolate, where surface hydrophobicity was increased by ultrasonic treatment, thereby improving the emulsifying properties of the protein.

Between protein molecules, the ultrasound can partially disrupt the hydrophobic interactions, as shown in our study. This could lead to increased exposure of hydrophobic groups on the surface of the protein molecules and whey protein isolates previous studies reported consistent findings, showing that different pH solvents cause the unfolding of major whey protein components, resulting in protein aggregation (Khem et al., 2016). This suggests that ultrasound can affect protein structure and denaturation, leading to changes in protein behaviour (Bacigalupe et al., 2015) and an increase in the ultrasound power, molecular motion accelerates. The increasing protein surface hydrophobicity is due to the transfer of hydrophobic amino acid residues embedded within the molecule to the surface of the protein molecule (Jiang et al., 2010), suggesting that a longer ultrasonic treatment time would lead to reformation of the hydrophobic binding between the protein molecules.

To balance the exposure of hydrophobic groups without causing significant aggregation or oxidation, moderate power levels (e.g., 200 W) with longer treatment times must be applied. Aggregation and reduced hydrophobicity are led by longer treatment times, generally increasing surface hydrophobicity for P150 and P200, but excessive treatment exposure, particularly at high power at P250 and P300.

In this study, the effect of ultrasound treatment on protein surface hydrophobicity is highlighted. To harness the benefits while minimising adverse effects, the need for precise control over power and time is emphasised. By exposing hydrophobic regions of the protein, surface hydrophobicity is significantly enhanced by ultrasound treatment. This can also improve solubility and functional properties. However, excessive treatment at high power or prolonged exposure may

lead to protein denaturation or aggregation, reducing hydrophobicity. Additionally, the final protein structure and hydrophobicity are determined by the solvent and pH conditions used during treatment. The ultrasonic treatment modifications can be attributed to the emulsifying and foaming properties of BSF, as will be discussed in sections 6.4.8 and 6.4.9.

6.4.6 Surface Charge (Zeta Potential)

How well proteins dissolve, emulsify, and foam depends on an important property called ζ -potential, or surface charge (Shevkani et al., 2014). Additionally, the presence of charged amino acids that change how proteins spread out and stick together is what it measures (Jiang et al., 2014; Nazari et al., 2018). The isoelectric point (pI) of BSF larvae protein isolates fluctuated with ultrasonic power: 4.4–5.0 (150W, **Figure 6.5A**), 4.5–6.5 (200W, **Figure 6.5B**), 3.2–7.0 (250W, **Figure 6.5C**), and 2.2–6.5 (300W, **Figure 6.5D**). The ζ -potential for the control ranged from 25.6 ± 1.6 mV at pH 2 to -29.7 ± 1.5 mV at pH 12. Below pH 4.4, there were positive charges, while above pH 4.4, there were negative charges. Ultrasound treatment had a significant impact on ζ -potential at pH values below 6, presumably due to the disruption of protein structure, which facilitated increased charge accumulation on the molecular chain (Zhao et al., 2009). Research indicates that ultrasonication influences ζ -potential by augmenting the quantity of negatively charged amino acids relative to positively charged ones, thereby reducing the absolute ζ -potential. Jiang et al. (2014) found that the ζ -potential went up when the sonication periods were longer, and the power was lower.

A protein solution usually has a positive ζ -potential if it has more positively charged amino acids than negatively charged ones (Bouzid et al., 2008). The surface charge of a particle influences both its stability and its interactions with other particles (Shen et al., 2017). A high ζ -potential indicates strong electric repulsion that prevents aggregation, whereas a low ζ -potential leads to higher attraction, causing coagulation. The ultrasound-treated samples' ζ -potential values did not always go over the ± 20 mV level that indicates strong colloidal stability (Schmitt et al., 2007; Akbari & Wu, 2016). Values generally ranged from about -30 mV to $+20$ mV, depending on the treatment settings and pH. Some samples were stable, while others were below the stability threshold.

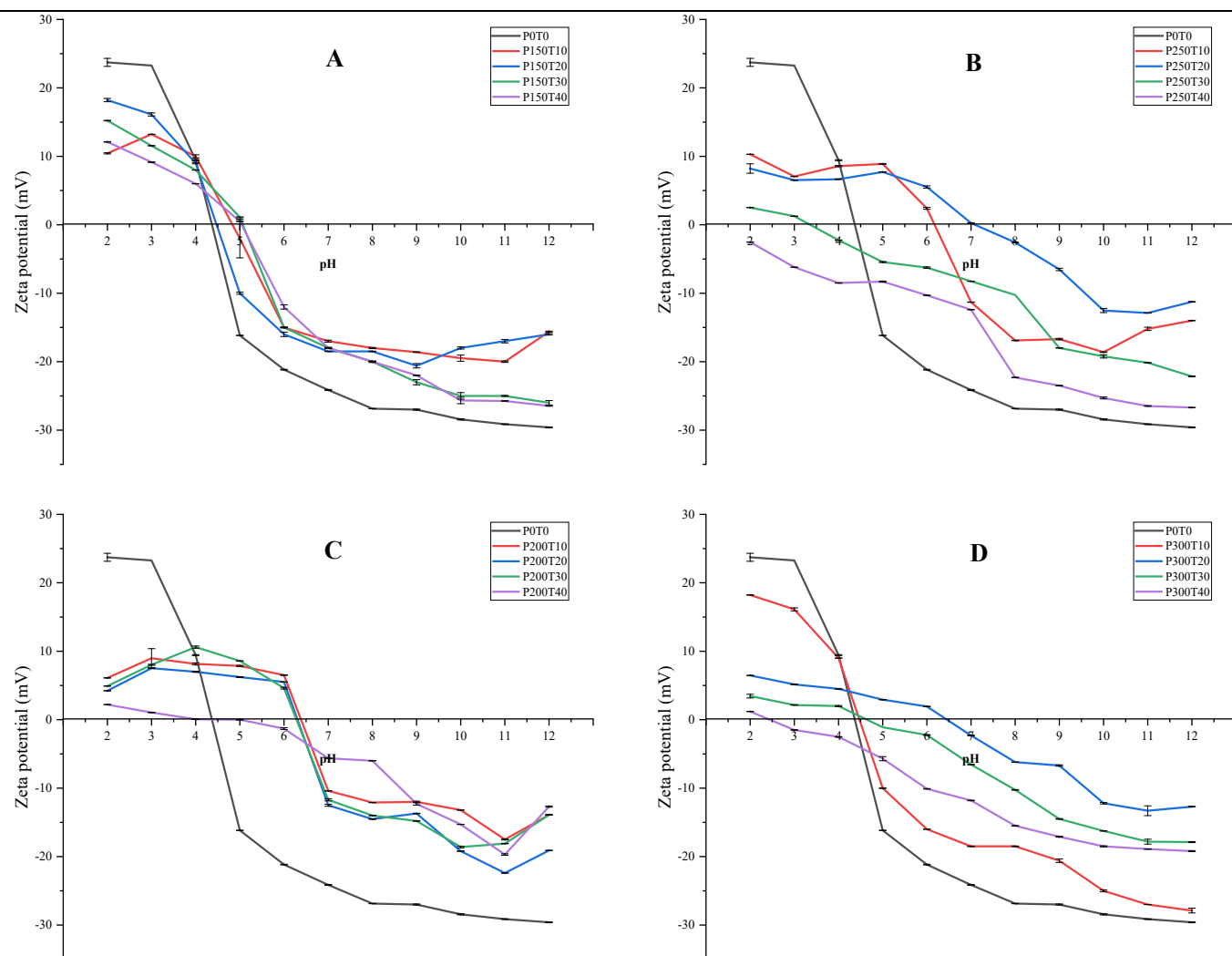


Figure 6.5. Zeta potential of black soldier fly larvae protein isolates (A – P150, B – P250, C – P200, and D – P300) as a function of pH over time. Lines indicate 0 min (black), 10 min (red), 20 min (blue), 30 min (green) and 40 min (purple). Data points are mean $\pm$ standard deviations.

An increase in the absolute ζ -potential was noted under specified conditions, including P250 (10 and 20 minutes at pH 10, 11, and 12) and P200 (30 minutes at pH 10, 11, and 12), as well as P200 at 10, 20, and 40 minutes at pH 10 and 12. Sonication may have caused this rise by exposing more charged groups on the surface of the protein. However, outside of these conditions, particularly at lower pH or with prolonged or excessive treatment, the absolute ζ -potential tended to decrease, possibly due to protein reaggregation, resulting in reduced effective surface charge and potentially lower emulsion stability (see **section 6.4.9**).

Positively charged groups exposed during treatment neutralize the negative charges on the protein surface (Chen et al., 2019). The balance between hydrophilic and hydrophobic groups affects the surface charge; ultrasound exposure alters this balance by exposing more hydrophobic groups, thus reducing the surface charge (Xiong et al., 2016). The control sample showed a negative ζ -potential value at neutral pH, which was lower than that of the BSF larvae, likely due to different salt concentrations and pH adjustments.

Factors such as salt concentration, pH, and temperature significantly affect the ζ -potential of protein (Ryan et al., 2012). The solubility (**Figure 6.3**) of the proteins correlated well with the ζ -potential, with larger net surface charges corresponding to higher solubility. As the ultrasonic power increased, the ζ -potential decreased, likely because of the disruption of the balance between electrostatic repulsion and attraction, causing re-aggregation and reduced emulsion stability. Ultrasonic-treated samples (P250 and P300) exhibited lower absolute ζ -potential values, similar to the control, particularly at higher pH levels, when compared to P150 and P200. On the other hand, our results showed that ultrasonication at low and medium powers decreased the negative surface charge on proteins, enhancing interparticle electrostatic repulsion, disrupting existing aggregates, and improving protein dispersion stability, leading to increased solubility (**Figure 6.3**). However, high-power sonication formed aggregates, decreasing the ζ -potential (**Figure 6.5**). In conclusion, ultrasound treatment significantly altered the ζ -potential of BSF larval proteins, enhancing their solubility and dispersion stability under optimal conditions.

6.4.7 Water binding capacity and oil binding capacity

The importance of proteins in absorbing water and oil (or fat) is crucial in food formulations, as it directly influences the organoleptic characteristics of the final product, such as the juiciness and softness of minced meat products, as well as the desirable textural properties of products such as

bread and cheese (Zhu et al., 2010). Ultrasound treatment may affect the water-binding capacity (WBC) and oil-binding capacity (OBC) properties of extracted proteins.

Following ultrasonic treatment, the WBC and OBC of BSF larval isolates showed varied responses when compared to the control (P0T0). Interestingly, in **Figure 6.6C**, the WBC and OBC of BSF larvae are already high, reaching 4.45 g/g and 2.17 g/g, respectively, significantly higher in comparison to alkaline acid obtained protein fractions of *Grylloides sigillatus* and *Tenebrio molitor* as reported by Zielińska et al. (2018). The maximum WBC (6.40 g/g) was observed in isolate P250T40, while the maximum OBC (6.30 g/g) was observed in isolate P300T10. The increased WBC of BSF larvae may be due to the cavitation effect, which expands protein molecules and increases the contact area and interaction force between proteins and water molecules, enhancing the WBC. Ultrasound cavitation causes protein molecules to unfold and expose internal hydrophobic groups, explaining the gradual increase in OBC with increasing ultrasound power, which is consistent with the surface hydrophobicity results. The surface hydrophobicity of the BSF larvae was in line with ultrasonic power, indicating enhanced hydrophobicity and improved oil-binding ability. Additionally, introducing salt into the extract disrupted the original zeta potential of the protein solution, thereby exposing many hydrophobic groups. Nonpolar side chains of amino acids readily combine with fatty hydrocarbon groups (Withana-Gamage et al., 2011).

As depicted in **Figure 6.6D**, with prolonged ultrasound treatment time, the WBC initially increased and then decreased, reaching a peak at 20 min of treatment at 300 W ($p < 0.05$). WBC of P150 dropped from 4.45 g/g in the control (P0T0) to approximately 1.89 g/g at P150T10 and gradually increased to 2.34 g/g at P150T40, but it still did not surpass the control. A similar trend was observed in isolate P200, where the WBC decreased from 4.45 g/g in the control to 1.84 g/g at P200T10 and increased to 2.84 g/g at P200T40. This phenomenon can be attributed to the ultrasound treatment causing the peptide chain skeleton to become porous and form ionized polar groups, resulting in increased water-binding capacity (Zou & Jiang, 2016). However, if the ultrasound power is prolonged for too long (> 250 W), a strong cavitation effect can cause bubble collapse due to ultrasound treatment, damaging both hydrophilic and hydrophobic groups and leading to decreased WBC (Zupanc et al., 2019). The aliphatic chains in oil bind to the nonpolar side chains of amino acids, leading to interactions between oil and protein. Consequently, the higher the protein hydrophobicity, the better is the OBC (Li et al., 2021).

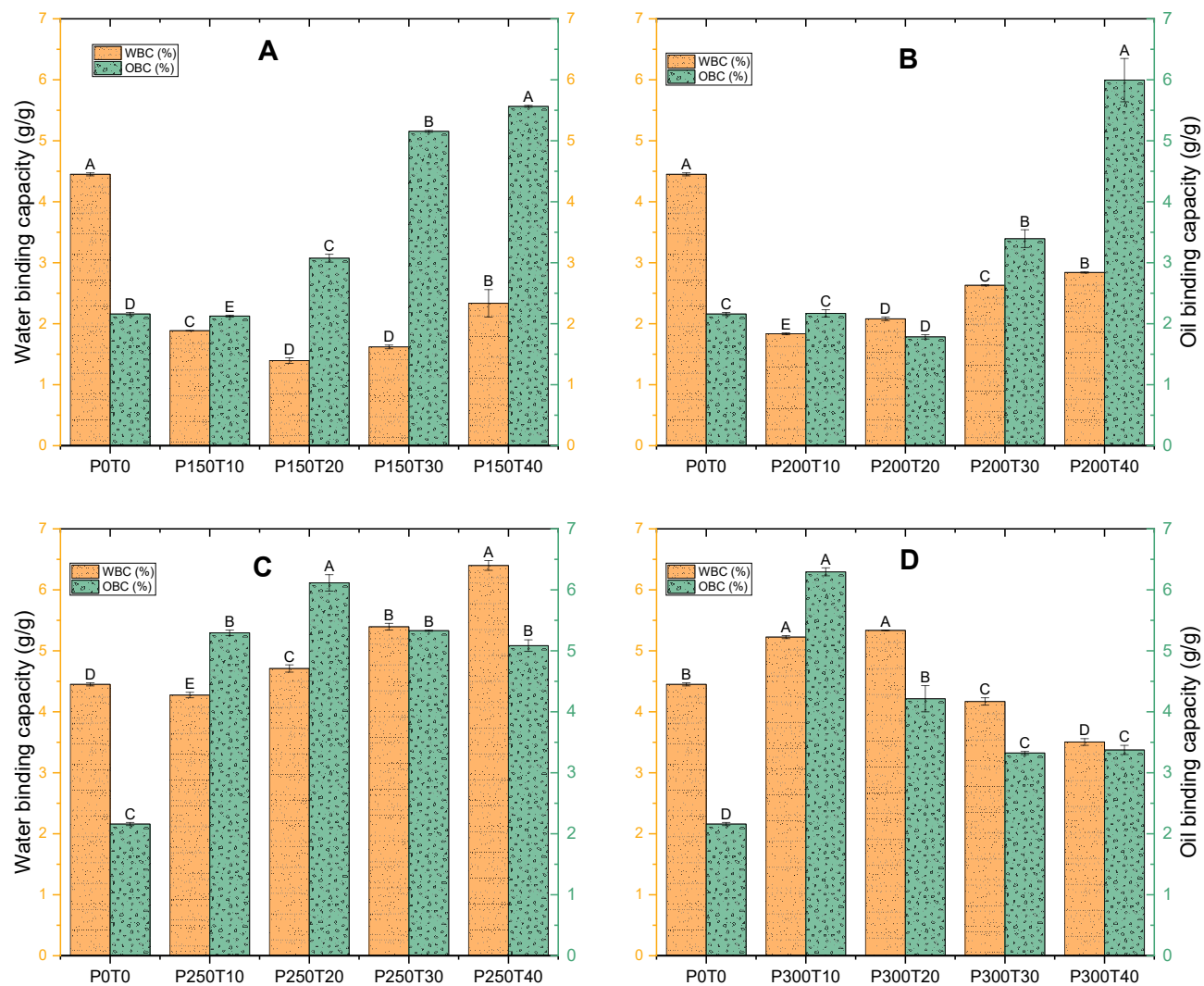


Figure 6.6. Water and oil binding capacity of black soldier fly larvae isolates (A – P150, B – P200, C – P250, and D – P300). Values are mean $\pm$ standard deviation; means with different superscripts are significantly different ($p < 0.05$).

The OBC of BSF larvae extracted without ultrasonic treatment (P0T0) was lower than that of larvae extracted with ultrasonic treatment. The enhanced OBC in ultrasonic-treated samples can be attributed to the structural modifications of the protein induced by ultrasonic treatment. Ultrasonication disrupts protein aggregates and unfolds protein structures, exposing more hydrophobic regions and functional groups, which can interact more effectively with oil molecules (Jambrak et al., 2014). For P150 (Figure 6.6A), the OBC steadily increased with ultrasonic treatment, starting from the control value of 2.16 g/g and reaching 3.08 g/g at 10 min (P150T10), ultimately peaking at 5.57 g/g after 40 min (P150T40). Similarly, for P200 (Figure 6.6B), OBC increased consistently with treatment time, rising from 2.16 g/g in the control to 6.00 g/g at P200T40. For P250 (Figure 6.6C), OBC initially increased, exceeding the control and reaching a maximum of 6.12 g/g at 20 min (P250T20).

However, further treatment reduced the OBC to 5.33 g/g at 30 min (P250T30) and 5.02 g/g at 40 min (P250T40), though these values remained higher than the control. These results suggest that the intensity and duration of ultrasonic treatment have a significant effect on OBC. Shorter treatment times or moderate intensity, such as P250T40, appear to maximize OBC, likely due to optimal disruption of protein structures and improved interaction between oil molecules and hydrophobic sites. However, prolonged treatment or higher intensities may lead to structural over-modification, reducing the effectiveness of oil binding. So, BSF larvae protein can be utilised to make a variety of foods, such as yoghurt, mayonnaise, and ice cream, by mixing them together. Recent research by Paglarini et al. (2019) showed that ultrasound can significantly increase the OBC of BSF larvae because of the partial unfolding and denaturation of proteins. This exposes many hydrophobic groups, facilitating the formation of an appropriate network to entrap oil droplets.

Ultrasound exposure also affected the WBC of *Tenebrio molitor* isolates, and Huang et al. (2023) said that the *Tenebrio molitor* protein had a great WBC of 6.89 g/g, which was achieved through alkaline extraction followed by acid precipitation. This value rose to 10.98 g/g when an ultrasonic power of 400W was applied, and Amiri et al. (2018) found that the WBC of myofibrillar proteins went up a lot when the ultrasonic power and time went up. After sonication, the protein surface area gets bigger, this could lead to an increase in white blood cells since the size of the protein particles gets much smaller. Li et al. (2018) documented a decrease in WBC for isolated

quinoa protein resulting from ultrasound therapy, exhibiting a progressive reduction in WBC as ultrasound power escalated (200, 400, and 600 W).

Flores-Jiménez et al. (2019) employed ultrasonic waves to extract canola protein, resulting in a notable increase in OBC relative to the control sample that lacked ultrasound treatment. The control, 15-minute, and 30-minute ultrasound-treated samples had OBC values of 2.84, 3.06, and 3.26 g/g, respectively. Li et al. (2018) also found that ultrasound-assisted extraction made isolated quinoa protein's OBC better, which suggests that higher protein hydrophobicity leads to higher OBC. They noticed that as the ultrasound strength was raised, the oil absorption went up as well. Zhang et al. (2023) documented a decrease in the OBC of *Tenebrio molitor* when subjected to high extraction times above 50 minutes, indicating that this reduction may result from the physical interactions between protein molecules and fat droplets.

These data demonstrate that while ultrasonic therapy induced a considerable shift in WBC and OBC, the functional qualities of isolates P150 and P200 did not improve beyond the values found in the control for WBC. For isolates P250 (**Figure 6.6C**) and P300 (**Figure 6.6D**), however, both WBC and OBC steadily rose with ultrasonic power, exceeding the control levels at elevated ultrasonic powers. In general, these results show that ultrasound technology could be used to improve the function of proteins and create better food products.

6.4.8 Foaming capacity and stability

The creation of foam happens when proteins unfold to make an interfacial layer. This lets air bubbles grow in suspension and keeps them from collapsing (Boye et al., 2010). Foaming capacity (FC) and foam stability (FS) are two common ways to measure how well proteins foam. FC tests how well a protein can make foam in certain parameters, like pH, temperature, and protein concentration. FS tests how well the protein can keep the foam volume over time (Lv et al., 2019). Data on the foaming properties of BSF larval proteins are shown in **Figure 6.7A-D**. **Figure 6.7A** displays that the FC of P150T10 is comparable to the control (P0T0). However, during the 20–40-minute interval, the FC increases significantly ($p < 0.05$), indicating a notable enhancement within this time. In **Figure 6.7B**, the FC of P200T10 is initially lower than the control, but this trend reverses during the 20–40-minute period, showing an increase in FC relative to the control. For P250 (**Figure 6.7C**), the FC across the 10–40-minute interval exhibits some fluctuations but does not differ significantly ($p > 0.05$) from the control.

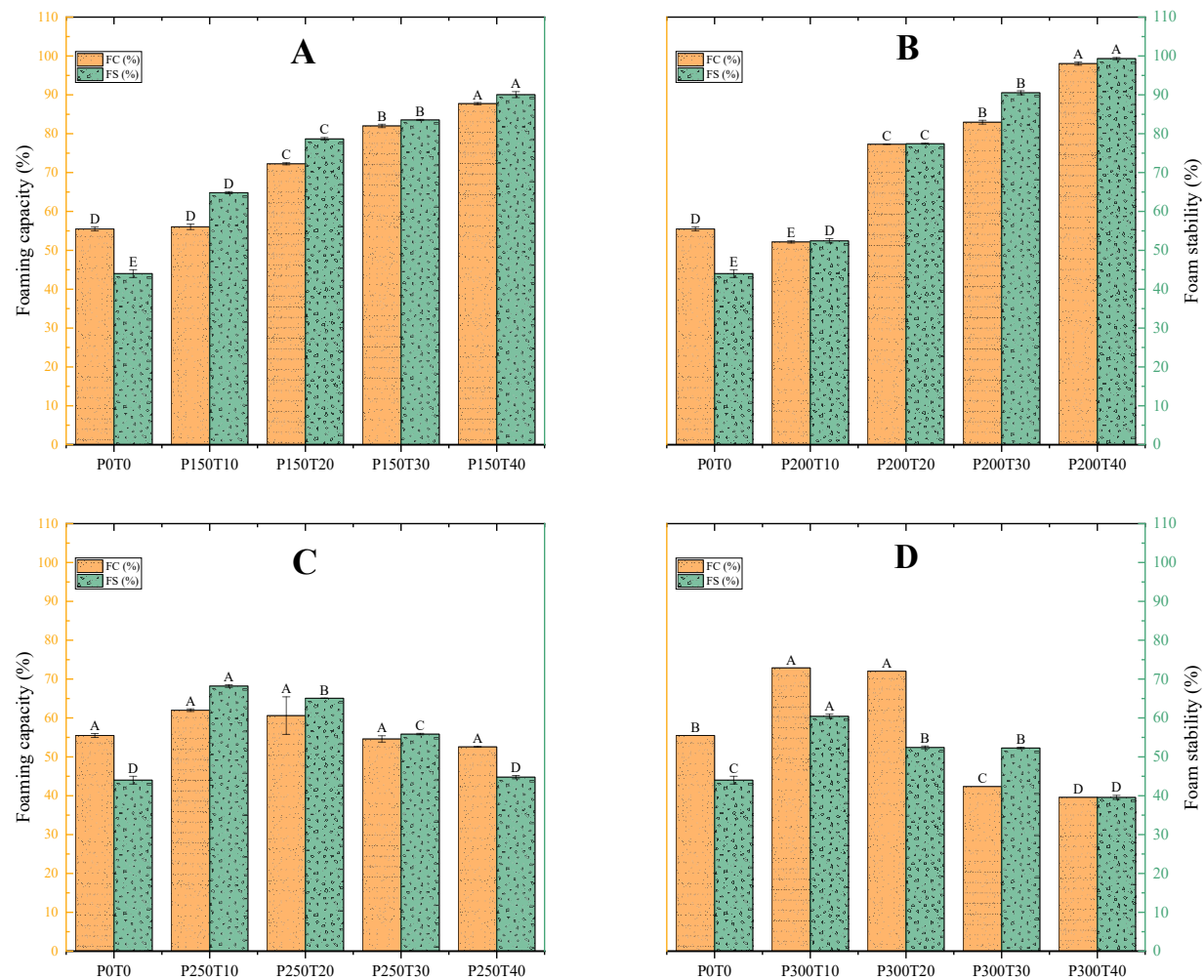


Figure 6.7. Foaming capacity and foam stability of black soldier fly larvae isolates (A – P150, B – P200, C – P250, and D – P300). Values are means $\pm$ standard deviations ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by different letters above the data points.

In **Figure 6.7D**, the FC of P300T10 initially surpasses the control, but this early increase is followed by a reduction during the 20–40-min period, resulting in a mixed trend. As illustrated in **Figure 6.7**, FS for both P150 and P200 demonstrates a consistent increase relative to the control throughout the 10-40 min interval. Conversely, an opposing trend is observed for P250 and P300, where FS initially exceeds the control at 10 minutes but subsequently declines during the 20-40-minute period. Our findings reveal that P200T40 (**Figure 6.7B**) exhibits the highest FC at 98.03% and FS at 99.33%. In contrast, P300T40 (**Figure 6.7D**) records the lowest values for both FC (39.60%) and FS (39.56%). According to Qu et al. (2018), the ultrasound treatment decreases the particle size of BSF larvae proteins and thus enhances their foaming performance. By reducing particle size, solubility improves, allowing more protein to be adsorbed at the air-water interface, thus enhancing foaming ability. The soluble fraction of a protein primarily determines the FC; however, insoluble particles can also improve the surface viscosity, contributing to stable foam formation.

However, the gradual decline in FC and FS at P250 and P300 aligns with the work of Karki et al. (2009), who found that the FC and FS of soy protein isolate (SPI) from ultrasonicated defatted soy flakes significantly decreased with increasing processing time from 30 to 120 seconds. The decline in FC and FS with higher power is likely due to changes in surface hydrophobicity and zeta potential. Ultrasound initially increases surface hydrophobicity by exposing internal hydrophobic groups at a very high power; too many hydrophobic groups are exposed, potentially leading to protein aggregation and decreased ability to stabilize foams. Previous studies indicate that ultrasound can enhance foaming properties for time treatment intervals up to 25 min before the trend reverses due to the formation of larger protein aggregates, which reduce the availability of hydrophobic groups (Kingwascharapong et al., 2021; Zhang et al., 2023; Mintah et al., 2020; Zielińska et al., 2018). Despite this, Mishyna et al. (2019) reported improved foaming properties in sonication of *S. gregaria* and *A. mellifera* flours under conditions of 70% amplitude at 6 min. This improvement was observed after the alkaline protein solubilization, followed by the acid precipitation method. This supports the idea that the functional properties of insect-based proteins vary depending on the type, source, ultrasound parameters, and processing conditions. Jambrak et al. (2008) also found that ultrasound treatment improved the FC and FS of whey protein, with FC increasing and then decreasing after 25 min, consistent with findings from several researchers (Malik et al., 2017; Mir et al., 2019).

Zhang et al. (2011) found that sonicating wheat gluten for 10 min denatured proteins, exposed hydrophobic groups, and increased adsorption at the air-water interface. Extended sonication increases the bubble size and desorbs protein molecules from the gas-liquid interface, which then interact to form aggregates (Li et al., 2017). Exposure of hydrophobic groups during ultrasound treatment enhances the likelihood of protein aggregation, decreasing FS to levels similar to those of untreated BSF larval proteins. The ultrasonic treatment causes the particle size of the protein molecules to become smaller and more flexible, whereas homogenization disperses protein and fat particles evenly, improving protein diffusion, absorption, and initial stability, thus increasing FC (Jambrak et al., 2008). The exposure of hydrophobic regions within the protein enhances air-protein interactions and improves foamability. Although ultrasound can enhance protein functional properties, excessive treatment time and intensity can lead to denaturation and loss of functionality. These findings suggest that ultrasound could be used to improve the industrial applicability of proteins by enhancing their functional properties.

6.4.9 Emulsifying properties

The ability of a protein to absorb oil to stabilize a two-phase system is known as its emulsifying capacity, which is influenced by the ratio of hydrophobic/hydrophilic amino acid groups as well as the protein's structural conformation, such as the ability to unfold and form a film around dispersed oil droplets. Proteins function as emulsifiers and help prevent negative structural changes such as coalescence, creaming, flocculation, and sedimentation. According to Boye et al. (2010), the emulsifying capacity of a protein is evaluated using two indices: emulsifying activity (EA) and emulsion stability (ES). EA measures the amount of oil that can be emulsified per unit of protein, whereas ES assesses the ability of the emulsion to resist structural changes over a specific period of time (Pongsawatmanit et al., 2006; O. A. Higuera-Barraza et al., 2017; Thiansilakul et al., 2007).

Figure 6.8 illustrates the EAI trends for the tested isolates. P150 (**Figure 6.8A**) and P200 (**Figure 6.8B**) exhibit a continuous increase in EAI from 10 to 40 min. A similar trend is initially observed for P250 (**Figure 6.8C**) and P300 (**Figure 6.8D**); however, a decline is evident from 30 to 40 min. Despite this decrease, the EAI values for P250 and P300 remain higher than the control (P0T0). Furthermore, the ESI for P150 and P200 shows a consistent increase between 20 and 40 min. In contrast, P250 and P300 demonstrate an initial increase at 10 min, followed by a decline at 20, 30, and 40 min to levels below the control.

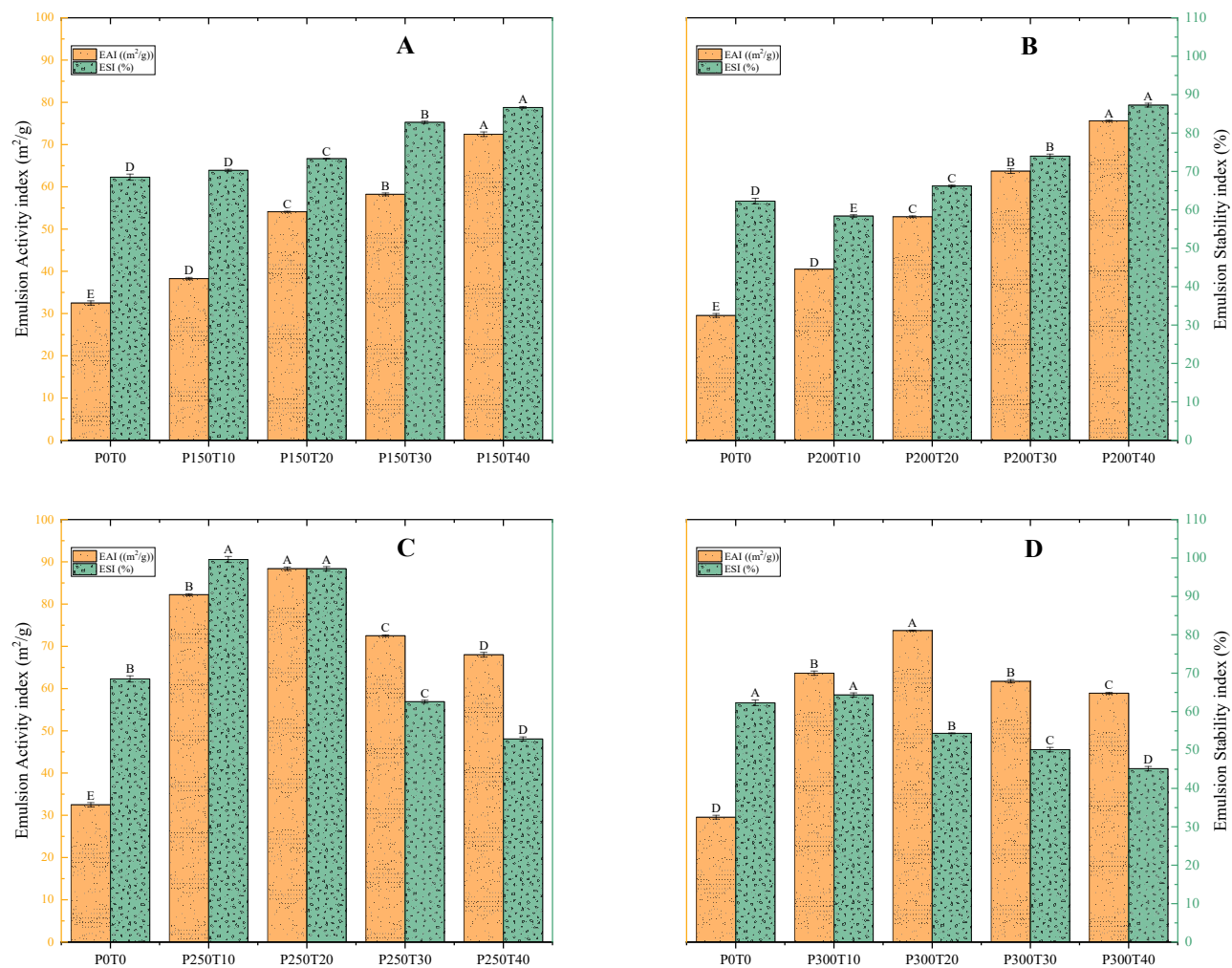


Figure 6.8. Emulsion activity index (EAI) and emulsion stability index (ESI) of black soldier fly larvae isolates (A – P150, B– P200, C – P250, and D – P300). Values are means $\pm$ standard deviations ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by different letters above the data points.

Our results demonstrate that P250T20 (**Figure 6.8C**) exhibits the highest EAI at 88.34%, while P0T0 shows the lowest EAI at 32.50%. Additionally, the highest ESI is observed for P250T10 at 90.55%, whereas P300T40 yields the lowest ESI at 45.15%. The increase in emulsifying activity was attributed to ultrasound treatment, which increased the surface-to-volume ratio by changing the structure of the BSF larval protein, allowing more protein to participate in the formation of the interfacial layer, thus increasing emulsifying efficiency.

Additionally, the increase in surface hydrophobicity and molecular flexibility while denaturing the protein leads to more effective absorption of protein molecules at the water-oil interface. It has been suggested that the increase in ESI due to ultrasound treatment is a result of the appropriate orientation of the proteins caused by the physical force produced by cavitation (Mitidieri & Wagner, 2002). The ultrasound enhanced the emulsifying properties of BSF larvae proteins, consistent with findings by Huang et al. (2023) on *Tenebrio molitor* proteins. The study found that while the ultrasonic treatment decreased the particle size, it also increased molecular mobility.

The cavitation effect of ultrasound depolymerizes protein aggregates, reduces particle size, and accelerates the diffusion rate of ultrasound-treated BSF larval proteins at the water-oil interface. The formation of a rigid film membrane around droplets at the interface with lower equilibrium interfacial tension stabilizes the oil droplets against the main instability mechanisms, such as creaming, sedimentation, coalescence, and flocculation (Gharibzahedi & Mohammadnabi, 2017). Soria and Villamiel (2010) reported that denatured proteins exposed more hydrophobic groups, often resulting in protein aggregation. Therefore, a balance must be established between the exposure of hydrophobic groups and the aggregation of protein molecules. However, Mu et al. (2010) and Karki et al. (2009) found that the ESI of high-intensity ultrasound-treated soy protein isolate was lower than that of untreated soy protein isolate. Ultrasonication also reduces the EAI and ESI of peanut protein isolates (Zhang et al., 2014). Higuera-Barraza et al. (2016) suggested that, as high intensity ultrasound time increases, EAI consistently increases. Zhou et al. (2016) found that the EAI of soy glycinin decreased after 40 min of increasing the high-intensity ultrasound time. This decrease in EAI was likely due to the increase in surface hydrophobicity value and solubility index at high intensity ultrasound times, which resulted in the reduced flexibility of glycinin.

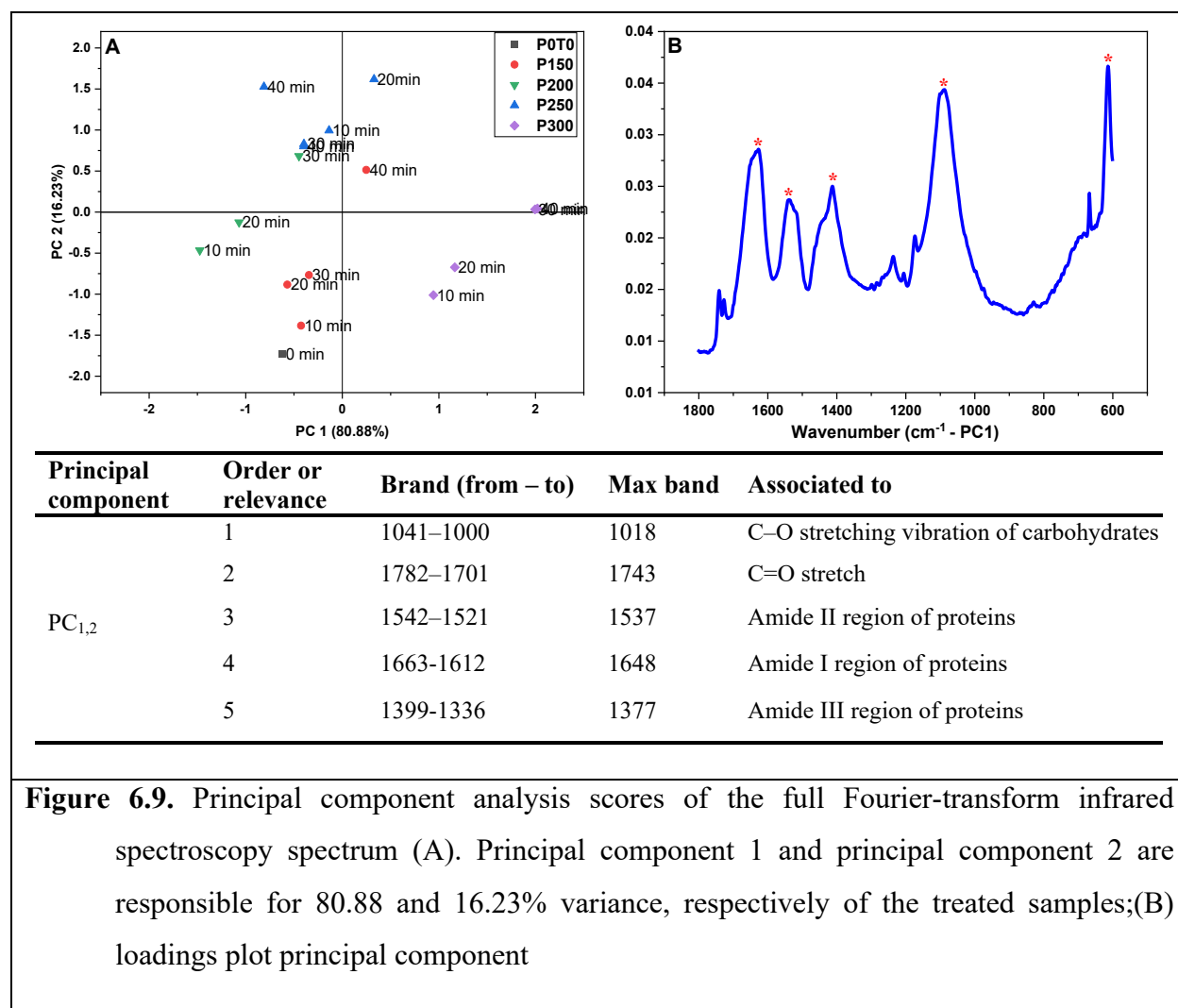
In our work, the decrease in EAI did not adversely impact ESI. This underscores the preeminent influence of surface hydrophobicity and solubility in this phenomenon. Jiang et al. (2017) also showed that pea protein isolate's higher amphiphilicity and structural flexibility in very high alkaline environment (pH 12.0) made it easier for protein molecules to spread around oil droplets at the interface. The experimental findings demonstrate that ultrasound treatment can markedly improve the emulsification activity and stability of BSF larval proteins.

6.4.10 Mid-range FTIR spectroscopy of native and ultrasonicated BSF larvae proteins.

FTIR can be used to study the secondary structure of proteins by looking at how molecules vibrate (Ellepola et al., 2005). The FTIR spectrum of protein consists of three groups of characteristic absorption bands: amide I band, amide II band and amide III band. Model studies employing polyamino acids have correlated the intensity of infrared adsorption in this region with α -helix ($1645\text{--}1660\text{ cm}^{-1}$), β -sheet ($1665\text{--}1680\text{ cm}^{-1}$), and random coil ($1660\text{--}1670\text{ cm}^{-1}$) concentration (Gülseren et al., 2007). Figure 6.9 shows the findings of a Fourier-transform infrared spectroscopy (FTIR) test on proteins from BSF larvae that were treated with different kinds of high-intensity ultrasound.

The FTIR spectra were analyzed using Principal Component Analysis (PCA) to visualize the effects of these treatments on the protein structure. The PCA plot shows distinct clusters based on ultrasound pressure (P150, P200, P250, and P300) and the ultrasound duration (represented by different colours), indicating that these factors significantly influence the FTIR spectral profiles. The distribution of samples is based on the first two principal components (PC1 and PC2), which explain 80.88% and 16.23% of the variance, respectively. PC1 primarily differentiates samples according to ultrasound pressure, with a gradient of changes in protein structure observed as pressure increases. Negative PC1 values were generally associated with lower pressures, while positive PC1 values corresponded to higher pressures, suggesting that PC1 reflects changes in protein structure or composition in response to the applied pressure. PC2 separates samples based on both pressure and ultrasound duration, reflecting changes in protein conformation or interactions induced by these factors. The PCA loadings indicate that specific wavenumbers are crucial in explaining the variance along the principal components. Positive loadings for PC1 around 1018 cm^{-1} and 1743 cm^{-1} correspond to the C-O stretching vibration of carbohydrates and

the C=O stretch, respectively, suggesting that variations in carbohydrate and carbonyl-containing compounds significantly contribute to sample separation along PC1. PC2.



On the other hand, shows positive loadings around 1537 cm^{-1} and 1648 cm^{-1} , associated with the Amide II and Amide I regions of proteins. This indicates that changes in protein secondary structure, particularly α -helices and β -sheets, contribute to the distribution along PC2. Similar results were found by Kingwascharapong et al. (2021) in their study on ultrasound-assisted extraction of proteins from Bombay locusts, they showed a shift from α -helical to random coil structures, with a decreased presence of ordered structures.

The PCA analysis shows that high-intensity ultrasonic treatment has a big effect on the FTIR spectra of BSF larvae proteins. This is mostly because of changes in the structure and

composition of the proteins and the presence of carbohydrates. When the pressure of ultrasound increases, the spectrum varies in a way that suggests changes in the shape or grouping of proteins. The stronger C-O stretching vibrations at lower pressures may mean that there is more carbohydrate in the sample or that the way they interact with proteins has changed. The impact of ultrasonic duration is less significant than that of pressure; yet it still induces minor spectral alterations, potentially indicating time-dependent protein modifications or breakdown. The results indicate that high-intensity ultrasound can alter the protein structure of BSF larvae, potentially affecting their functional characteristics and nutritional value. The PCA analysis clearly shows that FTIR spectroscopy could be a useful tool for keeping an eye on changes in proteins during food processing. This shows that more research is needed to fully understand how these changes happen and how to use these results in real life.

6.4.11 Secondary structure analysis of native and ultrasonicated BSF larvae using circular dichroism (CD) spectroscopy.

The CD is a useful tool for figuring out the secondary structure of proteins. This study employed circular dichroism (CD) to assess the secondary structure of BSF larvae isolates obtained by varying ultrasonic treatment strengths and durations. Table 6.4 displays the ratios of α -helix, β -sheet, β -turn, and random coil in both untreated control and sonicated BSF larvae protein samples

The data show that sonication changed the proteins' secondary structure a little bit. In particular, raising the power and length of sonication caused a big drop in the α -helix content ($p < 0.05$ for most comparisons) and a big rise in the β -sheet. A big rise in random coil was only seen at P300. These structural changes suggest that sonication induces conformational rearrangements in the proteins, which are statistically significant and reflect the impact of ultrasonic treatment on the stability and flexibility of the protein structures. Compared with the control sample (P0T0, α -helix: $22.20 \pm 0.55\%$, β -sheet: $22.80 \pm 0.12\%$), the negative peak intensity of the CD spectra for BSF larvae protein isolates subjected to different ultrasound treatments (P150, P200, P250, and P300) at varying time points (10, 20, 30, and 40 min) varied. For instance, at P150T40, the α -helix content decreased to $20.98 \pm 0.54\%$, while the β -sheet content increased to $24.11 \pm 0.30\%$. Similarly, at P300T40, the α -helix content was further reduced to $20.30 \pm 0.33\%$, with the β -sheet content increasing to $25.11 \pm 0.12\%$. These changes indicate a consistent decrease in the α -helix

content and a corresponding increase in the β -sheet content across the treatments and time points, which aligns with the data presented in **Table 6.4**.

Table 6.4. Impact of ultrasound-assisted extraction on the secondary structure composition of black soldier fly larvae protein isolates. Values are given as means $\pm$ SD from triplicate determinations.

Samples	α -helix	β -sheet	β -turn	Random coil
P0T0	22.20 $\pm$ 0.55 ^h	22.80 $\pm$ 0.12 ^a	15.90 $\pm$ 0.66 ^{cdef}	39.10 $\pm$ 0.23 ^{ef}
P150T10	22.01 $\pm$ 0.21 ^h	22.99 $\pm$ 0.35 ^b	15.82 $\pm$ 0.41 ^{cd}	38.94 $\pm$ 0.31 ^{cd}
P150T20	21.98 $\pm$ 0.23 ^{gh}	23.23 $\pm$ 0.51 ^c	15.98 $\pm$ 0.12 ^{cdef}	38.81 $\pm$ 0.06 ^{cd}
P150T30	21.82 $\pm$ 0.58 ^g	23.42 $\pm$ 0.01 ^d	16.22 $\pm$ 0.51 ^{cdef}	38.54 $\pm$ 0.02 ^{bc}
P150T40	20.98 $\pm$ 0.54 ^d	24.11 $\pm$ 0.30 ^e	16.41 $\pm$ 0.55 ^{def}	38.50 $\pm$ 0.12 ^{bc}
P200T10	21.22 $\pm$ 0.32 ^{ef}	23.44 $\pm$ 0.02 ^d	16.80 $\pm$ 0.46 ^{ef}	38.54 $\pm$ 0.71 ^{bc}
P200T200	21.85 $\pm$ 0.11 ^g	24.41 $\pm$ 0.05 ^g	15.48 $\pm$ 0.54 ^c	38.66 $\pm$ 0.51 ^c
P200T30	21.45 $\pm$ 0.84 ^f	24.23 $\pm$ 0.55 ^f	16.31 $\pm$ 0.12 ^{def}	38.01 $\pm$ 0.22 ^b
P200T40	21.20 $\pm$ 0.51 ^e	24.81 $\pm$ 0.14 ⁱ	16.88 $\pm$ 0.23 ^f	37.11 $\pm$ 0.11 ^a
P250T10	21.01 $\pm$ 0.65 ^d	24.23 $\pm$ 0.11 ^f	17.55 $\pm$ 0.33 ^{def}	37.21 $\pm$ 0.07 ^a
P250T20	19.11 $\pm$ 0.75 ^a	24.45 $\pm$ 0.25 ^g	18.03 $\pm$ 0.50 ^g	38.41 $\pm$ 0.55 ^{bc}
P250T30	19.88 $\pm$ 0.88 ^b	24.53 $\pm$ 0.22 ^{gh}	16.61 $\pm$ 0.44 ^{def}	38.98 $\pm$ 0.06 ^c
P250T40	19.45 $\pm$ 0.60 ^a	24.66 $\pm$ 0.41 ^h	16.68 $\pm$ 0.10 ^f	39.21 $\pm$ 0.04 ^{cd}
P300T10	21.43 $\pm$ 0.22 ^f	24.61 $\pm$ 0.15 ^h	13.86 $\pm$ 0.15 ^b	40.10 $\pm$ 0.22 ^{fg}
P300T20	21.31 $\pm$ 0.53 ^{ef}	24.80 $\pm$ 0.55 ⁱ	13.38 $\pm$ 0.12 ^b	40.51 $\pm$ 0.21 ^g
P300T30	20.35 $\pm$ 0.35 ^c	24.99 $\pm$ 0.52 ^j	13.33 $\pm$ 0.01 ^b	41.33 $\pm$ 0.31 ^h
P300T40	20.30 $\pm$ 0.33 ^c	25.11 $\pm$ 0.12 ^k	12.33 $\pm$ 0.02 ^a	41.82 $\pm$ 0.23 ^h

Our results suggest that sonication may have preferentially disrupted certain types of hydrogen bonds, thereby causing some of the α -helix structures to appear to be converted into β -sheet structures across most conditions, while a significant increase in random coil content was only observed at P300. For β -turn structures, significant alterations relative to the control were predominantly noted around P300, suggesting that structural transitions were contingent upon the specific conditions rather than uniform across all treatments. Similar results have also been reported by researchers who have applied sonication to other types of proteins, such as black soldier fly (Xu et al., 2023), *Tenebrio molitor* proteins (Zhang, Sun, et al., 2023), and cricket

protein (Edward et al., 2023), where sonication-induced size reduction was linked to alterations in secondary structures.

The ultrasound treatment probably made the BSF larvae protein structure partially expand, which changed the way hydrogen bonds interacted with each other. This caused the hard α -helix structure to become weaker and the more flexible structures, including β -sheets, to become stronger. This changed the protein's secondary structure (Wang et al., 2020). Zhang et al. (2023) also found similar results when they looked into how ultrasound-assisted alkaline extraction affected the secondary structure of *Tenebrio molitor* protein isolate. They observed a decrease in α -helix and random coil content, along with an increase in β -sheet content. However, Xiong et al. (2016) discovered that ultrasonic therapy did not influence the secondary structure of ovalbumin; this was likely attributable to several factors, including protein kinds, predominant secondary structure types, and ultrasound settings.

Particularly at higher power levels, where the conversion of β -turn into random coil becomes more obvious, the ultrasonic pulses used during ultrasound-assisted extraction may cause irregular protein reassembly (Ding et al., 2021). Part of the random coil may then reassemble into β -sheet structures, which could explain the structural transitions observed in the current study under these high-power conditions (P300). Once more, this implies that the impact of sonication on protein structure is influenced by the type of protein, the solution, and the sonication conditions. Our knowledge of how ultrasound can be applied as a non-thermal processing technique to produce particular structural alterations in novel protein sources—specifically BSF larvae—is expanded by this work.

6.4.12 Powder X-ray Diffraction

The crystal structure changes of BSF larvae protein isolates that were exposed to ultrasound treatment were examined using X-ray diffraction (XRD). The purpose of the study was to investigate these protein isolates' crystalline characteristics. As seen in **Figure 6.10**, the XRD spectra of the protein samples from BSF larvae that were not treated and those that were showed a noticeable peak at $2\theta = 20^\circ$ and a less noticeable peak at $2\theta = 10^\circ$. Cao and Tan (2005) define crystalline region I as the peak at $2\theta = 10^\circ$ and crystalline region II as the peak at $2\theta = 20^\circ$. The α -helix and random coil structures, with diffraction angles of approximately 12.2° , 24.7° , 27.9° , and 28.2° , are the main characteristics of crystalline region I.

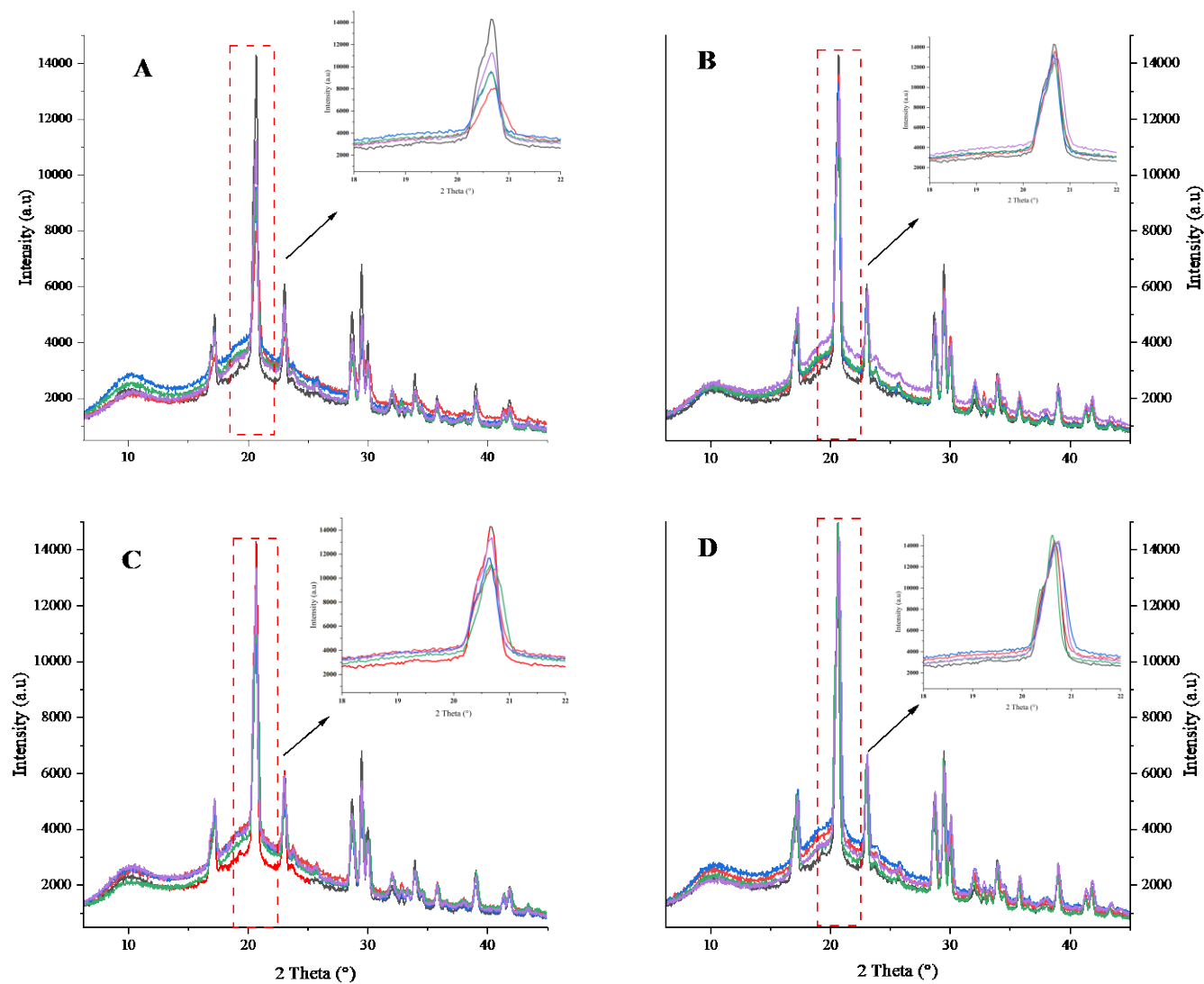


Figure 6.10. Effect of high-intensity ultrasound on the XRD of black soldier fly larvae proteins (A – P150, B– P200, C – P250, and D – P300). Line colours correspond to ultrasound duration: black (0 min), red (10 min), blue (20 min), green (30 min), and purple (40 min).

On the other hand, crystalline region II is primarily composed of β -sheet structures, which are concentrated at 16.7° , 20.7° , 24.0° , 25.5° , and 30.9° (Batra et al., 2021). The prominent peak observed at $2\theta = 20^\circ$ in the P150 sample (**Figure 6.10A**) demonstrates a consistent increase in peak intensity with extended treatment times, from 10 min (red) to 40 min (green).

Notably, the P150T40 sample exhibited a peak intensity comparable to the control (black), indicating that ultrasound treatment may enhance the activity or stability of the protein with longer exposure. This increase in diffraction intensity suggests an increase in crystal size, consistent with the findings of Yu et al. (2015), who reported that higher diffraction intensity corresponds to larger crystal size (Yu et al., 2015). The intensity profiles of the P250 and P300 samples (**Figures 6.10B-C**) closely align with that of the control sample, suggesting minimal alterations in structure or composition following treatment. The cavitation forces generated during ultrasound treatment likely altered the conformation of protein molecules, leading to the formation of new chemical bonds and a disruption of the regular molecular arrangement. Joshi et al. (2011) noted that proteins with higher crystallinity exhibit higher diffraction intensity and vice versa. This relationship was observed in the present study, where ultrasound treatment altered the crystallinity of BSF larvae protein isolates. Little research has been conducted on the effect of ultrasound on the crystalline structure of edible insects, particularly BSF larvae. To the best of our knowledge, this study is the first to explore the impact of ultrasound treatment power as a function of time on the crystalline structure of insect-derived proteins, offering new insights into protein modification and the potential use of BSF larvae as a novel functional ingredient.

Contrarily to our results, Yang et al. (2023) and Malik et al. (2018) observed significant effects of ultrasound treatment on the crystal structure of wild silkworm and sunflower protein isolates, respectively, with diffraction intensity decreasing progressively with increased sonication time. In contrast to the 300W or less used in the current study, the higher ultrasound power used in their studies (500W by Malik et al. 2018) and up to 800W by Yang et al. (2023) may be the cause of this disparity. Longer sonication times have been shown to convert random coil structures into stable β -sheet structures with water-insoluble characteristics (Samal et al., 2013). Further supporting earlier results from FTIR and CD analyses were minor variations and the emergence of new peaks in sonicated samples, which indicated compositional changes in the proteins' β -sheet and α -helix components.

6.4.13 Effect of Ultrasound on thermal properties of BSF larvae proteins

In the field of food science, where it is essential for clarifying protein denaturation, crystallisation, and other thermally induced transitions, the DSC technique is a basic tool for studying thermal behaviour in materials. The impact of ultrasound treatment on the thermal characteristics of BSF larvae proteins was examined in this study using DSC. The thermal profiles of several protein samples (P150, P200, P250, and P300) treated with ultrasound for varying lengths of time (0, 10, 20, 30, and 40 minutes) are shown in **Figure 6.11 (A–D)**. **Figure 6.11A** shows the T_{max} and ΔH values for the control sample, and **Appendix E1** shows the corresponding data for the other treatments.

Endothermic peaks in the DSC curves are a sign of thermal processes like melting or denaturation. These endothermic peaks most likely correlate to structural transformation or protein denaturation events in the context of BSF larvae proteins. Two separate endothermic peaks are present in every sample, according to the analysis of the DSC data. The loss of loosely bound water molecules linked to the protein matrix, or the denaturation of less stable proteins, is likely the cause of the first peak, which was seen below 100°C. Around 300°C, the second, more noticeable peak indicates the beginning of protein degradation, which includes thermal breakdown and the disintegration of more stable protein structures. The structural integrity and hierarchical thermal stability of the BSF larvae proteins under the effects of ultrasound treatment are reflected in these transitions. The position and intensity of these peaks vary across the different samples and ultrasound treatment durations, reflecting modifications in protein structure or composition.

At the lowest intensity (P150), the first peak fluctuates slightly with ultrasound duration, ranging from 58.06°C to 61.97°C (**Figure 6.11A, Appendix E1**). A slight decrease is observed at P150T40 (57.99°C), suggesting some degree of protein destabilization with prolonged ultrasound exposure. The corresponding enthalpy values also show significant variation. This variability reflects structural changes such as partial unfolding or aggregation of less stable protein fractions. For P200, values show a wider range, decreasing from 59.76°C at P200T10 to 53.30°C at P200T20, followed by a recovery to 58.36°C at P200T40 (**Appendix E1**). The values reveal pronounced fluctuations, with a significant drop in thermal activity at P200T30 (36.21 J/g), suggesting partial unfolding or degradation of protein structures. However, at P200T40, the value recovers to 174.08 J/g, indicating that longer ultrasound treatment may promote structural reorganization and stabilization. At higher intensities, more stable thermal profiles are observed.

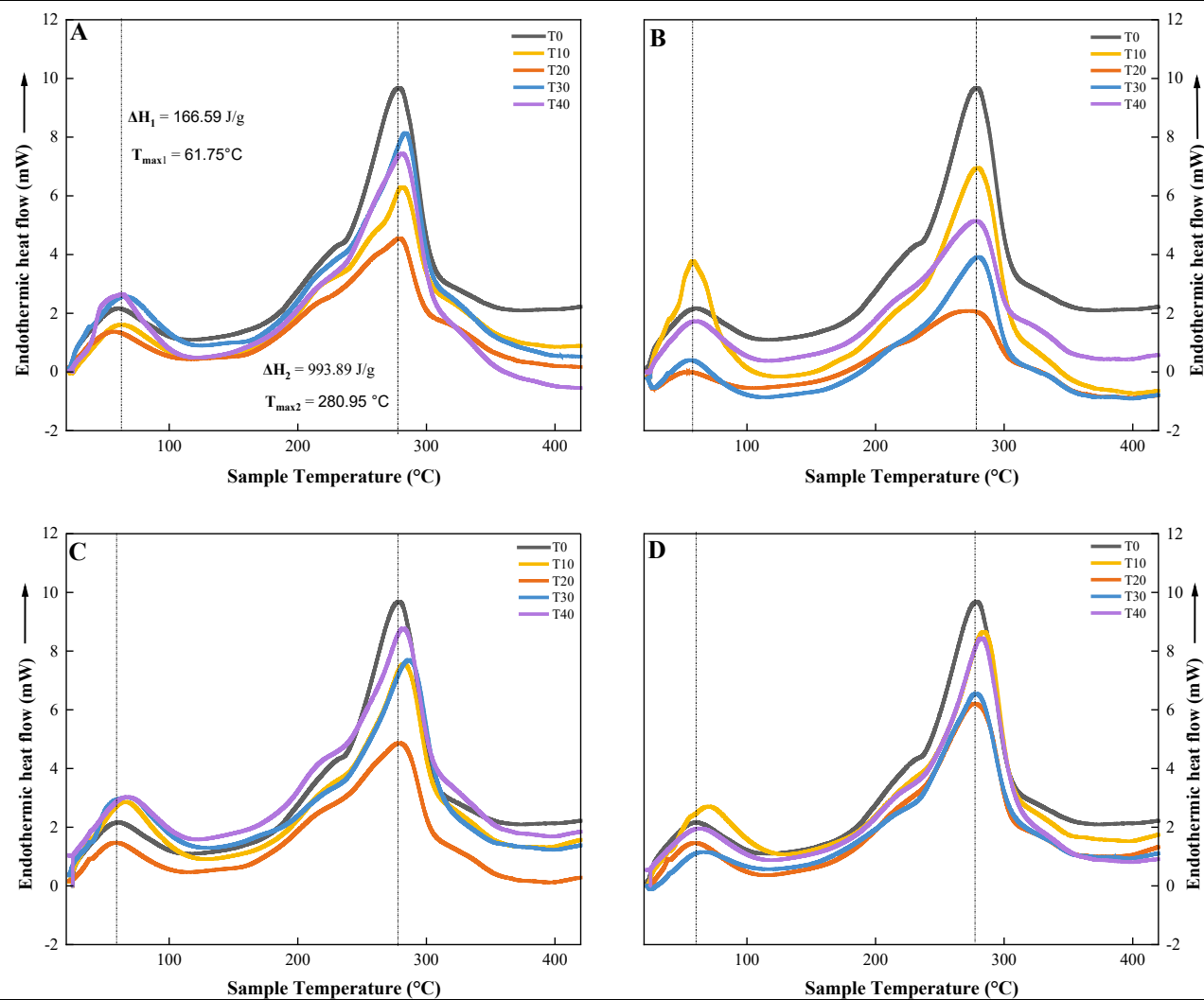


Figure 6.11. Effect of high intensity ultrasound on the DSC of black soldier fly larvae proteins (A – P150, B– P200, C – P250, and D – P300). Line colours correspond to ultrasound duration: black (0 min), yellow (10 min), red (20 min), blue (30 min), and purple (40 min).

For P250, the value steadily increases from 61.12°C (P250T10) to 65.12°C (P250T40), suggesting enhanced thermal stability of the protein structures under ultrasound treatment (**Figure 6.11C**). The values generally increase from 72.13 J/g (T10) to a peak of 173.87 J/g (T30), although an intermediate drop is observed at P250T20. This is followed by a marked decrease at T40 (90 J/g), suggesting possible structural degradation or denaturation after prolonged treatment. At the highest intensity (P300), the values increase steadily from T10 (104.17 J/g) to P300T40 (131.53 J/g), suggesting cumulative stabilization effects over time. For the second peak, the thermal profile increases significantly from 696 J/g (P300T10) to 787.26 J/g (P300T40). Across all samples, the second peak exhibits variations that align with the degree of ultrasound treatment. Regardless of power level, a similar pattern is seen across all power levels (P150, P200, P250, and P300): a decrease from T0 to T20, followed by an increase from T30 to T40. This suggests that the response to treatment duration varies.

Mir et al. (2019) reported similar outcomes for protein isolates from album (*Chenopodium album*) seed treated with high-intensity ultrasound (HIUS). The impact of ultrasound treatment on the thermal stability of BSF larvae proteins is highlighted by the observed changes in the heat enthalpy across all samples. The particular protein sample and the length of the ultrasound exposure both affect how much of an impact this has. The differences in peak intensity and position demonstrate how ultrasound treatment significantly affects the thermal characteristics of BSF proteins, with longer treatment times producing more noticeable structural changes. It appears that less energy was needed to unfold the ultrasonicated BSF larvae sample because of the decrease in denaturation temperature brought on by the ultrasound treatment. This is attributed to the destruction of intramolecular bonds in the BSF larvae, caused by the shear forces generated by cavitation bubbles. The current study's findings are consistent with those of Nazari et al. (2018) regarding HIUS-treated millet protein concentrates.

There are a lot of chances for food processing innovation because ultrasound can be used to modify the thermal and structural characteristics of BSF proteins. The production of new protein ingredients from BSF larvae with specific functional characteristics may have a variety of uses, such as the development of dairy alternatives, protein-fortified snacks, and meat alternatives. The DSC results demonstrate that ultrasound treatment significantly affects the thermal stability and structural properties of BSF larvae proteins. Higher power levels encourage stabilisation, whereas lower power levels cause more structural changes. These results offer important new information

about how to optimise ultrasound parameters to improve the characteristics of proteins in BSF larvae.

6.4.14 Influence of ultrasonication on molecular weight of BSF larvae proteins.

Using SDS-PAGE, the impact of ultrasonic-assisted extraction on the molecular weight distribution in BSF larvae proteins was ascertained. The SDS gels under reducing conditions are shown in Figure 6.12 (A-D), with molecular weights varying from 10 to 250 kDa. The most dominant bands were observed at 26, 30, 38, 50, 82, and 95kDa. β -Mercaptoethanol was used to reduce disulfide bonds in the protein samples before SDS-PAGE.

High-resolution mass spectrometry was used by Leni et al. (2020) to identify BSFL proteins. Through database comparison, they discovered myosin heavy chain (235 kDa), hexamerin F1 (81 kDa, common in insects, used for protein storage), actin-87E (42 kDa), and troponin C (18 kDa). Rabani et al. (2019) suggested the presence of structural and muscle proteins at 75 kDa. Ultrasound treatment affected the protein bands' pattern and intensity. As the length of sonication increased, some bands grew louder, while others weakened or vanished. In some isolates, especially those treated for longer periods of time or with higher power, these alterations were more noticeable. The ultrasonicated BSF larvae proteins' SDS-PAGE profiles showed distinct differences from the untreated control samples (P0T0), primarily in band intensity throughout the gel. Only slight alterations were seen in the region below 30 kDa, indicating that the treatments had less of an impact on lower molecular weight proteins.

This finding implies that neither detectable fragmentation nor disruption of the molecular distribution of soluble proteins was caused by ultrasonic treatment. Our findings were consistent with those of other studies that used ultrasound to extract the protein from rice bran (Zhang et al., 2018) and rapeseed (Li et al., 2020). Similarly, Zou et al. (2017) showed that ultrasound-assisted alkaline extraction had no discernible impact on the protein structure of the chicken liver protein isolate, and Xu et al. (2023) proposed that the ultrasonic conditions (4.57 W/cm^3 , 1 h) did not break the protein bonds in BSFLP (black soldier fly larvae proteins). Zhang et al. (2023) reported that ultrasonic cavitation effects at 4 W/cm^3 and pH 10 led to the degradation of high molecular weight proteins into smaller peptides during the extraction of *Tenebrio molitor* larvae proteins. In contrast, the ultrasonic conditions used in this study (150–300 W at neutral pH) did not result in such fragmentation, as evidenced by the consistent SDS-PAGE profiles.

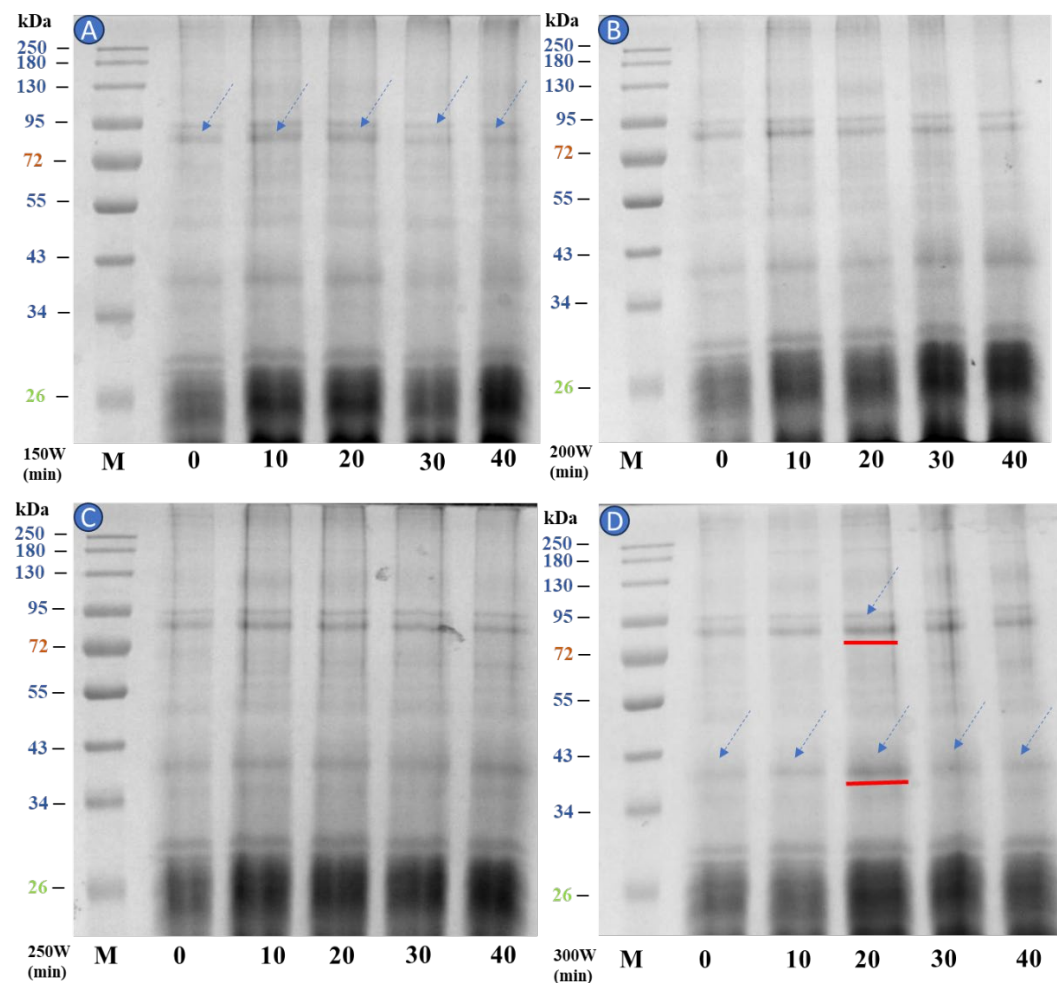


Figure 6.12 Effect of ultrasound on the reducing SDS-PAGE profile and molecular distribution of soluble proteins from black soldier fly larvae proteins (A – P150, B – P200, C – P250, and D – P300 at different treatment times of 10, 20, 30 and 40 min). Blue arrows indicate protein bands whose intensity or presence changed following ultrasound treatment. Red stripes highlight specific bands that are notably intensified or diminished.

The milder cavitation effects under these conditions may have minimized protein degradation, highlighting the importance of both ultrasonic power and pH in determining the extent of protein structural changes. In contrast to the control (P0T0), the concentration of each band in the P150 sample (Figure 6.12A) increased gradually as the ultrasound treatment time increased. This implies that rather than seriously breaking down protein subunits, ultrasonic treatment improved the solubility of proteins. Ultrasound probably breaks up insoluble protein aggregates by introducing mechanical shear and cavitation effects, which causes the previously aggregated proteins to be released into the soluble phase (Li et al., 2020). More noticeable protein bands in the SDS-PAGE profiles are in line with this phenomenon. Additionally, because of the cavitation and catalytic effects of ultrasound, the strength of protein bands first increased and then decreased with longer ultrasound times (Figure 6.12D) (Yang et al., 2019).

The SDS-PAGE profiles show that, in all samples (P150 at **Figure 6.12A**), except those treated at 250 W (**Figure 6.12C**), the intensity of particular protein subunits, particularly those around 38 kDa and 82 kDa, varies with treatment time. This suggests that ultrasonic power at 250 W may not generate sufficient cavitation energy to significantly affect the solubilization or aggregation state of these subunits. In contrast, at 300 W, the intensity of the bands near 38 kDa and 82 kDa (P300T20 in **Figure 6.12D**) was notably enhanced, indicating increased solubilization or disaggregation of these proteins under higher power conditions.

As shown in **Figure 6.12B**, ultrasound treatment at P200 resulted in visible changes in band intensity, particularly in regions above 55 kDa, while it relatively increased at approximately 38 kDa, with the maximum band strength noted at 30 min. This indicated that ultrasound treatment resulted in the degradation of large molecular weight BSF larvae proteins into smaller molecular structures due to the alteration of the molecular structure by the cavitation effect (Li et al., 2020).

Overall, the SDS-PAGE profiles indicate that ultrasound treatment altered the solubility and molecular distribution of BSF larvae proteins in a power- and time-dependent manner, primarily through changes in band intensity rather than the appearance or disappearance of specific bands. This suggests partial unfolding and reorganization of protein structures without major degradation.

6.4.15 Scanning electron microscope (SEM)

The microstructure of food components greatly influences their techno-functional properties (Mshayisa et al., 2021). To investigate these effects, SEM micrographs of BSF larvae proteins were captured both before and after ultrasound treatment (**Figure 6.13** (control)). The SEM of the control larvae protein shows a compact and irregular surface structure with densely packed protein aggregates. The morphology appears coarse, with clusters of aggregated particles and limited porosity. This structure suggests the proteins exist predominantly in an aggregated, insoluble form before any treatment.

SEM images of BSF larvae proteins subjected to varying ultrasound power and duration are shown in **Table 6.5**, with images obtained at 2000× magnification. Notably, the microstructure of ultrasound-extracted BSF larvae protein displayed significant differences compared to untreated samples. Heterogeneous particles, consisting of clumps of various sizes and shapes, were observed. At P150T10, the protein structure remains largely compact, with minimal disruption of protein aggregates. This could be because the probe did not exert enough cavitation forces under these treatment conditions, which resulted in a lack of turbulent forces and micro-streaming.

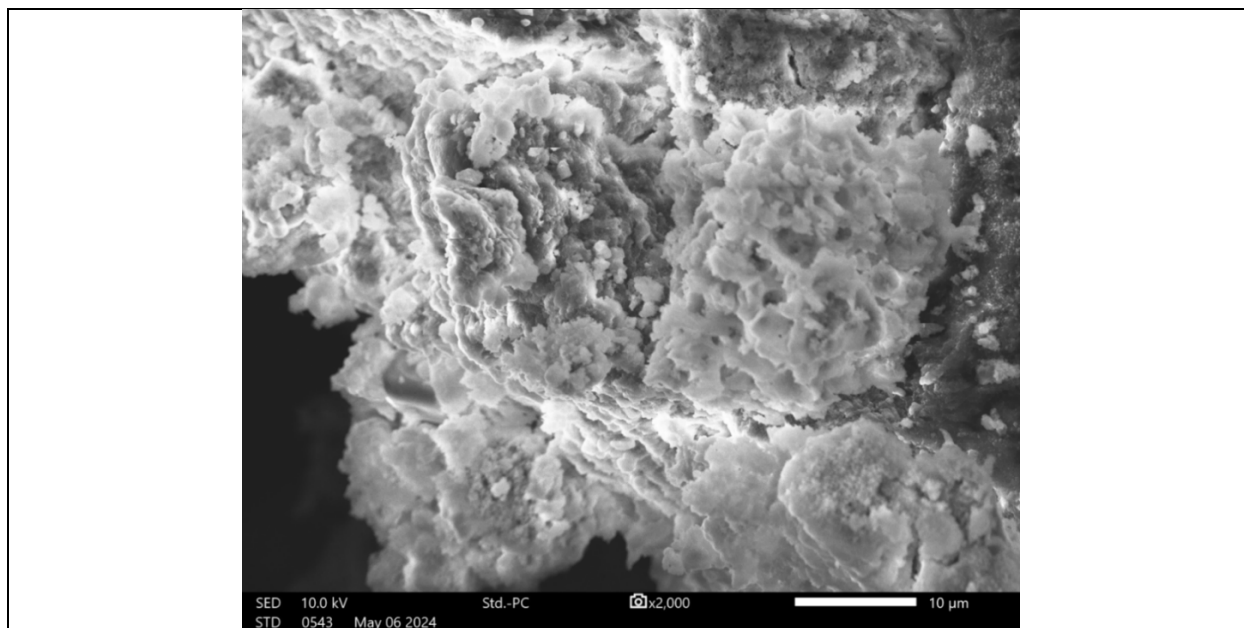
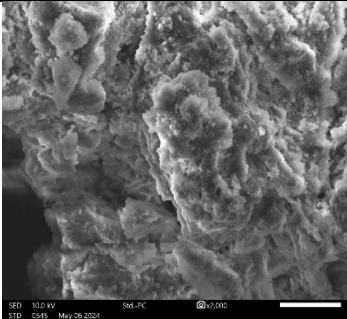
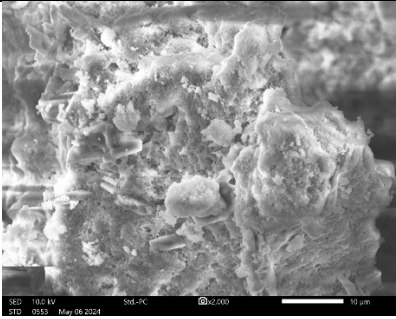
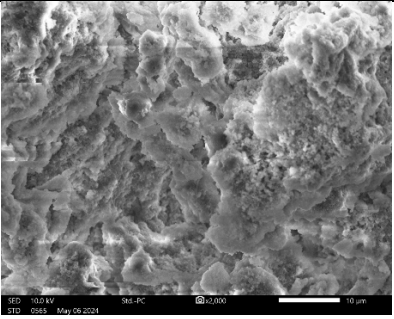
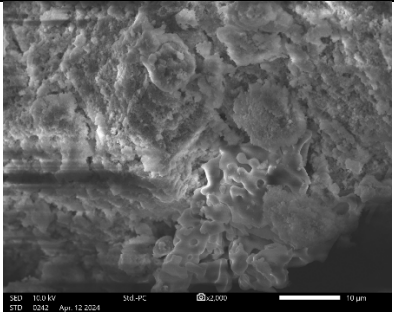
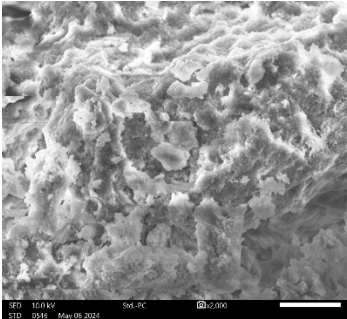
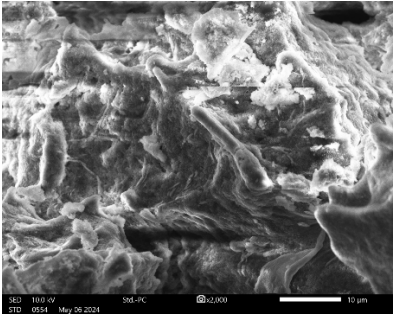
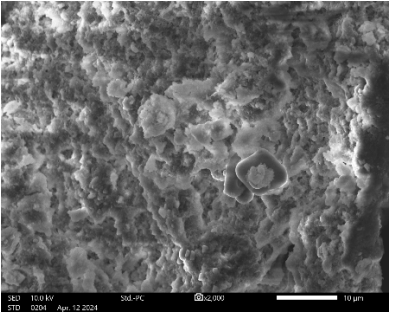
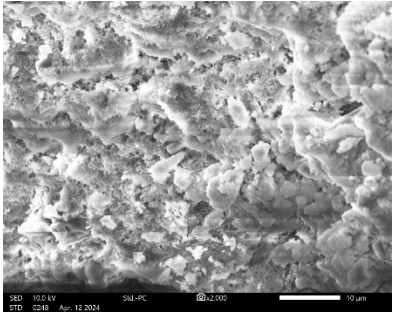
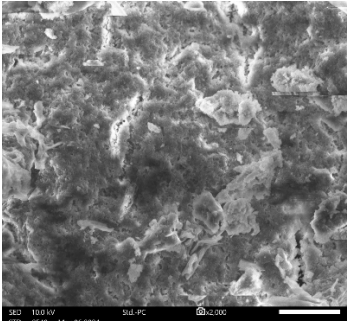
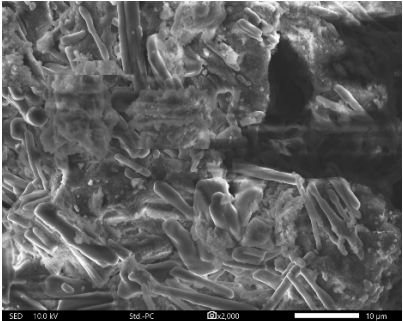
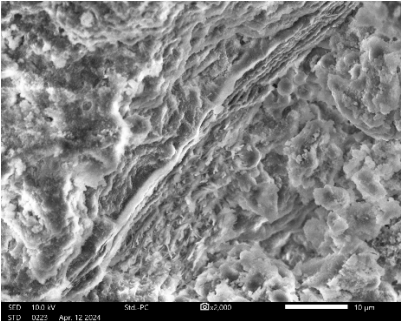
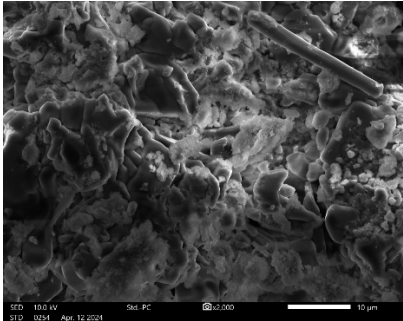
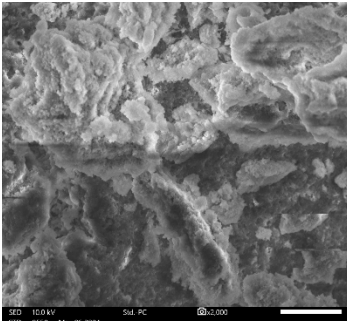
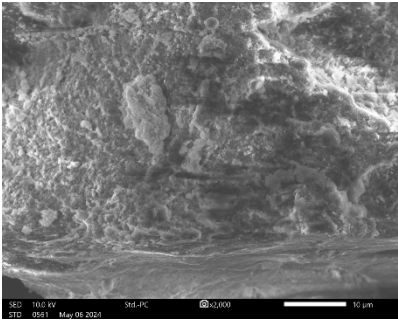
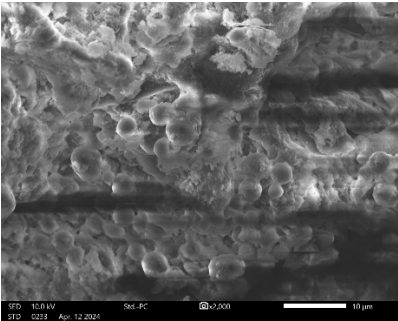
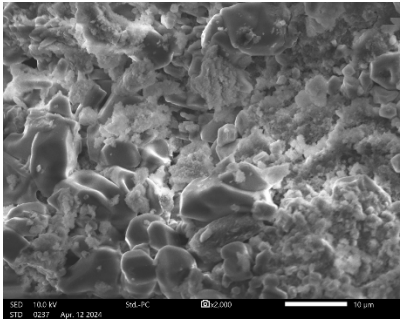


Figure 6.13. Scanning electron micrograph of the untreated black soldier fly larvae protein. Images are displayed at 2000x magnification (scale bars of 10 μm).

Small cracks and increased surface porosity, which are signs of partial disaggregation, appear as the structure gradually loosens as the treatment time is extended to 20 to 40 minutes. The globular structure seemed to change into a mesh structure due to long ultrasonic cavitation effects. The beginning of structural alterations is indicated by the slight disaggregation and surface roughness that are visible at P200T10. A more noticeable level of structural breakdown is indicated by the protein structure becoming more porous and fragmented after 20 to 40 minutes of treatment, along with the appearance of fibrous features and smaller aggregates. Similar results were noted by Huang et al. (2023) using *Tenebrio molitor*, who attributed the formation of irregular and smaller fragments to turbulent flow and cavitation. According to Zhao et al. (2014), ultrasonic treatment reduced the number of protein aggregates by breaking the cross-linking in chicken breast myofibrillar proteins. This result is consistent with our findings, which showed that samples P200T30, P250T40, and P300T40 produced more uniform structures when exposed to medium ultrasonic power (Table 6.5). Protein unfolding brought on by ultrasonication and an increase in surface hydrophobic groups in BSF larvae molecules may be the cause of these homogeneous structures.

At P250T10, the surface begins to show noticeable disruption. Between 20 and 40 min, the structure transitions into a more fibrillar and fragmented form, with visible network-like patterns that suggest substantial structural rearrangement and breakdown. At P300T10, the disruption of protein aggregates is markedly more pronounced compared to lower power treatments. As the treatment time progresses to 20–40 min, the protein structure undergoes extensive disaggregation, forming a highly fibrous and porous network. This reflects significant protein solubilization and disintegration, driven by the higher cavitation energy at 300 W. Li et al. (2021) found that ultrasound-assisted extraction produced more irregular and smaller fragments from krill protein, attributing this to cavitation and turbulent flow. Stefanović et al. (2018) reported similar results with egg white protein, where ultrasonic treatment disrupted cross-linking, resulting in fewer protein aggregates. Interestingly, sample P200T40 displayed the densest and smoothest surface, potentially due to protein aggregation under prolonged treatment (**Table 6.5**). The acceleration of protein extraction was linked to microstructural changes induced by ultrasound, as observed in ultrasound-assisted walnut protein treatment systems (Golly et al., 2019). Microstructure influences the chemical and physical properties and functionality of the protein.

Table 6.5. Scanning electron micrographs of ultrasound treated black soldier fly larvae proteins as a function of power and time. Images are displayed at 2000x magnification (scale bars correspond to 10 μ m).

Time/Power	150W	200W	250W	300W
10 min				
20 min				
30 min				
40 min				

The bulk density of the protein's loose structure, resulting from the synergistic effect between ultrasound and enzyme, was smaller than that of the dense structure extracted solely by ultrasound, which improved protein solubility (Hu et al., 2013). As ultrasound power increased, protein macromolecule aggregates broke into smaller, more uniformly distributed fragments, enhancing the dispersive effect on BSF larvae proteins. This increased surface area improved hydration, enhancing surface hydrophobicity and solubility. Smaller fragments also increased the contact area of protein molecules with water and oil, allowing better absorption at the air/water interface and lowering the interfacial energy barrier during homogenization, thus enhancing emulsification and foaming properties (Resendiz-Vazquez et al., 2017). High-power ultrasonication led to partial unfolding of black-bean proteins, exposing functional groups that interacted to form aggregates and networks, which typically improve solubility (Tang et al., 2009). Overall, these results suggest that the changes in protein microstructure might enhance protein functionality, making sonication a valuable technique in food processing.

6.4.16 Pearson's correlation analysis

Pearson's correlation matrix (**Figure 6.14**) provides insights into the interrelationships between various functional properties of BSF larvae protein isolates under different ultrasound treatment pressures and durations. WBC showed a moderate positive correlation with OBC ($r = 0.57$, $p < 0.05$) and EAI ($r = 0.67$, $p < 0.01$), suggesting that proteins with higher water absorption also exhibit enhanced oil-binding and emulsifying capabilities, potentially due to increased hydrophobicity or expanded surface area. However, WBC exhibited a weak negative correlation with surface hydrophobicity ($r = -0.24$, $p < 0.05$), indicating that hydrophobic residues were not significantly exposed to facilitate water absorption during ultrasound treatment. Additionally, a slight positive correlation with exposed SS groups ($r = 0.21$) suggests that minor protein unfolding may reduce water retention efficiency.

OBC demonstrated a positive correlation with surface hydrophobicity ($r = 0.31$, $p < 0.05$), implying that hydrophobic proteins interact better with oils, while a strong positive correlation with EAI ($r = 0.79$, $p < 0.001$) indicates that proteins with high oil-binding capacity may also dissolve better in alkaline conditions. This aligns with the results showing that high ultrasound intensities enhance OBC by exposing hydrophobic residues. FS positively correlated with solubility ($r = 0.70$, $p < 0.001$), confirming that ultrasound disrupts disulfide bonds and exposes sulfhydryl groups. FC showed weak or negative correlations with WBC ($r = -0.29$, $p < 0.05$) and OBC ($r = 0.35$, $p < 0.05$), indicating that while these structural changes enhance solubility and emulsification, they may reduce binding capacities.

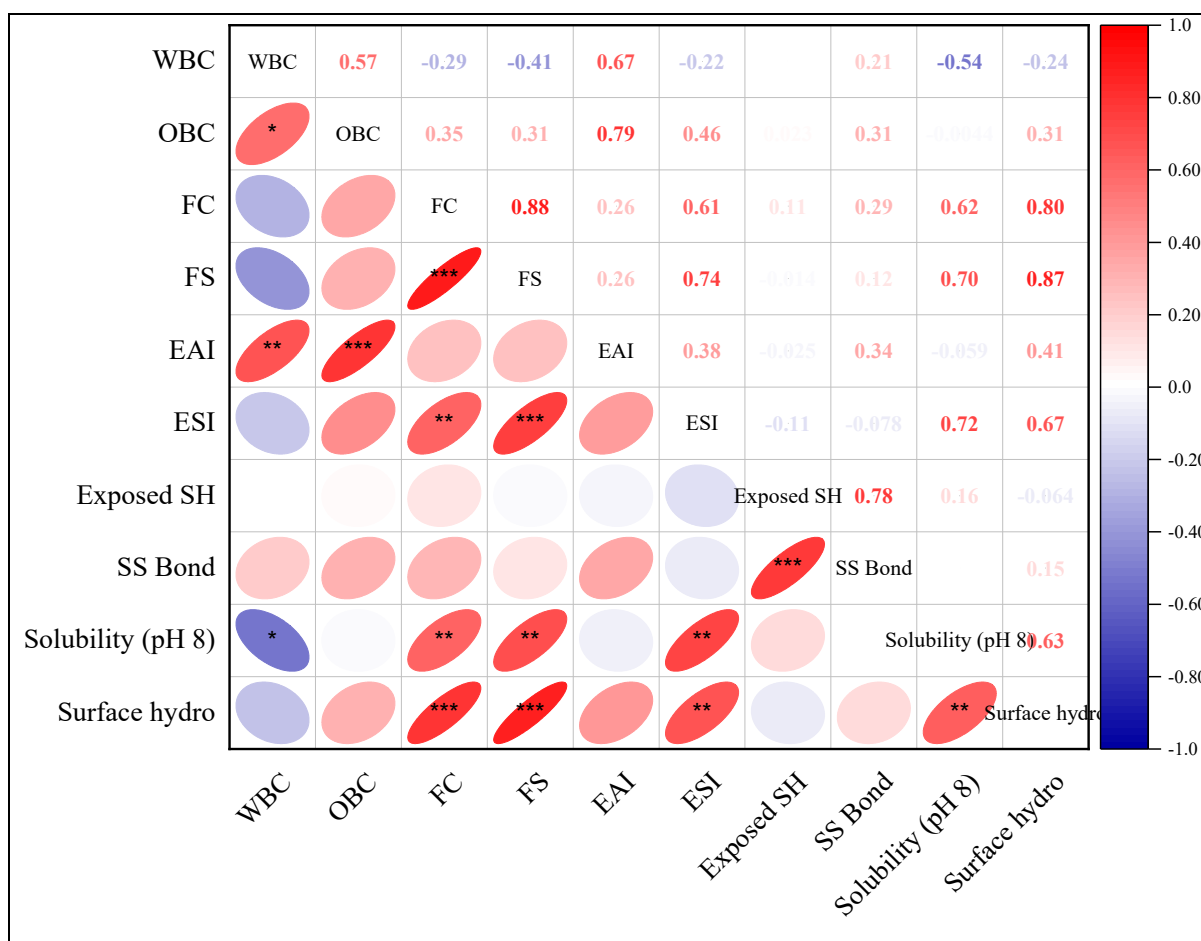


Figure 6.14. Correlation matrix between the black soldier fly larvae protein isolates functional properties via Pearson's linear correlation analysis; the colour gradation and the size of the circle represent the correlation coefficient (r value). Significance levels: *p < 0.05 (moderate significance), **p < 0.01 (higher significance), ***p < 0.001 (highest significance), blue (represents negative correlation) red (represents positive correlation).

Solubility exhibited a positive correlation with surface hydrophobicity ($r = 0.63$, $p < 0.001$), suggesting that exposure of hydrophobic residues enhances protein solubility under extraction conditions. A moderate positive relationship with ESI ($r = 0.72$, $p < 0.05$) linked increased protein unfolding to improved solubility. Surface hydrophobicity strongly correlated with most functional properties, including solubility, FS, FC, and ESI, underscoring its central role in determining protein functionality. However, its negative correlation with exposed SH indicated that reduced disulfide cross-linking increases hydrophobic residue exposure. Finally, EAI and ESI correlated positively with surface hydrophobicity, suggesting that structural flexibility and hydrophobic residue exposure enhance emulsification properties. Weak correlations with SS Bond content suggest that excessive cross-linking reduces emulsifying ability.

Overall, structural alterations brought about by ultrasound, such as protein unfolding, the exposure of hydrophobic residues, and the disruption of disulphide bonds, are responsible for the functional gains in solubility, emulsification, and binding capacities. This analysis lays the groundwork for treatment optimisation by highlighting the ways that ultrasound durations and intensities affect the protein functionality of BSF larvae.

6.5. Conclusions

The functional and structural characteristics of BSF larval proteins have been profoundly improved by ultrasound treatment, making them attractive candidates for use in the food, feed, and biotechnology sectors. The physical, chemical, and structural characteristics of the proteins are greatly impacted by the treatment, which optimises ultrasonic parameters like power and duration.

The BSF larvae protein isolates' colour characteristics were greatly affected by the ultrasound treatment; under particular circumstances, like P200T10, greater lightness (L^*) was noted. Although the protein content varied, protein recovery yields improved dramatically with ultrasound-treated samples reaching up to 77.56% (P250T40) compared to 62.74% for the control. The intricacy of ultrasound's effects on protein isolates was demonstrated by the lack of a consistent trend of colour variation with increasing sonication power or duration, despite these advancements. The essential amino acid profile of BSF larval proteins was enhanced by ultrasound, especially under ideal circumstances such as P250T10. The overall quality of the protein was improved, despite minor decreases in the lysine content. The variations in lysine and other amino acids under various ultrasonic treatments point to structural and functional alterations in proteins brought on by ultrasonic processing. These findings highlight the potential of ultrasound-assisted extraction to enhance amino acid profiles.

The amount of free sulfhydryl (SH) and disulphide (SS) bonds in the proteins of BSF larvae was greatly affected by ultrasound-assisted extraction. By oxidising SH groups and revealing buried groups during protein unfolding, increased ultrasonic power and duration initially increased the content of SS bonds. However, because of the oxidative effects of the hydrogen peroxide produced by cavitation, extended sonication resulted in a decrease in both SH and SS content. Protein functionality, including hydrophobicity, is impacted by these structural changes, highlighting the significance of exact control over sonication parameters. By dissolving protein aggregates and unfolding molecules, ultrasound treatment dramatically improved the solubility of BSF proteins. However, protein repolymerization could undo this

effect if power is applied excessively or treatment is prolonged. In general, the treatment enhanced surface hydrophobicity, revealing more hydrophobic groups, which enhanced functional qualities like foaming and emulsification. Ultrasound's cavitation effects exposed hydrophobic groups and enlarged protein molecules, greatly increasing WBC and OBC. Sonication that is too intense or prolonged, however, may reduce these abilities. BSF proteins treated with ultrasound showed enhanced FC, FS, EA, and ES. Optimal conditions, such as 200 W for 40 min (foaming) and 250 W for 10-20 min (emulsification), maximized these properties. Excessive treatment, however, could lead to protein aggregation and reduce functionality. Pearson's correlation matrix revealed how ultrasound treatment influences the functional properties of BSF larvae protein isolates, demonstrating how ultrasound treatment optimizes protein functionality through structural modifications.

FTIR and CD spectroscopy revealed significant shifts in BSF protein structure due to ultrasound treatment, particularly a reduction in α -helix content and an increase in β -sheet and random coil structures. These changes are indicative of hydrogen bond disruption and structural reassembly, aligning with patterns observed in other insect and plant proteins. XRD analysis showed no significant changes in crystal size or crystallinity, even with extended sonication times. Similarly, SDS-PAGE results indicated that ultrasound did not break down high molecular weight proteins into smaller fragments. As a result, there was no observable effect on protein solubility or reaggregation. DSC analysis showed a decrease in the heat enthalpy of BSF proteins after ultrasound treatment, indicating a reduction in thermal stability, which could be advantageous in specific food processing applications. Ultrasound treatment changed the microstructure of BSF proteins, according to SEM imaging. More homogeneous structures were produced by higher ultrasonic power, and protein aggregation from extended treatment improved solubility and emulsification even more.

A promising technique for enhancing the structural and functional characteristics of BSF larval proteins is ultrasound-assisted extraction. Researchers can use these proteins' enhanced solubility, emulsification, foaming, and binding capabilities to customise them for particular industrial uses, like in food formulations, by carefully modifying ultrasound parameters. Although more research is required to optimise these conditions for large-scale production, the technology's scalability and precise control make it suitable for commercial use. Additionally, the growing consumer demand for "clean labelling," where minimal processing and natural methods are preferred over conventional chemical treatments, is in line with the use of ultrasound as a non-thermal processing technique. According to this study,

ultrasound has the potential to be a very useful tool in the food industry, providing new ways to formulate products and add protein ingredients to satisfy particular dietary and functional needs.

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CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

“It’s time to break the taboo and embrace insects as a delicious and sustainable food source. Entomophagy is redefining culinary boundaries and paving the way for a more resilient food future.”

(Arnold van Huis, 2024)

7.1. General summary and conclusions

This PhD work examined the extraction, characterization, and enhancement of BSF proteins through varied extraction methods, focusing on their functional, structural, and physicochemical properties. The general objective was to purify native BSF larval proteins and evaluate their potential for food industry applications. The increasing demand for sustainable alternative protein sources was addressed. Through exhaustive methods consisting of chemical, thermal, structural, and techno-functional analyses, this study yielded significant awareness into the potential of BSF larval proteins for innovative food formulations and expansive industrial applications. The following sections summarise the findings of each research chapter.

The first research chapter examined the purification of BSF larval proteins using various extraction methods: alkaline extraction and acid precipitation, salting-in at 1% NaCl, salting-out at 80% $(\text{NH}_4)_2\text{SO}_4$, and ultrafiltration at 3kDa for the removal of salts, and characterised their structural, physicochemical, and thermal properties. These methods significantly influenced protein yield, purity, and structural integrity. Protein extraction methods, particularly salting-out-assisted treatments, substantially alter the properties of BSF larval proteins. These methods enhance protein yield and induce changes in available amino acid composition, rendering them more suitable for potential food applications. The total sum of all the essential amino acids was 321.95 mg/g for AAF, 300.98 mg/g for AAFSI, 286.31 mg/g for AAFSO, and 281.84 mg/g for AAFSISO. These values meet the adult daily requirements established by the World Health Organization, which specify a minimum of 277 mg/g. Notably, the proteins obtained through salting-out exhibited higher thermal stability, as demonstrated by the TGA analyses. AAFSO and AAFSISO had the highest content of ordered protein structures and exhibited the largest ΔH values, measured at 141 J/g and 137 J/g, respectively. This improved thermal stability is attributed to the higher level of disulfide (SS) bonding, which may have contributed to the maintenance of folded conformations and reduced conformational entropy. FTIR analysis provided insights into the molecular composition and structural elements of proteins, indicating the presence of β -sheet structures and various functional groups. Zeta potential measurements also suggested increased electrostatic repulsion, contributing to greater protein solubility in the salting-out-treated samples.

The second research chapter focused on evaluating the techno-functional properties (solubility, emulsifying properties, foaming capacity, foam stability, and water- and oil-binding capacities, surface hydrophobicity and bulk density) of BSF proteins and how these properties are influenced by different salt-based extraction methods. The use of $(\text{NH}_4)_2\text{SO}_4$ as a salting-

out agent demonstrated a significantly positive impact on protein solubility, with increased solubility at pH 2, indicating its potential for application in acidic food systems. Additionally, a strong, positive correlation was noted between solubility and foam stability ($r = 0.85$, $p < 0.01$), as well as with foaming capacity ($r = 0.98$, $p < 0.001$) and water binding capacity ($r = 0.84$, $p < 0.01$). Additionally, a strong, positive correlation was observed between solubility and foam stability ($r = 0.85$, $p < 0.01$), as well as with foaming capacity ($r = 0.98$, $p < 0.001$) and water binding capacity ($r = 0.84$, $p < 0.01$). Additionally, the influences of salt treatment on surface hydrophobicity displayed lower hydrophobicity values with salting-out methods, affecting the functional properties of proteins. Bulk density, a critical characterization of protein products, varied among BSF larval isolates. Salting-in (NaCl) decreased bulk density, while salting-out ($(\text{NH}_4)_2\text{SO}_4$) increased it. The significance of salt treatment methods in optimizing protein extraction based on specific properties of interest is emphasised by this finding. The bulk density of AAF was 1.23 g/mL, while the salt-treated isolates had values of 0.99 g/mL (AAFSI), 1.15 g/mL (AAFSO), and 1.52 g/mL (AAFSISO), respectively. Based on the outcome of the first two research chapters, the AFSISO (salting in/out extraction) was pursued in the third research chapter.

In the third research chapter, the potential to enhance protein extraction efficiency and functionality was explored. The effects of ultrasound treatment on the structural and functional properties of BSF larval proteins using the salting-in/out extraction method were investigated. As the salting-in/out extraction method had previously been established as the most effective in isolating target proteins with high purity and yield, this method was favoured for its ability to selectively precipitate proteins based on their solubility, while sustaining their native structure and function. The protein solubility, surface hydrophobicity, emulsifying activity, and foaming capacity were considerably improved by ultrasound treatment. These properties were maximised by optimal conditions, such as 200 W for 40 min (foaming) and 250 W for 10-20 min (emulsification). Excessive treatment, however, could lead to protein aggregation and reduce functionality. When an ultrasonic power of 300 W was applied, the WBC and OBC of the BSF larvae reached maximum values of 6.12 g/g and 6.30 g/g, respectively, at the P30010 isolate. Techniques such as FTIR, CD spectroscopy, SEM, and XRD revealed key changes in protein structure, including shifts from α -helix to β -sheet structures and reductions in crystallinity. For example, long ultrasonic cavitation effects appeared to transform the globular structure into a mesh structure. The surfaces of samples treated at P200T10 exhibited a more integrated form, while those at P300T10 were full of holes, suggesting that higher ultrasonic

intensity results in smaller structures. These structural modifications enhance protein solubility, rendering the extracts more versatile for food processing applications. Pearson's correlation matrix revealed significant interrelationships between the functional properties of BSF larvae protein isolates under ultrasound treatment. WBC showed positive correlations with OBC ($r = 0.57$, $p < 0.05$) and EAI ($r = 0.67$, $p < 0.01$), suggesting enhanced water and oil-binding capabilities. Foaming properties ($r = 0.87$, $p < 0.001$) were strongly influenced by surface hydrophobicity, as were solubility ($r = 0.63$, $p < 0.01$) and emulsifying properties ($r = 0.67$, $p < 0.001$) to a lesser degree, indicating its critical role in determining the functional behavior of BSF protein isolates. Ultrasound-enhanced hydrophobic interactions improve emulsification as shown by OBC's strong correlation with EAI ($r=0.79$, $p < 0.001$).

In conclusion, the purification and characterization of native BSF larval proteins manifests their functionality and significant potential as an alternative protein source for diverse food applications. The use of ultrasound-assisted extraction has proven particularly effective. Compared to conventional extraction methods, key functional properties such as solubility, foaming capacity, and emulsification have been enhanced. These functional improvements further contributed to the structural changes induced by ultrasound, making BSF proteins highly suitable for industrial use. This study highlights the sustainable and nutritionally beneficial nature of BSF proteins and their auspicious application in the food and feed industries. By employing such advanced extraction techniques, the functionality of BSF proteins can be adapted to meet specific industry needs. The critical role of extraction methods in optimizing protein performance for various food formulations is emphasized in this study.

7.2. Novelty of the study

The novelty of this study is based in its extensive examination of BSF larval protein purification, characterization, and functional enhancement, offering significant progress for the food and biotechnology sectors. The research presents several groundbreaking features:

7.2.1 Purification of BSF proteins via diverse extraction methods

Salting-in, salting-out, ultrafiltration, and ultrasound-assisted extraction are introduced in this study as innovative methods to purify BSF larval proteins. This is the first study to use these techniques to study the BSF larvae proteins. In addition, the comparative analysis of these purification techniques is employed to identify how each method influences protein yield, purity, and quality. The optimization of protein extraction for specific applications is provided

in this multi-method approach. Examples are the improvement of amino acid profiles and the attainment of high-purity protein isolates that are more suitable for food systems.

7.2.2 Elucidation of protein structure and composition

In this study, advanced structural analyses, including Fourier Transform Infrared (FTIR) Spectroscopy, Circular Dichroism (CD) Spectroscopy, X-ray Diffraction (XRD), and Scanning Electron Microscopy (SEM), are employed to examine protein structure at the molecular level. Changes resulting from various extraction methods and ultrasound treatment would be observed in protein secondary structures, decreased crystallinity, and microstructural alterations. This detailed structural elucidation provides novel insights into how these alterations improve protein solubility, emulsification, and thermal stability, enhancing their functionality in food formulations.

7.2.3 Use of ultrasound-assisted extraction to enhance protein functionality.

A key innovation is the application of ultrasound to improve BSF protein functional properties. The resultant benefits of this treatment were not only a boost in solubility and an increase in the lightness of colour (improving visual appeal for food use) but also an improvement to the amino acid profile and breakdown of protein aggregates. These enhancements led to improved water- and oil-binding capacities. This cutting-edge protein extraction and processing approach offers a scalable and environmentally friendly method for obtaining highly functional protein isolates.

In summary, the study's novelty stems from the use of advanced extraction techniques and ultrasound technology in its holistic approach to enhancing functional properties. This objective was continuously referenced in the elucidation of the BSF larval protein purification. These findings suggest innovative applications for BSF proteins in food and biotechnology. A sustainable, nutritionally valuable, and functional alternative is presented as an alternative to conventional protein sources.

7.3. Future Studies and Recommendations

While this study has provided a comprehensive understanding of BSF larval proteins, further exploration is required to fully realize their full potential in a range of applications:

7.3.1 Optimization of extraction techniques: The combination of the various techniques, that is, salting-in/out, ultrafiltration, and ultrasound-assisted extraction, should focus on

optimising for maximum protein yield, purity, and functional properties. Regulating these methods for augmentation in commercial settings is essential for endorsement in the food industry. Selection of the optimal extraction technique to align with desired techno-functional properties is a possible approach to achieve functionality for specific applications. Additionally, future studies must include the evaluation of the environmental impact of extraction processes, including energy use, water consumption, and waste production. The cost of production per kilogram should also be assessed. A comparison to existing protein sources, both plant- and animal-derived, should ensure competitive pricing and market feasibility.

7.3.2 Acceptance by food matrices: Future studies should assess the behaviour of BSF proteins incorporated into different food matrices, particularly in real-world food systems, such as bakery products, dairy alternatives, and meat equivalents. Acceptance studies for sensory and by consumers are an essential measure for successful market integration. The formulation of stable, nutritious products that meet consumer preferences can be promoted only once the interaction of BSF proteins with other food ingredients is analysed and fully understood. Furthermore, the sustainability and cost-effectiveness of incorporating BSF proteins into various food matrices should be analysed and judged. This will ensure that such applications can meet the growing demand for sustainable food products.

7.3.3 Nutritional and health impacts: This study focused on the functional and structural properties of BSF protein; however, the nutritional value, including digestibility, bioavailability of amino acids, and potential health benefits or risks associated with the consumption of insect-derived proteins, is required for health implications. Investigating the role of BSF proteins in addressing nutrient deficiencies, such as lysine deficiency in certain populations, would be highly relevant, especially in regions with limited access to traditional protein sources. Cost-effectiveness analysis should also be performed to compare the nutritional value of BSF proteins against conventional protein sources to highlight their potential for tackling global food security challenges at a lower environmental cost. Protein quality metrics should also be considered here or as part of future studies in line with standard dietary guidance. For protein, these guidelines remain based on the protein's essential amino acid profile and digestibility scores, thus are nutritionally and physiologically centric. Considering the shift toward plant-based protein and now insect-derived protein, the definition of protein quality is becoming increasingly short-sighted from a wider societal perspective.

Going forward, protein diversity, consumption, and environmental outcomes must be considered simultaneously for a sustainable future.

7.3.4 Comparative proteomes across taxonomic groups: Future research should focus on conducting comparative proteomic analyses across different insect taxonomic groups to uncover species-specific adaptations and conserved protein profiles. This comparative approach can reveal how ecological niches, feeding habits, and evolutionary traits shape the proteomes of various insects, particularly those used for food and feed. Understanding these differences could aid in the selection process of species with optimal nutritional and functional properties for industrial applications. Additionally, as insects from a variety of habitats manifest unique adaptations, it is recommended to explore their proteomic diversity, such as aquatic (*Hermetia illucens*) and terrestrial (*Acheta domesticus*) environments, for a better understanding. Proteins linked to specific ecological or functional features should be identified in order that researchers can refine species preference for targeted applications.

7.3.5 Life stage variations in proteomic profiles: The determination of the optimal life stage for harvesting, although based on nutritional value and functional properties, is a priority in the proteomic profiling of insects across their developmental stages (e.g., larva, pupa, adult). For example, larvae often possess higher reserves of proteins and lipids compared to adults, whose structural proteins are associated with mobility and reproduction. The identification of the maximum yield and functionality harvest points is related to the variations in the studied life stages. Post-translational modifications (PTMs) can influence protein solubility, digestibility, and functionality, making their study essential for optimizing insect proteins for food applications. The impact of PTMs, such as glycosylation and phosphorylation, across developmental stages requires further research. The combination of proteomic data with functional testing will enable the development of stage-specific insect-derived ingredients processed for different food matrices. In addressing these areas in the future, research will enhance our knowledge of insect biology and contribute to the development of sustainable and efficient insect protein production systems. The outcome will meet the growing global demand for alternative protein sources.

7.3.6 Regulatory and safety considerations: Regulatory approval and safety assessments must be conducted to ensure that these insect-derived proteins are safe for consumption as they enter the food chain. It is recommended that the focus is expanded to allergenicity, toxicity,

and microbiological safety. These studies require the involvement of inter- and transdisciplinary teams to achieve regulatory clearance and impact on the marketability of the protein. Furthermore, the comprehensive cost analysis must include the safety and regulatory requirements in the comparative study between traditional protein sources and insect-based protein production. The waste source of insect consumption determines the microbiome of the larvae, essential in the efficiency of biomass breakdown and metabolism, which indirectly influences the protein yield, quality, and safety considerations.

7.4. Final statement

The research executed in this study will enhance the relevance and commercial potential of BSF larvae proteins. The findings contribute to the development of a sustainable protein source in response to the objective of meeting global food security challenges.

APPENDICES

APPENDIX A

Appendix 1A Correlation matrix between the black soldier fly larvae protein isolates functional properties via Pearson's linear correlation analysis.

	WBC	OBC	FC	FS	EAI	ESI	Sol. pH 2	Sol. pH 4	Sol. pH 6	Sol. pH 8	Sol. pH 10	Surface hydro	Bulk density
WBC	1.00***												
OBC	-0.47	1.00***											
FC	0.89**	-0.52	1.00***										
FS	0.66	-0.18	0.89	1.00***									
EAI	0.91**	-0.66**	0.98	0.79*	1.00***								
ESI	-0.42	0.90	-0.61	-0.40	-0.72	1.00***							
Sol. pH 2	0.50	0.40	0.21	0.21	0.14	0.56	1.00***						
Sol. pH 4	0.67	-0.76**	0.88	0.74*	0.92**	-0.90**	-0.25	1.00***					
Sol. pH 6	0.85**	-0.77***	0.93	0.72*	0.98***	-0.82*	-0.01	0.96***	1.00***				
Sol. pH 8	0.84**	-0.64***	0.98	0.85**	0.98***	-0.75*	0.04	0.95***	0.97***	1.00***			
Sol. pH 10	-0.16	-0.07	0.28	0.53	0.20	-0.42	-0.58	0.48	0.24	0.35	1.00***		
Surface hydro	-0.82**	0.74***	-0.95	-0.76*	-0.98***	0.83*	0.05	-0.98***	-0.99***	-0.99***	-0.32	1.00***	
Bulk density	0.91**	-0.48	0.65	0.31	0.73*	-0.30	0.52	0.45	0.68	0.60	-0.46	-0.62	1.00***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; bold indicates the negative correlation; WBC – water binding capacity, OBC – oil binding capacity, FC – foaming capacity, FS – foam stability, EAI – emulsion activity index, ESI – emulsion stability index, Sol – solubility, Hydro - hydrophobicity

APPENDIX B

Appendix 2B. Effect of ultrasound on the solubility properties of black soldier fly larvae protein isolates. Error bars show standard deviation (*p < 0.05; **p < 0.01; ***p < 0.001).

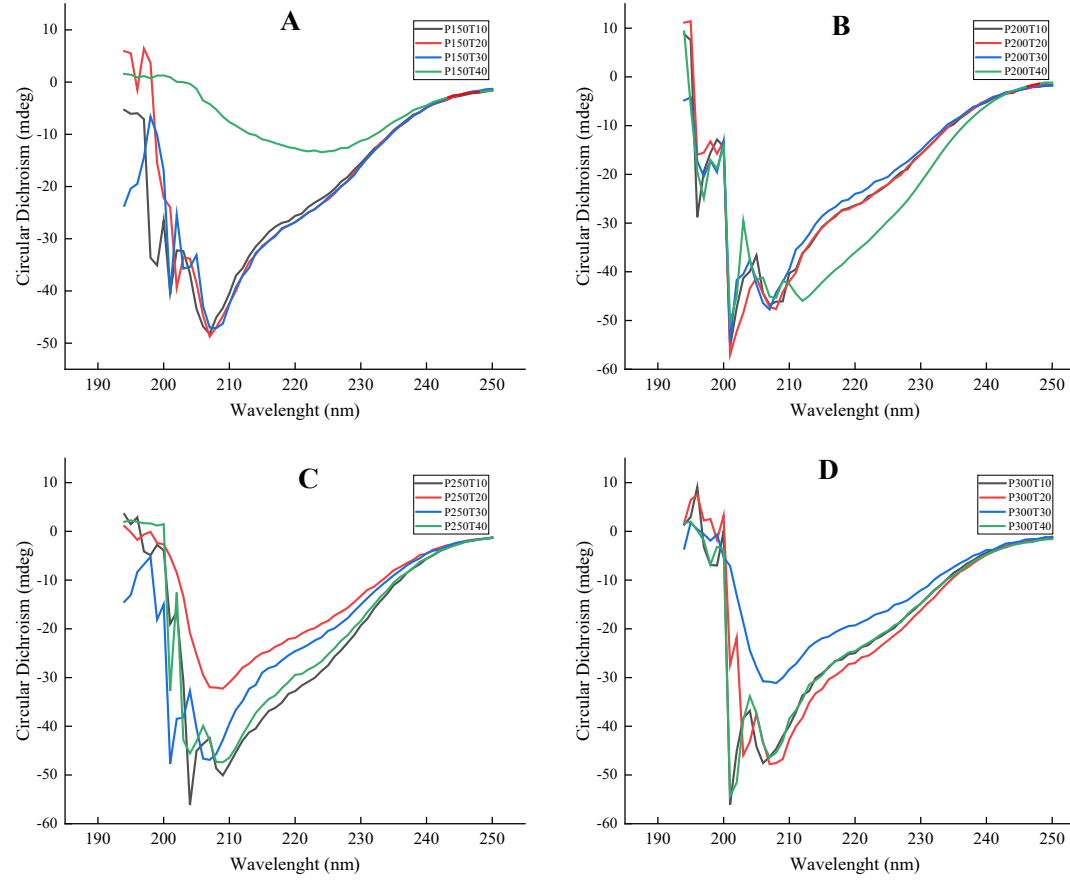
Treatment power	Treatment time	Solubility at pH 8
P0	0	71.76 ± 0.34 ^d
P150	10	75.00 ± 7.06 ^d
	20	85.60 ± 0.14 ^{fg}
	30	86.59 ± 0.12 ^{fg}
	40	87.95 ± 0.07 ^{fg}
P200	10	84.14 ± 0.59 ^{ef}
	20	88.12 ± 0.63 ^{fg}
	30	89.18 ± 0.86 ^g
	40	72.74 ± 0.06 ^d
P250	10	85.77 ± 0.70 ^{fg}
	20	81.11 ± 0.16 ^c
	30	59.49 ± 0.71 ^b
	40	56.04 ± 0.45 ^b
P300	10	72.62 ± 0.07 ^d
	20	65.39 ± 1.97 ^c
	30	56.07 ± 0.40 ^b
	40	45.84 ± 0.71 ^a

APPENDIX C

Appendix 3C. Techno functional properties values of the black soldier fly larvae protein extracts. Values are means $\pm$ standard deviations (n = 3).

Treatments	WBC (g/g)	OBC (g/g)	FC (%)	FS (%)	EAI (m ² /g)	ESI (%)	Exposed SH (μ M/g)	SS Bond (μ M/g)
P0T0	4.45 $\pm$ 0.03	2.16 $\pm$ 0.03	55.50 $\pm$ 0.50	44.00 $\pm$ 1.00	32.50 $\pm$ 0.50	62.28 $\pm$ 0.73	8.17 $\pm$ 0.03	3.24 $\pm$ 0.01
P150T10	1.89 $\pm$ 0.01	2.13 $\pm$ 0.02	56.04 $\pm$ 0.70	64.82 $\pm$ 0.27	38.28 $\pm$ 0.28	63.91 $\pm$ 0.27	7.73 $\pm$ 0.11	6.23 $\pm$ 0.74
P150T20	1.40 $\pm$ 0.05	3.08 $\pm$ 0.07	72.28 $\pm$ 0.28	78.68 $\pm$ 0.34	54.10 $\pm$ 0.14	66.68 $\pm$ 0.04	7.56 $\pm$ 0.01	7.75 $\pm$ 0.01
P150T30	1.62 $\pm$ 0.03	5.16 $\pm$ 0.02	82.06 $\pm$ 0.45	83.56 $\pm$ 0.12	58.23 $\pm$ 0.34	75.27 $\pm$ 0.28	7.13 $\pm$ 0.06	8.62 $\pm$ 0.15
P150T40	2.34 $\pm$ 0.23	5.57 $\pm$ 0.02	87.74 $\pm$ 0.29	90.06 $\pm$ 0.77	72.44 $\pm$ 0.55	78.78 $\pm$ 0.21	6.76 $\pm$ 0.05	8.97 $\pm$ 0.05
P200T10	1.84 $\pm$ 0.15	2.17 $\pm$ 0.06	52.17 $\pm$ 0.29	52.45 $\pm$ 0.56	44.56 $\pm$ 1.00	58.41 $\pm$ 0.34	7.73 $\pm$ 0.11	6.51 $\pm$ 0.74
P200T20	2.08 $\pm$ 0.03	1.79 $\pm$ 0.04	77.31 $\pm$ 0.03	77.43 $\pm$ 0.13	58.22 $\pm$ 0.23	66.22 $\pm$ 0.34	6.76 $\pm$ 0.01	7.35 $\pm$ 0.01
P200T30	2.63 $\pm$ 0.01	3.40 $\pm$ 0.15	82.96 $\pm$ 0.49	90.55 $\pm$ 0.51	70.09 $\pm$ 0.61	73.99 $\pm$ 0.54	7.00 $\pm$ 0.06	6.72 $\pm$ 0.15
P200T40	2.84 $\pm$ 0.01	6.00 $\pm$ 0.36	98.03 $\pm$ 0.40	99.33 $\pm$ 0.38	83.16 $\pm$ 0.17	87.28 $\pm$ 0.50	5.62 $\pm$ 0.05	6.50 $\pm$ 0.05
P250T10	4.28 $\pm$ 0.05	5.30 $\pm$ 0.05	62.01 $\pm$ 0.34	68.22 $\pm$ 0.35	82.22 $\pm$ 0.23	90.55 $\pm$ 0.72	7.74 $\pm$ 0.09	6.01 $\pm$ 0.74
P250T20	4.71 $\pm$ 0.06	6.12 $\pm$ 0.14	60.62 $\pm$ 0.84	65.08 $\pm$ 0.02	88.38 $\pm$ 0.40	88.38 $\pm$ 0.49	6.14 $\pm$ 0.11	6.55 $\pm$ 0.01
P250T30	5.40 $\pm$ 0.06	5.33 $\pm$ 0.01	54.61 $\pm$ 0.83	55.86 $\pm$ 0.08	72.51 $\pm$ 0.18	56.90 $\pm$ 0.35	6.14 $\pm$ 0.11	5.65 $\pm$ 0.05
P250T40	6.40 $\pm$ 0.08	5.09 $\pm$ 0.10	52.58 $\pm$ 0.11	44.78 $\pm$ 0.42	68.01 $\pm$ 0.56	48.07 $\pm$ 0.49	4.66 $\pm$ 0.11	4.66 $\pm$ 0.11
P300T10	5.23 $\pm$ 0.03	6.30 $\pm$ 0.07	72.88 $\pm$ 0.35	60.44 $\pm$ 0.55	70.01 $\pm$ 0.56	62.34 $\pm$ 0.55	8.50 $\pm$ 0.15	12.73 $\pm$ 0.28
P300T20	5.34 $\pm$ 0.05	4.22 $\pm$ 0.22	72.06 $\pm$ 0.17	52.43 $\pm$ 0.36	81.11 $\pm$ 0.12	54.35 $\pm$ 0.12	6.29 $\pm$ 0.06	11.06 $\pm$ 0.50
P300T30	4.17 $\pm$ 0.06	3.33 $\pm$ 0.03	42.38 $\pm$ 0.08	52.27 $\pm$ 0.20	67.90 $\pm$ 0.45	50.10 $\pm$ 0.56	6.29 $\pm$ 0.06	8.55 $\pm$ 0.01
P300T40	3.51 $\pm$ 0.06	3.38 $\pm$ 0.08	39.60 $\pm$ 0.61	39.57 $\pm$ 0.52	64.78 $\pm$ 0.22	45.15 $\pm$ 0.58	5.14 $\pm$ 0.01	7.90 $\pm$ 0.11

APPENDIX D



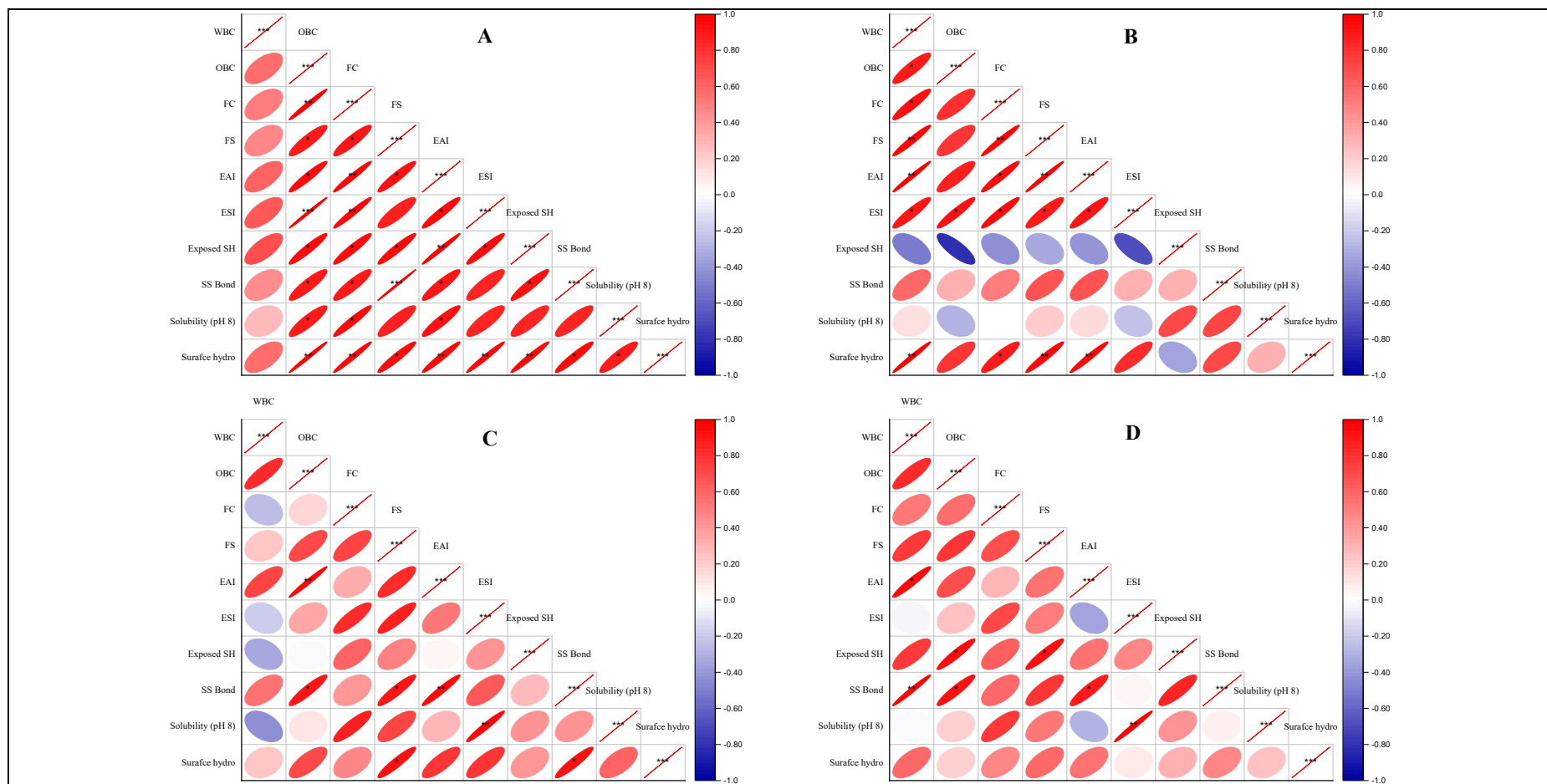
Appendix 4D. Effect of high-intensity ultrasound on the circular dichroism of BSF larvae proteins: A – P150, B – P200, C – P250, and D – P300. Line colours correspond to ultrasound duration: black (10 min), red (20 min), blue (30 min), and green (40 min).

APPENDIX E

Appendix 5E. Thermal properties of the black soldier fly larvae protein isolates. Values are means $\pm$ standard deviations (n = 3).

Test parameters	Peak 1		Peak 2	
	T _{max} (°C)	ΔH (J/g)	T _{max} (°C)	ΔH (J/g)
P0T0	61.75 $\pm$ 2.14 ^c	166.585 $\pm$ 4.25 ^l	280.95 $\pm$ 0.52 ^{bcd}	993.89 $\pm$ 2.45 ^p
P150T10	58.06 $\pm$ 0.01 ^b	55.315 $\pm$ 0.62 ^b	280.76 $\pm$ 0.72 ^{bcd}	391.55 $\pm$ 1.97 ^a
P150T20	59.54 $\pm$ 0.02 ^c	181.33 $\pm$ 1.47 ^h	281.88 $\pm$ 0.80 ^{def}	547.62 $\pm$ 2.31 ^c
P150T30	61.97 $\pm$ 0.07 ^{ef}	109.7 $\pm$ 0.78 ⁱ	279.86 $\pm$ 0.55 ^{ab}	912.71 $\pm$ 0.81 ^o
P150T40	57.99 $\pm$ 0.37 ^b	156.70 $\pm$ 0.78 ^k	280.03 $\pm$ 0.32 ^{ab}	559.86 $\pm$ 0.56 ^d
P200T10	59.76 $\pm$ 0.70 ^{cd}	111.57 $\pm$ 0.76 ⁱ	280.60 $\pm$ 0.74 ^{bcd}	707.88 $\pm$ 0.74 ^l
P200T20	53.30 $\pm$ 0.91 ^a	100.11 $\pm$ 0.20 ^g	281.23 $\pm$ 0.25 ^{bcde}	491.50 $\pm$ 1.53 ^b
P200T30	54.55 $\pm$ 0.08 ^a	36.21 $\pm$ 0.44 ^a	278.82 $\pm$ 1.00 ^a	631.23 $\pm$ 1.17 ^g
P200T40	58.36 $\pm$ 0.23 ^b	174.08 $\pm$ 0.81 ^m	280.69 $\pm$ 0.84 ^{bcd}	678.17 $\pm$ 0.59 ^j
P250T10	61.12 $\pm$ 0.14 ^{de}	72.125 $\pm$ 0.64 ^d	280.36 $\pm$ 0.33 ^{bc}	613.84 $\pm$ 2.47 ^f
P250T20	61.24 $\pm$ 0.01 ^c	69.35 $\pm$ 0.23 ^c	281.96 $\pm$ 0.10 ^{def}	608.28 $\pm$ 0.97 ^e
P250T30	64.24 $\pm$ 0.42 ^{gh}	173.87 $\pm$ 0.54 ^m	280.54 $\pm$ 0.72 ^{bcd}	640.68 $\pm$ 0.57 ^h
P250T40	65.12 $\pm$ 0.05 ^h	90.00 $\pm$ 0.36 ^e	282.67 $\pm$ 0.49 ^{ef}	672.66 $\pm$ 0.54 ⁱ
P300T10	64.10 $\pm$ 0.07 ^{gh}	104.17 $\pm$ 0.49 ^h	282.85 $\pm$ 0.24 ^f	887.81 $\pm$ 0.66 ⁿ
P300T20	62.78 $\pm$ 0.35 ^{fg}	109.08 $\pm$ 0.71 ⁱ	279.75 $\pm$ 0.71 ^{ab}	696.09 $\pm$ 0.23 ^k
P300T30	54.72 $\pm$ 0.47 ^a	95.025 $\pm$ 0.25 ^f	281.63 $\pm$ 0.11 ^{cdef}	708.95 $\pm$ 0.44 ^l
P300T40	63.70 $\pm$ 0.78 ^{gh}	131.535 $\pm$ 2.10 ^j	281.56 $\pm$ 0.05 ^{cdef}	787.26 $\pm$ 1.97 ^m

APPENDIX F



Appendix 6F. Correlation matrix between the black soldier fly larvae protein isolates (A - P150, B – P200, C – P250 and D – P300) functional properties via Pearson's linear correlation analysis; the colour gradation and the size of the circle represent the correlation coefficient (r value). Significance levels: *p < 0.05 (moderate significance), **p < 0.01 (higher significance), ***p < 0.001 (highest significance), blue (represents negative correlation) red (represents positive correlation).

