UNIVERSIDADE FEDERAL DE VIÇOSA

LUCAS SALES QUEIROZ

EXPLORATORY STUDY OF COLLOIDAL PROPERTIES OF BLACK SOLDIER FLY (*Hermetia illucens*) LARVAE PROTEINS OPTIMIZED BY SUSTAINABLE APPROCHES

VIÇOSA - MINAS GERAIS
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Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*

Advisor: Italo Tuler Perrone

Co-advisors: Antônio Fernandes de Carvalho
Federico Casanova

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Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

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2022

Queiroz, Lucas Sales, 1991-
Exploratory study of colloidal properties of black soldier fly (*Hermetia illucens*) larvae proteins optimized by sustainable approches / Lucas Sales Queiroz. - Viçosa, MG, 2022.
1 tese eletrônica (175 f.): il. (algumas color.).

Orientador: Ítalo Tuler Perrone.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, 2022.
Referências bibliográficas: f. 153-175.
DOI: https://doi.org/10.47328/ufvbbt.2022.172
Modo de acesso: World Wide Web.

1. *Hermetia illucens*. 2. Insetos comestíveis - Teor proteico. 3. Proteínas na nutrição animal. 4. Alimentos funcionais. 5. Sustentabilidade. I. Perrone, Ítalo Tuler. II. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

CDD 22. ed. 664.66

Bibliotecário(a) responsável: Alice Regina Pinto CRB6 2523

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APPROVED: March 29th, 2022

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ACKNOWLEDGEMENTS

This thesis is a substantiation of hypothesis and ideas that finally turned into reality.

First to GOD, for providing me health and wisdom.

To my family, my biggest gratitude for their unparalleled love and support.

To Priscila de Lima Paula, you supported me with patience and motivation.

To professor Antônio Fernandes de Carvalho who always believed on his students generating all the necessary tools for us to fight for our goals.

To Federico Casanova for teaching me how to do science from a new perspective, for sharing your knowledge and your office! Thank you for all your help and friendship.

To professor Italo Tuler Perrone for accepting me as a PhD student and giving all the green light for this ambitious and challenging project.

Special Thanks to Ipek, (Pana) Giota, Efthymios, Angelique, Gustavo and Paulin.

To all professors, technicians and students that directly or indirectly worked in this project.

To Federal University of Viçosa (UFV) and Technical University of Denmark (DTU) as well as all the partners, specially nextProtein company.

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for granting the scholarship.

To the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), for granting the scholarship.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), to granting the scholarship.

For millions of years, mankind lived just like the animals. Then something happened which unleashed the power of our imagination. We learned to talk… (*Stephen Hawking*)

ABSTRACT

QUEIROZ, Lucas Sales, D.Sc., Universidade Federal de Viçosa, March, 2022. **Exploratory Study of Colloidal Properties of Black Soldier Fly (*Hermetia illucens*) Larvae Proteins Optimized by Sustainable Approches**. Advisor: Italo Tuler Perroni. Co-advisors: Antonio Fernandes de Carvalho and Federico Casanova.

The black soldier fly larvae (BSFL), *Hermetia illucens* (Linnaeus), has been largely utilized for animal feed. Due to its interesting composition, BSFL has shown great potential to be further implemented in the human diet. Herein the main colloidal properties of BSFL protein extract were investigated, gelling, foaming and emulsifying ability were deeply studied. To improve knowledge on protein profile SDS-page electrophoresis, surface hydrophobicity, particle size and ζ-potential Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) and other important methodologies were utilized. Interfacial rheology, shear and dilatational, were evaluated to understand the role of BSFLP when used as surfactant in oil-in-water emulsions. In addition, the physical and oxidative stability of fish oil-in-water (O/W) emulsions were investigated using BSFL protein extract as an emulsifier. Alternative treatment such as ohmic-heating and sonication were applied to verify their impact on protein extraction and functionality. Finally, a study evaluating protein hydrolysis on emulsifying property of BSFL was considered. As a result, the best foam stability was reached at 85 °C with 15 min of treatment. About the gelling behavior, 15 min of ultrasound was able to improve gel-like structure and the gel strength. Ultrasound treatment induced modifications on physicochemical and functional properties of BSFL protein. In emulsion systems, ohmic heated samples had the most interesting physical stability, however, the oxidative stability was not improved under the explored parameters. Considering the emulsions stabilized with hydrolysed protein samples (BSFLPH), it was concluded that, limited hydrolysis had the best physical stability considering the TSI value for 2 days. Both BSFLPH and BSFLP demonstrated surfactant properties as they decreased interfacial tension and created a viscoelastic protective film.

Keywords: Black soldier fly. Techno-functionality. Protein extract. Sustainability.

RESUMO

QUEIROZ, Lucas Sales, D.Sc., Universidade Federal de Viçosa, março de 2022. **Estudo exploratório das propriedades coloidais das proteínas da larva mosca do soldado negro (*Hermetia illucens*) otimizado por abordagens sustentáveis**. Orientador: Italo Tuler Perroni. Coorientadores: Antonio Fernandes de Carvalho e Federico Casanova

A larva da mosca do soldado negro (BSFL), *Hermetia illucens* (Linneaus), é bastante utilizada na alimentação animal. Devido à sua composição nutricional, BSFL demonstrou ótimo potencial para ser implementada na dieta humana. As principais propriedades coloidais do extrato proteico da BSFL foram investigadas, como por exemplo, propriedades gelificantes, formação de espuma e as propriedades emulsificantes. Importantes metodologias de caracterização também foram utilizadas, tais como eletroforese com gel de SDS, hidrofobicidade de superfície, tamanho de partícula, potencial zeta, espectroscopia de infravermelho e calorimetria. Para entender o papel das proteínas como surfactante na interface de emulsões, técnicas reológicas foram avaliadas. As propriedades físicas e a estabilidade oxidativa de emulsões (O/W) contendo óleo de peixe e proteínas da BSFL como emulsificante, foram investigadas. Metodologias de aquecimento ôhmico e ultrassom foram aplicados para verificar seus impactos na eficiência extrativa e na funcionalidade de proteínas. Por fim, um estudo avaliando a hidrólise de proteínas sobre a propriedade emulsificante da BSFL foi considerado. Como resultado, a melhor estabilidade da espuma foi alcançada na temperatura de 85 °C com 15 min de tratamento. O tratamento com ultrassom por 15 min foi capaz de melhorar a estrutura e a resistência do gel. As amostras submetidas ao tratamento ôhmico apresentaram a estabilidade física mais interessante, porém, a estabilidade oxidativa não foi melhorada sob os parâmetros explorados. Considerando as emulsões estabilizadas com amostras de proteína hidrolisada (BSFLPH), concluiu-se que a hidrólise limitada apresentou a melhor estabilidade física durante 2 dias de experimento. Tanto o BSFLPH quanto o BSFL demonstraram propriedades surfactantes, pois diminuíram a tensão interfacial e criaram um filme protetor viscoelástico.

Palavras-chave: Mosca do soldado negro. Tecno-funcionalidade. Extrato proteico. Sustentabilidade

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SUMMARY

1. GENERAL INTRODUCTION

Several projections have suggested an expressive growth in human population for the next decades[1,2]. According to some authors this number may reach over 9 billion[3] or even more, around 11 billion people until 2050[4]. This fact means an increasing pressure on the earth`s resources for food and feed. The Food Agriculture Organization of the United States (FAO) states that in order to provide adequate nutrients for the increasing population, the global food production should increase around 70% by 2050[5]. This perspective demands agricultural and livestock growth which will increase deforestation, water consumption, and greenhouse gas emission. Therefore, in order to overcome these undesirable changes and reduce the negative environmental impact the usage of insects as an alternative source of proteins, lipids and minerals to be included in the human diet has garnered increasing interest over the past years.

About 2 billion of people in 113 countries, most of them in Central America and Asia, regularly consume insect as part of their cultural traditions and diet. In these countries nearly 1900 insect species are eaten and they are typically collected in the wild and sold in the open market or even some restaurants[6].Insect farming for food and feed purpose have significantly increased in Europe and South America which means that people from this region of the globe started noticing how important and promising this alternative source will be for the next generations with an environmental, nutritional and economic value [7]. Although there are strong arguments on nutritional and sustainable facts related to insect incorporation in human diet, people, mainly in the western culture, are more interested in a good eating experience and the general insect appearance generates a disgusting feeling that reduce consumers acceptance[8].

In order to alleviate these negative perceptions and keep the nutritional and sustainable interest over the insect consumption, different strategies and technologies must be applied. The idea is to incorporate insect ingredients such as proteins, lipids, minerals, and chitins as non-recognizable form such as insect flower, which, according to some studies, can be a potential gateway for wider acceptance by people in the West [9,10].Thus, the right preparation of this insect flour or protein concentrate extract is of great importance for the food industry.

Among the nutritional compounds of insects, proteins are highlighted as one of the most attracting and promising to be soon implemented in the human diet. Studies based on protein quality, protein content, nutritional value and the amino acid composition of insects have been largely stated in the literature[3,11,12]. Zielinska, [13], have stated a protein content of 70%, 52.35% and, 76% of dry matter for *Grylloids sigillatus*, *Tenebrio molitor*, and *Schistocerca gregaria* respectively. Queiroz, 2021[11] have reported a study on nutritional and functional characterization of black soldier fly larvae (BSFL) and the protein content was equal to 61% of dry matter.

Hermetia illucens Linnaeus 1758 (Diptera: Stratiomyidae), popularly known as black soldier fly (BSF), has been considered as a possible commercial source of proteins that contains all the essential amino acids (EAA) for human consumption[11]. The presence of specific enzymes in their gut makes it possible to feed on a variety of substrates and then convert it into high quality proteins[14]. Recent studies reported the capacity of BSF to feed on abattoir waste, spent grains, restaurant organic waste and general waste from crop agriculture[15]. This ability unveils BSF as a crucial biotechnological tool for the near future which can possibly lower the cost of farming, reduce organic waste worldwide and finally address the expected nutritional quality in food.

Researchers have been focusing their attention on the larvae and prepupae stage of BSF, as this is the period when the insect feeds and accumulate biochemical compounds within the structural spaces between their organs where nutrients are stored for the latest non-feeding stage of their life cycle[16]. Therefore, changes in insect feed may generate different larvae biomass with inconsistent nutritional quality and content[17]. In addition, not only the nutritional aspect should be considered but also the food safety element as when reared on unknown organic waste the risks of uptalking higher number of heavy metals and presence of potential pathogenic microorganism increases. Overcoming this setback will be important in the future studies when considering a large-scale production of animal feed and further incorporation of insect into human diet.

In order to include insect as an ingredient into conventional food system, the functional properties of their proteins need to be fully understood. In this context, the term functionality refers to any property, except the nutritional ones, of food or food ingredient, that address its utilization, e.g., viscosity, water and oil holding capacity, solubility, emulsification, gelling and foam capacity[18]. Comprehending the functional properties of new ingredients can indicate the best application for their use in a food system and facilitate the replacement of conventional and non-sustainable functional food.

There are few studies over the literature exploring the functionalities of BSFL proteins. The protein charge, size and conformation are some of the features that can directly affect their functionality. Rabani et al.[19] have identified proteins with molecular weight of 75 and 50 KDa in BSFL samples, which has been linked to muscular proteins, exoskeleton proteins and enzymes in other insect species. Those muscular proteins might be interesting for the gelling properties and other functionalities[20]; besides they could possibly replace soy and meat protein as an alternative protein source. Conversely, it is necessary to consider that the protein extraction, defatting, drying, feed and killing methods applied to BSFL can provide a different protein profile which will result in changes of functionality[21]. A preliminary study on the functionality of BSFL flour and protein extract was performed by Buβler et al.[22], the authors evaluated the water and oil holding capacity, protein solubility and emulsifying capacity. The study elucidated the influence of high fat content in the BSFL samples over its functionalities. The study reported that the presence of fat negatively affected the BSLF functionality and when samples were defatted through different methods of extraction an improvement of protein functionalities was noticed.

BSFL protein extract demonstrated to have advantageous water and oil holding capacities. This ability of proteins to absorb and retain both water and oil is linked to the emulsifying properties which can indirectly affects the mouthfeel, taste and the texture of food[18]. The foam capacity and foamability of BSFL protein extract was also evaluated in another study where the authors previously heated the samples with different temperature, 75°C and 85°C for 15min, where the higher temperature revealed a more interesting foam stability. Combining all the data evaluated by the authors, including DLS and ζ-potential, it was suggested that the stability was related to the presence of a bigger protein net which could form a more stable and viscoelastic film on the air-water interface[11].The data is interesting as in general, the foaming capacity of insect proteins is normally found to be very poor, revealing unstable foams[18,23]. Conversely, more studies and data about BSFL protein interfacial activity is needed, in order to better elucidate how these proteins will behave when applied in food formulations such as foam and emulsions.The increasing trend of replacing synthetic and non-sustainable sources of emulsifiers with natural and more eco-friendly protein sources, has placed edible insects as a promising alternative source of proteins with a very low environmental footprint[24,25].

A recent study comparing BSFL protein concentrate with WPI at three different concentrations, 0.1%, 0.5% and 1.0%, as emulsifier, reported that at the lowest protein concentration BSFL revealed a significant higher emulsifying activity compared to WPI. When the concentration was raised to 0.5% BSFL protein concentrate was able to emulsify all oil fraction, whereas WPI only reached such result at concentration of 1.0%[26]. The reported functionality is dependent on protein surface, distribution of hydrophobic groups and molecular flexibility. Thus, some techniques can be considered as a pre-treatment aiming to cause protein structural changes and consequently improved techno-functionality. Ohmic heating (OH), microwave, ultrasound and pulsed electric field (PEF) are the main techniques applied to increase yield of protein extraction and protein functionality by causing structural changes[27–30].

Ohmic heating has been proved to influence unfolding and aggregation patterns of whey protein by changing the distribution of secondary protein structures[31]. The method has garnered attention from researchers because of its more uniform and faster heating rate when compared to conventional heating methods. In sum, the technique involves the passage of an alternating electrical current that will pass through the food, and according to Joule’s law the current and the resistance of food will generate an increase in temperature due to internal energy dissipation[32].

Queiroz et al.[24] have performed the first study concerning BSLF protein and its emulsifying property after OH treatment. The study evaluated the physical and the oxidative stability of oil-in-water emulsion stabilized by BSLF protein extract before and after ultrasound and Ohmic heating treatment. The authors noticed modification in the secondary structure of BSFL proteins after each treatment and the emulsions stabilized with BSLF protein pre-treated with OH had the best physical stability over 7 days of storage.

Another relevant technique that can be applied to increase the yield of protein extraction and may cause structural changes in proteins , is called Ultrasound. The technique works through a probe that emits a frequency of 20-100 KHz causing quick formation and collapse of gas bubbles during microseconds, a phenomenon that is named ultrasonic cavitation[33]. Even though there has been a lack of information correlating ultrasound treatment with saprophagous edible insects, a recent study aimed to describe the intrinsic changes in BSFL protein structure caused by sonic-treatment, fixed and sweep-frequency mode. Significantly increase in sulfurhydride value, dispersibility and ζ-potential were observed. A decrease in particle size and alterations in the secondary structure, mainly β-sheet and random coil, were also observed[34].

To date, gelation, which is another important functional property, has been detected in different insect protein fractions suggesting that those proteins have strong gelling capacity and can possibly be an alternative to conventional non-sustainable protein source used by the food industry[35].Until the present moment, there was no study reporting the gelling properties of BSFL proteins[18]. Although it can be assumed that due to its protein profile, BSFL would follow a similar outcome as other insect species, further studies and analysis must be performed to fully understand the specific gelling properties of BSFL protein fractions.

2. LITERATURE REVIEW

2.1 EDIBLE INSECT AS AN ALTERNATIVE PROTEIN SOURCE: A REVIEW ON THE CHEMISTRY AND FUNCTIONALITIES OF PROTEINS UNDER DIFFERENT PROCESSING METHODS

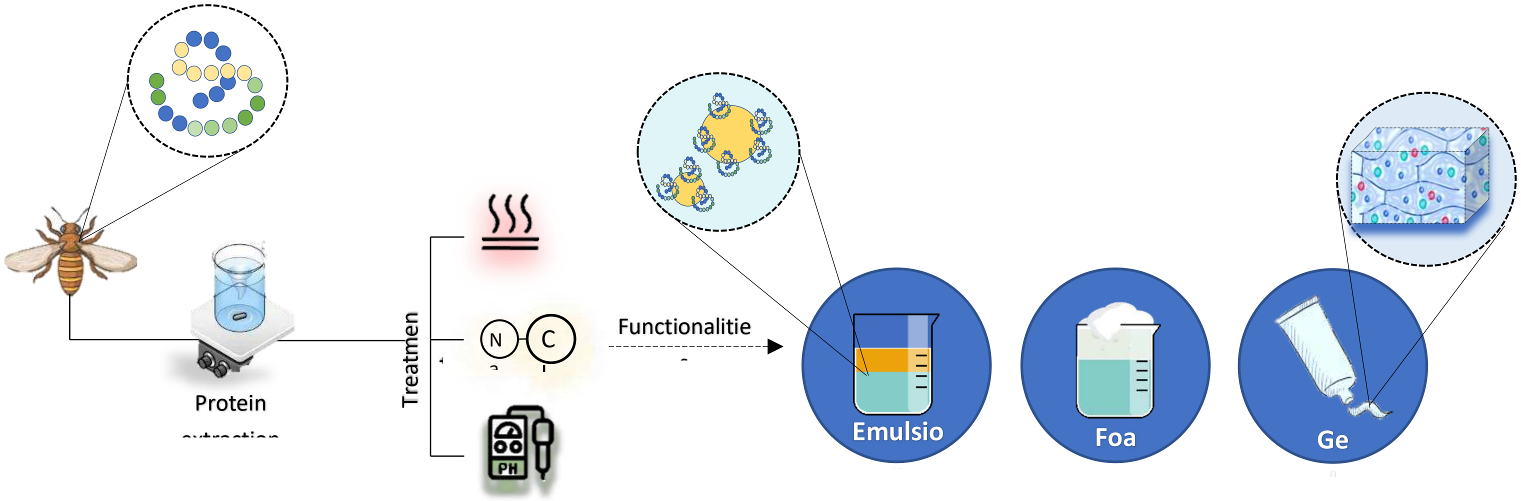
The content of this chapter will be submitted at:

Journal of Food Science

Lucas Sales Queiroz, Naaman Francisco Nogueira Silva, Flemming Jessen, Mohammad Amin Mohammadifar, Rodrigo Stephani, Antonio Fernandes de Carvalho, Ítalo Tuler Perrone, Federico Casanova*

This work has been performed in collaboration with Federal University of Juiz de For a, from department of pharmacy and department of chemistry; Research Group for Food Production Engineering, National Food Institute, Technical University of Denmark; Centro de Ciências da Natureza, Universidade Federal de Sao Carlos (UFSCar) and Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa (UFV)

Graphical abstract



2.1.1 Introduction

Sustainability has been one of the main topics discussed by all the United Nations Member since they adopted a resolution to “shift the world on to a sustainable and resilient path”. As a result of this discussions a plan was created “The 2030 Agenda for Sustainable Development”. The plan formulated the 17 Sustainable Development Goals (SDGs) which is an urgent shared action by either developing and developed countries that questions the way we explore our natural sources [36].The following three SDGs are closely interconnected and requires a great innovative solution to be achieved: zero hunger (SDG2) aims to reach greater food security and improved nutrition through a sustainable agriculture system; responsible consumption and production (SDG12) aims to reduce waste generation by recycling and reuse; and climate action (SDG13) which claims to combat climate changes and its consequences and impacts. All reported goals can be achieved if we start exploring new alternative and more sustainable food source.

Currently, the world’s population counts more than 7.8 billion of people and in 2050 this number is expected to reach the mark of 9-10 billion [37]. The increase in population and consequent food demand inevitably means agricultural and livestock growth which elevates deforestation, water consumption and greenhouse gas emission [38]. The number of resources needed to process and produce animal protein-enriched products, such as meat and milk, are not considered efficient since the feed conversion rate (FCR), kg of dry matter/ kg of edible weight, of beef is 25 and the FCR for whole milk is around 0.7 [39]. Even with an inefficient food system, the FAO (2011) projects that the consumption of livestock meat is expected to increase around 173% until 2050, this fact goes in the opposite direction of the expected SDGs making it an alarming problem for future generations. The reported numbers highlight the importance to adopt a more rebalanced and sustainable food system, focusing on biodiversity, food safety, and a more effective distribution of high-quality proteins for the world’s population [40,41].

Some examples of emergent and sustainable food source include vegetables (e.g wheat, chia, moringa, beans, lentils) microorganisms (e.g., fungi and microalgae), and insects (*Hermetia illucens*, *Tenebrio molitor*, etc). In the recent years plant-based products, including those protein-enriched, have gained great popularity mainly due to consumer’s growing interest in a more ecofriendly diet, as well as the rise of veganism and vegetarianism in western cultures [42]. The most common example is soy protein linked to the fabrication of simulated meats and tofu, since soy proteins present a good gelation property [43,44].

Nonetheless, proteins from plants have been found to lack certain essential amino acids, mainly leucine, methionine, and lysine [45](Gorissen, et al.,2018) and to show less digestibility, such as soy and wheat, compared to animal-based proteins, which makes it necessary to combine or blend various plant-based proteins to obtain a complete and nutritious diet [45]

Insects are invertebrate animals that belongs to the *Phylum Arthropoda*, their biomass represents 95% of the animal kingdom with an enormous biodiversity, however only a few studies have reported their application in food system either for the nutritional and technological aspects. Insects are a very interesting source of proteins with a nutritional quality comparable to that offered by cattle [46] with the advantage that insect farming presents a substantially smaller impact in the environment in terms of land, water usage, deforestation and greenhouse gases production [47]. Part of this advantage comes from the efficient feed to meat conversion ratio of insects, which in case of crickets, only 2.2kg of feed is required to produce 1Kg of edible insect, whereas for beef this value can be more than 10 times higher [46,48,49].

Some organizations have been evaluating the prospect of using insects as food source and feed. The Food and Agricultural Organization of the United Nations (FAO), have extensively studied and reported the advantages of consuming insects, considering the cultural, economics, safety, production, and nutritional aspects, such as the adequate amount of essential amino acids. In general, insect proteins meet all the requirements of the WHO for amino acid composition showing high values for phenylalanine + tyrosine and being also rich in tryptophane, lysine and threonine (Van Huis, 2016). The Orthoptera order is one of the most valuable alternative protein sources and most of the edible insect fulfill the required essential amino acids (EAAs) for human consumption [23,50].

They have been more commonly consumed in Asia, Africa and some countries in Latin America and although there is a negative cultural impact in the western world against the use of insect as protein food source, it has been part of the diet in many tropical and subtropical countries [51,52].

Although insect as human alternative food can bring all the reported advantages, consumers acceptance remains the largest barrier to be overcome, since entomophagy is constantly associated with a disgusting feeling [53,54]. Therefore, a good way to improve insect acceptance by consumers would be to process the whole insect as insect flour or protein extract powder.

The presence of legislations that supports the production and marketing of insects as human food, is another fact that can affect consumer's acceptance. Recently, *Tenebrio molitor, a* beetle of the Tenebrionidae family, has been authorized as the first insect as novel food for human consumption. An advantageous breakthrough done by the Commission Implementing Regulation 2021/882. This legislation opens new market opportunities and can increase people's interest on insect as food [55].

Beyond nutritional aspects, insect species can provide promising proteins to be further explored about their functionalities. For that, it is important to understand that the physicochemical properties of these proteins and their structure will play a fundamental role on their functionality and that those features can be modified by the processing parameters used before and during the protein extraction [47,56].

Recent studies have unveiled the technological potential of insect proteins by exploiting their solubility, gelling ability, foamability and emulsifying properties and also by characterizing the proteins according to the physicochemical properties [10,23,56–60].

Gravel & Doyen[43] have described a common 5-step food protein processing starting with pre-treatment, followed by defatting, protein solubilization and recovery, protein purification and finally drying process as the fifth step, which can be set at the beginning and at the end of the process. An extra additional step can be added at the begging, the insect farming and fasting period, as they might affect the future explored functionalities and insect protein content (Fig 1.). It is important to notice that all reported steps are optional and will depend on the starting material and the final purpose of the product.

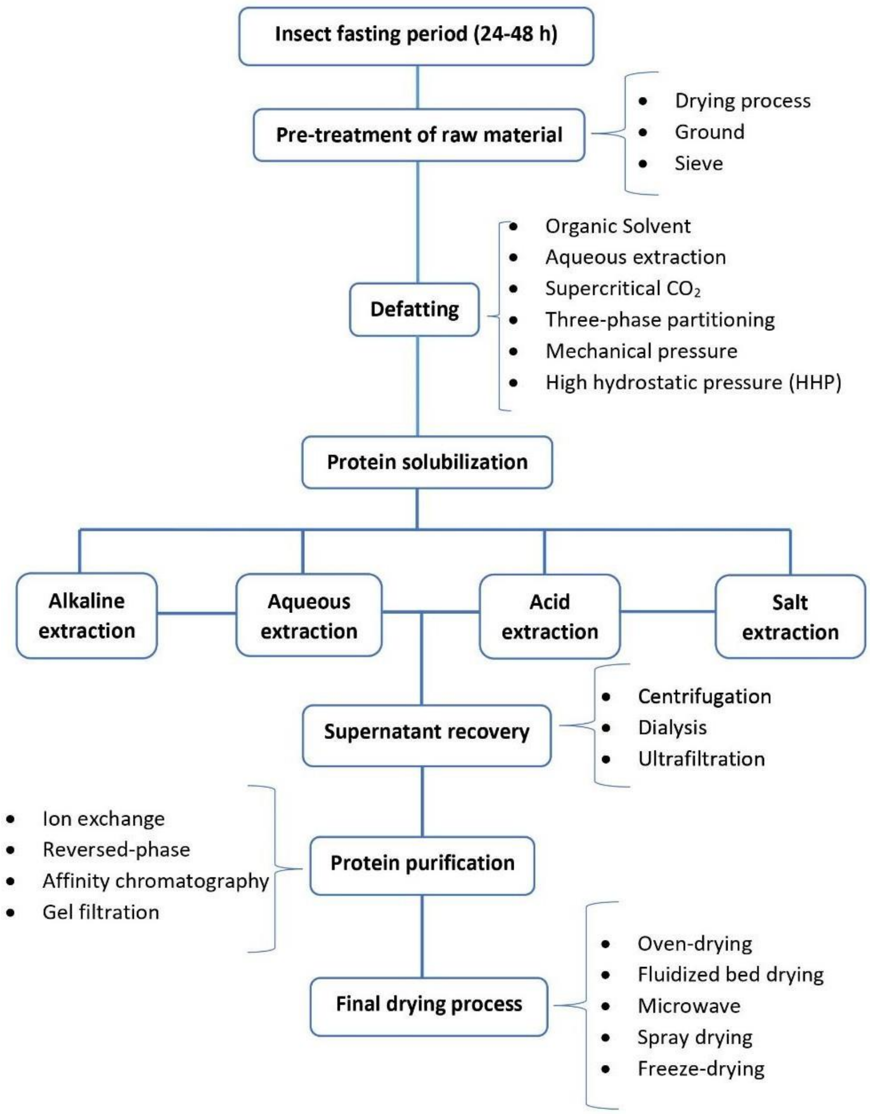


Figure 1- Flowchart of insect processing for protein extraction

To support the studies, this review aimed to highlight that insect may be an interesting protein-enriched source with promising technological attributes and, the importance of each methodology used to extract proteins and its impact on protein's quality and quantity after extraction. The focus was also to discuss how some pre-treatments such as salt addition, pH alteration and heat, when applied to protein matrix, can impact the yield of extraction and protein applicability in a food system.

2.1.1.1 Insect processing methods applied for protein extraction

2.1.1.1.2 Insect feed and fasting time

Black soldier fly larvae (BSFL) are considered as the insect converter of organic waste into edible biomass, and the composition of such material relies on the substrate. Spranghers et al. [61] have grown BSFL on four different substrates: chicken feed, vegetable waste, biogas, digestate, and restaurant waste. The authors reported that the total biomass of the harvested prepupae differed substantially among the four substrates, where the total prepupal biomass was highest for chicken feed and the lowest for biogas digested. In addition, a slow development of larvae feed on restaurant waste, was observed. Protein content varied from 399 to 431 g/Kg dry matter among the four feeds. Differences between amino acid composition was not significant but, ether extract (EE) and ash content significantly changed.

Since invertebrates are considered as farmed animals when industrially reared, the use of animal manure, catering waste or former foodstuffs containing fish and meat as feeding substrates are completely prohibited. Therefore, research performed by [62] evaluated the nutritional composition of BSF reared on vegetable mix only, 49 different substrates divided in three groups according to their seasonal availability, showed that protein content of BSF prepupae ranged between 35 and 49% DM, revealing the highest values in the Autumn group of substrates.

Considerable effort has been placed on the development of an optimized diet for insects that would provide a maximized biomass with highly nutritious larvae. A study performed by Rumbos et al.[63] A. Evaluated the usage of various commodities for the development and nutritional quality of *Tenebrio molitor*. The work stated that protein content of *T. molitor* fed on the different substrates tested ranged between 41 to 68% and the lipid content also fluctuated from 14 to 38%, and interestingly the study showed through a linear regression analysis that the protein content in the tested substrates did not show a strong correlation with the protein content of the larvae.

Insect fasting time is another parameter to be considered before handling insects to processing. This process is important as the number of contaminants, including vegetable proteins, can be reduced to a small trace and then not interfere in further analysis and protein composition (Zielińska et al., 2018). To avoid this contaminant Mishyna et al. [58], reared in a controlled environment, adult grasshopper (*S. gregaria*), larvae and pupae of honeybee (*Apis mellifera*) and before submitting the insect to protein extraction process the insects were fasted during at least 24 hours. A similar procedure was performed by Khampakool et al. [64] when larvae of *Protaetia brevitarsis* were fasted for 24h in order to clear any gastrointestinal feces and residuals.

2.1.1.1.3 Insect pre-treatment

As the first step of crude protein obtention, the raw material needs to be processed and this first treatment depends on the characteristics of the raw product. For example, legumes such as peas and soybeans are generally dried, when necessary, and dehulled, making the next grinding or milling process easier and efficient. This initial step can greatly increase protein content as the majority of fiber-rich parts are removed [43] as insects are also full of fibers, they can be directly compared to legumes in this context. Drying can increase the shelf-life of foods by reducing water content and therefore, its availability for degradative reactions [65].

Therefore, in the first step insects are usually dried, ground and finally sieved to produce a fine powder that provides larger surface area of contact with the solvents in the next stages [22,43,60,66]. The drying step can occur either in the beginning or at the last stage of protein isolation/purification process. A review performed by Melgar-Lalanne et al. [65] used oven-drying and showed how the parameters of some insects were altered using different temperatures, from 40 to 80 ℃ during 7 to 48 hours of treatment, or until a constant weight was attained. The Freeze-drying treatment is another common method used as an insect drying step. Through this process frozen specimen are dehydrated by sublimation and vacuum, with no significant changes in color, flavor, smells, and physical form [67]. These two methods are constantly compared in order to verify the influence of different drying methods over the chemical composition of insect [68].

The microwave drying technique is another common method that was reported for some insect as *Tenebrio molitor and Hermetia illucens* [69,70]. Even though fluidized bed is not the most common drying technique, it was also studied as an alternative drying process for insect flour production [70]. Spray drying has been largely used for protein solutions or suspensions, basically the technique requires proteins to be atomized into a small droplet and pass through a hot air feed that will dries the samples. Only few studies have documented this technique as insect pre-treatment [71]. A study on *T. molitor* has applied spray-drier to produce spray-dried mealworm powder, the method allowed the formation of a rounder shape powder with high homogeneity of particle size [72].

2.1.1.1.4 Insect powder defatting step

The process before protein extraction is the removal of lipid content. Different methods of defatting can yield protein extracts with different techno-functional properties. Hexane has been a commonly used solvent in defatting; however, it has become somehow unpopular due to negative environmental impact, safety issues and effects on protein functionality [73]. A work by Kim et al. [74] evaluated the effect of aqueous fat removal and defatting by organic solvents, such as hexane, methanol and ethanol, on the protein functionality of *Protaetia brevitarsis,* the defatting process was based on the method previously described by Yi et al. [23]. The experiment intended to understand how the two methods may affect the nutritional, color and the techno-functional aspects of insect proteins. The amino acid composition, protein solubility, and functional properties (foamability, and emulsifying capacity) had the most interesting result when proteins were defatted with hexane. In addition, Kim et al.[74] reported that ethanol extraction showed the lowest oil residue (0.13 ± 0.06 %) in the protein extract while aqueous extraction had the highest oil content (14.82 ± 0.16 %). Conversely, Ndiritu [75] studied *Acheta domesticus* and stated that the aqueous extract, with no hexane defatting step, was more interesting when the techno-functionality was the focus, and the hexane extraction is more desirable if the intention is to obtain acceptability and nutritional aspects with a greater protein content. In addition, the efficiency of defatting with aqueous extraction was compared to various solvent-based extraction methods for four insects and the authors stated that aqueous extraction gave the lowest oil yield of all reported techniques [76]. Three-phase partitioning (TPP) and the mechanical pressing are other fat removal methods reported in the literature, both are usually studied for legumes defatting, such as soybeans [43,77]. The TPP technique was originally used for protein purification, and the process consist of simultaneous addition of *t*-butanol and ammonium sulphate (w/v) to a slurry or crude preparation, where protein will form an interface between upper organic layer and the lower aqueous phase. However, different from protein, during the defatting process the oil phase goes to the upper layer (organic) which will be evaporated on a rotary evaporator to obtain the extracted oil [77].

An alternative method, called supercritical CO_2 (SC-CO_2) extraction has been used to defat silkworm chrysalis [78] and according to Purschke et al.[79] CO_2 method showed promising results giving 95% oil recovery from *T. molitor*. When compared to conventional solvent extraction, SC-CO_2 showed some advantages such as, reduced oxidation; possibility to extract thermal sensitive metabolites as the process works under low critical temperature; solvent-free residues; and the adequate properties as it is chemically inert, non-toxic, and non-explosive [79,80].

Moreover, another interesting technology has been documented in the literature for the same purpose, the high hydrostatic pressure (HHP), the method was recently applied for insect defatting for *Acheta domesticus and T. molitor* [81]. This method was already reported for metabolites extraction from plants, which causes air in the plant cell vacuoles to leak and damage the cell membrane allowing the contact with the extracting solvent (Sadus,1992). The method was compared to traditional hexane extraction and the results showed the influence of HHP process over the techno-functional properties of mealworm and cricket powders. The study concluded that application of high hydrostatic pressure (500 MPa), during 15 min, resulted in changes in the functional parameters but the data was interesting to enable HHP to be used as alternative method. However, further studies will be necessary mainly to verify its possible commercial use, comparing the operating costs with other conventional techniques [81].

Nongonierma & FitzGerald [82] reported that the organic extraction of lipids may imply protein losses, as some proteins that have more affinity to the solvent can be partitioned to the solvent phase being further removed. However, it is not commonly reported in the literature. Therefore, when a more sustainable and ecofriendly performance is seeking, keeping protein integrity and reduction of solvent usage, SC-CO_2 seems to be the gentlest extraction, using low temperatures which contributes to the conservation of native protein structure. However, it is still unclear which method will give the best oil removal, protein quality and protein content, since the result depends on the matrix studied and the final research interest. As there are not many studies stating that over the literature, each insect or derivative product need to be evaluated under the reported conditions of extraction.

2.1.1.1.5 supernatant recovery and extraction

Protein extraction is influenced by previous processing and can be carried out using a variety of methods. The extraction rate and features are also species dependent, and these parameters can influence the final functional properties of protein groups. In order to obtain protein from insect flours, protein solubility might be optimized by varying some parameters during the process.

The proteins are frequently separated from the lipids and other components by varying the pH and ionic strength of the media to control protein solubility. As the isoelectric point of most food matrices, including insects, are between pH 4 and 5, at high values of pH proteins surfaces are mostly charged and it promotes electrostatic repulsion between proteins and protein-water interaction, resulting in higher solubility [83,84]. Each insect species presents a different group of proteins then for protein precipitation/ extraction the specific isoelectric point (pI) needs to be reached by altering the pH in the medium, which is generally below pH 5.0 [10]. Some different pH range has been used for protein precipitation, i.e., around pH 4.0-5.0 for yellow mealworm proteins [66,85] pH 5.0 for silkworm chrysalis [78]. The pI can be determined for the whole group of proteins or even for specific protein fractions, as it was determined for *B. mori* proteins, at pH 2.5 (albumins), pH 2.7 (globulins), pH 4.0 (glutelins) and pH 4.5 (prolamins)[86].

Ionic strength is another parameter that might be modified to promote a better protein solubility and it can be alter by the addition of ions in the medium. At low concentrations of salt (NaCl), ionic strength of 0.5 - 1M, salt ions will act as a “shield” on the protein surface promoting higher protein-water interaction and water solubility, this method is also called “salting-in” [87]. As the charge and electrical repulsion favors protein solubility the addition of salt (ions) such NaCl may increase protein solubility in water at high pH and it can be used as a great strategy during protein extraction [85]. Studying the influence of NaCl concentration upon *T. molitor* protein extraction Yi et al.[85] found that at pH 8.0 the proportion of water-soluble proteins raised from 59.2% to 94.7% (w/w) with 0.1 and 1.0 M NaCl, respectively. The non-water-soluble proteins can be found concentrated in the pellets. Yi [88] have shown by LC-MS/MS, that pellets from aqueous extraction mainly contained muscle proteins, i.e., actin, myosin, tropomyosin and troponin

The most common method for insect protein solubilization is based on alkaline addition followed by acid precipitation of the proteins at their isoelectric point as stated by Mintah et al.,[3]. The author sought to establish an optimized procedure for protein extraction from microwave-dried larvae of *Hermetia illucens* (HIL), using ethanol as defatting agent. The authors explored three extracting parameters: temperature, alkaline solution to sample ratio and time. An optimal condition, using a mathematical polynomial model was used to illustrate the influence of extraction parameters over protein yield. The extraction condition was studied at 52.23℃, 24.85:1.00 (v/w) of NaOH and 59.43 min protein yield 63.12± 1.05%. Experimentally, the protein extraction using the optimized protocol was compared with a modified protocol (60 min, 15:1 alkaline solution to sample ratio, and 40℃) protein yield for optimized protocol was 80.42 ± 0.90% and 76.91 ± 0.91% for modified protocol. The study is important to show how mathematical models can be successfully applied to investigate the optimum parameters for protein extraction and how the results might change under practical study.

A similar alkaline process has been explored by other authors using black cricket [89]. Intending to provide chitin separation, Purschke et al.[90] mixed a defatted Migratory locust coarse meal with deionized water and adjusted the pH to 9.0 (NaOH 4M) and the slurry was finally filtered through a fine-mesh cheese filtering cloth. Zhao et al.[66] also reported a protein extraction method for mealworm larvae but based on four different factors: NaOH concentration, NaOH to defatted larvae ration using ethanol, temperature and time. The results showed that under optimized extraction conditions, 0.25 M NaOH, a 15:1 mL: g NaOH: fat -free larvae ratio, 40℃ for 1h of extraction, had a yield of 70% of protein content.

Yi et al. [23] reported a different protein extraction method using an eco-friendlier system by adding demineralized water and ascorbic acid in a N_2-frozen insect for further sieve and centrifugation. Three fractions were obtained: the residue, the pellet and the supernatant. The pellet fraction ranged from 65 % to 75 % being the highest protein content when compared to other fractions. All chitin-bound nitrogen was present only in the pellet fraction since it is not soluble in the aqueous solvents.

An alternative and specific protein extraction method was carried by Okagu [91] in order to obtain non-cuticular yellow mealworm proteins. The samples were washed and frozen in liquid nitrogen, blended with 1.6% w/v sulfite, frozen and lyophilized. The grounded powder was defatted, using hexane 1:5 (w/v), and the non-cuticular protein was extracted using potassium tetraborate decahydrate (1%) at pH 9.1, the supernatant was removed from the residue containing the cuticular protein after centrifugation. The sample was lyophilized and then a dialysis system took place for salt and other non-protein component removal.

Intending to improve protein extraction, some steps might be altered during alkaline extraction, from example, ultrasonic technique has been successfully applied by Yang [78]. The method is generally implemented replacing the shaker step, after adding the alkaline solution. Mishyna et al. [58] have compared the use of sonicated extract to conventional defatting and alkaline extraction. Among the three methods the highest protein extraction rate was obtained with the sonication method. The highest values of foaming and emulsifying capacity of grasshopper powder, was also found when the ultrasound method was applied.

Soluble proteins can be usually recovered by precipitation techniques. The method is commonly achieved by adjusting pH and the pI (4-5) through isoelectric precipitation in a centrifugation process. In some situations, protein denaturation might be avoided, then, the supernatant can be freeze-dried or further purified in a membrane filtration system. Ultrafiltration (UF) can reduce the occurrence of protein denaturation and the method can produce protein concentrates with great content (65%) or even protein isolate with 90% purity if coupled with diafiltration [43,71]. However further studies are necessary about insect protein size and properties as these methods were not yet reported for insect proteins in the literature.

2.1.1.2 Insect protein properties under processing

2.1.1.2.1 Processing parameters

Each insect species has its proteins profile and peculiarities, even insects in the same specie but at different life stage can produce another group of proteins and then show a contrasting behavior over the explored techno-functionalities [47,92]. Herein we understand the importance of having a greater knowledge about physicochemical characteristics of insect proteins and how the variation of heat, pH and NaCl concentration can impact their intrinsically properties such as solubilization, ζ-potential, hydrodynamic diameter, hydrophobic surface, free sulfhydryl group, water holding capacity, oil holding capacity, gelling ability, emulsifying, foamability, rheological and texture ability (Table 1).

A common practice in industrial food processing is the use of salt, pH alteration and heat variation over food matrixes [93]. Sodium chloride (NaCl) is largely applied as a preservative, spice, color maintenance, texture agent, flavor agent and a regulator of bacteria and yeast growth. In the same way variation of pH can impact different aspects of food such as flavor, consistency and shelf-life, as pH is a logarithmic measurement, even small changes in values means significant alterations [94]. Heat variation, another commonly applied process, can be used for microorganism control and consequently extending shelf life. Thermal techniques such as bleaching, pasteurization and ultra-high temperature (UHT) process are generally applied during food processing and aim to improve color, texture, food quality, stability and safety [95]. Intending to preserve and study the matrix behavior all reported processing parameters can be extended to alternative food source such as the insects. Some studies have been demonstrated how these processes can alter protein functionality and cause structural changes [22,66,89].

Table 1- Insect characterization and functionalities over NaCl, pH and Heat treatment

Source	Treatment			Characterization								Colloidal properties			Reference
	NaCl	pH	Heat	Sol.	ζ	Size	Hyd.	-SH	WHC	OHC	R/T	Emul.	Foam	Gel	
Acheta domesticus											X		X	X	Yi et al., 2013
Acheta domesticus				X					X	X		X	X		Ndiritu et al.,2017
Allomyrina dichotoma		X										X	X		Kim et al.,2020
Alphitobius diaperinus											X		X	X	Yi et al., 2013
Anacridium melanorhodon		X	X	X					X	X					El Hassan., 2008
Apis mellifera			X	X		X	X	X			X	X	X	X	Mishyna et al., 2018 Mishyna et al., 2019
Blaptica dubia											X		X	X	Yi et al., 2013
Bombyx mori												X	X		Chatsuwan., 2018
Bombyx mori									X	X		X		X	Omotoso, 2015
Chondracris roseapbrunner		X		X			X	X				X	X		Chatsuwan et al., 2018
Cirina forda									X	X		X	X	X	Osasona and Olaofe, 2010
Cirina forda		X		X					X	X		X	X	X	Omotoso, 2006
Gryllus assimilis	X		X		X	X	X	X					X	X	Santiago et al.,2021
Gryllodes sigillatus				X					X	X		X	X		Zielińska et al., 2018
Gryllodes sigillatus				X								X	X		Hall et al., 2017
Hermetica illucens	X	X	X	X			X		X	X					Buβler et al., 2018
Imbrasia oyemensis	X	X							X	X		X	X		Akpossan et al., 2015
Locusta migratoria	X	X		X								X	X	X	Purschke et al.,2018
Locusta migratoria	X	X		X					X	X		X	X	X	Purschke et al.,2018
Oryctes owariensi									X	X		X	X		Assielou et al.,2015
Patanga succincta		X		X			X	X				X			Chatsuwan, et al., 2018
Protaetia brevitarsis		X		X			X					X	X		Kim et al.,2021
Rhynchophorus phoenicis									X	X			X		Koffi, et al., 2017
Schistocerca gregaria			X	X			X					X	X		Mishyna et al., 2018

															Mishyna et al., 2019
Schistocerca gregaria				X					X	X		X	X		Zielińska et al., 2018
Sphenarium purpurascens			X	X					X	X		X	X	X	Torruco-Uco et al., 2018
Tenebrio molitor	X	X	X	X			X		X	X					Buβler et al., 2018
Tenebrio molitor		X										X	X		Kim et al.,2020
Tenebrio molitor	X		X	X	X		X	X				X	X		Jiang et al., 2021
Tenebrio molitor			X				X	X	X	X		X	X	X	Lee et al.,2019
Tenebrio molitor	X	X	X	X					X	X	X				Zhao et al., 2016
Tenebrio molitor	X		X		X	X						X			Gould & Wolf, 2018
Tenebrio molitor				X					X	X		X	X		Zielińska et al., 2018
Tenebrio molitor											X		X	X	Yi et al., 2013
Zophobas morio											X		X	X	Yi et al., 2013

Sol.= Solubility; ζ =Zeta potential; Dh = hydrodynamic diameter; Hyd. – Hydrophobic surface -SH -*sulfhydryl group* WHC= Water holding capacity OHC = Oil holding capacity Gel = gelling ability Emul. = Emulsifying Foam= foamability R/T= rheological and texture ability

2.1.1.2.1 Protein solubility

Protein solubility, an important functional property, directly depends on amino acid composition (hydration and surface hydrophobicity), protein size, three-dimensional structural conformation and also extrinsic factors, including ionic strength, pH changes and temperature [96]. Most of the techno functionalities mentioned beforehand depends on how the protein interacts with the solvent and therefore its solubility [60].

Buβler et al. [22] investigated the influence of pH, NaCl and temperature changes over techno-functionalities of *T. molitor* and *H. illucens* protein fractions. Protein solubility of these insects (pI around 4) was highly dependent on pH, showing higher solubility in the alkaline region, ph 10 for *T. molitor* and ph 12 for *H. illucens* and at extreme acid conditions, pH2. Through the measurements of exposed hydrophobic tryptophan residues, under extreme pH conditions (acidic or alkaline), the authors reported that protein may unfold and then expose the hydrophobic groups. The result showed that even extreme acid or extreme alkaline pH can alter protein structure. Buβler et al. [22] also verified the protein profile under the influence of pH by SDS-PAGE analysis. At pH 7 for protein extraction, the result showed that *H. illucens* had only two major protein bands, in the molecular weight region of 14.3 KDa and 80.5 kDa, the protein extract was composed of 75.9% of high molecular weight fraction and 24.1% of low molecular weight fraction. However, when taking extreme pHs (2 and 12) an expressive increase in low molecular weight protein was observed. The data shows that extreme pH conditions led to a great proteolysis of the 80.5 KDa band to 14.3 KDa or lower.

Increasing in NaCl molarity also improved protein solubility for both insects. Increasing ionic strength from 0 to 0.4M, reduced the solubility however, NaCl molarity of 3 and 4 maximized protein solubility to 55% (*T. molitor*) and 70% (*H. illucens*), the results are expressed for insect flour, for *T. molitor* protein fraction, the increase in ionic strength decreased protein solubility. Altering the extraction temperature from 20 to 60 ℃ clearly influenced protein yields by 20 % and 10 % for *T. molitor* and *H. illucens*, respectively [22]. Purschke et al.[97] also evaluated *Locusta migratoria* L. protein extract solubility upon pH change. Protein solubility showed low results, ranging from 10 to 22% with minimum and maximum at pH 5 and pH 9, respectively. The protein pI was registered at pH 4. Over the literature, pH alteration is one of the most commonly parameter to study insect protein solubility [60,98].

High hydrostatic pressure (HHP) was firstly reported over the literature by Bolat et al. [81] as a defatting process for later comprehension how the process can affect the functionalities of insect proteins extract. The author compared untreated group with HHP group for two species of insects *T. molitor* and *A. domesticus*. The pressure had no effect on primary or secondary structure, but it is known that tertiary and quaternary structure are highly sensitive to HHP, which can cause changes on the solubility. Pressure by itself was not able to cause changes in cricket protein solubility but in *T molitor*. The association between temperature and pressure was enough to cause changes in protein solubility for both insects. The difference in number of hydrophobic groups for each insect protein group, can be one good explanation for the reported outcome.

Commonly methods applied to conventional food processing have been switched to understand insect proteins behavior, therefore, some other treatments, such as ultrasound, ohmic heating, and also UHT and pasteurization aligned with pH and NaCl alterations might be further explored to verify how they can affect protein solubility, as these methods are frequently applied in the industry.

2.1.1.2.2 Surface hydrophobicity and -SH groups

Other physicochemical parameters can be evaluated after food processing, and they can directly affect the techno functionalities. One important intrinsically parameter is the presence of free sulfhydryl groups (*SH*). Proteins that have cysteine and cystine groups can suffer polymerization during heat process by means of thiol-disulfide exchange reaction forming then a strong polymer structure. During denaturation, proteins can unfold and reveal the thiol groups which will be available for intermolecular interactions with other proteins [94,99].

In globular tertiary protein structures, generally hydrophilic amino acid side groups are predominantly at the surface, and hydrophobic ones stay in the core of the structure (Leu, Ile, Val, Phe, Trp). Upon unfolding, due to food processing, many hydrophobic residues become exposed which usually leads to a reduced solubility as the nonpolar groups will form intermolecular hydrophobic bonds and cause further increase in protein aggregation [34,100].

The effect of heat treatments and NaCl concentration over the secondary structure of protein extract from black crickets was tested for hydrophobic surface exposure and presence of sulfhydryl groups. Santiago et al.[89] noticed that when the heat increases up to 85℃ for 15 min there was a progressive enhancement in surface hydrophobicity. This can be explained by the unfolding process and consequent exposure of hydrophobic patches. The same thing was also observed by Mishyna et al. [56] when tested proteins from honeybee brood.

Santiago et al. [89] also stated that surface hydrophobicity decreased under temperatures above 90 ℃ for 15 min, and it was explained by the fact that initial aggregation between proteins started to happen, which was guided by the intermolecular hydrophobic interactions. When NaCl was add to the protein extract no significant result was observed, but when both treatments heat (95 ℃ for 15 min) and 0.5 M NaCl were add to the experiment, the result showed that the higher NaCl concentration, the lower the surface hydrophobicity, indicating that the treatment of heat and increasing concentration of NaCl favors the thermo-induced aggregation of black cricket proteins. The author confirmed the reported results through hydrodynamic diameter (D_h) and ζ- potential. The D_h analysis revealed an increase in particle size from 20.08 $\pm$ 1.66 nm to 303.08 $\pm$ 13.45 nm after heat and 0.1 M NaCl. ζ-potential was calculated to endorse the D_h results and a decrease in electrostatic repulsion (at pH 7) between the proteins from untreated dispersion – 44.0 $\pm$ 1.3 mV to – 17.5 $\pm$ 0.8 mV (Santiago et al., 2021). No significant free *SH* groups were identified which is explained by the low content of cysteine residues in proteins of black cricket [53,89].

Jiang et al. [101] tested the physicochemical and techno functionality of *T. molitor* protein extract under different condition of heat and salt addition (salting-in/out). The presence of SH was evaluated under salting-in treatment and compared to untreated group; no significant differences were found at levels of exposed SH patches. It is known that higher levels of exposed SH groups can mean proteins self-association or aggregation resulting in reduced solubility. However, when the author evaluated the disulfide bond content (SS) under salting-out treatment, protein samples showed higher

SS content than the untreated and the salting-in group. It is known that SS bonding can stabilizes the protein folded structures and then decrease the conformational entropy, which improves thermal stability. ζ-potential was calculated for all systems, the salting-out ζ-potential showed higher values when compared to untreated and salting-in ones. It indicated that the salting-out treated proteins contained more electrostatic charges due to the higher salt addition. The higher the ζ-potential the lower is the chance for the system flocculates as each particle tends to repel each other [102].

Lee et al. [103] demonstrated how the defatting process, as mentioned before, can directly affect the protein structure, exposing hydrophobic amino acids through denaturation. The author obtained protein extract from *Protaetia brevitarsis* by hexane defatting method. To confirm protein structure alteration, the author also performed another interesting method, the Tryptophan fluorescence test [86]. Through this method, it was possible to prove that the buried residues of tryptophan were exposed to the protein surface after defatting process which corroborates with an increase in surface hydrophobicity. Corroborating, Chatsuwan et al.,[86] noticed how surface hydrophobicity may vary according to the protein extraction protocol, beside the intrinsic factors. The data was compared with a previously paper in the literature by Azagoh et al.[102] Chatsuwan et al [104] also evaluated the total sulfhydryl content and the free sulfhydryl content for protein extracts from and *Chondracris roseapbrunner* (WSPC) (1.06 and 0.48 μmol/g) and *Patanga succincta* (WSPP) (1.14 and 0.48 μmol/g). The results showed a similar content for both insect samples which was expected based on the amount of sulphur-containing amino acids in the two protein extracts.

2.1.1.3.4 Water holding capacity/oil holding capacity

Another important functional property of a protein matrix is the ability to retain water against gravity, per gram of sample material, and it also includes bound water, hydrodynamic water, capillary water and entrapped water. The amount of water linked to a protein is directly affected by the amino acid profile, the number of charged residues, protein conformation, hydrophobicity, and extrinsic factors such as pH, temperature, and ionic strength which can be altered by the addition of salt. The ability to retain water has been reported with different names over the literature, water-holding capacity (WHC), water-binding capacity (WBC), and water absorption capacity (WAC) [22,43]. The water holding capacity positively affects the texture and moisture of food and it is highly connected to gelation properties.

Oil-holding capacity (OHC), Oil binding capacity (OBC), and Oil absorption capacity are the commonly found names referring to the amount of fat that can be held by a specific mass of protein powder. This property is crucial for better understanding the emulsifying ability of proteins. Generally, small, and low-density proteins tend to absorb more oil than the big and high-density ones [43,105]. The presence of hydrophobic amino acid in the structure and the food processing method can affect the OHC of the protein matrix. This property is largely explored in food systems to improve texture and flavor which makes food more palatable [105,106]. The comprehension of protein OHC tends to be an interesting property to enhance insect supplementation in food formulations and, therefore, provides greater consumer acceptability.

Tenebrio molitor is one of the most explored insects for its functional property. The WHC of *T. molitor* protein fractions, when compared to *T. molitor* flour, decreased by 0.41 g/g_{DM} for high protein fraction, and 0.37 g/g_{DM} for Low protein fraction. In addition, the OHC of protein extract compared to the flour decreased by 0.21 g/g_{DM} for the low protein fraction whereas it significantly increased by 0.26 g/g_{DM} when the high protein fraction was tested [22](Buβler et al.,2018). In contrast to this study, Zielinska et al., [60] have shown that protein preparation had a higher WHC (3.95 g/g) and OHC (2.74 g/g) than the whole insect flour (1.29 g/g and 1.71 g/g, respectively). Further studies are necessary to better comprehend how the different methods of protein extraction and flour production might affect the WHC and OHC properties. However, it is known that the presence of fat in the flour and the protein size and conformation, which might be different on each fraction, will act on the functional property of those proteins.

T. molitor proteins were studied for their WHC under heat variation by Lee et al.[103], and the protein matrix did not differ significantly concerning temperature and heating time (1.02 to 1.18 g/g), as the extraction followed the method of Zhao et al.[103] (WHC= 1.29 g/g), the result of WHC for the non-treated group for both authors were consistent. Zielinska et al.[60] have also reported the WHC of *T. molitor* whole insect, 1.29g/g, and *T. molitor* protein preparation, 3.95 g/g. The results showed that the highest value of WHC was reported for protein preparation which is an important fact to be considered for future application in the food industry. Besides *T. molitor*, Zielinska et al.[60] have also reported the WHC and OHC for two other insect species, *Gryllodes sigillatus* and *Schistocerca gregaria*, and the protein preparation for all insects showed higher WHC and OHC when compared to the insect flour. Comparing protein preparation for *T. molitor* in the study performed by Lee et al.[72] and Zielinska et al. [60], different results are stated, however, as the amount of water/oil absorbed by proteins depends on a variety of intrinsic and extrinsic parameters, being the extraction method one of them, it is difficult to compare the results between different processes. Lee et al., 2019 have also stated the OHC of *T. molitor* protein preparation under heating treatment. The result showed that the OHC was dependent on the temperature variation. Protein extract was exposed to three different temperatures (55 ℃, 75℃, and 95℃) for different heating times (20, 40, and 60 min) and compared with the control group (no temperature exposure). The OHC decreased in all tested temperatures getting lower as the time increased, a similar result was found for *Sphenarium purpurascens* Ch. (grasshopper) [107]. It was interesting to notice that in all tested temperatures for 20 min of heating time, there was no significant difference between these groups and the control. Lee et al.[107] explained that the temperature treatment collapses the protein network which might decrease the OHC. In addition, the longer heating time promotes protein aggregation due to conformational changes leading then to a decline in the protein-oil holding capacity.

2.1.1.3 Colloidal properties

A colloid system is generally formed by two mixed parts, where one part is dispersed throughout another but does not form a solution. The system is composed by a dispersed phase and a continuous phase and there are many types of colloidal system based on the form and composition of the two parts. According to the states of matter constituting the two phases, colloidal system in food is classified as a multicomponent system such as, sols, gels, emulsions, and foam. Food colloids are responsible for the structure, texture and mouth-feel characteristics of many different food products and therefore will impact in the final technological aspects and consumer acceptance [94,108].

The gelling capacity is a protein functionality of great importance, used in food formulations such as jellies, desserts, yogurts, meat products, etc. The gelling agents, such as proteins, and other polymers are food additives employed as thickening and stabilizer agents that provide food texture through the formation of a gel. Several proteins have been employed as a gelling agent, these include zein from corn, soy proteins, and some animal proteins like egg and whey protein [109].

Macroscopically, a gel is a material that behaves as an elastic solid, suffering partially reversible deformation under certainly applied tension, and at the same time as a viscous liquid that can partially drain. Microscopically, a gel is a colloidal dispersion in which the solid phase (the network of a polymer chain) forms a structured three-dimensional matrix in the interstices of which are lodged with molecules of the liquid phase, water in case of hydrogels [110]. In the case of protein gels, during gel formation it is necessary to unfold the native protein structure followed by protein aggregation, this process will create a three-dimensional organized network cross-linked by non-covalent and covalent bonds [105]. Gel formation is a two-step process; the first one is the input of energy which leads to protein unfolding and exposure of hydrophobic amino acid residues; the second is the approximation and protein aggregation, due to covalent disulfide S-S bridges and non-covalent bonds like hydrophobic interactions, hydrogen bonds and/or van der Wall interaction. The size of the gel network directly impacts on the functional role of proteins, for example, the large-pore networks show a low WHC [105]. Gel formation depends on the nature of the gelling agent such as molecular weight, amino acid composition, and also protein concentration. External factors are also needed to provide the energy required to form the gel network, such as physical, temperature and pressure, and chemical factors, pH, ionic strength, and the presence of an enzyme [40,111,112].

2.1.1.3.1 Gelling properties

The gelling capacity is measured by the least gelation concentration, or the critical minimum concentration, being specific for each hydrocolloid. Thus, if the concentration is in the range of the critical minimum concentration, the higher the molar mass the faster is the process of gelation [113]. Even though the gelling property of animals and plant proteins has been well documented over the literature, there have been only a few papers investigating the gelling agents from edible insects and their application in food formulations. Yi et al.[23] have studied the gelling property of protein fraction of five insect species, *Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus, and Blaptica dubia*. In this study, the insect supernatant solutions were heated for 10 min at 86 ± 1 ℃ and the concentration of 3 and 30% (w/v) was evaluated at pH 3, 5, 7, and 10. Although the initial gel formation is characterized by a rheological study where an increase in the storage modulus (G') over the loss modulus (G") is observed, it might also be evaluated under visual observation in a tube, as firstly described in the literature by Beveridge, Jones, & Tung. [114] and then followed by other authors. The authors reported that 30% (w/v) of supernatant protein fractions of all insects evaluated formed a gel at pH 7 and pH 10. House cricket fraction (*Acheta domesticus*) was the only one to form a gel at 3% (w/v) and pH 7. Mishyna et al.[115] have reported the least gelation concentration of raw powder from honeybee brood, the data showed the lower concentration of 5% (w/v) at pH 7 and the highest concentration at 11% (w/v) at pH 3. Protein concentrates from *Locusta migratoria* showed gel formation at a similar pH of 7.9 and a concentration of 20% or 16.5 g of proteins/ 100mL [97]. The least gelation concentration of *Sphenarium purpurascens* Ch., grasshopper, was evaluated under a range of temperature and the concentration of 14% was identified as the minimum to start forming the gel structure in the tube [115].

A few other insects were also evaluated for their protein gelling properties. Black cricket (*Gryllus assimilis*) protein isolate (BCPI) was tested for its critical gelling concentration (CGC) at a fixed pH 7 and suspended in Milli Q water, the protein isolate was heated in a water bath at different temperatures (80,85 and 90℃) for 15min [89]. Whey protein isolate (WPI) was used as a comparative parameter. The critical gelling concentration was determined as 6.5% w/w at 90℃. The value was interesting when compared to WPI, a conventional gelling agent with a minimum gelling concentration of 6%, and also compared to other insect-based protein-rich ingredients, for example, Osasona & Olaofe [116] reported a higher value of 14% w/w for *Cirina forda* larvae.

Santiago et al. [89] also evaluated the influence of NaCl addition over the gelation capacity of BCPI. It was observed that NaCl concentration between 0.3-0.5 M impaired the gelation. Lee et al [103] evaluated the gel strength of protein extract of yellow mealworm under different heat treatment (55, 75, and 95℃) and exposure time (20, 40, and 60 min). The higher gel strength was obtained at the maximum time, 60 min, and higher temperature 95 ℃. It can be inferred by the action of temperature over the protein unfolding process that will expose the hydrophobic groups and possibly facilitate the net formation between proteins. The ability of a protein to form gels is useful in the development of new formulations as this property provides the retention of water, flavor, sugar, and food ingredients in the structural matrix [107].

In general, the gelling capacity of insect proteins revealed promising results even when compared to whey protein isolate (WPI). It was stated that beyond intrinsic parameters, the presence of NaCl, and pH alterations in the medium influence gelation, which implies that electrostatic interaction, directly participates in the formation of gel network of insect proteins. Protein concentration and temperature are other two important parameters to be considered in order to understand the gelling capacity of insect proteins, as they can accelerate the three-dimensional structure formation and the sol-gel transition.

Complementary to the gelling capacity of insect proteins, the rheological properties have been evaluated in different studies where the analyzes highlight the development of storage modulus (G’) and loss modulus (G’’). The G’ is a measure of the elastic energy stored reversibly in a gel during deformation and characterizes its stiffness. G” is a measure of the energy dissipated during deformation as a result of viscous friction (Yi et al., 2013). Under heating and cooling treatment, the rheological properties of gels from five insects (*Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus and Blaptica dubia*) were described by Yi et al.[23]. The evolution of G’ and G” was studied during temperature ramp at a concentration of 15% w/v and pH 7. The G’ kept increasing for the first and second phases, when the temperature was constant at 90 ℃. During the cooling phase, G’ and G” sharply increased which characterize the presence of hydrogens bonds between structural elements in the gel. The authors reported the gelation temperature for all five insects, *T. molitor* 61.7 ℃, *A. diaperinus* 58.2 ℃, *Z. morio* 51.2 ℃, *A. domesticus* 56.2 ℃, *B. dubia* 63.2 ℃. Compared to the other insects *A. domesticus* had the hardest gel and although *B. dubia* had the lowest actual protein content (7.4%), it formed the second hardest gels among all other tested species. In addition, the adult stage showed stronger gels than the larvae ones, then apparently the insect growth influence the protein body composition [23]. At a supernatant fraction concentration of 15% w/v, all insect species had a comparable maximum linear strain, a value of around 50%. In conclusion, Yi et al.[23] reported that the detailed rheological study shows that insect proteins can form gels with similar properties as those formed by conventional food proteins. A complementary study of the rheological properties from *T. molitor* protein extract showed the dependence of the elastic modulus by the sample concentration, pH, temperature, addition of NaCl, and transglutaminase [66].

The thermal liquid-solid transition of honeybee brood powder was evaluated through a compact rheometer by Mishyna et al.[58] The study exhibited the gradual increase of G' and the moment when it crossed the loss modulus at approximately 50℃, which means the onset of protein denaturation and further aggregation leading to a sol-gel transition. As stated before, noncovalent forces probably initiate the network formation, and this step was followed by the formation of sulfhydryl/disulfide interchange reactions, where the elastic modulus G' sharply increase indicating a gel hardening as previously demonstrated for ovalbumin [117]. Honeybee brood powder gel hardness increased as a function of pH showing maximum results at pH 7 and 9. The microstructure analysis by scanning microscopy corroborates with the above data as an acidic pH environment caused the formation of gels with a larger porous structure, higher heterogeneity, and more irregular area. On the other hand, at more alkaline condition, gel microstructure was more regular, continuous, and had smaller voids. The authors concluded that protein solubility at different pH directly affects the gel formation and textural properties, and soluble proteins from honeybee brood demonstrated great similarities to conventional proteins, such as whey [58]. The fact that the soluble fractions contained protein groups capable of forming gel structure is a promising result for future food applications.

The rheological parameters of insect proteins were analyzed through the evolution of the storage modulus G' and loss modulus G''. The studies intend to demonstrate how temperature and other extrinsic parameters may impact gel strength and how protein unfolding can lead to a sol-gel transition, due to exposure of hydrophobic groups and formation of sulfhydryl/disulfide interchange reactions which contributes to protein aggregation. The life stage also affects gel strength, where the adult stage appears to show the harder ones. Therefore, a more detailed study is necessary to understand not only if the whole protein extract can contribute to the gelling property but also if protein groups from the insect profile can enhance this function. The data also reveal that different insects demonstrated a different gelling capacity and gel strength which is dependent on the temperature, pH, protein concentration, insect life stage.

2.1.1.3.2 Foaming properties

Food foams are composed of an aqueous continuous phase that surrounds a gaseous (air) dispersed phase and these air bubbles can be stabilized by a viscoelastic film of proteins or other compounds [118]. In foam formulations, proteins spontaneously migrate from a bulk liquid to an air/water interface which implies that the free energy of proteins is lower at the interface when compared to the bulk aqueous area [94]. Due to the interfacial properties of proteins, these amphiphilic molecules are the main surface-active agents that help with bubble formation and stabilization. Foam-type foods have been well explored on the market such as whipped cream, meringue, marshmallow, ice cream, bread, and many other formulations [94,105]. The foaming property will rely on proteins' ability to form thin stable film at gas-liquid interfaces. Thus, it will depend on intrinsic parameters such as, protein composition, the number of hydrophobic and hydrophilic residues over protein surface, structural form as globular or non-globular protein, size, steric effect, flexibility, charge density and distribution, and amino acid composition [105,119,120]. In addition, extrinsic parameters might also affect the foaming property such as changes in ionic strength, heating, the addition of salt, sugars, and lipids, and the protein concentration. In short, for a protein to perform a good foaming agent, it must follow three basic requirements: (i) it needs to quickly adsorb to the air-water interface, (ii) it must rapidly unfold and rearrange at the interface and, (iii) it needs to form a viscous cohesive film through intermolecular interaction [105,121].

Regarding foam structure, two parameters are generally evaluated, foamability and foam stability. The foamability or foam capacity of a protein is related to the amount of interfacial area that can be created by a protein. Complementary, foam stability (FS) expresses the capacity of the protein to stabilize foam against the opposite force as the gravitational and mechanical ones [60,104].

Kim et al. [74] have reported different defatting processes using methanol, ethanol, or *n*-hexane of proteins extract from *Protaetia brevitarsis* larvae and the effect of organic solvents over the functional properties. The study revealed that aqueous extract showed the lowest foam capacity (20.0 ± 7.1%) while methanol, ethanol, and hexane extract showed higher foam capacity, 72.2 ± 7.9; 56.3 ± 8.8; and 88.9 ± 15.7%, respectively. The foam capacity and foam stability for *P. brevitarsis* larvae protein extracts showed a positive correlation with surface hydrophobicity and protein solubility as was also stated by Medrano et al.[122]. Besides, the high-fat content in the aqueous extract may hamper foam formation [123].

The foam capacity of grasshopper meal was studied by Torruco-Uco et al. [107]. To obtain the meal Grasshoppers (*S. purpurascens* Ch.) insects were dried at 55℃ and then ground in a coffee mill to reach a uniform particle size of 0.420 mm. Foam capacity was equal to 7.17%, and the author compared with) *A. domesticus* (1.42%) [75]. However, it is important to notice that Torruco-Uco et al. [107] studied the whole ground insect meal and Ndiritu et al. [75] reported the results for an aqueous extracted residue, which are different materials and therefore, making the studies difficult to compare.

The foamability and foam stability of black cricket protein isolate (BCPI) were reported by Santiago et al.[89] under different treatments. As the temperature increased the foamability increased, reaching 1070% at 95℃. Untreated BCPI showed foamability of only 190% and foam stability (the remaining foam) between 50-70%. The group with the higher NaCl concentration (0.5M) and heat treatment of 95℃ showed 35% of foam remaining after 30 min (the results were compared to WPI). The greater foam property upon NaCl addition can be explained by the capacity of the proteins to form a more elastic and cohesive film at the air bubbles [89].

Other studies performed by Hall, Jones, O'Haire, and Licega [10], have also reported the foam properties of another cricket species, *Gryllodes sigillatus*, which showed lower foamability (100-155%), but better foam stability, up to 90 min of standing. However, different devices were used to prepare the foam and another insect processing methods were applied. Water-soluble protein extract from *Patanga succincta* (WSPP) and *Chondracris roseapbrunner* (WSPC) was tested for its functional properties, including FC and FS [104]. The results were compared to BSA (81.23% of FC and 67.41% of FS) WSPP and WSPC exhibited lower FC values when compared to BSA, 8.57 and 25.71%, respectively. However, the FS of both insects was significantly higher than BSA, 98.72

and 86.41% for WSPP and WSPC, respectively. The results indicated the promising capacity of both water-soluble protein extract to stabilize foam against collapse. It is important to mention that the devices used for foam production and the methodology applied both for insect processing and foam formation will have a strong impact on the final result, which can make it difficult to elucidate comparations between some studies.

A Recent study performed by Queiroz et al.[11] evaluated the FS and FC of black soldier fly larvae protein extract after heating treatment of 15min. The data showed that FC was similar even after 85℃/ 15min compared to untreated sample, but the FS showed more interesting response at 85℃/ 15min, and the stability was statistic significant after 20min of storage. The study emphasizes the possibility of applying BSFL proteins as stabilizer agent and the optimization of proteins functionality after simple heating treatment.

In general, the capacity to form foam is reduced when the aqueous extraction is used, and the defatting process might improve this functionality. It can be explained by the fact that aqueous extraction did not remove a great number of lipids that can compete with proteins during air bubbles stabilization and hamper foamability and stability. Extrinsic treatment such as heat might cause protein unfolding which causes fast protein adsorption at the air/water interface. Temperature combined with NaCl addition also revealed to be interesting for foam formation as they prevented protein drainage and/or form a more elastic and cohesive film. Intrinsic parameters such as amino acid composition and their position in the protein structure will also affect the reported functionalities.

All data reported opens the possibility for edible insect proteins to be used in suitable food applications. The studies also demonstrated how insect proteins are affected either by intrinsic and extrinsic parameters and knowing how food processing can affect protein foamability is an essential tool for further research. The foaming property of insect proteins might be further implemented in food formulations; however, a more detailed investigation needs to be performed in order to identify which proteins are responsible for such functionality and how treatments can optimize that.

2.1.1.3.3 Emulsification properties

Emulsions are a homogeneous mix of, at least, two immiscible liquids (colloidal dispersion), which can be characterized according to the relative spatial distribution of both liquids [120]. The presence of oil droplets in a continuous phase of water is called oil-in-water (O/W) emulsion and a water droplet in the continuous oil phase is referred to as water-in-oil (W/O) emulsion [94]. As the energy associated with the interface is proportional to the interfacial area ($\Delta_{interf}G = \gamma\Delta_{\mathrm{interf}}A$), during emulsion formation, there is an increase in energy due to the bigger interface system. Even though, smaller droplets can assume more positions in the system, which generates a higher configurational entropy ($\Delta_{config}S$), the energy associated with emulsion formation is much higher than the configurational entropy variation, $\Delta_{interf}G > \Delta_{config}S$. The higher energy associated makes the whole system thermodynamically unstable. The amphiphilic character of proteins allows the formation and stabilization of emulsions by reducing surface tension at the oil-water interface, as they create a film that prevents coalescence, flocculation, creaming, sedimentation, and Ostwald ripening [43]. Therefore, proteins play an important role as an emulsifier in many natural and processed food, such as egg yolk, soy milk, butter, mayonnaise, etc. In homogenized milk, for example, casein micelles and whey proteins replace the lipoprotein membrane with a stronger protein film, which is more stable against creaming than natural milk [94,119].

As foamability and other proteins functionalities, the emulsifying property is also influenced by intrinsic (hydrophobic groups) and extrinsic (pH, ionic strength) factors [74]. Protein size directly affects emulsion formation and stability as small proteins enhance diffusion and generally form a better emulsion, whereas larger proteins tend to have lower diffusion but greater emulsion stability once the layer surrounding the oil-water interface has formed [94,119]. The emulsion properties of some edible insects have been explored over the literature. Different materials were tested for emulsion capacity and stability such as insect flour, protein extract obtained through different solvents extraction, and all under a variety of extrinsic factors.

Emulsion capacity (EC) is a measure of the number of grams of oil per gram of protein that can be emulsified, and emulsion stability (ES) measures the resistance of the emulsion over a specific time [124]. Protein concentrate of edible crickets (*Acheta* *domesticus*) was evaluated for its emulsifying capacity and emulsion stability using corn oil and water to form oil-in-water (O/W) emulsion. Three different materials were evaluated, hexane extracted proteins (HE), aqueous extracted precipitate (AE precipitate), and aqueous extracted residue (AE residue). The aqueous extraction revealed a high emulsion capacity and stability, and AE precipitate recorded the highest value, 41.7 % (v/v) and 33.6 % (v/v) for EC and ES, respectively [75]. EC and ES of *T. molitor* (TM)*, Allomyrina dichotoma* (AD)*, Protaetia brevitarsis seulensis* (PB) were explored under three different processes, ground (G), defatted (D), and extraction (E). Only EPB had a significantly higher EC, around 100%, compared to all other insects and treatments which showed a similar result for all insects and parameters, around 70%. The study reported that as the process evolved, following ground, defatting, and finally extraction, emulsion stability changed for the worse except for PB [125]. Previous studies have also reported that the emulsion capacity and stability of edible insects might reduce after the defatting step as the process might increase surface hydrophobicity and further protein aggregation [126–128]. However, Kim et al. [125] concluded that the emulsion stability of PB (oil-in-water emulsion) did not reduce like the other insects, which suggests that protein aggregation did not occur for PB, which makes PB a promising functional protein source.

Recent study evaluating the emulsifying property of BSFL and its physical and oxidative stability, reported the advantageous usage of sustainable treatments such as ultrasound and ohmic-heating to optimize protein functionality [11].The study reported that over seven days of storage samples treated with ohmic-heating showed the most interesting stability compared to untreated and ultrasound-treated sample. The interfacial tension (IFT) analysis by pendant drop corroborates with the storage test as the ohmic-heated treated sample showed the lowest IFT. It was the first time that ohmic-heating was applied for the optimization of BSFL protein functionalities. The data stated the importance in using alternative techniques in order to improve protein functionalities and how BSFL proteins show advantageous potential to be applied in food systems.

Mishyna et al. [58] have exhibited the emulsion capacity and emulsion stability of different fractions from *S.gregaria* and *A. mellifera* (raw powder, defatted powder, the powder obtained by alkaline extraction, and the powder obtained by alkaline and sonication extractions). The study showed that emulsions stabilized by raw and defatted fractions of both insects showed significantly lower values for both insects. The highest emulsion stability was detected for protein fraction from *S. gregaria*, and more than 80% of the emulsified layer remained stable after 120 min when the sonication fraction of *S. gregaria* was tested. The higher ES values from *S. gregaria* alkaline and sonication fraction can be due to the high surface charge of proteins from these fractions, preventing droplets approaching and aggregation, as Wang et al., [86] have reported similar results for barley protein extract. The EC of protein fractions (alkaline and sonication fractions) showed the highest values (100%). Raw and defatted powder of *S. gregaria* showed EC (oil-in-water emulsion) values of 39.5 and 45.0%, respectively. The higher values of EC in the protein fractions might be due to the greater protein content, improved solubility, and the presence of hydrophobic groups in protein-enriched fractions of insects. Damondaran,[129] have concluded that low molecular weight surfactants might easier adsorb and reduce the interfacial tension, which directly improves emulsion formation. This statement correlates with higher ES of *S. gregaria*, as the protein extract was characterized by low molecular weight proteins when compared to *A. mellifera*. Gould et al.[57] have also reported a synergistic enhancement of surfactant efficiency by the presence of small proteins. In addition, emulsifying capacity and stability were improved in the presence of hydrolysates from *Gryllodes sigillatus* [10]. It is also important to consider the protein composition, based on hydrophobic amino acids presence and position during protein structure, these intrinsic parameters can directly contribute to the emulsion formation, and it will vary according to each insect and the respective protein profile.

In conclusion, insect products such as protein concentrate, or insect flour, showed promising features to be used in food formulation as an emulsifying agent. Emulsion capacity and emulsion stability of insect proteins have been recently more explored over the literature, and it was stated that the defatting process, grounding, and extraction method, directly modify protein functionality beyond its intrinsic aspects [3,89].The aqueous extraction revealed greater emulsion property and the defatting process reduced this property, which might be explained by the protein/solvent interaction during the defatting step causing protein surface hydrophobicity increase and possible protein aggregation. The result is interesting as it reinforces how the physicochemical characteristic of proteins and their interaction with added ingredients, should be completely understood and controlled, as it can be considered as a key step for the development of insect food formulation and improve consumer acceptance.

3. OBJECTIVE AND STRATEGIES

3.1 General Objective

The present study aimed to understand the role of black soldier fly larvae as a food additive considering the nutritional and functional aspect of its proteins. In addition, part of the work investigated how some alternative green technologies, such as ultrasound and ohmic-heating, could improve the protein extraction and modify the final functionalities. This strategy sought to open new possibilities to apply insect protein as food ingredient and finally increase consumer's acceptance.

3.2 Specific objectives

- The first chapter aimed to evaluate the viability of protein extraction from BSFL, using a conventional chemical extraction. Therefore, the nutritional composition of both insect flour and protein extract were evaluated. In this chapter the objective was also to compare the protein profile (SDS-PAGE and FTIR) and the thermal stability of both samples. The final part of the study aimed to investigate the foaming stability and capacity of BSFL protein extract after and before heating treatment.
- The second chapter aimed to bring the hypothesis that BSFL protein extract would perform an interesting gelling property, based on previous studies that used other insects' proteins for the similar purpose. The idea is that BSFL could be a good candidate to replace conventional proteins sources currently used as gelling agent in the food industry. As it was the first time that the gelling properties of BSFL proteins was explored, the work aimed to evaluate it under different conditions of ultrasound treatment.
- The third chapter aimed to use BSFL protein as an alternative and sustainable source of emulsifiers in order to investigate the physical and oxidative stability of O/W emulsions. Fish oil was used as a reference as it is rich in polyunsaturated fatty acids (PUFA) that oxidizes fast in normal conditions. Aiming to understand how alternative and green technologies can affect the protein extraction and the functionality of BSFL proteins, Ultrasound and ohmic heating were used separate and in combination in BSFL samples (Figure 1), before the protein extraction.

- In the fourth chapter the study aimed to understand the impact of protein hydrolysis on the physical stability of O/W emulsion prepared with BSFL protein as emulsifier; The interfacial properties of BSFL proteins and BSFL hydrolyzed protein samples. The study aimed to better understand how different degree of hydrolysis can impact the interfacial properties or O/W emulsion, using Double Wall Ring rheology and pendant drop static/oscillatory mode in order to measure it.

4. RESULTS AND DISCUSSION

4.1 PHYSICO-CHEMICAL AND COLLOIDAL PROPERTIES OF PROTEIN EXTRACTED FROM BLACK SOLDIER FLY (*Hermetia illucens*) LARVAE.

The content of this chapter has been published at:

International Journal of Biological Macromolecules

doi.org/10.1016/j.ijbiomac.2021.07.081

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This work has been fully performed at the Group for Food Production Engineering, National Food Institute, Technical University of Denmark in collaboration with Federal University of Juiz de Fora Research; Agrocampus Ouest, Research Group for Analytical Food Chemistry; Technical University of Denmark, Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN); Department of Health Technology, Technical University of Denmark and nextProtein, Genopôle Campus.

The world's population is expected to reach 9 - 10 billion in 2050 [130]. This perspective directly impacts food consumption by means of agricultural and livestock growth which might increase deforestation, water consumption, and greenhouse gas emission [131]. To mitigate the risks of climate change caused by traditional agriculture and livestock production, there has been an increase in the search for new sustainable protein sources. In recent years, insects have gained increased attention by the FAO as an underexploited sustainable protein source [132] due to their high protein content and the presence of all required amino acids for human consumption [133]. Insects have a high feed conversion efficiency and a low environmental footprint when compared to livestock production and most conventional plant agriculture practices [134]. Among all edible insects, *Hermetia illucens*, popularly known as black soldier fly (BSF), has been considered as one of the most promising insects for the commercial production of proteins, with an effective sustainable approach for human consumption [135]. Due to the presence of a selection of enzymes in their gut, BSFL can consume a variety of substrates and then reduce agricultural wastes by feeding on them and synthesize high-quality proteins [136]. BSFL have revealed the ability to feed on abattoir waste, animal waste, spent grains and general organic waste from crop agriculture [16,133]. This ability can lower the cost of farming and unveil BSFL as a sustainable biotechnological tool for waste valorization [15]

Besides livestock and poultry, the larvae of black soldier fly have been applied in feed formulation in the aquaculture sector [137]. the insect meal was used to feed salmon in their early stage and no difference in fish zootechnical performance was observed compared to traditional diets [138] BSFL meal also offered a good alternative to fish meal and soya when tested for rainbow trout, catfish, tilapia and Pacific white shrimp [139].

Protein content of this insect is reported to be between 37 to 63% of dry matter, and fat content can vary from 7 to 39% [140].The variation in nutrient content in BSFL can come from a variety of factors, including diet quality and quantity and larvae density [140]. *H. illucens* are rich in saturated fatty acids, in particular, lauric acid (C12:0) [141]. A large amount of structural and muscular proteins have been identified in BSFL, corroborating with the possibility of using insect proteins as an alternative to replace meat and soybean proteins [142,143]. Moreover, antimicrobial peptides and some enzymes such as trypsin and chymotrypsin were identified, which could be, in the near future, of industrial interest [19]. Besides protein and fat, BSF larvae contain of around 2 to 9% of chitin, a modified polysaccharide that contains nitrogen [133].

Despite the reduced negative impact on the environment as well as the interesting nutritional properties in insects, the visual aspect and the association of insects with vermin and as disease vectors [144] have been considered the main limiting factors, particularly in the western world, for consumer acceptance of insects as a food [145]. An interesting, proposed alternative to increase user's acceptance is the extraction of insect proteins to be further used as food fortification ingredients. To extract a high protein content, different methods can be applied, and each process can cause protein modifications resulting in changes of the final techno-functional properties, protein thermal stability and its subsequent application [22,23,135,146]. Some authors have sought to optimize the protein extraction of BSF, exploring a common alkaline extraction method after microwave oven drying process. The optimization was considered for alkaline solution to sample ratio, temperature of extraction and time. The authors also reported that defatting also enhanced the protein content of BSF meal [135]. Buβler et al.[22] investigated the influence of protein extraction and defatting process in the functional properties of BSFL and *Tenebrio molitor*. The authors produced three different samples, high protein fraction, low protein fraction (insoluble) and defatted meal. The study suggested that the techno-functionalities can be effectively manipulated depending on the method of protein extraction and consequently the protein composition.

Therefore, knowing the protein profile, the protein functionality and consequently its quality can be an important strategy to increase consumer's acceptance of insect ingredients used in food formulation development. Some papers in the literature have focused on the nutritional composition of insects, including the use of BSF flour and protein extract for feed ingredients [147,148]. The techno-functional properties of some insects have also been highlighted over the literature, based on the water/oil holding capacity, gelling, foaming, and emulsifying properties [10,23,57–60]. However, considering BSFL protein extract, there is still a lack of information on its protein characterization, thermostability and functional properties. Herein, we aimed to evaluate black soldier fly larvae (BSFL) flour and its protein extract composition, considering the nitrogen-protein conversion factor of 5.62 for protein extract and 4.67 for larvae flour, instead of the general value of 6.25, according to Janssen et al [149]. Furthermore, in order to investigate the nutritional value of BSFL flour and BSFL protein extract, amino acid analysis and mineral composition were evaluated. Beyond nutritional aspects, the thermal stability by differential scanning calorimetry (DSC) and the protein structure by Fourier-transform infrared spectroscopy (FTIR) was studied for BSFL flour and protein extract. Finally, the foaming properties after heat treatment was evaluated, to evidence the advantage of protein extraction for food technological application.

4.1.2 Material and Methods

4.1.2.1 Preparation of defatted insect powder

The BSFL flour was kindly provided by nextProtein, Tunisia. Briefly, nextProtein raises BSFL in a plant located in Grombalia, Tunisia. BSFL were fed on formulas of feed mixtures obtained from biowaste, in compliance with EU regulations. Once larvae reached their harvesting stage insects were separated from the substrate by sieving. They were then blanched in hot water (70°C) for 5 minutes and softly dried for 3-4 hours until a moisture content <7% was obtained. Dried BSFL were then pressed to extract the oil and obtain a protein meal, partially defatted. The protein meal was then further ground to obtain a powder, named "protein flour" in this paper. The chemicals were purchased from

Sigma-Aldrich. To remove the remaining lipids, the BSFL flour was stirred in heptane extractor solvent (ratio 1:5, w/v) for 1h at 40 °C. The mixture was then decantated and the fat extract (liquid fraction) was recovered with a paper filtration (N° 00H, 7 cm, Sweden). After solvent evaporation by a rotary evaporator (Laborota 4000-efficient, Heidolph instruments GMBH & CO KG), the recycled solvent was used for a second extraction in order to remove any residual fat remaining in the insect flour.

4.1.2.2 Protein extraction

After fat extraction, the insect defatted flour was left in a fume hood overnight to evaporate the residual solvent. It was then dissolved in 0.25 M NaOH (ratio 1:15, w/v) according to Zhao et al. [66] and stirred (Polymix Buch & Holm, DK) at 300 rpm, 40 °C for 1h. After centrifugation (Sigma Laborzentrifugen GmbH 4-16KS, Germany) at 2493 ×g 4 °C for 20min, a 2nd alkali extraction followed by centrifugation was made on the solid fraction. This remaining solid fraction was stored and referred to as the chitin extract. The supernatants from both extractions were combined. The pH of this obtained liquid was adjusted to the isoelectric point 4.3~4.5 with 2M HCl to precipitate the proteins. After decantation, the solution was centrifuged at 1272 ×g 4 °C for 15 min to recover the precipitate. Finally, the precipitate was washed twice with distilled water and freeze-dried overnight.

4.1.2.3 Moisture, ash, and protein content

Moisture and ash of the flour and the protein extract were determined after drying in the oven for 24h, respectively at 105 °C and 600 °C, according to the AOAC standard method [150]. Dry matters (1) and ashes (2) were calculated as follows:

$$\text{Dry matter (\%)} = \frac{\text{dry matter weight}}{\text{initial weight}} \text{ x } 100 \tag{1}$$

$$\text{Ashes (\%DM)} = \frac{\text{ash weight}}{\text{dry matter weight}} \text{ x } 100 \tag{2}$$

The protein content in BSFL flour and protein extract was measured with Dumas method, a quantitative analysis of the protein content based on the amount of nitrogen. A nitrogen-to-protein conversion factor (Kp) of 4.67 and 5.62 were used for protein flour and protein extract respectively, according to Janssen et al. [149]. Kp value is given by the sum of all anhydrous amino acids and the percentage of nitrogen (Nt), provided by Dumas method. Nt for BSFL flour and BSFL protein extract were 7.70% and 12.06%. The protein content of the flour and protein extract were measured in duplicate, using 150 mg in crucibles. Aspartic acid and wheat flour were used to calibrate the instrument.

4.1.2.4 Amino acid composition

Acid hydrolysis (6 M HCl) in an oven for 18 h at 110 °C using 10 mg sample per mL HCl was applied for amino acid profile measurement. Samples were then cooled at room temperature (25 °C) and used both without dilution and after a 3-fold (1 + 2) dilution with 6 M HCl in the remaining procedure to quantify both high and low abundant amino acids. During acid hydrolysis, cysteine and tryptophan are destroyed and therefore they have not been reported in the results. Then 100 µL was diluted with 1.5 mL 1 M $NaCO_3$ and filtered in a 0.2 µm syringe filter (Q-max PTFE, Ø13 mm, Frisenette ApS, Knebel, Denmark) before derivatization using the EZ: Faast™ Amino Acid Analysis kit from Phenomenex® (Torrance, CA, USA). 50 µl of samples were then analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technology) [151].

4.1.2.5 Mineral composition

The mineral composition was measured by inductively coupled plasma mass spectrometry (Thermo iCAPq ICPMS, Thermo Electron, Bremen, Germany) following microwave-assisted digestion (Multiwave 3000, Anton Paar, Graz, Austria) using concentrated nitric acid (SPS Science, Paris, France). The sample digests were diluted with ultrapure water (milli-Q) before analysis. Quantification was performed using yttrium as the internal standard. All standards were prepared from certified stock solutions (SPS Science). Quality assurance of the analytical results was done by analysis of the certified reference material (DORM-4 fish protein, NRCC, Canada).

4.1.2.6 SDS-gel electrophoresis

Polyacrylamide gel electrophoresis was performed in order to determine the insect protein profile. The method was applied in a Mighty Small (Hoefer) slab cell using 12% acrylamide (C=2.6% (w/w)) slab gels (1.5 mm thick). 2mL 1% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT) and 60 mM Tris HCl (pH 8.3) were used for extraction of the dry sample (50 mg). The sample was previously prepared under shaking at room temperature for 1 h. Then it was homogenized (Polytron PT 1200, Kinematica) for 30 s, boiled for 2 min, and set at room temperature (25 °C) for 30 min. For supernatant collection, the sample had undergone a homogenization process, boiled again (2 min), and was centrifuged for 15 min at 20 °C at 20,000 ×g. Gel wells were loaded with 10 µL (40 µg protein), and the extract aliquot was diluted with sample buffer (125 mM Tris HCl (pH 6.8), 2.4% SDS, 50 mM DTT, 10% v/v glycerol, 0.5 mM EDTA) and bromophenol blue. 100 V for 15 min constant voltage was applied followed by 150 V for 1 h (max. 40mA per gel). Finally, the gel was stained using colloidal Coomassie Brilliant Blue as described by [152] Rabilloud and Charmont (2000). Molecular weight Mark12™ from Novex was applied for further analysis.

4.1.2.7 Differential scanning calorimetry

Differential scanning calorimetry DSC 250 (TA Instruments, New Castle, Delaware, USA), equipped with Refrigerated Cooling System 90, was used to understand insect products' thermal stability. Calibration required distilled water (melting point (m.p.) = 0 °C; DHm = 334 J/g) and indium (m.p. = 156.5 °C; DHm = 28.5 J/g). 30 L empty aluminum pans, hermetically sealed, were used as reference. Nitrogen at a flow rate of 50 mL/min was used as a carrier gas. The DSC was measured on the flour and protein extract using approximately 4mg of sample in the pans, in duplicate. The samples were cooled to -90 °C and then scanned from 10 °C to 250 °C at a heating rate of 5 °C/min, to obtain the heat flow in the function of the temperature. The thermograms present characteristic peaks, which can be associated with glass transition, thermal unfolding, and solid-melting according to Al-Saidi et al. [153]. The interpretation of the curves was made using Trios software, determining glass transition from the mid-point of the shift in the curve, and unfolding and melting with the enthalpy and maximum of the peak.

4.1.2.8 Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of insect flour and protein extract were recorded in using a Perkin-Elmer Spectrum 100 spectrometer (Waltham, Massachusetts, USA), based on a Universal Attenuated Total Reflectance sensor 125 (UATR-FTIR). ATR FTIR was used to record the FTIR spectra in the transmission mode over a range of 4000–650 cm^{-1}. Spectra were plotted as absorbance (AU) as a function of wavenumber (cm^{-1}). Automatic signals were recorded in 4 scans. The measurements were performed in triplicate.

4.1.2.9 Turbidity measurements

As an indicator of turbidity, the absorbance at 400 nm of protein dispersion was evaluated according to Shen et al. [154]. Measurements were performed in a quartz sample cell with a UV–Vis spectrophotometer (Hitachi, U-1500, Japan) Milli-Q water was used as a blank. All measurements of each sample were performed in duplicate.

4.1.2.10 Particle size by dynamic light scattering (DLS)

Particle size distribution was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (MPT-2, Malvern Instruments, Worcestershire, UK), according to Chili et al. [155], with modifications. Briefly, protein dispersions were diluted at 0.1% w/v with 10 mM phosphate buffer (pH 7), followed by filtration using 0.45 μm syringe filters (Pall, New York, USA). Measurements were carried out at 25 °C, at a scattering angle of 173°. Each sample was analyzed in triplicate, and the particle size was expressed as hydrodynamic diameter (d_h). Eq. (3).

$$d_h = \frac{KT}{3\pi\eta D} \tag{3}$$

Where D is the translational diffusion coefficient; K is the Boltzmann's constant; T is the thermodynamic temperature; η is the dynamic viscosity.

Polydispersity Index (PdI) was also reported for each sample, data was measured in triplicate, Eq. (4).

$$PdI = \left(\frac{\sigma}{2\alpha}\right)^2 \tag{4}$$

Where it is represented by the standard deviation (σ) of the particle diameter distribution divided by the mean particle diameter.

4.1.2.11 ζ-potential and isoelectric point

Protein extract was dissolved in Milli-Q water, and using a titration instrument (MPT-2, Malvern Instruments, Worcestershire, UK) the pH of the sample (10 mL) was adjusted in a range from 2 to 9 by adding 1M NaOH or 1M HCl. The analysis was performed on the 8 solutions using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) with capillary cells. The isoelectric point (pI) was determined graphically, as being the pH corresponding to a ζ-potential of zero. ζ-Potential was calculated from the electrophoretic mobility (μ) using the Henry equation, Eq. (5):

$$\zeta = \frac{3\eta\mu}{2\epsilon f(kRh)} \tag{5}$$

where η is the viscosity of the buffer (1.033×10^{-3} Pa s^{-1}), ϵ is the medium dielectric constant (dimensionless), Rh is the complex radius (nm) and f(kRh) is Henry's function. A value of 1.5 was adopted for f(kRh), referred to as the Smoluchowski approximation, as the analysis was performed in aqueous media.

4.1.2.12 Foaming properties

Foaming properties of the samples were measured according to Jarpa- Parra et al. [156], with some modifications. Insect flour and protein extract were rehydrated in distilled water (C = 10 g/l) and left for 12 h at 25 °C for swelling. Ultraturrax mixer (Colonial Scientific, DI 25 basic yellow line, Richmond, USA) was used to prepare foam through homogenization of 40 mL of solution at 9500 rpm for 1 min. The homogenized solution was poured into a 100 mL graduated cylinder sealed with parafilm. The foaming capacity (FC) was calculated according to Eq. (6). V_t and V_0 represent the formed foams after homogenization, and the volume of the protein solution, respectively. The foaming stability (FS) was evaluated based on Eq. (7), V_{foam} is the foam volume at specified times (t_n) of 5, 10, 15, 30, 60 min at 20 °C and V_f is the foam volume immediately after mixing at time zero (t_0).

$$FC = \frac{V_t - V_0}{V_0} \, x \, 100\% \tag{6}$$

$$FS = \frac{V_{foam}\ (t_n)}{V_f\ (t_0)} \, x \, 100\% \tag{7}$$

The whole experiment was performed by the same person and conducted in triplicate at room temperature (25 °C), 75 °C, and 85 °C.

4.1.2.13 Statistical analysis

All statistical tests were done using ANOVA, the level of significance was $p < 0.05$, followed by Tukey’s test using the Statistical Package for the Social Sciences software (SPSS 22.0, SPSS Inc., Chicago, IL, USA).

4.1.3 Results and Discussion

4.1.3.1 Nutritional composition

The nutritional composition of BSFL flour and protein extract were analyzed including dry matter, ash, and protein content, and are reported in Table 1.

Table 1- Dry matter, ash, protein content, and mineral composition for BSFL flour and protein extract.

Composition	Elements	Flour	Protein extract
Dry matter (%)	-	94.8	97.4
Ash (%)	-	9.2	14.4
Protein (%)	-	37.3*	61.1**
Macrominerals (g/kg)	Na	0.90	20.2
	Mg	3.04	0.17
	K	12.4	1.34
	Ca	15.6	0.49
Microminerals (mg/kg)	Cr	0.84	1.05
	Mn	124	6.65
	Fe	916	776
	Co	0.10	0.06
	Ni	0.57	0.55
	Cu	12.1	32.9
	Zn	92.4	29.7
	As	0.13	0.02
	Se	0.41	0.71
	Sr	150	8.41
Heavy metals (mg/kg)	Cd	0.14	0.09
	Pb	0.49	0.27
	U	0.02	0.03
	Hg	0.01	0.02
Total minerals (g/kg)		33.2	23.0

* nitrogen-to-protein conversion factor of 4.67

** nitrogen-to-protein conversion factor of 5.62

The dry matter of the flour and protein extract were 94.8% (w/w) and 97.4% (w/w) respectively. The ash content of flour was 9.2% (w/w) and 14.4% (w/w) for the protein extract. The results for flour and protein extract ashes were similar to those reported by Mintah et al. [135], at 8.5% and 12.4% (w/w), respectively. After extraction, 50g of BSFL flour yield 22.7% of protein extract, 18.9% of chitin extract and 9% of lipid extract. The lipid and chitin extracts were stored, and the protein extract was considered in this study for protein quantification and characterization. Using a conversion factor of 4.67 and 5.62 for insect flour and protein extract, based on Dumas's method, the protein content of raw BSFL flour was 37.3% and after alkaline extraction, protein extract had 61.1% of proteins. The data was in accordance with the results found by Janssen et al. [149], at 36.7% and 67.6% for BSFL flour and BSFL protein extract, respectively. The mineral content was lower in the protein fraction (23.0 g/kg) compared to the flour (33.2 g/kg) (Table 1), as the extraction process separates the proteins from the chitin and minerals. For most individual minerals, the content decreased after the protein extraction process (e.g. Mg: 3.04 g/kg in the flour, 0.17 g/kg in the protein extract). Further studies need to be performed in order to understand how different methods of extraction can alter the mineral content. Regarding the flour, the comparison with other studies suggests that the mineral content could vary depending on the feeding substrate of the larvae, yet this variation might be minor according to Spranghers et al. [157,158]. It is known that BSFL accumulate calcium and manganese during life, but not sodium or sulphur which is in accordance with the results herein reported [158]. In general, different parameters might alter the nutritional composition of BSF in different stage of life, for example, a study using a mixture of waste and specific amount of protein/fat showed that using more protein in their diet produce a more proteinaceous insect, however this correlation was not very clear for fat [159]. The processes used to prepare the flour also can influence the nutritional composition such as the defatting step can be performed by some methods and using a variety of solvents such as n-hexane, ethanol, ethyl acetate, etc. Ravi et al. [21] showed that each method will affect the lipid composition and the quality of proteins at the end of extraction. Therefore, the obtained results for nutritional composition here presented, is directly related to all the parameters of processing applied in this study and can be altered if modifications occur.

4.1.3.2 Amino acid composition

Table 2 reports the amino acid composition, expressed in mg of amino acid per gram of crude protein where the essential amino acids: threonine, methionine, valine, histidine, lysine, leucine, phenylalanine, and isoleucine, can also be found. The flour and the protein extract contained all essential amino acids (EAA) measured (tryptophan was not measured). Quantitatively, the sum of all the EAA, 295.8 mg/g, and 424.2 mg/g, for flour and protein extract, respectively, meets the requirement set out by the World Health Organization (minimum of 277 mg/g) [160]. BSFL flour showed low values for some EAA such as threonine, methionine, lysine, and leucine, according to the FAO [23,133]. However, except for threonine, BSFL protein extract showed higher levels for all EAA which makes the method of extraction interesting when nutritional value is considered. Spranghers et al. [157] have shown that concentration of each amino acid might have small differences according to the BSFL diet. Herein, we have reported that the amino acid content might also vary after protein extraction and previous processing such as the killing method [133]. Mintah et al. [6] has also reported changes in the amino acid (AA) content based on different protein extraction methods for BSFL. When compared to other protein sources such as meat and chicken, BSFL protein extract can be considered as a sustainable alternative source, based on the greater values for some EAA. In developing countries, lysine is of particular interest as it shows low content in people’s diet [133], and the recommended concentration (45 mg/g) can be easily reached by using BSFL protein extraction as a food fortification ingredient.

Table 2- Amino acid (AA) composition (mg/g of crude protein) and total AA content (w/w %dry weight) of the Black Soldier Fly larvae flour and protein extract, compared with the adult daily requirements

Amino acid	Flour	Protein extract	Adult daily requirements 1985 FAO/WHO/UNU
Histidine	14.5	23.6	15
Leucine	49.9	78.9	59
Isoleucine	45.1	64.8	30
Lysine	39.7	47.1	45
Methionine	11.8	18.1	22 (Met + Cys)
Phenylalanine + Tyrosine	51.6 (24.4+ 27.2)	91.1(44.8+ 46.3)	38 (Phe + Tyr)
Threonine	21.5	21.7	23
Valine	61.7	78.9	39
Tryptophane	nd	nd	6
Sum essential AA	**295.8**	**424.2**	**277**
Alanine	59.2	67.4	
Arginine	20.9	20.9	
Aspartic acid	52.4	75.6	
Glutamic acid	80.5	77.4	
Glycine	39.9	44.8	
Hydroxyproline	1.3	0.7	
Proline	62.2	65.2	
Serine	26.7	26.8	
Sum non-essential AA	**343.1**	**378.8**	
Sum AA	**638.9**	**803**	

(Met: methionine, Cys: Cysteine, Tyr: Tyrosine, Phe: Phenylalanine, nd: not detected)

4.1.3.3 Protein composition SDS-page

SDS-PAGE technique was performed under reducing conditions, with dithiothreitol (DTT) reagent (Fig. 1). In Figure 1 the image of the marker, from the same gel, was added on the side of protein extract and flour line, in order to make it easier to identify the bands. Two main regions could be noticed for protein extract distribution, one band right below 66 kDa (according to the marker reference) and another region below 31 kDa. Ravi et al. [161] have already reported a similar protein distribution under different product processing (conventional drying, frozen, microwave drying, scalding, and blanching). The authors matched the lower protein bands to the cleavage of disulfide bonds, under reducing conditions, which guides to protein denaturation and further breakdown into peptides. Rabani et al. [19] have also stated the large presence of structural and muscular proteins showing stronger bands in the region between 75 and 50 kDa. This range of molecular weight, for insect samples, has been linked to enzymes, muscle proteins and exoskeleton proteins in other previous works for *H. illucens* and *Tenebrio molitor* [21,23]. The low molecular weight proteins, under 31 kDa might also refer to glutelin proteins from actin family, with Troponin C the most abundant according to Leni et al. [162]. Hexamerin F, a common protein in larvae of insects that functions as storage protein[163], was identified by Leni et al.[162] as the main protein between 116 kDa and 66 kDa bands. The protein extraction, defatting, drying, and killing methods applied to BSFL samples are known to exhibit a different protein profile and must be considered for further analyzes [21,161]. A darker area is seen on the top of the gel in the protein extract area, which might correspond to proteins that did not fully run through the gel, due to protein aggregation that caused an increase in size. Gels performed with bigger pores might facilitate the separation of such proteins and identification, in the future. According to BSFL flour and protein extract, it is proposed that insects could be a promising source for fortification and technological investigation purposes [19,142,143].

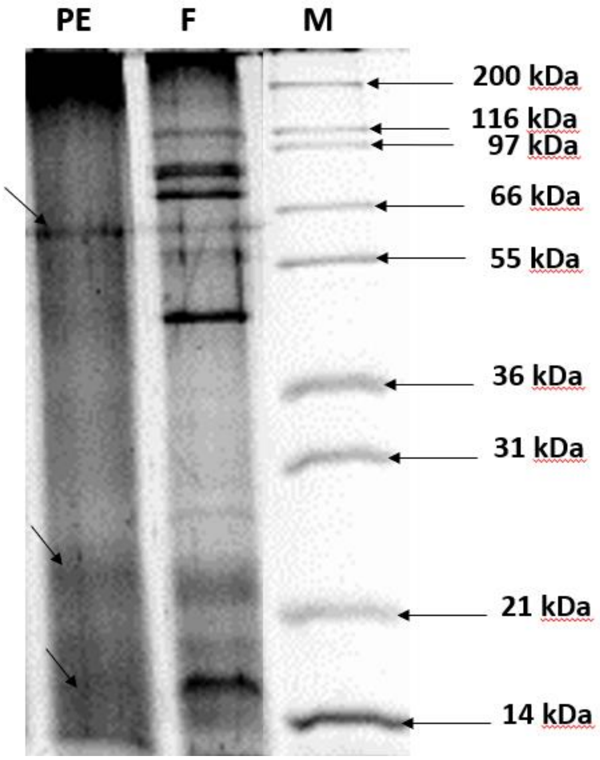


Figure 1. SDS-PAGE analysis of BSFL protein extract and flour, where PE= Protein extract; F= Flour and M= Marker.

4.1.3.4 Turbidity

Absorbance at 400 nm was applied to calculate the turbidity (optical density, OD) of the dispersion. Three different treatments of temperature, untreated (25 °C), 75 °C, and 85 °C were used to observe any change in turbidity. An increase in turbidity might be due to protein aggregation that remained suspended in the liquid matrix [89,164]. Fig. 2 shows that OD clearly increases after each treatment and the difference was statistically significant according to Tukey's test for $p<0.005$. Even though the concentration used was low (1% w/v) it was enough to reveal some protein aggregation and increase in turbidity. Untreated group had OD of 0.297± 0.001, 75 °C group had OD of 0.368 ± 0.004 and 85 °C 0.414 ± 0.001. All measures were performed in duplicate In accordance, we reported an increase in particle size after temperature treatment which can be directly correlated to the increase in turbidity, where the highest turbidity was verified in the sample treated at 85 °C that showed the highest particle size. In addition, LaClair and Etzel [165] have reported a complete study on protein aggregation and increase in turbidity upon heating treatment of whey protein in beverages over storage.

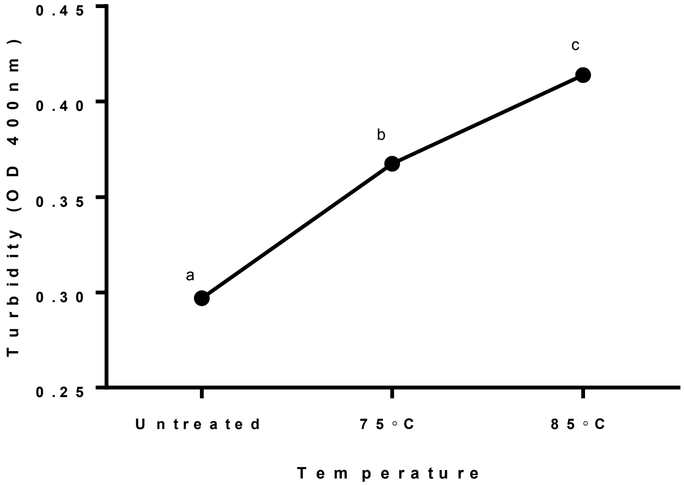


Figure 2. Turbidity OD 400nm of BSFL protein extract dispersion (1% w/v), untreated, heat-treated (15 min) at 75 °C and 85 °C. Different letters mean a significant difference for $p < 0.05$ among samples.

4.1.3.5 Thermal stability

DSC analysis gives detailed information about the qualitative and quantitative thermal properties of a solid material such as the melting and the degradation temperature, and specifically heat-induced denaturation of proteins [165]. The thermograms present the heat flow (W/g) in function of temperature (°C). The DSC graphs for the BSFL flour (A) and BSFL protein extract (B) are shown in Fig. 3. All thermograms are marked with an X, Y, and Z letters for glass transition (Tg), unfolding, and solid melting, respectively. Tg was observed as a shift in the curve between 20 to 30°C for the BSFL flour and 30 to 50 °C for BSFL protein extract, and the results are similar to the previous data reported for Tg of *H. illucens* protein extract by Barbi et al. [166]. The range in Tg temperature might be due to different protocols of protein extraction. An exothermic peak, right before the unfolding peak (Y), temperature range between 110 °C to 140 °C, appears in the flour graph (Fig. 3A) but not in the protein graph (Fig. 3B). This led to the hypothesis that this peak was characteristic for remaining components such as chitins and lipids rather than the proteins from BSFL flour [167,168]. BSFL protein extract thermogram, Fig.

3B, showed an endothermic peak (Y), which is defined at 150 °C, resulting from the thermal unfolding of the proteins [151,169]. Both the BSFL flour and protein extract also showed an evident endothermic peak (Z) at around 200 °C, which has been previously reported as the melting point [151], and restrained weight loss due to moisture content [166]. The complete degradation of BSFL proteins can be reached at 500 °C according to Barbi et al. [166] and Wang et al. [170].

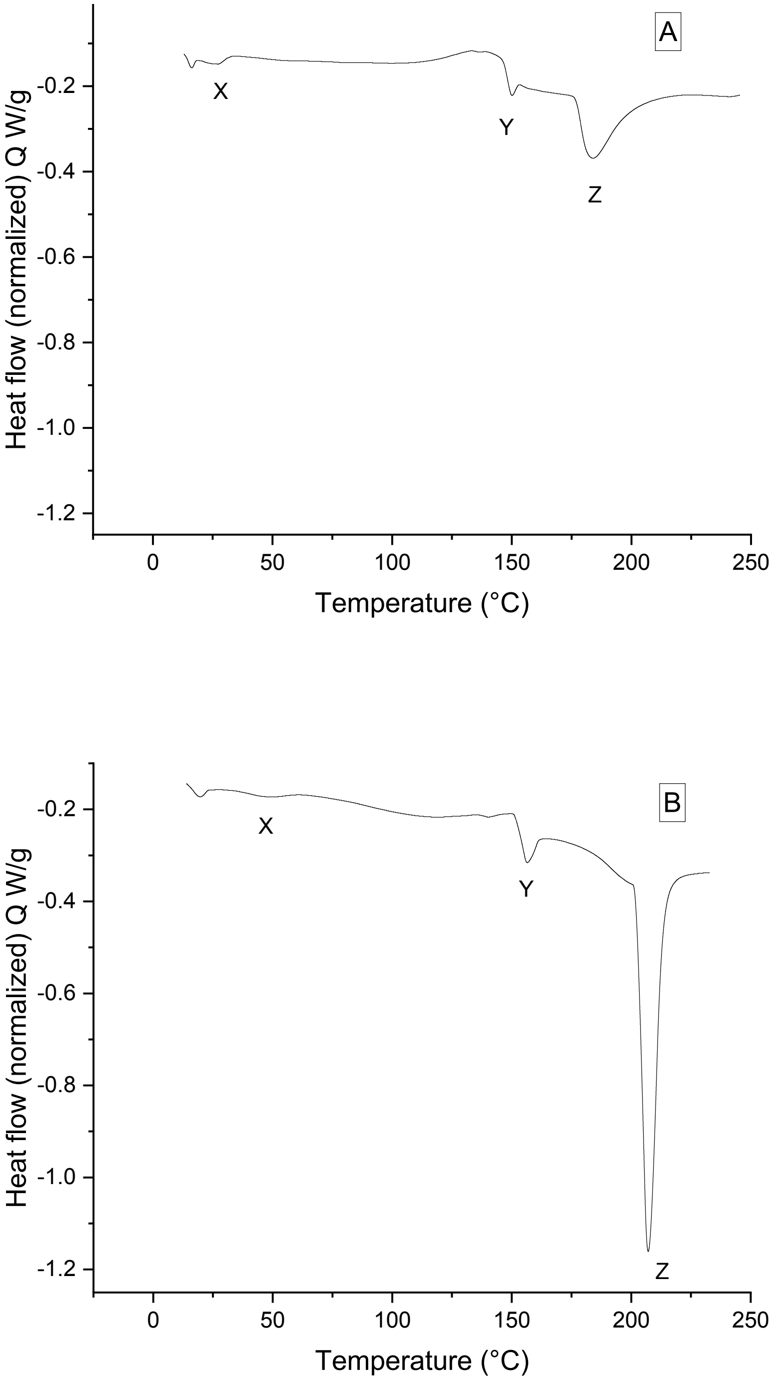


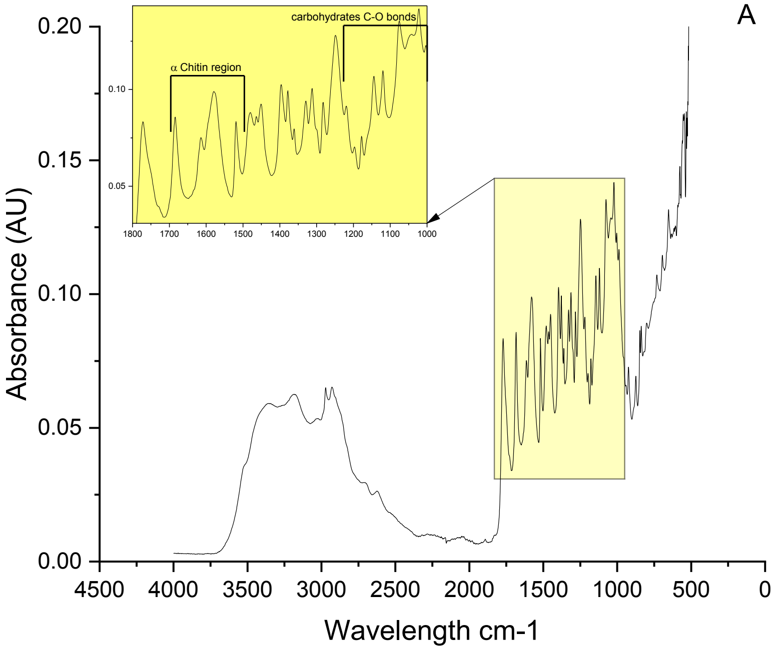
Figure 3- DSC thermograms of (A) BSFL flour and (B) BSFL protein extract.

4.1.3.6 FTIR spectra

In order to obtain a biochemical fingerprint of BSFL proteins and to verify if the extraction process might have altered the molecular bonds from BSFL proteins, FTIR analysis was applied in the flour and in the protein extract (Fig. 4). The technique might provide solid information about molecular composition, functional groups, and the secondary structure of proteins. In general, five distinct FTIR spectra regions can be identified corresponding to the Amide group due to the presence of peptide bonds between amino acids (Table 3). For FTIR protein extract analyses, a clear N-H free stretching vibration could be noticed in the range of 3500 – 3200 cm^{-1}. However, referring to insect powders some authors have linked the peaks in the same range to vibrations of O-H group stretching [171,172]. Amide B group, 3100 – 2900 cm^{-1} seems to be represented by a split of two peaks. This wavelength result represents the asymmetric and symmetric stretching of C-H. The next Amide I and II bands are the most prominent and characteristic vibrational bands from the protein back-bone in the FTIR spectra [173]. Amide I band is identified in the range of 1700 – 1600 cm^{-1} which is caused by the stretch vibrations of carbonyl group C=O from peptide linkage. The frequency of Amide I is also correlated to the secondary structure of proteins [173]. The result herein reported (Fig. 4 B) shows high absorbance at 1634 cm^{-1}, and according to Kong & Yu [173], the region 1640-1620 cm^{-1} has been assigned to β-sheet structure. In contrast, Amide II band in the range of 1550 – 1500 cm^{-1} derives from in-plane N-H bending and CN stretching vibration and although it might be correlated to protein secondary structure, it shows less protein conformational sensitivity compared to Amide I [173]. C-O stretching vibrations between 900 -1200 cm^{-1} are normally caused by carbohydrates [171]. The band near 1040 cm^{-1} was observed to correspond to some polysaccharides such as galacturonic acid and glucuronic acid [171]. Amide III band was identified at 1233 cm^{-1} according to Nagarajan et al [171]. This region represents the combination between peaks of C–N stretching vibrations and N–H deformation from amide linkages, and also absorptions from CH_2 wagging vibrations, arising from two amino acids, glycine and proline side-chains. The FTIR spectra of the protein extract were well defined according to the reported bands. However, due to the more complex composition, the flour FTIR spectra had more bands in the same range (Fig. 4 A). In the insect flour FTIR spectra the absorbance intensity in the range of 900-1200 cm^{-1}, which is associated with carbohydrates C-O bonds, was higher for flour than for the protein extract. In addition, the sharp bands between 1500 and 1700 can be related to the presence of α-chitin, as previously reported for other insects [167,174]. BSFL flour has a very complex composition, with high content of chitin, lipids and other elements that made it difficult to point out the specific region for proteins in the FTIR spectra. However, all amide bands where well identified in the protein extract showing the efficiency of the extraction process.

Table 3-Characteristic infrared band frequencies and their corresponding functional groups in *H. illucens* protein extract.

Designation	Found frequency (cm^{-1})	Description
Amide A	3274	N-H stretching
Amide B	2929	C-H stretching
Amide I	1634	C=O stretching
Amide II	1514	C-N stretching, N-H bending
Amide III	1233	C-N stretching, N-H bending



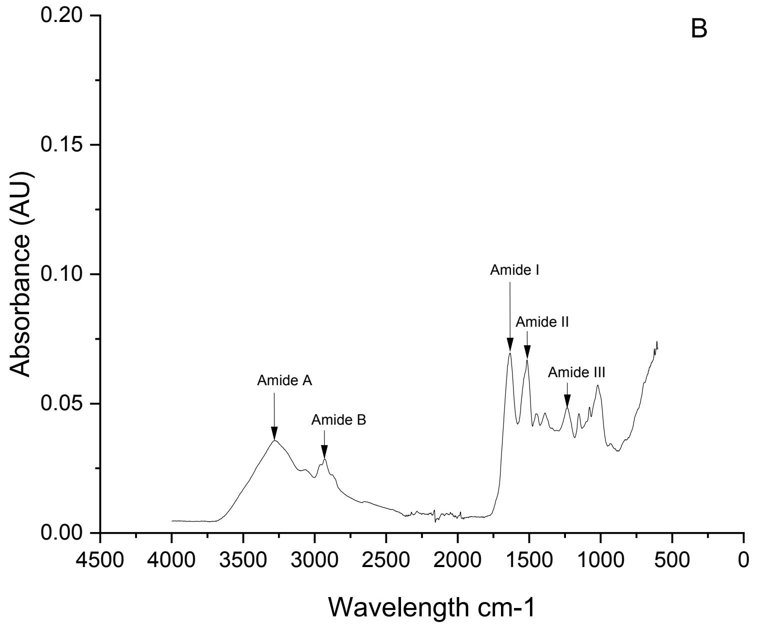


Figure 4. FTIR spectra of (A) BSFL flour and (B) BSFL protein extract.

4.1.3.7 Foam capacity and foam stability

Fig.5 shows the protein extract foamability and foam stability at 1% w/v of protein content, under different temperature treatments. The lowest foamability (Fig. 5B) was found for untreated dispersion (65%) and the highest value for samples treated at 85 °C (72%). However, no statistical difference between all groups was observed ($p < 0.05$). Foam stability is reported in Fig. 5A. The result represents how foam stability might increase according to the heating treatment on the protein matrix, due to the capacity of unfolded proteins with exposed hydrophobic groups, and form a more cohesive surface film at the air-water interface [175]. After 5 min, the lowest foam stability was found for untreated samples (66%) followed by the sample heated at 75 °C/ 15 min (83%) and finally the sample at 85 °C/15 min (96%). A statistically significant difference could be noticed between the untreated and 85 °C/15 min samples, during the first 20 min of the experiment. According to Lawal et al. [176], high foam stability could be a result of the formation of stable molecular layers at the air-water interface which will consequently cause benefits in texture, stability, and elasticity. This result was in accordance with the size and ζ-potential data, which suggests charge decrease and protein aggregation under heat treatment causing the particle size to increase. The graphic shows results for foam stability up to 60 min. However, the experiment was carried out for more than 12 hours, and no reduction in foam volume was observed for all groups after this point (data are not shown). Santiago et al. [89] have also reported the foamability and foam stability for black cricket protein isolate. The authors showed an increase in foamability after heat treatments of 75 °C/ 15 min and 95 °C/ 15 min, with the highest value for samples treated at 95 °C. The foam properties of *H. illucens* from two different protein extracts under different pH, were previously reported by Mintah et al [135]. The study revealed the best foamability at pH 6 and foam stability at pH 4, and in the Isoelectric point region, no temperature treatment was mentioned.

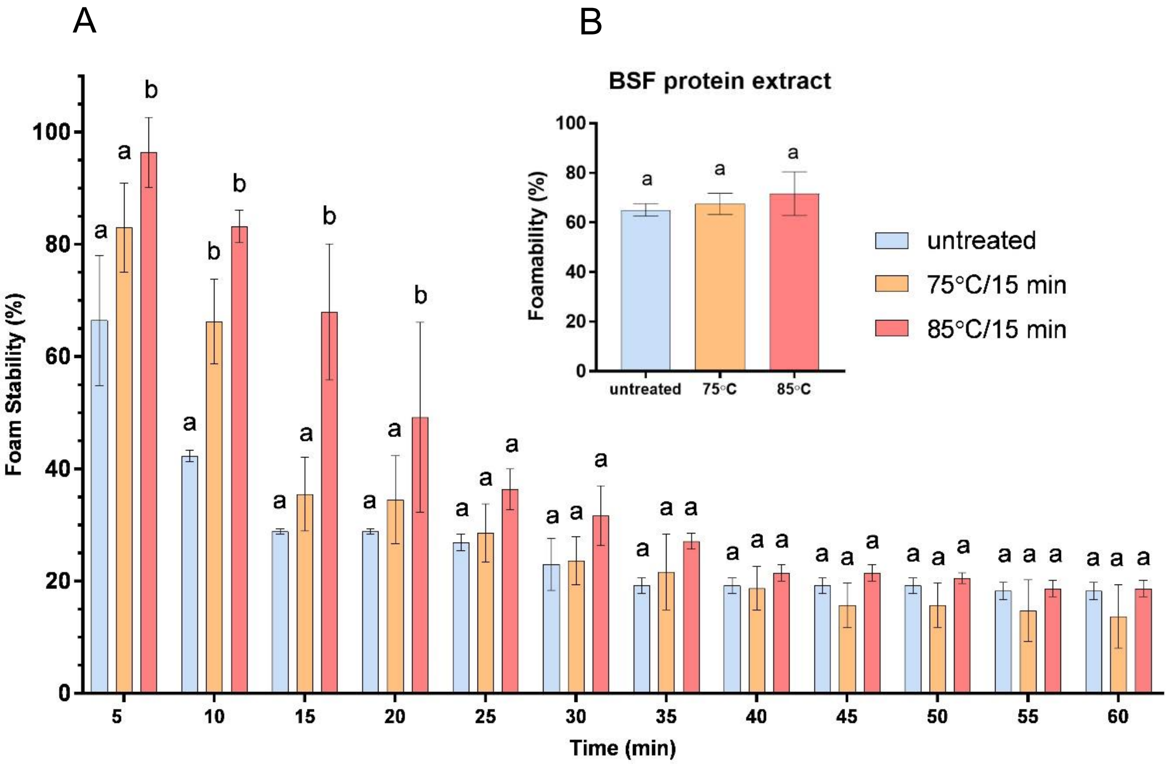


Figure 5- Foam stability (A) and foamability (B) of BSFL protein extract (1% w/v) concentration under three different conditions: untreated, 75°C/ 15 min and 85°C/ 15 min.

4.1.3.8 Particle size distribution and ζ-potential

The particle size was expressed as the hydrodynamic diameter (d_h) of proteins in an aqueous dispersion, and it was determined by the dynamic light scattering method, stated in Table 4. Samples were considered as a polydispersed system which suggests that there were a few populations within the sample. Polydispersity index (PdI) was also reported in Table 4 in order to estimate average uniformity of samples. Samples are considered as polydisperse when PdI is higher than 0.1, which matches with the data herein stated for all samples [177]. It might be confirmed by the d_h of 174.76 ± 1.61 nm for the untreated sample, which was larger than the upper size for typical proteins. However, after the heating treatments, it was clear to see how aggregation was induced and particle size extensively increased. Samples heated for 15 min at 75 °C showed an increase up to 202.80 ± 7.30 nm and the highest d_h was found for samples heated at 85 °C, 251.56 ± 5.50 nm. Upon heating, treated proteins might unfold and cause exposure of hydrophobic side chain groups. This may lead to a reduced solubility and formation of intermolecular hydrophobic bonds that cause further protein aggregation and particle size to increase [100,135,178]. Mishyna et al. [58] have shown the heat-induced aggregation of proteins for *Apis millifera* protein extract at temperatures of 55, 70, 85, and 100 °C, with the highest protein coagulation reached at 85 °C. ζ-potential is the difference between the mobile dispersion medium and the stationary layer of fluid, the slipping plane, attached to the dispersed particle [179]. A numerically high ζ-potential, close to ± 30.00 mV, is considered as an indication of stability and lower values, close to zero, indicates protein instability as it might favor hydrophobic-hydrophobic interactions and cause protein aggregation [151,169].

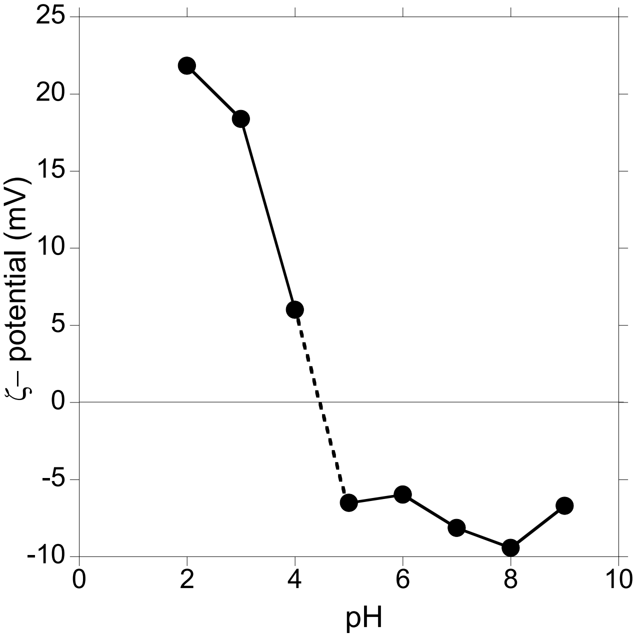


Figure 6. ζ-potential as a function of the pH.

The ζ-potential data showed that all samples were negatively charged, and the highest values were found for the untreated sample -15.63 ± 0.4 mV. After the heat treatment, a significant difference was observed between untreated and treated samples, where samples 75 °C/ 15 min and 85 °C/ 15 min showed a very low value of -5.96 ± 0.40 mV and -6.51 ± 0.7 mV, respectively. This reduction in charge might help to explain the previously reported particle size increase, and lower ζ-potential indicates less repulsion between proteins and more probability of protein aggregation [180]. Fig.6 revealed the ζ-potential variation according to the pH range from pH 2 to 9. The data showed that all samples were positively charged in pH range 2 - 4 and negatively charged from pH 5-9. The isoelectric point (p*I*) value was established by the intersection of the curve on X-axis at 0 mV. The Isoelectric point was defined at 4.5 which was similar to other previous studies of insect protein values [60].

4.2 GELLING PROPERTIES OF BLACK SOLDIER FLY (*Hermetia illucens*) LARVAE PROTEIN, AFTER ULTRASOUND TREATMENT.

The content of this chapter was submitted and is under approval at:

Food Chemistry

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4.2.1Introduction

Most of the research on functional properties have been conducted with non-sustainable animal-based protein, which includes dairy, meat, poultry, and fish [181]. However, the consumption of animal proteins has a substantial effect on the environment, which is directly correlated to climate changes, higher emission of greenhouse gases and depletion of natural resources [182]. As the world population is predicted to reach 10 billion by 2050 as well as the rise of the dairy and meat consumption is expected to be incremented by 58% and 73% respectively, sustainable consumption and production of protein should be considered [183].

Amongst the several feasible and sustainable solutions, insect proteins have gained increased attention by FAO, since they release less greenhouse gases and ammonia, cause less pollution, require reduced space and water and have a high protein content with the presence of all required amino acids for human consumption [182]. The feed conversion ratio *i.e.*, FCR (kilogram feed: kilogram live weight) was found to be 1.7 for black soldier fly larvae as opposed to 2.5 for chicken, 5 for pork and 10 for beef. This indicates that the insect is more efficient to convert feed into body mass since they are cold blooded and lack the demand of feed to sustain their body temperature (van Huis et al. 2013).

Hermetia illucens, popularly known as black soldier fly (BSF) has been considered one of the most promising alternatives of proteins source [18]. BSF can feed and digest on various types of food, including abattoir waste, animal waste, spent grains and turn them into a nutrient rich food, reducing agricultural and other organic wastes [18]. From a nutritional aspect, BSF larvae (BSFL) is mainly rich in protein (37 to 63% dry matter) and lipid (7 to 39% dry matter) [11]. Additionally, the amino acid composition of BSF meets the requirements set by WHO, which makes BSF a good quality alternative source of protein [11,18]. A great amount of structural and muscular proteins have been identified in BSFL, which corroborates with the possibility of applying its proteins as an alternative to replace meat and soybean proteins[142] (Altmann et al. 2018).

Even though insects showed interesting nutritional characteristics and reduced impact in the environment, the visual aspect of whole insects still causing an aversion for the consumption amongst consumers, in the western world [135]. However, this fact can be mended by the extraction of insect protein and production of insect powder, which can be further incorporated as food fortification ingredients. Therefore, processed form of insects can be incorporated in foods to slowly transition the consumers to take the preliminary step to taste insect-based foods. In order to increase consumer acceptance, comprehensive knowledge should be gained on the techno functional properties of insect proteins which can help in introducing new food products with insect proteins [11].

Insect processing and protein extraction can directly affect the final protein content and its techno-functionality. Recent studies have investigated how defatting followed by alkaline extraction of proteins can impact of physicochemical and functional properties of BSFL proteins [11,135].

Recently, there have been a few studies exploring the different techno-functional properties of insect protein (solubility, water holding capacity, oil absorption capacity, gelling, foaming and emulsifying properties), which uncovers their technological potential and further use in food applications [89]. In order to improve these techno-functionalities, different treatments can be applied such as ultrasound technique [28].

Ultrasound is an acoustic wave method that involves frequency higher than 20 kHz, which causes a quick formation and collapse of gas bubbles, recognized as ultrasonic cavitation. The technique is normally employed to either chemical or physical food alterations [186]. The cavitation phenomenon causes hydrodynamic shear forces and a rise in temperature at the site of bubble collapse which affects the physicochemical properties of protein. With a of reduced processing time and the low amount of solvent needed, ultrasound treatment has been considered as a green technology that can enhance protein application in food systems [187].

There have been many studies on improving the physicochemical and functional properties of proteins due to the application of high intensity ultrasound such as solubility, surface hydrophobicity, water holding capacity, gelling, emulsifying, and foaming properties [188]. In addition, the use of high energy ultrasound has an effect on the particle size, microstructure, secondary structure, and tertiary conformation of proteins [188]. Ultrasound has also been applied to some insect species such as *Tenebrio molitor*, *Gryllus bimaculatus* and *Bombyx morri* for improved protein extraction [189]. A previous study investigated the influence of ultrasound treatment in the control of silk fibroin gelation. It was stated that sonication initiated the β-sheet formation and accelerated the formation of physical cross-links involved in gel stabilization [190].

However, no studies have reported the use of ultrasound treatment on gelling properties of BSFL protein extract and there has been a lack of information of how sonication can affect the physicochemical properties of BSFL protein extract. Therefore, the aim of this study is to investigate the gelling properties of protein isolated from BSFL (Black soldier fly) larvae flour and evaluate the impact of ultrasound treatment on the size, structure, and functionality of proteins. The characterization of the dispersions and gels made from BSFL protein extract was investigated. The preparation of BSFL gels was done to evaluate the mechanical and viscoelastic properties of the gel by oscillatory rheological measurements. Furthermore, the effect of ultrasound treatment on the physicochemical properties and the possibility of improving the gelling properties was also carried out. Finally, the pore size by confocal laser scanning microscopy (CLSM) and image analysis was studied.

4.2.2 Materials and methods

4.2.2.1 Raw materials

Black soldier fly (BSFL) larvae flour was supplied by nextProtein (Paris, France). n-Heptane and Hydrochloric acid, 37% were purchased from VWR Chemicals (Gilwice, Poland). Sodium hydroxide (pellets), Sodium dihydrogen phosphate dihydrate, di-Sodium hydrogen phosphate dihydrate were obtained from Merck KGaA (Darmstadt, Germany). Rhodamine B base (dye content: 97%) and 8-Anilino-1-naphthalenesulfonic acid (ANS) were also purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2.2 Protein extraction

For the lipid extraction, the BSFL flour was stirred with heptane (ratio 1:5, w/v) for 1 h at 40 °C. The mixture was then decanted, and the lipid extract (liquid fraction) was recovered with paper filtration (N° 00H, 7 cm, Sweden). After evaporation of the solvent by a rotary evaporator (Laborota 4000-efficient, Heidolph instruments GMBH & CO KG), the recycled solvent was used for a second extraction in order to remove any residual fat remaining in the insect flour. After the lipid extraction, the defatted flour was left in the fume hood overnight to evaporate the residual solvent. It was then dissolved in 0.25 M NaOH (ratio 1:15, w/v) according to [66] and stirred (Polymix Buch & Holm, DK) at 300 rpm, 40 °C for 1 h. After centrifugation (Sigma Laborzentrifugen GmbH 4-16KS, Germany) at 2493×g 4°C for 20 min, a 2nd alkali extraction followed by centrifugation was done on the solid fraction. This remaining solid fraction was stored and referred to as the chitin extract. The supernatants from both extractions were combined. The pH of this obtained liquid was adjusted to an isoelectric point of 4.3-4.5 with 2 M HCl to precipitate the proteins. After decantation, the solution was centrifuged at 1272×g 4 °C for 15 min to recover the precipitate. Finally, the precipitate was washed twice with distilled water and freeze-dried overnight.

4.2.2.3 Protein content

The protein content in BSFL flour and protein extract was measured with Dumas method, a quantitative analysis of the protein content based on the amount of nitrogen. According to [191], the nitrogen conversion factor that was used for BSFL flour and BSFL protein extract were 4.67 and 5.62 respectively in order to avoid the overestimation of protein content due to the presence of non-protein nitrogen in insects. Kp value is given by the sum of all anhydrous amino acids and the percentage of nitrogen (Nt), determined by the Dumas method. The percentage of nitrogen for BSFL flour and BSFL protein extract were 7.70% and 12.06%. The measurements of each sample were performed in duplicates, using 200 mg in crucibles. Aspartic acid and wheat flour were used to calibrate the instrument

4.2.2.4 Ultrasound treatment

The 10% (w/v) dispersions were prepared by stirring BSFL protein in 0.1M phosphate buffer at pH 7 for two hours at 20 °C. 30 ml volume of 10% (w/v) protein dispersions were put in an ice bath to reduce the increase in temperature during ultrasound treatment. The probe was inserted inside the solution and the ultrasound treatment (Branson Sonifier, Emerson, St. Louis, MO, USA) was fixed at 20 kHz, 500 W, pulse ON and OFF for 5 s at different exposure time for 5, 15 and 30 min. In order to determine the influence of ultrasound intensity in a process, it is necessary to measure the actual ultrasonic energy transferred to the medium. The temperature of the liquid being sonicated was recorded versus time with a thermocouple (Pico Technology, St Neots, UK) connected to a temperature recorder. The dissipated acoustic power in the liquid was calculated with the help of equation (1) according to [28] with slight modifications. The temperature rise was estimated from the slope of the straight portion of the line that was obtained during the first 60 s of the experiment. The acoustic power intensity, I (W/cm^2) was calculated by equation (2) based on [128]:

$$P = mCp\frac{dT}{dt} \tag{1}$$

$$I = \frac{P}{S_A} \qquad (2)$$

where P (W) is the acoustic power, S_A is the surface area of the ultrasound emitting surface (cm^2), m is the mass of ultrasound treated solution (g), Cp is the specific heat of the medium (4.18 J/g °C) and $dT\ /\ dt$ is the rate of temperature change with respect to time, starting at t=0 (°C/s). The acoustic power of the BSFL protein dispersions was calculated to be 20.38 W (II), 24.54 W (III) and 29.14 W (IV) respectively. The acoustic power intensity of the BSFL protein dispersions were found to be 29.53 W/cm^2 (II), 35.56 W/cm^2 (III) and 42.23 W/cm^2 (IV) respectively. The treatments were labelled: sample without ultrasound treatment (I), sample after 5 min (II), sample after 15 min (III) and sample after 30 min (IV) of ultrasound treatment.

4.2.2.5 Particle size and zeta potential by dynamic light scattering (DLS)

The dynamic light scattering (DLS) analysis, which included the particle size (nm) and zeta potential (mV) was measured with Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) followed according to [89]. The 10% (w/v) dispersions, including the ultrasound treated samples were diluted to 0.1% (w/v) with 0.01M phosphate buffer at pH 7 and was kept for stirring overnight. All the dispersions were filtered using filters of 0.45 µm (Thermo Fisher, Massachusetts, USA) and were finally filled in the capillary cells (Malvern Instruments, Worcestershire, UK) to measure the particle size and polydispersity index at 25 °C as well as the zeta potential at 23 °C. The measurements of each sample were performed in triplicates.

4.2.2.6 Turbidity

The filtered protein dispersions were filled in the cuvettes (Eppendorf, Hamburg, Germany). The absorbance was measured at 400 nm based on [89] in a UV–Vis U-1500 spectrophotometer (Hitachi, Tokyo, Japan) and the measurements of each sample were performed in triplicates.

4.2.2.7 Surface hydrophobicity

The surface hydrophobicity was determined using 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe, according to the method described by [89] with slight modifications. The dispersions were diluted to reach different concentration (0.0025, 0.005, and 0.01% w/v) with 10 mM phosphate buffer at pH 7. Then, 20 µL of 8 mM ANS solution was prepared in the same buffer and was added to 4 mL of each protein dispersion and further mixed. The dispersions were kept in the dark for 15 min at room temperature. The extrinsic fluorescence intensity was measured using a SPECTRAmax GEMINI spectrophotometer (Molecular Devices, CA, USA) according to [58] with an excitation wavelength of 390 nm and the emission spectrum was collected at 470 nm. The blanks were labelled buffer with ANS and protein dispersions without ANS. All experiments were performed in triplicates. The surface hydrophobicity was determined as a slope of the linear regression of the relative fluorescence intensity and protein concentration.

4.2.2.8 Preparation of gels

After the ultrasound treatment, the 10% (w/v) dispersions were transferred in tubes (Nerbe Plus, Winsen, Germany) of 5 ml each for every sample to heat at 85 °C for 30 min in the water bath (Julabo, Seelbach, Germany) and then cooled down in an ice bath for 2 hours at 4 °C, which was based on [192] with slight modifications. The gel formation was determined through visual observation. The tubes were inverted and was checked if the gel did not slip or fall.

4.2.2.9 Water holding capacity

The water holding capacity can be defined as the ratio between the remaining mass after centrifugation and the initial mass of the samples, expressed in percentage. The weight of the dispersions before heating in the water bath (Julabo, Seelbach, Germany) as well as before centrifugation was noted. Initially, BSFL protein was suspended in 0.1M phosphate buffer at pH 7, total volume of 30 ml, which were stirred on the magnet stirrer for two hours, followed by ultrasound treatment. The dispersions were transferred to centrifuge tubes and heated at 85 °C for 30 mins in the water bath. Samples were then cooled down in an ice bath for 2 hours at 4 °C when gels were formed. Finally, the gels were put in a centrifuge (Sigma 4-16KS, Osterode, Germany) for 15 min, 3000×g at 4 °C according to [193] to verify the presence of supernatant. After the centrifugation, the supernatant was drained carefully and weighed. The weight of the pellet was also noted. The measurements were performed in triplicates for each sample.

4.2.2.10 Rheology

The viscoelastic properties of 10% (w/v) dispersions, including the ultrasound treated samples were studied using Discovery HR-2 Hybrid Rheometer (TA Instruments, USA) based on [23,194] with some modifications. The geometry used was concentric cylinder and 15 ml of sample was loaded. To prevent dehydration, a cap was placed over the samples. The protein dispersions were successively subjected to a temperature ramp, frequency sweep, and stress sweep, during which G' (elasticity modulus), G" (viscosity modulus), and δ (phase angle) were measured. All the rheological measurements were performed in triplicates, each time on a freshly prepared protein solution. A temperature ramp was initiated immediately after loading the samples. The heating was done from 25 °C to 85 °C at 5 °C/min, 85 °C for 10 min and cooling from 85 °C to 25 °C at 3 °C/min. During the temperature ramp, a constant strain of 0.05% and frequency of 0.1 Hz was maintained. A frequency sweep was performed after the temperature ramp, where the frequency was varied from 0.5 to 20 Hz at a constant temperature of 25 °C and a constant strain of 0.01%. Finally, the samples were subjected to an amplitude sweep, where the strain was increased from 0.1% to 5000% at a constant frequency of 1 Hz and at a constant temperature of 25 °C.

4.2.2.11 Confocal laser scanning microscopy

The structure of the gels was observed by confocal laser scanning microscopy (CLSM) according to [195] with slight modifications. A solution with a concentration of 85 g/L with rhodamine B base in dimethylsulfoxide (DMSO) was prepared followed by stirring for 10 min to ensure a homogeneous solution. The suspensions were labelled by rhodamine B base at 0.085 g/L. The suspensions were put in petri dishes in the water bath at 85 °C for 30 min in the water bath followed by cooling in an ice bath for 2 hours at 4 °C. Finally, the samples were imaged using a 100x lens (Nikon CFI plan achromat

NA1.45) on a spinning disc confocal microscope constituted by an inverted microscope (Nikon Ti2) equipped with a laser source (405 / 488 / 561 / 640 nm), a confocal spinning disc module (Yokogawa CSU-W1, 50um pinholes), a quad-band emission filter (440 / 521 / 607 / 700 nm) and an sCMOS camera (Photometrics Prime95B). The average pore size was measured from the confocal images after being analyzed by 2-D correlation analysis using ImageJ® software, as described by [193]. The use of correlation function can be performed when there is a dependence between a physical parameter over an extension. Briefly, the images were adjusted to 16 bits, where each pixel had a greyscale ranging from 2^0 (white) to 216 (black). The proteins appear as white spots while the empty regions as black spots. The unclear patterns were calculated based on a spatial collection function. If the color of a pixel located at the coordinates $\vec{r} = (x, y)$ is set as $I\ (\vec{r})$, the function, $C(\vec{\delta})$, is defined by equation 3:

$$C(\vec{\delta}) \equiv \frac{I(\vec{r})I(\vec{r}+\vec{\delta})}{I^2} - 1 \tag{3}$$

where the result stands for the mean of coordinates over the whole image.

4.2.2.12 Statistical analysis

All measurements were carried out in triplicate unless stated otherwise. The results were shown as the average value ± standard deviation (SD). The values given in the tables and figures are the means of triplicates, and error bars indicate the standard deviation. The values having different lower-case letters within each column are significantly different. The statistical analysis was performed by one-way ANOVA with Tukey’s post-test with a level of significance of $p < 0.05$ using GraphPad PRISM software.

4.2.3 Results and discussion

4.2.3.1 Particle size and zeta potential

The protein content of BSFL flour and the BSFL protein extract was evaluated and found to be 35.67% and 62.24% respectively. The particle size of the BSFL protein dispersions is displayed in Table 1. All the treated dispersions were found to be statistically significant ($p<0.001$) when compared to the untreated dispersion. The particle size significantly increased to 245.3 ± 1.0 nm till 15 min of ultrasound treatment. The results obtained for the increase in particle size are comparable to [196] for the dispersions of black bean protein isolate. This can be attributed to turbulent forces and micro-streaming due to ultrasound treatment that can increase the speed of collision and aggregation, and results in the formation of soluble aggregates. The aggregation can be the outcome of non-covalent bonds such as hydrophobic interactions [197]. Although, the particle size decreased to 197.1 ± 1.1 nm after 30 min of ultrasound treatment which are similar to the results reported by [198] where the particle size of soy protein concentrates and isolates dispersions decreased after 30 min at 20 kHz, 600 W. At a higher power and longer time of ultrasound treatment, the aggregates are violently broken into smaller soluble protein aggregates by the forces of cavitation which can disrupt the non-covalent interactions [196].

The ζ-potential of all the dispersions lies within the range of -20 to -30 mV as shown in Table 1, which indicates that they are moderately stable [199]. The ζ-potential of the treated sample after 5 min and the untreated sample had no significant difference ($p>0.05$). The ζ-potential after 15 min of ultrasound treatment in contrast to the untreated sample increased significantly ($p<0.001$) which can be compared to the results obtained by [196] for the dispersions of black bean protein isolate. This could be attributed to the increase in the exposure of hydrophobic groups to the surface of the protein molecules [200]. Although, after 30 min of ultrasound treatment, the ζ-potential decreased from -26.5 ± 0.5 to -22.4 ± 0.6 mV. At a higher power and longer duration of ultrasound treatment, the observations are similar to the results reported by [197,201] for ovalbumin and whey protein isolate respectively. The reason for the decrease in ζ-potential might be due to the presence of smaller aggregates which prevents the negative amino acids to come to the surface and decreases the exposure of hydrophobic groups to come to the surface [201].

Table 1. Particle size (nm), zeta potential (mV), absorbance and surface hydrophobicity of untreated (A) and treated (B-D) samples.

Sample	Hydrodynamic diameter (d, nm)	ζ-potential (mV)	Absorbance	Surface hydrophobicity
A	141.1 ± 0.7^{a}	-23.6 ± 0.3^{e}	0.178 ± 0.004^{a}	184.7 ± 8.73^{a}
B	181.5 ± 1.0^{b}	-24.3 ± 0.3^{e}	0.212 ± 0.003^{b}	267.7 ± 10.28^{b}
C	245.3 ± 1.0^{c}	-26.5 ± 0.5^{f}	0.554 ± 0.005^{c}	617.9 ± 17.59^{c}
D	197.1 ± 1.1^{d}	-22.4 ± 0.6^{g}	0.249 ± 0.004^{d}	432.5 ± 14.13^{d}

4.2.3.2 Turbidity

The turbidity of the BSFL protein dispersions demonstrated a similar trend as the particle size as illustrated in Table 1. Ultrasound treatment had a significant effect ($p<0.001$) on the turbidity in contrast to the untreated dispersion for all the treated samples. The absorbance of the protein dispersion remarkably increased to 0.554 ± 0.005 till an exposure time of 15 min of ultrasound treatment. This can be attributed to the increase in particle size and the formation of aggregates which led to a rise in the turbidity, as observed by [154] in the study of whey protein isolate dispersions treated with high intensity ultrasound. However, after 30 min of ultrasound treatment, the absorbance considerably decreased to 0.249 ± 0.004. Lower turbidity after 30 min of treatment was due to the decrease in particle size and diameter due to high shear energy waves and turbulence from cavitation which resulted in smaller particles of protein [202]. Due to the decrease in the particle diameter, the surface area available for light scattering increases which results in the decline in turbidity [154]. The turbidity of the BSFL protein dispersions demonstrated a similar tendency as particle size.

4.2.3.3 Surface hydrophobicity

The surface hydrophobicity of the BSFL protein dispersions is shown in Table 1. Ultrasound treatment had a significant impact on the surface hydrophobicity ($p<0.001$) when compared to the untreated sample. The increase in surface hydrophobicity can be attributed to a certain degree of unfolding of the protein molecules due to ultrasound treatment, which causes the rise in the exposure of previously embedded hydrophobic groups to the surface [196]. These results are similar to [28] where the rise in surface hydrophobicity was observed for whey protein concentrate, soy protein isolate and egg white protein dispersions treated with high intensity ultrasound. The surface hydrophobicity was found to markedly increase to 617.9 ± 17.59 till 15 min of ultrasound treatment. These observations can be supported by [203] where the surface hydrophobicity of the dispersions prepared from soybean protein isolate increased to approximately 200 and 225 for 5 and 15 min as opposed to the untreated sample which was approximately at 95. Although, after exposing for 30 min of ultrasound treatment, the surface hydrophobicity considerably decreased to 432.5 ± 14.13. These results are comparable with [196,201] for black bean protein isolate and whey protein isolate respectively. At higher power and longer time of ultrasound treatment can lead to partial denaturation of proteins which can reduce the surface hydrophobicity [196]. As previously stated by the particle size results, the longest ultrasound treatment might cause the breakdown of big aggregates resulting into smaller ones by the intermolecular hydrophobic interactions, thus hiding most of their hydrophobic amino acid groups in the inner part of the aggregates [201].

4.2.3.4 Water holding capacity

The water holding capacity of the treated samples (5, 15 and 30 min) were significantly ($p<0.001$) higher than that of the untreated sample as shown in Fig 1. These results can be due to the effect of ultrasound treatment that led to the change in protein conformation and structure resulting in the exposure of hydrophilic amino acid residues towards water [198]. As a result, it led to better retention of water in the gel which was further confirmed by the reduction of centrifugal loss. The untreated gel had a water holding capacity of 60.35 ± 0.09% whereas after 5 min of ultrasound treatment, it was noted to have a huge increment in the water holding capacity of 93.95 ± 0.13%. The water holding capacity of the gel after 30 min of treatment was evaluated to be the highest at 96.02 ± 0.28%, which is similar to the results obtained by [204] for myofibrillar proteins treated with high intensity ultrasound at 20 kHz, 300 W where the water holding capacity was found to be the greatest after 30 min of treatment. Although, after 30 min of treatment, the slight increment in the water holding capacity of the gel from 95.35 ± 0.42% to 96.02 ± 0.28% was found to be not significant ($p>0.05$) when compared to 15 min of treatment. These results were comparable to [205] where the difference between water holding capacity after 20 min and 40 min of ultrasound treatment of gels from whey protein isolate did not change significantly.

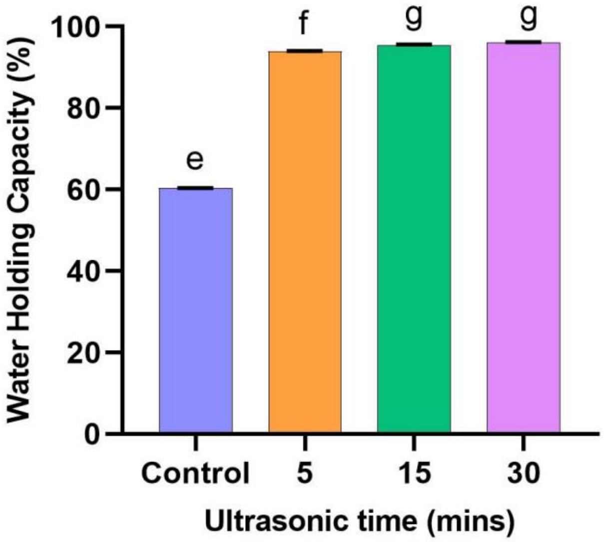


Fig. 1. Effect of ultrasound treatment on water holding capacity (%) of untreated (I) and treated samples II (5 min), III (15 min) and IV (30 min).

4.2.3.5 Rheology

4.2.3.6 Temperature ramp

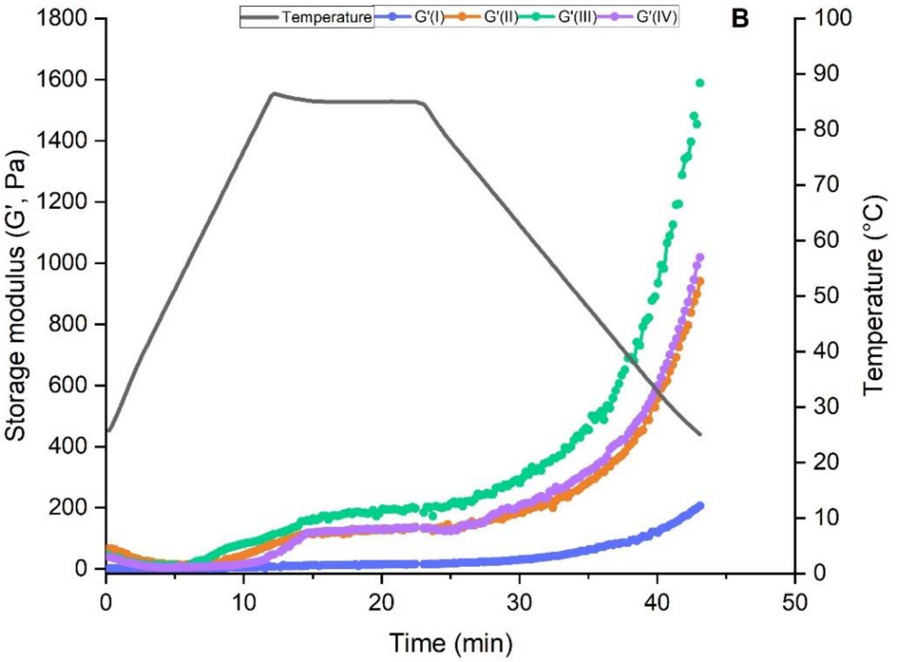
For sample I and III, the evolution of the storage modulus and loss modulus was determined during the temperature ramp to compare between treated and untreated samples as shown in Fig 2A. During the heating period from 25 to 85 °C, there was a slight increase in both the elastic and loss modulus. The storage modulus was also witnessed to cross the loss modulus during this period which implies the onset of protein denaturation [115]. In the constant heating period at 85 °C, elastic modulus was observed to increase gradually. This showed that the BSFL gel started to form due to the formation of aggregates and the transition from sol to gel [23,115]. Finally, in the cooling period, both the elastic and loss modulus had a sharp increase which can be due to the formation of hydrogen bonds for the development of the gel structure [23]. Thus, the cooling step had the most significant effect on the formation of the gel network due to the sharp increase in the elastic modulus [206]. The results obtained from the temperature ramp for BSFL gel both treated and untreated are similar to [23] where they inspected the gelling properties of the protein fractions from five insect species. The untreated and treated BSFL gels displayed a similar behaviour during the temperature ramp. Since the gels exhibited an elastic dominant behaviour, the course of the elastic modulus in the temperature ramp curve is displayed in Fig 2B for the samples. At the end of the cooling period, the ultrasound treated samples had a higher elastic modulus compared to the untreated gels. The magnitude of the elastic modulus increased till 15 min of treatment, which was found to be the highest. This can be attributed to the increase in particle size which led to the formation of aggregates due to the exposure of hydrophobic groups to build a firmer gel network [206]. However, after 30 min of treatment, the magnitude of the elastic modulus decreased. This can be explained due to the aggregates broken down smaller by the forces of cavitation which can disrupt the non-covalent interactions and prevent further aggregation [202]. These results are comparable to [206] which was reported for gels from soybean protein isolate treated with ultrasound. The tan delta during heating and cooling period is illustrated in Fig 2C for sample I and III. The results from tan delta were synonymous with the results of temperature ramp. Lower values of tan delta were found during the cooling period as well as towards the start of constant heating period, which indicated a dominant elastic behaviour.

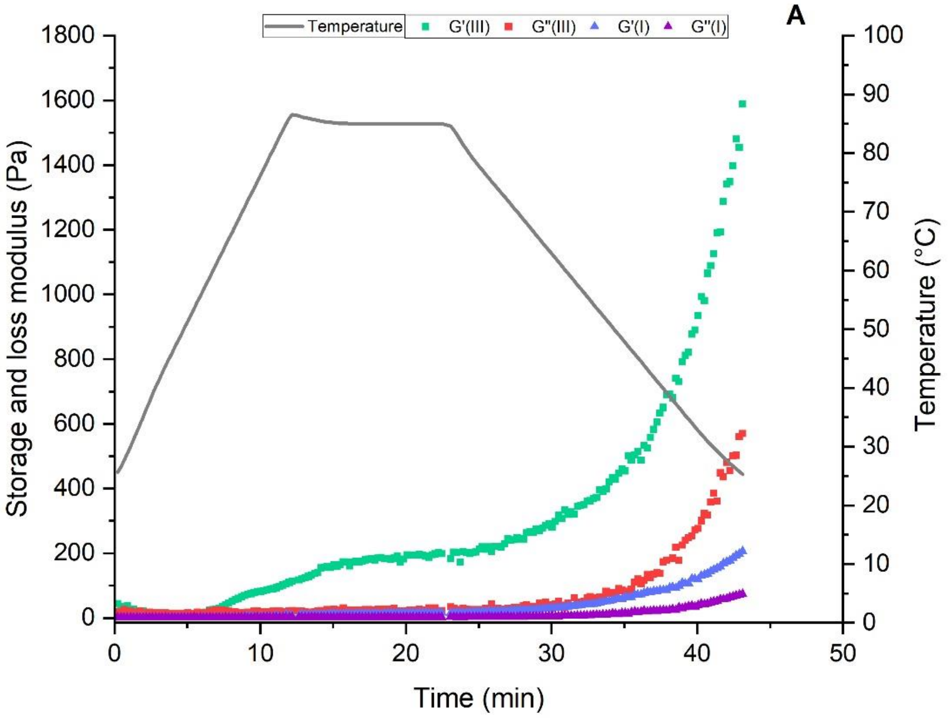
4.2.3.7 Frequency sweep

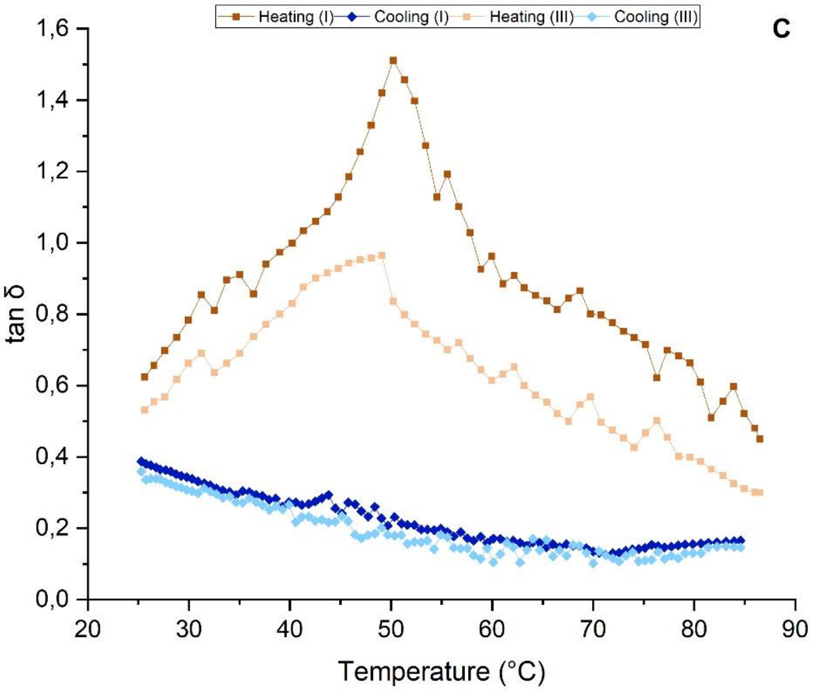
In the frequency sweep, both the elastic and loss modulus increased whereas the complex viscosity decreased with the increase in frequency for all the samples in Fig 2D. The elastic and loss modulus had a dependence on frequency and there was no crossover between them, which implies the formation of gels due to non-covalent bonds [207]. The elastic modulus was observed to be greater than the loss modulus indicating that the gel demonstrates solid-like behaviour. The trend was similar for both treated and untreated BSFL gels. These results are comparable to [206] for gels from soybean protein isolate treated with ultrasound where the elastic modulus was found to be higher than the loss modulus for the treated and untreated samples exhibiting elastic dominant behaviour. Since the gels exhibited predominantly an elastic behaviour, the course of the elastic modulus during the frequency sweep was plotted for all the samples. The curves for the treated and untreated samples were fit according to [207]. The slope of elastic modulus versus frequency for sample III and sample I was calculated to be 0.268 and 0.227 respectively. The slope of the curve was found to be the highest for sample III after 15 min of treatment as compared to sample I, the untreated sample, indicating that the ultrasound treatment increased the frequency dependence of the value of elastic modulus of BSFL gel [207]. The results are comparable to [205] where the effect of ultrasound treatment increased the dependence on frequency for gels from whey protein isolate. Since the slope is greater than zero for all the samples, which further corroborates that physical gels were formed due to non-covalent linkages [207]. The treated samples (II, III, IV) had greater values of elastic modulus than the untreated sample i.e., sample I which also confirms the firmer texture of the treated BSFL gels [206]. These results are similar to [205] where the elastic modulus after ultrasound treatment was found to be higher than the untreated gels of whey protein isolate.

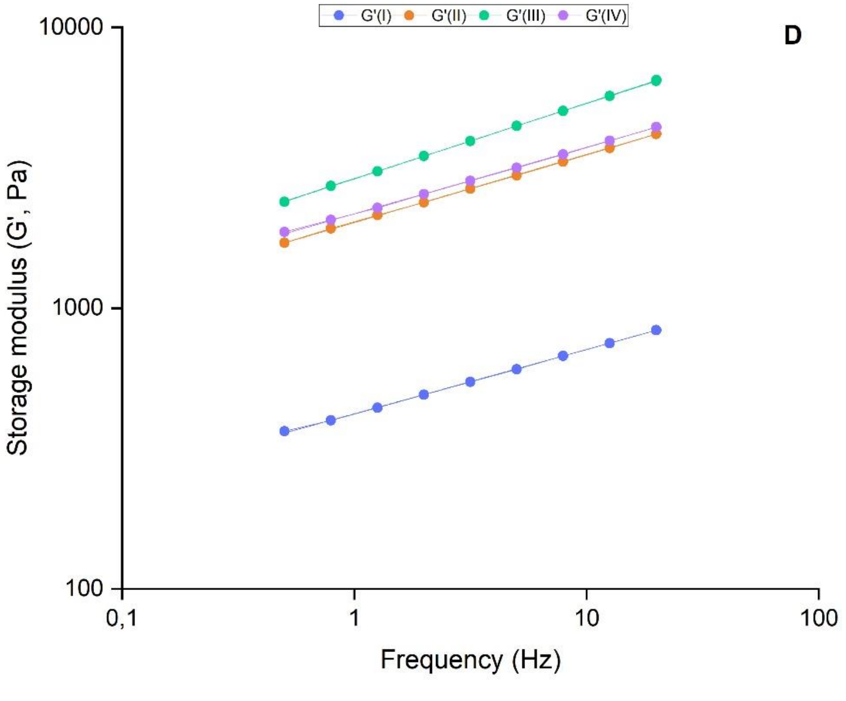
4.2.3.8 Amplitude sweep

At a lower strain for all the samples, the elastic modulus was observed to be greater than the loss modulus which suggested that the gel has an elastic dominant behaviour. During small deformations, the structure of the gel remains undisturbed in the linear viscoelastic region [208]. However, when the strain is increased, a crossover occurs between the elastic and loss modulus and finally, the loss modulus was found to be greater compared to the elastic modulus. Therefore, large deformations lead to breaking down the structure of the gel resulting in a viscous dominant behaviour [194]. The trend witnessed for the BSFL untreated gel are comparable to the results reported by [23]. The course of the elastic modulus was shown in Fig 2E for all the samples during the amplitude sweep. When compared with the treated samples, the magnitude of elastic modulus in the linear viscoelastic region was found to be higher in contrast to the untreated gel. The highest value of the elastic modulus was estimated for sample III which was around 2900 Pa whereas the lowest value was calculated for sample I which was around 365 Pa in the linear viscoelastic region. This indicates that sample III (treated gel after 15 min of treatment) was eight times more elastic than sample I (untreated gel). The major difference in the magnitude of the elastic modulus between treated and untreated samples implies that the ultrasound treatment had a significant effect on the elastic modulus. Therefore, a similar trend was observed where the magnitude of the elastic modulus increased with the increase in the exposure time until 15 min of treatment and when exposed to a longer time of 30 min, it decreased. This observation corroborates with the results obtained from both the temperature ramp and frequency sweep. The gel strength obtained for the ultrasound treatment after 15 min was found to be greater than the gels made from *A. domesticus*, *B. dubia*, *Z. morio*, *T. molitor*, and *A. diaperinus* supernatant gels where the gel strength was found to be 2500, 1600, 390, 100 and 140 Pa respectively [23]. Hence, BSFL protein extract gel after ultrasound treatment displayed remarkable gelling properties compared to other gels prepared from *A. domesticus*, *B. dubia*, *Z. morio*, *T. molitor*, and *A. diaperinus*.


B
Temperature
G'(I)
G'(II)
G'(III)
G'(IV)
Storage modulus (G', Pa)
Temperature (°C)
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50


A
Temperature
G'(III)
G''(III)
G'(I)
G''(I)
Storage and loss modulus (Pa)
Temperature (°C)
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50


Heating (I)
Cooling (I)
Heating (III)
Cooling (III)
C
tan δ
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
20
30
40
50
60
70
80
90
Temperature (°C)


G'(I)
G'(II)
G'(III)
G'(IV)
D
Storage modulus (G', Pa)
10000
1000
100
0,1
1
10
100
Frequency (Hz)

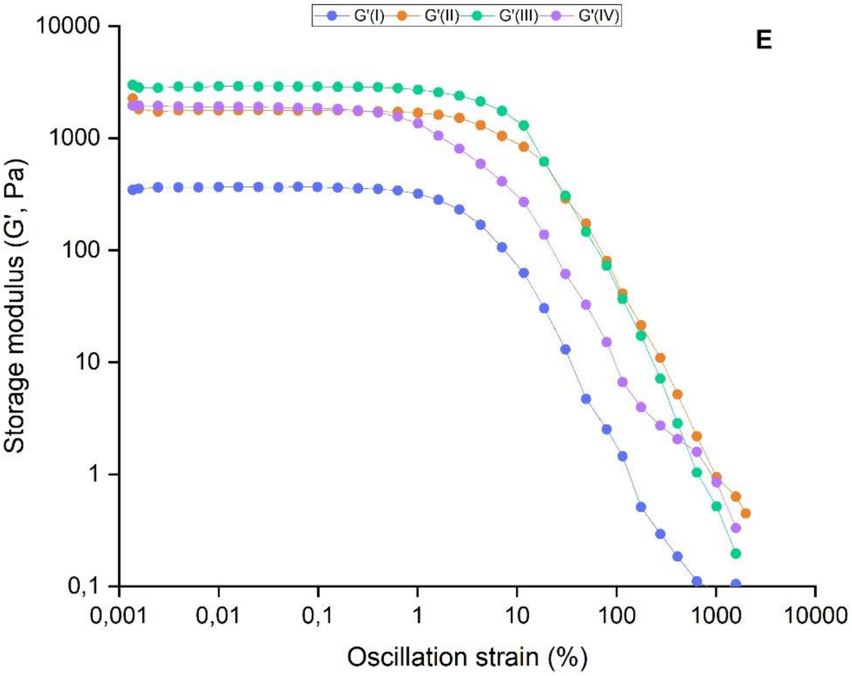


Figure 2- (A) Storage and loss modulus, temperature (°C) as a function of time (min) for sample I and III; (B) storage modulus (G') and temperature (°C) as a function of time (min) for I, II, III, and IV; (C) tan δ as a function of temperature (°C) for sample I and III during heating and cooling period; (D) storage modulus (G') as a function of frequency (Hz) (E) storage modulus (G') as a function of oscillation strain (%) for I, II, III, and IV.

4.2.3.9 Confocal laser scanning microscopy

The microstructure of BSFL gels was investigated with confocal laser scanning microscopy and the mean pore size average was estimated by 2-D correlation analysis (Fig 3). The protein network appears orange, while the pores appear dark. Sample I presented the biggest pore size mean (1.65 ± 0.04 μm) among all samples, showing smaller protein aggregates with relatively larger, distinguished, and non-homogeneous pores. After ultrasound treatment, a dense and compact network of protein clusters was formed. Sample II showed a faint definition with larger protein aggregates and smaller pores (1.21 ± 0.03 μm). For sample III, the gel attained a considerably fine structure which had the smallest-sized pores (0.54 ± 0.05) as compared to all the samples. Lastly, sample IV displayed larger aggregates with inhomogeneous clusters and bigger pores (0.78 ± 0.03 μm) compared to sample III. The more compact and homogenous microstructure of sample III is in agreement with the rheological results, where this sample presented the highest elasticity. A similar tendency was also observed by [195] for milk gels, where lower pore sizes were related to more elastic gels. Sample III had bigger aggregates with higher hydrophobicity, which was promoted by the exposure of hydrophobic amino acids on the surface of the aggregate. Thus, hydrophobic interactions were allowed to take place in higher extensions, which had direct reflexes in its microstructure.

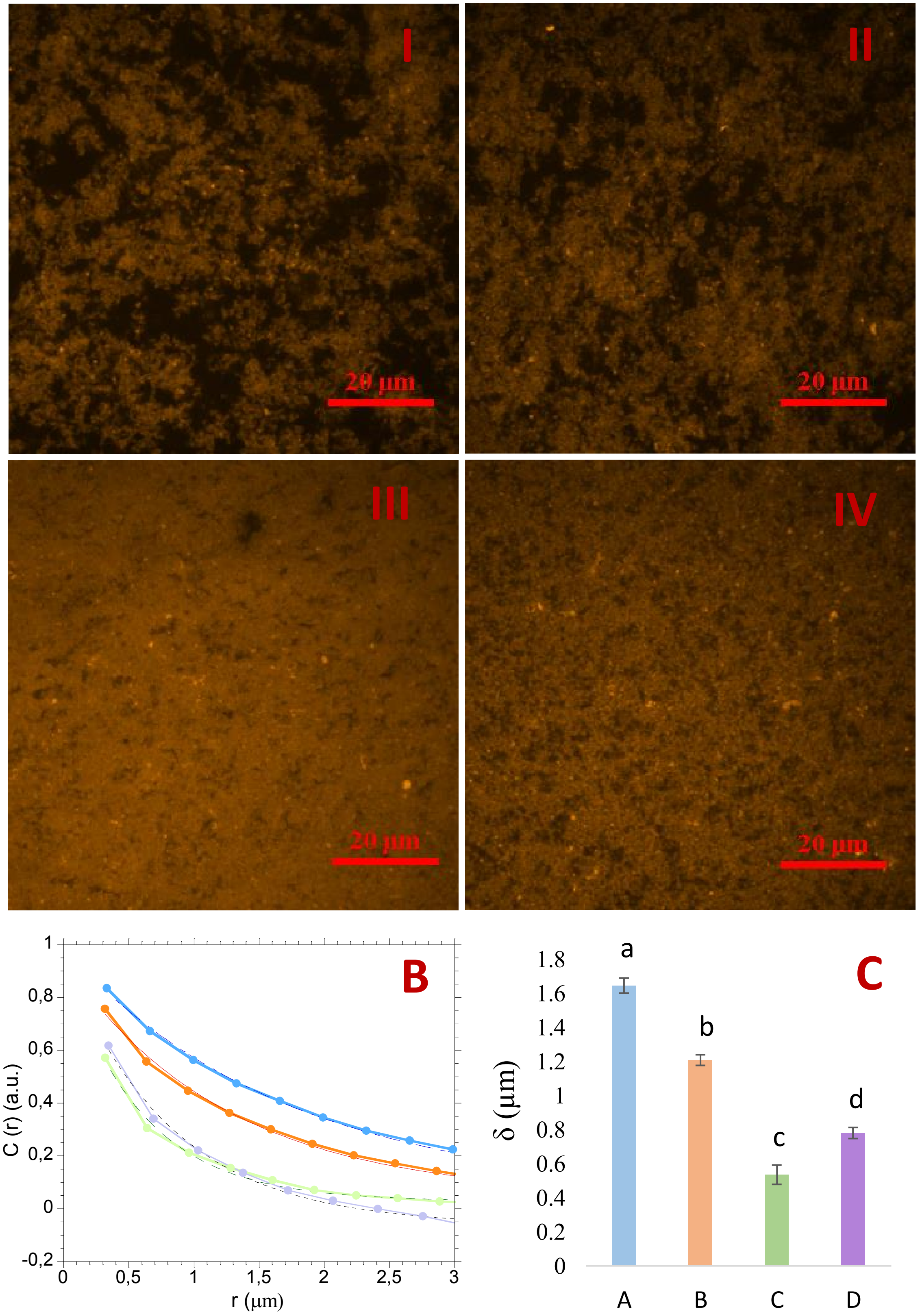

I
20 µm
II
20 µm
III
20 µm
IV
20 µm
B
C (r) (a.u.)
1
0,8
0,6
0,4
0,2
0
-0,2
0
0,5
1
1,5
2
2,5
3
r (µm)
C
δ (µm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
d
A
B
C
D

Fig. 3. (A) Confocal laser scanning microscope (CLSM) images of untreated (I) and treated (II, III, IV) samples. (B) Plot correlation as a function of pore diameter with exponential decay fit for I (●), II (●), III (●), and IV (●). (C) Gels mean pore size (μm), different letters on the top of the columns indicate significant differences at 5% significance by Tukey HSD test.

4.3 PHYSICAL AND OXIDATIVE STABILITY OF LOW-FAT FISH OIL-IN-WATER EMULSIONS STABILIZED WITH BLACK SOLDIER FLY (*HERMETIA ILLUCENS*) LARVAE PROTEIN CONCENTRATE.

The content of this chapter has been published at:

Foods

doi.org/10.3390/foods10122977

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This work has been performed in collaboration with Department of Pharmaceutical Science at Federal University of Juiz de Fora; Department of Food Science and Technology, Federal University of Viçosa; Food Production Engineering, National Food Institute, Technical University of Denmark; Research Group for Analytical Food Chemistry, Technical University of Denmark; Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN); Department of Health Technology, Technical University of Denmark; and nextProtein,

4.3.1 Introduction

Long-chain (LC) fatty acids such as docosahexaenoic acid (DHA), 22:6 (n-3) and eicosapentaenoic acid (EPA), 20:5 (n-3) can be found in fatty fish such as mackerel, tuna, cod fish and salmon, and they are collectively referred to as marine omega-3 (n-3) fatty acids [209]. Notwithstanding, n-3 PUFA-enriched foods have shown important benefits for human health, and they come with the challenge of negatively affecting oxidative stability, since n-3 PUFAs are very susceptible to oxidation.

Fish oil-in-water (O/W) emulsions have been used as an advantageous system for LC n-3 PUFA delivery in order to reduce lipid oxidation [210]. Although emulsions might protect n-3 PUFAs against oxidation, they are thermodynamically unstable [210]. The selection of an appropriate emulsifier plays an important role not only for the physical stability of the emulsion, but also for the oxidative stability. Oxidation in emulsions is initiated at the oil–water interface and factors such as the thickness and charge of the interface can affect the oxidative stability of the emulsion [211]. Therefore, comprehending the structure, properties and inner dynamics of the emulsion interface is essential to improve physico-chemical stability and potentially reduce oxidative effects on food emulsions.

Proteins have been found to protect the oil phase against oxidation due to their chelating and free radical scavenging properties and the ability to form a physical barrier with thick layers at the emulsion interface [212]. The industry has used many synthetic emulsifiers over the last years. Nevertheless, there is a growing demand for natural and more sustainable ingredients in food production due to public health concerns and climate change effects. This demand influences the increasing trend of replacing synthetic and non-sustainable sources of emulsifiers with natural and more eco-friendly protein sources [213].

Among the alternative food sources, edible insects have drawn the attention of the FAO and the scientific community, as it has been reported as a promising protein source with potential technological applications in food systems and a low environmental footprint [213]. *Hermetia illucens*, popularly known as black soldier fly (BSF), has been identified as one of the most promising insects for the commercial production of proteins, showing great benefits for humans and the environment [11]. The protein content of BSFL is stated to be between 37% to 63% of dry matter and is mainly composed of structural and muscular protein [11]. Edible insects can have an increased acceptance by consumers when they are not directly visible in food [214,215].Therefore, the production of insect powder and the exploration of their protein techno-functionalities in food systems have been highlighted as great alternatives to increase consumer's acceptance [3].

In order to improve these techno-functionalities by altering protein structure, several treatments can be applied, such as ohmic heating [30], microwave [29], ultrasound [28], pulsed electric field [27] and the combination of two or more systems, such as ultrasound and ohmic heating [111]. Ohmic heating (OH) has received increased attention due to its rapid and volumetric heating rates that enable high temperatures to be applied in the sample during a very short time period [216]. During OH treatment, an alternating electric current pass through the sample and its resistance generates an internal heat. A study on the effect of electric field on proteins has stated interesting conformational changes in the proteins' structure, as it resulted in protein films and gels with distinctive properties [217]. However, there is no particular study reporting the effect of OH on BSFL proteins.

The effect of ultrasound, an acoustic wave method with a frequency of 20–100 kHz, on food structure is explained by the quick formation and collapse of gas bubbles, called ultrasonic cavitation, which is generated through localized pressure differentials that occur in a few microseconds. [33]. Ultrasound treatment is reported to affect the structural, physical and functional properties of proteins [128]. In addition, ultrasound has also been applied to insect species such as *Tenebrio molitor*, *Gryllus bimaculatus* and *Bombyx morri* during the protein extraction step, and the study showed that protein yield increased for all insect samples after ultrasound treatment [189].

The objective of this study was to investigate the effect of OH treatment and the combination of OH and ultrasound on BSFL protein extraction and how it would affect the physical and oxidative stability of 5% fish oil-in-water emulsions prepared with BSFL protein concentrate as an emulsifier. Sodium caseinate (CAS) was used as a reference emulsifier in all tests. OH and ultrasound techniques were applied as an assisted extraction method based on the hypothesis that these techniques could increase the yield of protein during the extraction. In addition, both treatments are expected to alter the tertiary and secondary structure of proteins, which can influence the interfacial properties.

4.3.2 Materials and Methods

4.3.2.1 Material

Cod liver oil was provided by Vesteraalens A/S (Sortland, Norway) and kept at −40 °C until used. Cod liver oil showed peroxide value of 0.25 ± 0.07 meq peroxides/kg oil. The fatty acid composition (%, *w*/*w*) was found as: C14:0 (4.0), C16:0 (9.2), C16:1n-7 (8.3), C18:0 (2.2), C18:1n-9 (15.8), C18:1n-7 (4.1), C18:2n-6 (2.5), C18:3n-3(0.2), C20:1n-9 (11.4), C20:5n-3 (8.8), C22:1n-11 (5.4) and C22:6n-3(11.4). Alpha-, beta-, gamma-, and delta-tocopherol contents were 146 ± 7, 0 ± 0, 97 ± 2, 43 ± 0.3 μg toc/g oil, respectively. Sodium caseinate (Miprodan 30) with 93.5% protein content was provided by Arla Foods Ingredients amba (Viby J, Denmark).

The BSFL flour was kindly provided by nextProtein, Grombalia, Tunisia. BSFL were fed on formulas of different mixtures based on biowaste according to EU regulations. When harvesting stage was reached, the larvae were separated from the subtrate by sieving. Samples were blanched in hot water at 70 °C for 5 min and softly dried for 3–4 h to reach a moisture content of <7%. The oil fraction was then extracted by pressing the dried sample. A protein meal, partially defatted, was then obtained. Protein-enriched sample was then ground in order to produce the insect flour. The chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, US).

Black soldier fly larvae protein concentrate (BSFL) has gone through two different pretreatments in this work: BSFL pretreated with ohmic heating (BSFL-OH) and BSFL pretreated with ultrasound and ohmic heating (BSFL-UOH). All process and analysis are explained within the next sections.

4.3.2.2 Sample Preparation

4.3.2.3 Preparation of Defatted Insect Powder

In order to remove the remaining lipids from BSFL flour, it was mixed with ethanol (ratio 1:20, *w/v*) and stirred for 12 h at 40 °C. The sample and the liquid fraction (fat extract) were recovered with a filter paper and decanted, and the fat extract (N° 00H, 7 cm, Sweden) solvent was evaporated using a rotary evaporator (Laborota 4000 efficient, Heidolph Instruments GMBH & CO KG), solvent was recovered and reused in the second extraction to remove any residual fat. Residual solvent was removed by placing the sample under a fume hood overnight.

4.3.2.4 Ohmic Heating Pre-Treatment

As a pre-treatment step, laboratory scale ohmic heater (BCH Ltd.,Whitworth, Lancashire, UK) was used on the defatted BSFL sample. The equipment consisted of a control unit with a maximum supply of 230 V at 60 Hz and a rectangular polyethylene heating cell with two titanium electrodes placed at two ends. For this study, the distance between electrodes and inner width of cell were set to 12 cm and 9.5 cm, respectively. The electric current and voltage were registered by a built-in data logger system, and temperature was monitored using a K-type thermocouple connected with TC-08 data logger (Pico Technology, St. Neots,Cambridgeshire, UK).

Right after the defatting step, the BSFL powder was stirred for 1 h in a NaOH (0.25 M) solution, and 250 mL was transferred to OH cell. The applied voltage was set to 115 V, and the sample was heated to a temperature of 90 °C and held for 15 min. Immediately after the treatment, the treated BSFL was cooled down to room temperature, and the alkaline extraction took place (Section 2.2.4).

4.3.2.5 Ultrasound and Ohmic Heating Pre-Treatment

Defatted sample (Section 2.2.1) was stirred for 30 min in NaOH solution (0.25 M), followed by ultrasound treatment (Branson, Danbury, CT, USA) in a pulse mode (3 s ON, 3 s OFF) at 20 kHz, 440 W for 15 min.

The intensity of the treatment was determined for each sample using Equations (1) and (2) [218]:

$$Pa = M.Cp.\left(\frac{dT}{dt}\right) \tag{1}$$

$$I\alpha = \frac{Pa}{Sa} \tag{2}$$

Where Pa is power (W), M is the mass 10 g (g), Cp is the specific heat 4.18 (J/g °C) and dT and dt are the differential of temperature (°C) and time, respectively, during the first 30 s. $I\alpha$ is the intensity of treatment, which is equal to the power (Pa) divided by the surface area of 0.69 cm^2 (Sa) [200]. *Iα* The power and intensity of treatment were found to be 13 W and 18.9 W/cm^2, respectively.

After ultrasound treatment the sample went through ohmic heating treatment as reported in (Section 2.2.2), and finally, the alkaline extraction (Section 2.2.4) took place.

4.3.2.6 Alkaline Extraction of Protein

Alkaline protein extraction of BSFL occurred according to Queiroz et al. [11] with slight modifications. The extraction was performed for black soldier fly larvae ohmic heating-treated sample (BSFL-OH), black soldier fly larvae ultrasound and ohmic heating (BSFL-UOH) and the untreated black soldier fly larvae (BSFL). Briefly, the defatted powder was solubilized in 0.25 M NaOH (ratio 1:20, *w*/*v*) and stirred (Polymix Buch &

Holm, A/SHerlev, Denmark) at 300 rpm, 40 °C for 1h. After centrifugation (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 2500× *g* 4 °C for 20 min, a 2nd alkaline extraction was repeated on the remaining pellet. The isoelectric point, 4.3~4.5, was reached with 2M HCl, in order to promote protein decantation. The solution was left overnight for complete precipitation of the proteins. After decantation, sample was centrifuged at 1300× *g* 4 °C for 15 min to recover the pellet. The pellet was washed twice with distilled water and freeze-dried overnight.

4.3.1.7 Protein Content

The protein content of BSFL, BSFL-OH and BSFL-UOH was measured according to Dumas method. A nitrogen-to-protein conversion factor (Kp) of 5.62 was used according to Janssen et al. [149].

4.3.2.8 Protein Characterization

4.3.2.9 Differential Scanning Calorimetry (DSC)

DSC 250 (TA Instruments, New Castle, DE, USA), with a Refrigerated Cooling System 90, was used in order to comprehend sample's thermal stability. Distilled water (melting point (m.p.) = 0 °C; DHm = 334 J/g) and indium (m.p. = 156.5 °C; DHm = 28.5 J/g) were used for calibration. As reference, empty aluminum pans (20 µL volume), hermetically sealed, were used. Nitrogen was used as the carrier gas, with a flow rate of 50 mL/min. The DSC was used to measure the thermal stability of all protein concentrate samples produced in this study. An amount of 7 mg of sample was weighed in duplicate. First, the system was cooled to 0 °C and then scanned from 0 °C to 250 °C at a heating rate of 5 °C/min, The thermograms (heat flow as function of the temperature) all the characteristic peaks, glass transition, thermal unfolding and solid-melting were identified, according to Al-Saidi et al.[151]. Trios software® (New Castle, DE, US) was used for data interpretation.

4.3.2.10 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of all protein concentrate samples were stated using a PerkinElmer Spectrum 100 spectrometer (Waltham, MA, USA), based on a Universal Attenuated Total Reflectance sensor 125 (UATR-FTIR). The transmission mode was applied in a range of 4000–650 cm^{-1}. All the spectra were plotted as absorbance (AU) in function of wavenumber (cm^{-1}). All the measurements were performed in triplicate.

4.3.2.11 Interfacial Tension of BSFL Protein Concentrate at the Oil–Water Interface

The interfacial properties of each emulsifier in oil–water interface were verified by the pendant drop technique, using a drop tensiometer OCA20 (DataPhysics Instruments, Filderstadt, Baden-Württemberg, Germany). For that, 0.01% (*w*/*w*) of each protein concentrate, BSFL, BSFL-OH, BSFL-UOH and sodium caseinate (CAS) was prepared separately in 10 mM sodium acetate-10 mM imidazole buffer (pH 7.0) and was stirred for 2 h. The syringe was filled with the aqueous phase, and for each analysis a small drop was formed using the automated syringe with quartz glass cuvette. The cuvette contained MCT oil (WITARIX® MCT 60/40, IOI Oleo GmbH, Hamburg, Germany). The calculation of the interfacial tension was based on the shape analysis of a pendant drop according to the Young–Laplace equation, Equation (3)

$$\Delta P = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \tag{3}$$

where ΔP (mN/m^2) stands for the difference in pressure across the interface, γ (mN/m) is the interfacial tension, and R_1 and R_2 (m) are the principal radii of curvature of the pendant drop. All the measurements were performed in duplicate.

4.3.2.12 Emulsion Preparation and Storage Experiment

Emulsifiers (CAS, BSFL, BSFL-OH, BSFL-UOH) were dissolved in a 10mM sodium acetate-10 mM imidazole buffer and placed in a shaking water bath at 50 °C for 2 h. Then, solutions were left stirring overnight at 300 rpm at room temperature. Before emulsification, pH was adjusted to 7 using 2 M NaOH. Emulsions were produced with 5% oil and 0.2% protein in 220 g batches. To produce the emulsion each solution was pre-homogenized using ultraturrax (Polytron, PT120E, 18,000 rpm, 30 s) for 3 min at 16,000 rpm. All the oil was added during the first minute. Then, homogenization was carried out in a microfluidizer [219], pressure 9 Kpsi and 3 passes. In order to accelerate lipid oxidation and prevent microbial growth, iron solution (Fe^{2+}, 50 μM) and sodium azide (0.05% *w*/*v*) were added, respectively. Emulsions were stored in amber bottles at room temperature in darkness. Physical and oxidative stability data were obtained at days 0, 1, 3, 6 and 10.

4.3.2.13 Physical Characterization of Emulsions

4.3.2.14 Turbiscan Analysis

Physical stability was evaluated using Turbiscan Tower (Formulaction, Toulouse, France). The technology is based on the static multiple light scattering principle. Samples were illuminated by an infrared light source (λ = 880 nm), and two sensors collected the backscattered (BS) and transmitted (T) signals. The stability criteria were based on the Turbiscan Stability Index (TSI) according to a previous work [218]. The TSI is a number calculated at time *t* by summing up all temporal and spatial variations in a considered zone:

$$TSI_{(t)} = \frac{1}{N_h} \sum_{t_i=1}^{t_{max}} \sum_{z_i=z_{min}}^{z_{max}} |BST(t_i, z_i) - BST(t_{i-1}, z_i)| \quad (4)$$

with: t_{max} is the measurement point corresponding to the time t at which the TSI is calculated, z_{min} and z_{max} are the lower and upper selected height limits, respectively, N_h = $(Z_{max} - Z_{min})/\Delta h$ is the number of height positions in the selected zone of the scan, and BST is the considered signal (BS if $T < 0.2\%$, T otherwise). Therefore, the lowest the TSI is the most stable sample.

Samples were analyzed for the first 24 h and then from day 3 to day 7 in order to verify the stability over time. All samples were stored at room temperature and protected from light. A total of 67 scans were performed during the first 24 h, and 269 were performed from day 3 to day 7; the number of scans were more frequent during the first hours in order to see changes in more detail.

4.3.2.15 Hydrodynamic Diameter (Dh) and ζ-Potential

Droplet size of the emulsions was measured using laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) on days 0 and 10 according to Horn et al. [220]. Results were measured based on the surface area moment mean (D(3,2)), as given in Equation (5):

$$\mathrm{D}[3,2] = \frac{\Sigma n_i\, d_i^3}{\Sigma n_i d_i^2} \tag{5}$$

where n corresponds to number of droplets with measured diameter, d is the diameter of the droplet, and i represents the size class of the droplets. Samples were measured in duplicate.

The analysis was performed on the emulsions using Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK) with capillary cells. ζ-Potential was calculated from the electrophoretic mobility (μ) using the Henry equation, Equation (6):

$$\zeta = \frac{3\eta\mu}{2\epsilon f(kRh)} \tag{6}$$

where η is the viscosity of the buffer (1.033×10^{-3} Pa s^{-1}), ϵ is the medium dielectric constant (dimensionless), Rh is the complex radius (nm) and $f(kRh)$ is Henry's function. A value of 1.5 was adopted for $f(kRh)$, referred to as the Smoluchowski approximation, as the analysis was performed in aqueous media.

4.3.2.16 Oxidative Stability of Emulsions

4.3.2.17 Primary Oxidation Products—Peroxide Value (PV)

Primary oxidation products were determined on lipids extracted from the emulsion according to the Bligh and Dyer method with slight changes [221]. Lipids were extracted using 10 g of emulsion and 30.0 mL of methanol and chloroform, 1:1. First, the 30 mL of methanol was added, followed by 15 mL of chloroform, and mixed at 8,000 rpm for 10 s with an Ultraturrax (Polytron, PT120E). Another 15mL of chloroform was added and mixed again for more 10 s at 8,000 rpm. Finally, 15 mL of water was added and mixed for another 10 s at 8,000 rpm. Samples were centrifuged at 2800 at 18 °C for 10 min. The water phase was discarded, and chloroform phase was filtered and weighed, and 9 g was deposited into a previously tared beaker. The beaker with chloroform extract was left overnight in the fume hood and then dried in the oven at 105 °C for 2 h and weighed. PV was subsequently determined on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm [211]. In sum, Bligh and Dyer extracts were weighed and left to evaporate to dryness under N_2 flow. An amount of 10 mL of chloroform/methanol 7:3 (*v/v*) was added to all samples, including 2 blank tubes. Two other tubes with Fe (III) were included as standard samples. An amount of 50 µL of thiocyanate solution was added to all tubes, and then 50 µL of Fe (II) chloride solution was added to all tubes except for the blank ones. All tubes were incubated for 5 min at room temperature in darkness, and finally, the absorbance was measured in the previously mentioned spectrophotometer. Measurements were conducted in duplicate.

4.3.2.18 Tocopherol Content—HPLC

HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 µm Silica; 4.6 × 150 mm) was used to analyze tocopherols in all emulsions. Tocopherol analysis was performed according to the AOCS Official Method [222] using lipid extracts (Section 2.8.1), and samples were later evaporated and dissolved in heptane. All the nalyses were performed in duplicate. Tocopherol content was expressed in mg/kg of sample weight. The method analyzes all 4 tocopherol homologues. The quantification is based on the integrated area in the chromatogram.

4.3.2.19 Secondary Oxidation Products—Dynamic head space GC-MS

Volatile compounds were measured according to García-Morenoet al. [223]. Volatile compounds were trapped on Tenax GR tubes by dynamic headspace using the following purging conditions: temperature of 75 °C, 30 min and nitrogen flow 150 mL/min. The volatile compounds were separated in a gas chromatograph (Agilent Technologies, 6890 N Network GC System, DE, USA) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness; Agilent Technologies, J&W GC Columns, Palo Alto, CA, USA). In order to identify individual volatile compounds, the MS library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, 70 eV; mass-to-charge ratio scan between 30 and 250) were used. The volatile compounds 2-ethyl-furan,1-penten-3-one, pentanal, (E)-2-pentenal, 1-penten-3-ol, hexanal, (E)-2-hexenal, N-heptanal, (z)-4-heptenal, (E)-2-heptenal, 1-octen-3-ol, octanal, (E,E)-2,4-heptadienal, 2-octenal and *(E,E)*-2,4-decadienal were determined in emulsion samples. A stock standard solution was prepared with all aforementioned volatile compounds, and seven different concentrations of diluted stock solution were added into fish oil-in-water emulsion. This method was performed in order to maintain similar release conditions for standard volatile compounds. Analysis was performed in triplicate, and the results are stated as ng/g of emulsion.

4.3.2.20 Statistical Analysis

In the present study, ANOVA, followed by Tukey’s test ($p < 0.05$ as the level of significance) was used for all the statistical analyses. Data were treated by Statistical Package for the Social Sciences software (SPSS 22.0, SPSS Inc., Chicago, IL,USA).

4.3.3 Results and Discussion

4.3.3.1 Protein Content of BSFL before and after Pre-Treatments.

The conventional alkaline extraction method was able to provide samples with a protein content of 62 ± 0.2%. A similar result, using the same method of extraction, was obtained previously by Queiroz et al. [11]. In order to increase the yield of extraction and to improve the protein functionality, ohmic heating and ultrasound techniques were applied. Using ohmic heating as a pre-treatment on the insect flour (BSFL-OH), the yield of protein extraction reached 66.7 ± 0.1%, and using the combination of ohmic heating and ultrasound (BSFL-UOH), the protein content was equal to 66.2 ± 0.1%. A previous work using ultrasound was able to improve protein extraction from edible insects [189]. Therefore, herein we aimed to implement both techniques to optimize the protein extraction. However, when using ohmic heating or a combination of both ultrasound and ohmic heating, the yield of extraction was similar and not statistically different ($p < 0.05$). This was the first time that ohmic heating was applied to a BSFL sample in order to increase the protein content. Therefore, different parameters might play an important role in increasing the yield of extraction when using ultrasound and ohmic heating.

4.3.3.2 Differential Scanning Calorimetry (DSC)

Qualitative and quantitative information can be obtained through thermal stability analysis, and DSC has been largely used for the characterization and identification of phase transitions in food products [224]. Normally, phase transition is grouped in two types: first-order transition, which includes crystallization, protein denaturation, melting, condensation and evaporation, and second-order transition, commonly represented by the glass transition temperature [225]. Thermograms are reported as heat flow (W/g) in the function of temperature (°C). The DSC graph for the BSFL-OH and BSFL-UOH samples is reported in Figure 1. The thermogram was marked with T_g, T_u, T_m (BSFL-UOH) and T'_g, T'_u, T'_m (BSFL-OH) for the glass transition, unfolding and solid melting point, respectively. The glass transition was observed as a shift in the curve between 45–60 °C for both BSFL-UOH ($T_g = 48$ °C) and BSFL-OH ($T'_g = 56$ °C). The results are similar to those of previous studies reporting on *H. illucens* protein extract [11]. An endothermic peak, identified as an unfolding peak, T'_u and T_u, can be observed at 162 °C and 143 °C for BSFL-OH and BSFL-UOH, respectively. The same region has been stated as the thermal unfolding point for other proteins [151], including black soldier fly larvae, which has shown to have an unfolding point at 150 °C for BSFL protein extract [11]. Unfolding involves the dissociation of intramolecular bonds, which results in an endothermic process. On the other hand, protein aggregation involves the possible reformation of intra- and intermolecular hydrophobic bonds resulting from different treatments, such as sonication [226]. In this study, there was a decrease in the denaturation temperature for the sample treated with OH, after which sonication occurred. In addition, an increase in enthalpy was observed for the BSF-UOH sample, probably due to protein aggregation and bond formation. These data are consistent with a previous study on whey protein concentrate that reported protein aggregation and increase in enthalpy after prolonged sonication treatment [227]. The same pattern can be observed in the melting point (T_m), as an increase in enthalpy and decrease in temperature was observed for BSFL-UOH, which might be due to the long exposure time of the samples to ultrasound treatment, causing protein aggregation as similarly reported in a previous study for pea protein and fish skin gelatin [218].

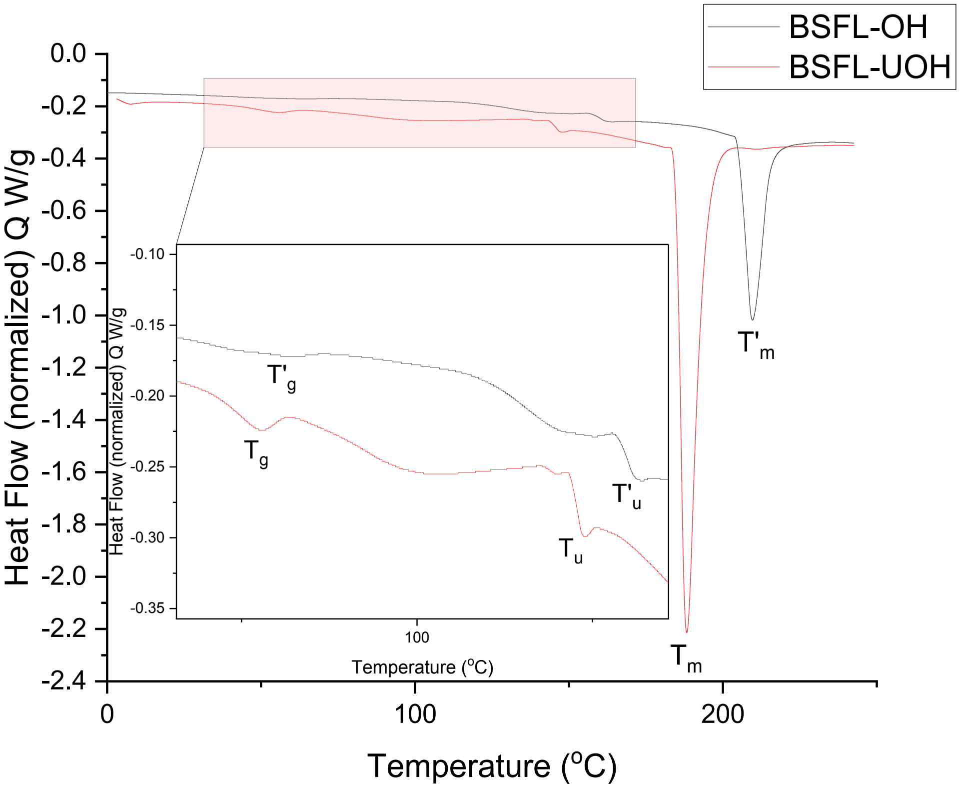

Figure 2- DSC Thermogram of BSFL-OH and BSFL-UOH protein concentrate

4.3.3.3. Fourier Transform Infrared Spectroscopy (FTIR)

In order to characterize the protein, concentrate samples and verify how the OH and ultrasound pre-treatment can alter the tertiary and secondary structure of BSFL proteins, the samples were analyzed by FTIR (Figure 2a). A previous work characterized the BSFL protein concentrate, obtained by the same alkaline extraction as herein reported, and a high absorbance was noticed at 1634 cm^{-1}, which was assigned to the β-sheet structure [11]. Another work reported the main amide bands and specifically the amide I, which is correlated to the secondary structure of proteins, highlighting the presence of α-helices (1650–1658 cm^{-1}), β-sheets (1610–1640 cm^{-1}), random coils (1640–1650 cm^{-1}) and β-turns (1660–1695 cm^{-1}) according to the peaks on each wavenumber range [228]. Similarly, the present work identified the amide bands, and amide I was reported to show some differences after the pre-treatments. BSFL and BSFL-UOH showed a more similar FTIR spectrum in the range of 1600–1700 cm^{-1}. However, BSFL-OH, instead, had a lower and diffuse peak distribution, which highlights changes in the secondary structure of the proteins after OH treatment only. The peaks at 1647 cm^{-1} (amide I) and 1538 cm^{-1} (amide II) are indicative of α-helices conformation, and the presence of shoulder bands at 1626 cm^{-1} and 1517 cm^{-1} also indicates some degree of β-sheet secondary structure [229]. A more detailed analysis can be seen in Figure 2b, where the Savitsky–Golay method and the second derivative were applied, similarly to a previous study on the protein concentrate of the black soldier fly [26]. The spectrum shows a qualitative analysis of the amide I region, correlated to C=O stretching. It was possible to state the different distribution of each secondary structure on the proteins extract where BSFL-OH showed a very clear different pattern of all secondary structure when compared to BSFL and BSFL-UOH. The peaks in the range of β-sheets are similar between BSFL-UOH and BSFL but showed a different pattern when both were compared to BSFL-OH. In addition, the intensity of peaks for α-helices and random coils appeared to be lower for BSFL-OH. The β-turns region was observed to be more affected by ultrasound treatment, whereas BSFL-UOH had a sharper peak with a different distribution when compared to BSFL and BSFL-OH. The secondary structure of proteins is directly connected to its functionalities, including its solubility and emulsifying properties [230].

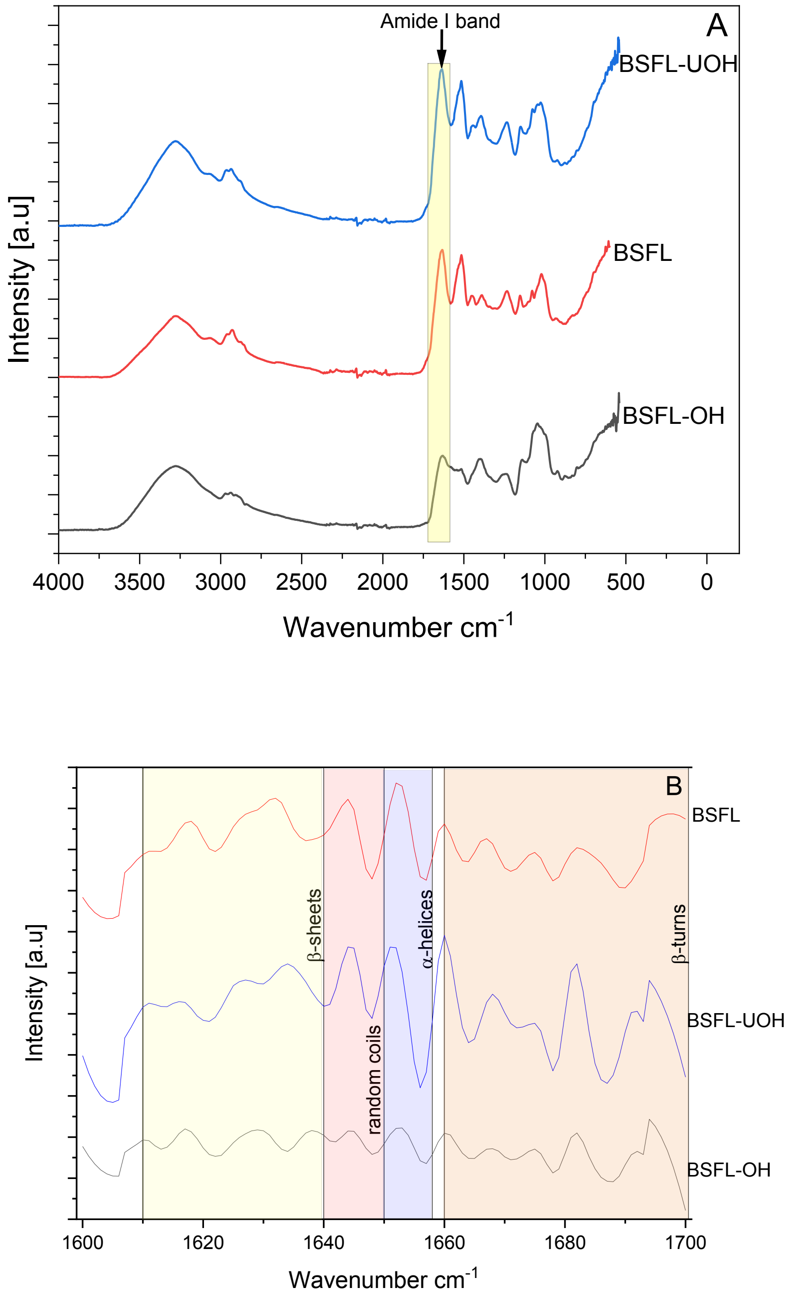

Figure 2. (A) FTIR spectra of BSFL, BSFL-OH and BSFL-UOH and (B) second derivative of amide I band, different colors yellow, red, purple, and orange represent the different secondary structure ranges β-sheets, random coils, α-helices and β-turns, respectively.

Amide II band at 1520 cm^{-1}, which derives from in-plane N-H bending and CN stretching vibration, could be noticed more clearly for BSFL and BSFL-UOH than for BSFL-OH. Amide III band was identified at 1234 cm^{-1} for BSFL and BSFL-UOH, but a slight shift was observed for BSFL-OH at 1250 cm^{-1}. This region is related to the combination of C-N stretching vibrations and N-H deformation from amide linkages, absorptions from CH_2 wagging vibrations, normally arising from glycine and proline side-chains [11]. Due to the complexity of each extract, other bands can be assigned to the presence of C-O bonds from carbohydrates (900–1200 cm^{-1}) and the presence of a-chitin by the bands between 1500 and 1700 cm^{-1} [167,174].

4.3.3.4 Emulsion Characterization

4.3.3.5 Interfacial Tension

Oil–water interfacial tension (IFT) was measured in order to understand the effects of different methods of protein extraction compared to CAS and conventional alkaline extraction. Figure 3 represents interfacial tension as a function of time for the different samples BSFL, BSFL-OH, BSFL-UOH and CAS. During the first 3 min, the IFT quickly dropped for BSFL-OH, from 17.0 to 14.4 mN.m^{-1}, which means the proteins had a fast adsorption to the oil–water interface, probably due to the smaller peptide size and the pattern of distribution of hydrophilic and hydrophobic groups on the protein surface. The BSFL-UOH and BSFL samples showed a slower reduction in interfacial tension and reached a higher IFT after 30 min, compared to BSFL-OH (12.9 mN.m^{-1}) and CAS (12.7 mN.m^{-1}). The results corroborate the data obtained from the Turbiscan emulsion stability analysis, where BSFL-OH showed the most promising result. The data were also comparable to CAS, which was the reference emulsifier. Besides protein size and the disposition of hydrophobic groups on the protein surface, lipid oxidation can also induce changes in the interfacial composition, as surface-active molecules are formed in the oil phase [231]. A more uniform heat provided by OH without inducing excessive denaturation or coagulation of proteins might have influenced the best performance of BSFL-OH [232]. The low interfacial tension found for BSFL-OH implies a superior interface activity and a minimized contact area between hydrophobic and hydrophilic regions. In general, the dynamics of the oil–water interfacial tension is managed by the diffusion rate of the emulsifier and the time it takes to reach and adsorb to the interface. In addition, the clearer differences in secondary structure for BSFL-OH, reported by FTIR analysis, might help us to understand the more promising results compared to BSFL and BSFL-UOH, as the disposition of different secondary structures can directly affect protein functionality.

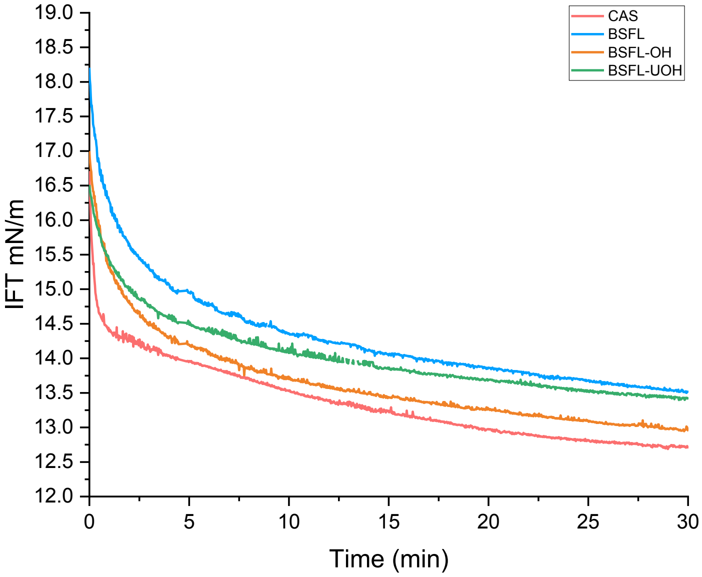

Figure 3. Interfacial tension of CAS (sodium caseinate), BSFL (Black soldier fly larvae), BSFL-OH (black soldier fly larvae after ohmic heating) and BSFL-UOH (black soldier fly larvae after ohmic heating and ultrasound) emulsifier samples. The oil–water interfacial tension without emulsifier was 25 mN/m during 30 min for MCT oil/water.

A previous work reported the interfacial properties of black soldier fly protein concentrate (BSFPC) using a sunflower oil–water system to measure the decrease in IFT. The study reported that BSFCP instantaneously decreased IFT to 8.4 mN/m and reached 3.4 mN/m after 90 min of experiment [26]. The author used a 10 times higher protein concentration, 0.1% (*w*/*w*), compared to the concentration used in this study, 0.01% (*w*/*w*). The higher protein concentration, time of analysis and the difference between the method of extraction in both works are some of the features that might have affected the dynamic of the interfacial properties of the studied proteins.

4.3.3.6 Turbiscan Emulsion Stability Analysis

In order to quantitatively compare the emulsion stability for all samples, the TSI as a function of time was applied (Figure 4). CAS emulsion was used as a reference and showed the highest stability during the whole experiment, followed by BSFL-OH, BSFL and BSFL-UOH emulsions. During the first 17 h, BSFL emulsion showed comparable stability with BSFL-OH emulsion; however, after this point, BSFL-OH emulsion showed improved stability but with no statistical difference ($p < 0.05$) compared to BSFL emulsion. BSFL-UOH emulsion showed the lowest stability over time, which was statically significant ($p < 0.05$) when compared to BSFL, BSFL-OH and CAS emulsions, possibly due to the exposure of hydrophobic groups and increase in surface hydrophobicity after the ultrasound treatment, which could cause an increase in protein aggregation, as previously stated by some studies [28,233]. This fact might have hampered the stabilization of the droplets in emulsion. BSFL-UOH emulsion reached a TSI equal to 3 after the first 8 h, while BSFL-OH emulsion and BSFL emulsion reached this value after 18 h and 17 h, respectively. A TSI lower than 3 is considered as a reference value for a stable system [218]. CAS emulsion reached the value of 3 only after 74 h. In conclusion, the more uniform heating and higher protein content obtained from OH treatment was good for providing better emulsifying stability that was statistically significant ($p < 0.05$) for O/W emulsions compared to sample treated by the combination of the ultrasound and OH and a slight improvement, which was not statistically significant ($p < 0.05$), when compared to the untreated sample at the end of the experiment. A previous study on soybean milk protein reported an increase of 38% in emulsifying activity after the sample was treated with OH-assisted extraction [234]. A study comparing black soldier fly protein concentrate (BSFPC) with whey protein isolate (WPI) showed that at a low concentration (0.1%), BSFPC has stronger emulsifying properties than WPI, and at higher protein concentration (2%), BSFPC revealed comparable emulsion activity to WPI. In addition, it has been reported that sunflower oil-in-water emulsion, stabilized by BSFPC, had a similar result to emulsion stabilized with WPI. Yet, BSFPC successfully reduced the mean droplet diameters of emulsions prepared with 20% to 40% of lemon oil compared to the same emulsions prepared with WPI [26].

The ability of a protein to stabilize emulsions could be an important characteristic required in many food formulations. Therefore, different strategies can be considered in order to improve this. A study reported on the emulsifying properties of BSFL protein sample and its hydrolyzed products by different enzymes. The study revealed that the non-hydrolyzed sample showed improved emulsifying stability compared to the sample hydrolyzed by alcalase, (DH = 18.4%), papain (DH = 15.34%) and pepsin (DH =9.8) [235].

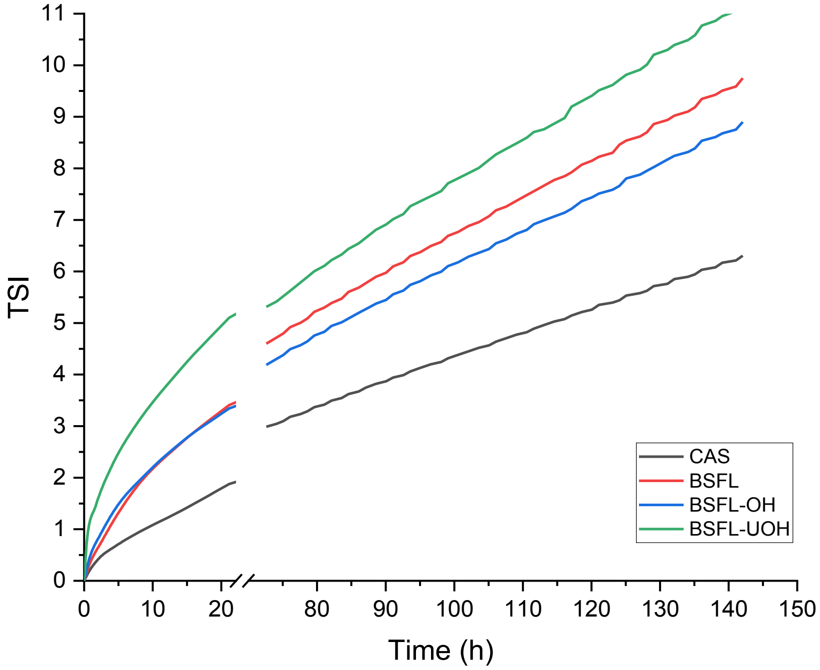

Figure 4. TSI analysis of CAS (sodium caseinate), BSFL (black soldier fly larvae), BSFL-OH (black soldier fly larvae after ohmic heating) and BSFL-UOH (black soldier fly larvae after ohmic heating and ultrasound) emulsions as a function of time (hours).

4.3.3.7. ζ-Potential and Hydrodynamic Diameter

The emulsion stability relies on a variety of factors, including particle charge and hydrodynamic diameter, which can play an important role. ζ-potential is defined as the potential difference between the surrounding medium and the stationary layer of fluid attached to the particle [179]. A numerically ζ-potential values close to ± 30.00 mV is considered an indication of stability, as particles will tend to repel each other and might avoid coalescence or general aggregation [169,236]. ζ-potential was calculated on day 1 and day 10 after emulsions were produced (Figure 5a). After the first day, all BSFL emulsions showed a high zeta potential, between −39 and −42 mV, which can be interpreted as sufficient electrostatic repulsion to prevent droplet coalescence. The values were similar to the reference CAS emulsion, −42 mV. A similar zeta potential value has previously been reported (−31 mV) for oil-in-water emulsion stabilized with CAS [237]. Wang et al., [26]studied emulsions stabilized with BSFL protein concentrate and reported that it had a strong surface repulsive effect, even after seven days of storage, with zeta potentials values between −40 mV to −35 mV. Similarly, no emulsions showed significant changes in zeta potential over 10 days of storage, except for BSFL-UOH emulsion, which increased the zeta potential to −58 mV. This increase in surface charge might be caused by the oxidation of the oil phase, which will release chemical compounds to the interface and can promote chemical changes and physical destabilization [218,238].

The droplet size results are reported in Figure 5b. Globally, droplet surface size has not exceeded 0.90 µm in the first day of storage. CAS emulsion showed the lowest droplet size (0.24 µm). After ten days of storage BSFL-OH emulsion showed a not statistically significant increase in particle size, from 0.57 µm to 0.68 µm, whereas BSFL, BSFL-UOH and CAS emulsion showed a larger increase reaching 0.96 µm, 1.08 µm and 0.53 µm, respectively. The droplet size herein reported corroborates with the stability data stated in the Turbiscan analysis (Section 3.4.2), where the CAS emulsion with the smallest particle size showed the best stability followed by BSFL-OH, BSFL and BSFL-UOH emulsions after 10 days of storage. The combination of the ultrasound and OH in BSFL-UOH emulsion provided the biggest droplet size compared to the alkaline extraction and OH treatment alone. A previous study by Jiang et al. [196] reported that high-power (450 W) ultrasound treatment on black bean protein isolate led to an increase in particle size due to the repolymerization of aggregates through noncovalent and covalent interactions. Similarly, other studies on egg protein and bovine serum albumin reported that high-intensity ultrasound treatment increased particle size through protein aggregation that was caused by hydrophobic interactions [28,233]. The presence of more protein aggregates can hamper the capacity of proteins to stabilize the O–W interface and then cause the decrease in emulsifying stability.

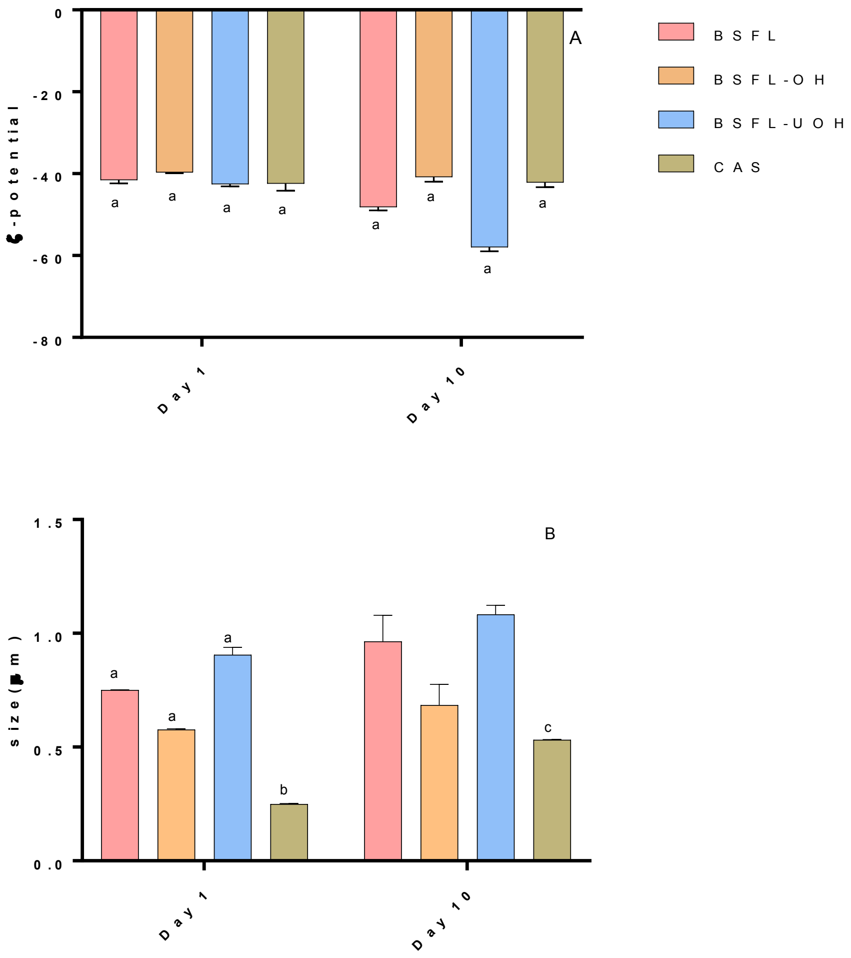

Figure 5. (A) ζ-potential (mV) measured during 10 days of storage and (B) size (μm) measured during 10 days of storage. Different letters mean a significant difference for $p < 0.05$ among samples.

4.3.3.8 Oxidative Stability of Emulsions

4.3.3.9 Primary Oxidation Products

The oxidation rate of each emulsion was compared during 10 days of storage based on peroxide value (PV) analysis, indicating the formation of lipid hydroperoxides (Figure 6a). After 24 h, CAS, BSF-OH and BSF-UOH emulsions showed similar results for oxidation, 7.12 ± 0.18, 9.36 ± 0.85, 8.50 ± 0.30 meqO_2/Kg oil, respectively, and BSFL emulsion had a higher value (11.35 ± 0.19 meqO_2/Kg oil) compared to the others. Until day 6, except CAS, all emulsions had a fast increase in PV and, in general, they had higher PVs compared to CAS emulsion. At day 10 of storage, BSFL-UOH (80.6 ± 0.87 meqO_2/Kg oil) had a significantly ($p < 0.05$) higher prooxidant effect when compared to BSFL-OH (64.4 ± 4.41 meqO_2/Kg oil) and BSFL (41.0 ± 1.11 meqO_2/Kg oil), and all the three emulsions had significantly ($p < 0.05$) higher PV compared to CAS (27.33 ± 0.32) at day 10. One possible explanation might be the low capacity of BSFL-UOH samples to prevent the metals in the aqueous phase from promoting lipid hydroxyl radicals and cause further oxidation of other lipids [50]. In addition, some studies reported that the electrical charge of the oil droplets can impact lipid oxidation, whereas negatively charged particles showed higher lipid oxidation compared to the positively charged ones. It was argued that the positive particles can repel the positive prooxidant ions, while negative particles will attract the same ions [239,240]. This might have contributed to the high oxidation in all samples. In addition, BSFL-UOH showed the largest interface area, which could lead to more oxidation. PV for BSFL (6.2 ± 1.0 meqO_2/Kg oil), BSFL-UOH (5.4 ± 1.1 meqO_2/Kg oil) and BSFL-OH (2.8 ± 0.2 meqO_2/Kg oil) protein concentrate was also evaluated and showed that ultrasound treatment might have led to a higher oxidation compared to OH only.

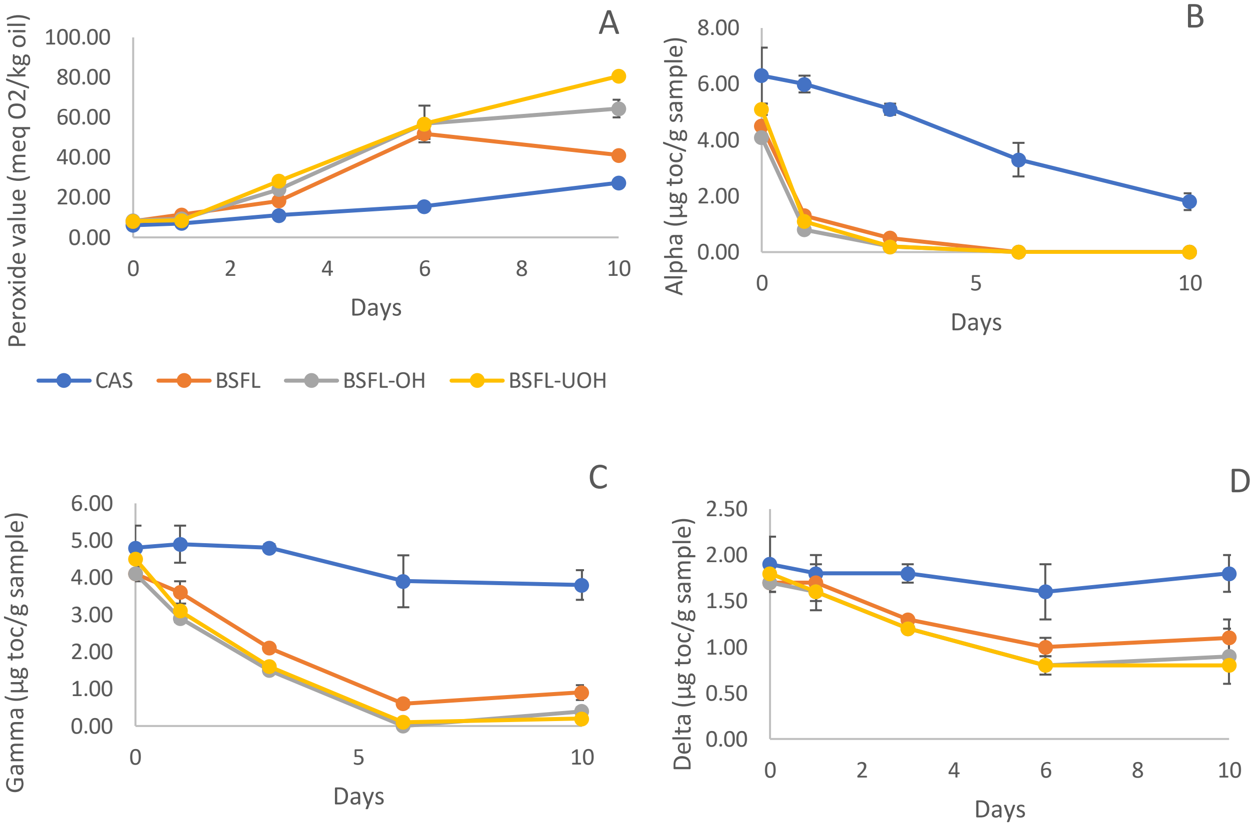

Figure 6. (A) Formation of primary oxidation products and consumption of (B) alpha-tocopherol, (C) gamma-tocopherol, and (D) delta-tocopherol content during 10 days of storage.

Conversely, a previous study reported the in vitro antioxidant activity of BSFL protein and protein hydrolysates using different models, including the radical scavenging model (DPPH and ABTS). The results showed that BSFL samples showed promising antioxidant properties, mainly the hydrolyzed ones [241]. However, different methods of protein extraction and treatments were used, which can cause protein structural changes that compromise the antioxidant activity. After 10 days of storage, the BSFL sample showed a decrease in PV from 51.08 ± 2.56 to 41.09 ± 1.11. This can be explained by the decomposition of hydroperoxides by the hemolytic scission of the double bond adjacent to the hydroxyl group to form the secondary oxidation products [242,243].

4.3.3.10 Changes in Tocopherol Content—HPLC

At day 0, tocopherol homologues levels in the emulsions showed the following range: alpha-tocopherol from 4.10 ± 0.1 to 6.30 ± 1.0, gamma-tocopherol from 4.10 ± 0.1 to 4.80 ± 0.6, and delta-tocopherol 1.70 ± 0.1–1.90 ± 0.3 mg toc/g of sample (Figure 6b, c, d). As the fish oil content was the same in all samples, the significant reduction in tocopherol contents in samples with BSFL proteins at day 0 was attributed to the consumption of tocopherol during emulsion production. Tocopherol consumption showed a similar behavior for all BSFL emulsions, and CAS emulsion had the highest tocopherol content after 10 days of storage. Alpha-tocopherol significantly decreased after 10 days of storage for all emulsions, with a sharp reduction even during the first day of storage. The results indicate that alpha-tocopherol was acting as an antioxidant in the presence of the BSFL samples and was the most consumed tocopherol when compared to gamma- and delta-tocopherol. Considering the CAS emulsion, the consumption of alpha-tocopherol significantly decreased during 10 days of storage, whereas gamma- and delta-tocopherol content had no significant difference ($p < 0.05$). After 6 days of storage, alpha-tocopherol decreased to 0 for BSFL, BSFL-OH and BSFL-UOH emulsions, and gamma-tocopherol reached 0 for BSFL-OH emulsion. These results are consistent with the increase in peroxide value previously stated for all BSFL protein containing emulsions after 6 days of storage. Therefore, the formation of primary oxidation products during the storage for these emulsions might have been higher if no tocopherols were present and consumed as antioxidants. BSFL emulsion had a slightly higher value for gamma- and delta-tocopherol, which also corroborates with PV value, whereas BSFL emulsion had the lowest oxidation among the three emulsions stabilized by insect protein.

4.3.3.11. Secondary Volatile Oxidation Products—DHS GC-MS

The formation of volatile compounds was measured during 10 days of storage for all emulsions. Figure 7 shows the concentration of some secondary volatiles present in the emulsions. 2-ethylfuran, penten-3-ol, hexanal and heptanal were chosen due to their higher concentration in the emulsions. 2-ethylfuran and penten-3-ol originated from the oxidation of n-3 PUFAs such as EPA and DHA, while hexanal and heptanal come from the oxidation of omega-6 and omega-9 fatty acids, respectively [244]. The secondary volatile compounds showed a low concentration until the third day of storage for all emulsions with insect protein as emulsifier. After day 3 of storage, a clear increase in the volatile content can be noticed with some differences between emulsions prepared with BSFL, BSFL-OH and BSFL-UOH. CAS emulsion had the lowest volatile content for all compounds analyzed and, for heptanal, CAS emulsion had the concentration, which was equal to 0 during the 10 days of storage. CAS is known for providing a good physical barrier at the oil–water interface and also for its chelating activity, both in the continuous phase and at the o–w interface [245]. This good and homogeneous physical barrier at the interface can efficiently hamper the diffusion of prooxidant molecules when compared to all emulsions containing BSFL proteins.

After 10 days of storage, BSFL emulsion had significantly lower volatiles content ($p < 0.05$) for 2-ethylfuran, penten-3-ol, hexanal and heptanal (including other volatiles shown in the supplementary material, Figure S1) compared to BSFL-OH and BSFL-UOH. The data corroborates the peroxide value previously reported and tocopherol consumption. The result suggests that OH and ultrasound treatments had an important effect in the BSFL proteins extract, since treated samples showed higher oxidation compared to untreated one. A previous study evaluating the effect of OH and ultrasound on whey protein concentrate stated that the application of both pretreatments may alter the protein structure and disrupt some covalent bonds [111]. The unfolding process of proteins and the exposure of hydrophobic groups might cause an increase in hydrophobic interaction between proteins and the formation of aggregates, which can also shield some of the sites with antioxidant activity [246]. The disposition of these aggregates on the oil–water interface can directly influence lipid oxidation since a nonhomogeneous distribution of proteins on the interface can leave spots, whereas prooxidants can enter and oxidase the lipids [246].

The exposure of some nucleophilic amino acids, such as histidine and lysine, can favor covalent reactions between secondary oxidation products and the protein, which cause chemical modifications altering their functional attributes and consequently, their ability to prevent droplet aggregation. Noncovalent interactions may also occur, for instance, hydrophobic interactions between volatile oxidation products and hydrophobic regions of proteins [243]. The higher concentration of volatile for all emulsions, using BSFL as emulsifier, might have contributed to the formation of residual lipid content in the extract. The high temperature and pre-treatment used for protein extraction could have accelerated the lipid oxidation and production of hexanal and n-heptanal, which were close to 0 for CAS emulsion. However, this is the first time that alkaline BSFL protein concentrate and BSFL subjected to OH and ultrasound treatment were evaluated for their ability to provide oxidative stability as emulsifiers. As previously stated, PV values for BSFL protein concentrate, after 10 days, were lower when compared with treated (OH and UOH) samples; however, further studies must be performed in order to fully comprehend how the different pre-treatments can cause lipid oxidation and lower the emulsion stability.

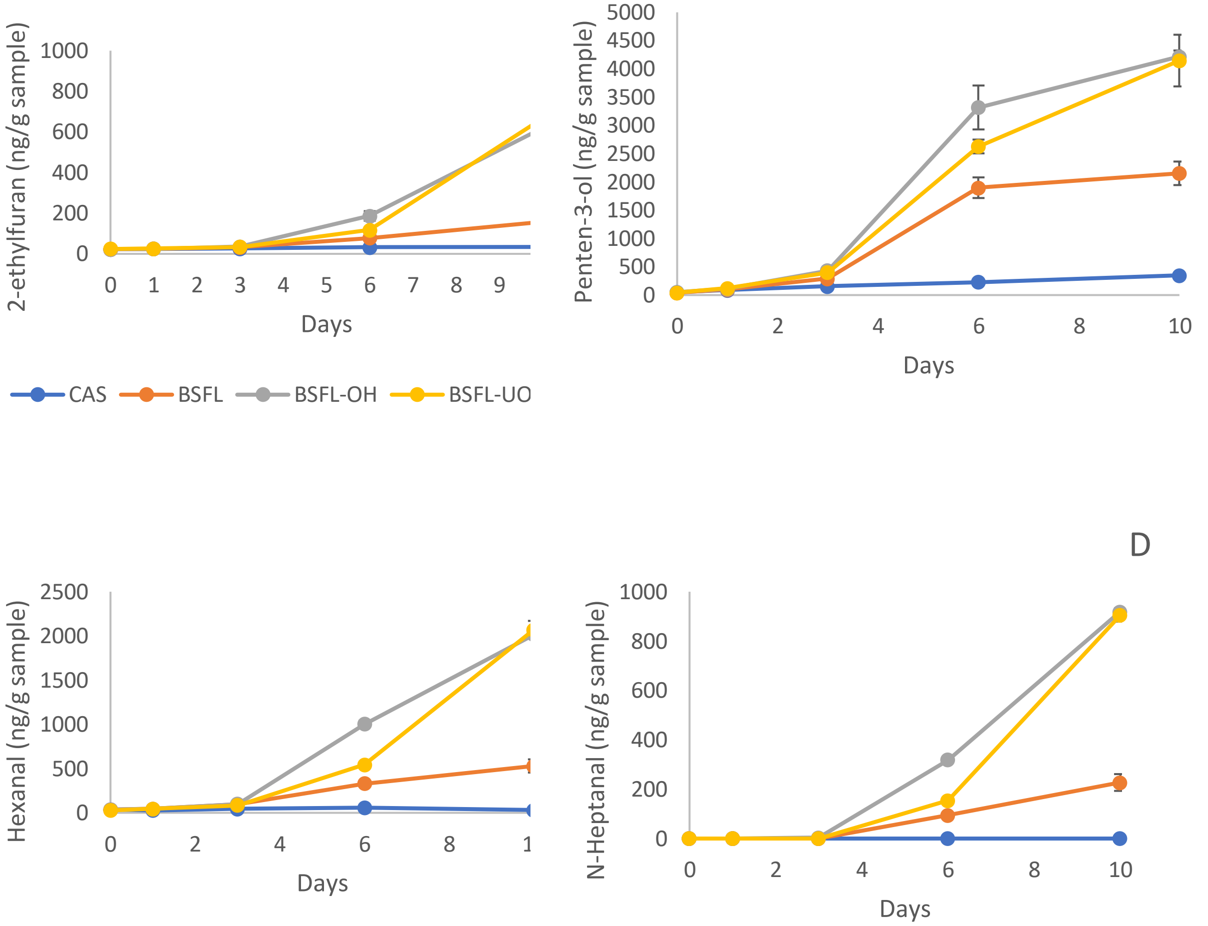

Figure 7. Content of secondary volatile in all emulsion during 10 days of storage: (A) 2-ethylfuran content; (B) Penten-3-ol content; (C) Hexanal content; (D) N-Heptanal content.

4.4 EMULSIFYING PROPERTIES AND INTERFACIAL CHARACTERIZATION OF OIL-IN-WATER EMULSION STABILIZED BY HYDROLYZED PROTEINS FROM BLACK SOLDIER FLY LARVAE.

The content of this chapter will be submitted at:

Food Hydrocolloids

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This work has been fully performed at the Group for Food Production Engineering, National Food Institute, Technical University of Denmark in collaboration with Federal University of Juiz de Fora Research; Línstitut agro Rennes Angers, Frence ; Research Group for Analytical Food Chemistry; Technical University of Denmark, nextProtein, Genopôle Campus ; Department of Food Science and Technology, Federal University of Viçosa.

4.4.1 Introduction

The agricultural resources reduction, increasing in world's population and consequent demand for food represent challenges for food supply for the next decades[38]. Therefore, it is relevant to extend the knowledge about new protein sources considering their functional properties, nutritional value and low environmental footprint [247,248].

Currently, more than 1900 insect species are characterized as edible[46] and some of them have been reported through the literature as a potential food source with high nutritional quality in terms of amino acid composition, high protein digestibility ,low-resource investment, and sustainable rearing[249]. Black soldier fly (BSF), *Hermetica illucens*, has been considered one of these insects of interest, due to its ability to convert organic waste into edible biomass what makes it a promising source of proteins to be further implemented as food in the human diet [18].

Hermetica Illucens larvae is composed of 36 to 63% of proteins on a dry basis depending on its rearing substrate, larvae age and way of killing [18,62,140,250]. It has been reported in the literature that protein content can reach higher amount when proteins are extracted by alkaline extraction and isoelectric precipitation, varying from 61.1 to

67.6%, using a nitrogen-protein factor of 5.62 according to [11,149]. BSFL can provide all essential amino acids (EAAs) in sufficient rates in accordance with FAO requirement. BSFLP have been reported to have high amounts of histidine, threonine as well as some hydrophobics amino acids such as valine, isoleucine or leucine [251,252].

In total, the hydrophobic amino acids residues stand around 30% of the total amino acids content. According to [129], this fact makes it impossible that the tertiary structure of BSFLP buries all the hydrophobic amino acids inside its core, thus some hydrophobic patches should exist at the surface and enhance formation of polymers through hydrophobic interactions [129]. Muscular proteins, in particular actin have been identified as the most abundant in BSFLP extracts, including enzymes, exoskeleton and storage proteins [24,253]. Secondary structure of BSFLP is mainly composed of β-turn and β-sheet structures according to [24,34].

However, sustainability and nutritional content are not sufficient attributes to reach relevant acceptability of insect in human diet [4]. The visual aspect and the association of insects with discussing memories and as disease vectors have been considered the main limiting factors to increase consumer acceptance particularly in the western world [254].Therefore, processing of insect proteins in order to produce a powder and disguising it in products, could be an alternative to integrate insect powder as an ingredient in food products [255].

Currently industrial food emulsions are mainly stabilized with synthetic surfactants or with milk or egg proteins, thus providing information about emulsifying properties of other proteins sources with a more sustainable approach, could give alternative ingredient choices for food formulations [43]. There is a relevant concern about how insect proteins can be better introduced as alternative food ingredient by exploring their functional properties[43].

Advantageous lipid and water absorption abilities have been identified for BSFLP, two characteristics that can contribute to emulsifying properties [18]. Moreover, BSFLP have been stated to show interesting foaming capacity and stability [11,18]. In addition, It has been concluded that BSFLP concentrate obtained by alkaline extraction possesses an interesting emulsifying properties [256]. All these analyses of functional properties of

BSFLP enable the possibility of using these proteins as a functional ingredient in food products with lower environmental footprint and interesting nutritional quality.

The effects of different modifications on the tertiary and secondary structures of proteins as well as their physicochemical parameters have been initially investigated for BSFLP aiming to improve its functional application [24].

Proteolysis is an important processing method for food industry in order to improve some parameters of interest such as, solubility, gelation, emulsifying, foaming characteristics, taste, production of bioactive peptides or reduction of allergenicity [129,257]. Indeed, these properties contributes to the flavors, texture, stability, and appearance of food products [255]. Whereas whole insects have low acceptability, the use of proteolysis, that has been shown as promising for insects, could be an alternative to insert high quality source of protein, extracted by a more sustainable way and with improved functionality in a food formulation[43,82].

The study aimed to evaluate the effect of hydrolysis on BSFLP physicochemical properties and its further impact over the surfactant properties of BSFLP and hydrolysates in oil-in-water emulsions. To deeply address this functionality and the participation of those proteins as emulsifier, the interfacial properties of BSFLP and hydrolysates were studied by the dilatational and shear interfacial rheology. The work sought to understand if hydrolyzation can be a good strategy to reduce the physical instability of water-in-oil when BSFLP are applied as emulsifiers.

4.4.2 Material and methods

4.4.2.1Preparation of BSFL protein and hydrolysates

Black Soldier Fly larvae partially defatted flour was provided by nextProtein, Tunisia. BSFL larvae were fed on biowastes before being separated from their feeding substrate by sieving when harvesting stage was reached. Then, a partially defatted BSFL flour was recovered after larvae processing: blanch, drying and pressing [24]. Alcalase® was provided by Novozymes, Denmark. For all dispersions, distilled water was used.

BSFL defatted flour was obtained by mixing twice the BSFL flour for 2 h at 40 °C with ethanol (ratio 1:20, w/v), recovered with a filter paper and evaporated under fume hood overnight. Then, BSFL protein (BSFLP) was extracted according to [24] method with slight modifications. Briefly, BSFL defatted flour was stirred in 0.25 M NaOH (ratio 1/20, w/v) during 1 h at 40 °C, 300 rpm. The resulting mixture was centrifuged at 2500x g, 4 °C for 20 min. This alkali extraction was repeated on the recovered pellet. Supernatants from both alkali extractions were combined and proteins were isoelectric precipitated by adding 2 M HCl. The precipitate was centrifuged at 1300x g, 4 °C for 15 min, washed twice with distilled water and freeze-dried.

A 1% (w/v) BSFLP (50 mL) dispersion was homogenized by stirring at 40 °C for 1 h at 450 rpm and overnight at room temperature. Temperature, and pH were adjusted by heating at 50 °C during 1 h and adding 0.5 M NaOH until pH8 respectively. The dispersion was hydrolyzed at 50 °C and pH8 with Alcalase (E/S ratio 3:100) at two DH: 4% and 12.5% by adding 0.337 mL and 1.165 mL of 0.5 M NaOH respectively, according to pH stat method. The DH was defined as, (Eq.1):

$$\mathrm{DH} = \mathrm{B} \times \mathrm{Nb} \times \frac{1}{\alpha} \times \frac{1}{\mathrm{MP}} \times \frac{1}{\mathrm{htot}} \times 100\% \qquad (1)$$

Where B (mL) was the amount of base added during hydrolysis, Nb (N) the normality of the base, MP (g) was the mass of protein, htot (mEq/g) the total peptide bonds equals to 10.53 mEq/g for BSFLP according to amino acid composition found by[11]. $1/\alpha$ was the average degree of dissociation of the α-amino groups, equals to 1.13 at pH8 and 50 °C (the extrinsic conditions used in this study) for protein according to [258] and used in other studies adapted to their own parameters[259].

Hydrolysis was stopped by heating the samples at 85 °C for 20 min. The hydrolysates were cooled in ice prior to be centrifuged at 10000 g, 10 °C during 15 min. Finally, supernatants were freeze-dried, DH4 and DH12.5 refer to the resulted powders at respective degree of hydrolysis and BSFLP hydrolysates (BSFLPH) to both hydrolysates.

Prior to all experiment, pH of solutions made for BSFLP was adjusted to pH8 using 0.5M NaOH and the solution was vacuum filtrated using qualitative filter paper (Advantec). All BSFLP and BSFLPH dispersions were homogenized by stirring at 40 °C for 1 h and overnight at room temperature, 450 rpm on a magnet stirrer.

4.4.2.2 Protein content

Protein content of BSFLP and BSFLPH was determined using Dumas method, considering a nitrogen-to-protein factor of 5.62 according to [259]. Measurements were performed in duplicates.

4.4.2.3 Size and ζ-potential

Particle average size (Hydrodynamic diameter), size distribution, polydispersity, and ζ-potential of BSFLP and BSFLPH were determined using Zetasizer Nano-ZS (Malvern Instruments). 0.1% (w/v) BSFLP and BSFLPH dispersions (10 mL) were syringed in capillary cells after being filtrated with Minisart® filter, with 0.45 µm pore size.

Size and size distribution were established using hydrodynamic diameter d_H (m) measured by Dynamic Light Scattering (DLS) technique, following Stokes-Einstein equation, based on the size of a sphere diffusing at the same rate as the particle (Eq.2) :

$$d_H = \frac{kT}{3\eta\pi D} \tag{2}$$

Where k was the Boltzmann's constant ($1.380649\times10{-}23$ m²kg/Ks²), T the absolute temperature (298.15 K), η the dynamic viscosity of the solvent (0.89 mPa.s) and D (m²/s) was the diffusion coefficient established from the Brownian motion of particles corresponding to speed of the particles.

Particle size distribution was analysed as a plot of the intensity distribution as a function of d_H. Polydispersity index (PdI) of the samples was defined as (Eq.3):

$$PdI = \left(\frac{\sigma}{2\alpha}\right)^2 \tag{3}$$

Where σ (m) was the standard deviation and 2α (m) the mean diameter of particles.

ζ-potential was defined using Henry equation linked to electrophoretic mobility µ (m²/V.s) defined as the speed of a particle reported to an applied external electric field (Eq.4):

$$\zeta = \frac{3\eta\mu}{2\epsilon f(\kappa\alpha)} \tag{4}$$

Where ζ (mV) was the ζ-potential, ϵ (F/m) the permittivity, η the medium viscosity (0.93 mPa.s), $f(\mathrm{ka})$ was the Henry function equals to 1.5 for polar medium according to Smoluchowski approximation, with ka the ratio of particle radius α (nm) to double layer thickness κ (nm^{-1}) (Debye length).All measurements are reported as the mean and standard deviation of 3 measured values.

4.4.2.4 Intrinsic fluorescence and surface hydrophobicity

Intrinsic fluorescence and surface hydrophobicity (H_0) were measured using a SpectraMax Gemini microplate fluorometer (Molecular Devices) according to [260] method with some adjustment. 200 µL of 1% (w/v) BSFLP and BSFLPH dispersion were poured in a 96-wheel plate. Emission spectra were recorded between 300 nm and 500 nm after applying an excitation wavelength of 280 nm at a constant slit of 2 nm. The fluorescence relative intensity (FRI) was reported as a function of the emission wavelength.

H_0 was measured using 1-anilino-8-naphthalene sulphonate (ANS) as a probe binding at protein surface to hydrophobic residues. Similar dispersions used for intrinsic fluorescence were diluted at 0.25, 0.5, and 0.75%. 100 µL of ANS (8 mM) were added in 4 mL of each concentration, vortexed 10 s and kept in the dark during 15 min. 200 µL of each solution were poured in wheel plate. FRI was recorded with an excitation and emission wavelength of 390 nm and 450 nm respectively. H_0 was defined as the slope of the fitting curve of the FRI plotted as a function of concentration.

Intrinsic fluorescence and surface hydrophobicity measurements were measured in duplicate.

4.4.2.5 Interfacial tension and dilatational properties

Interfacial tension (IFT) and dilatational measurements were measured with pendant drop method using an optical contact angle and contour analysis OCA 25 (Dataphysics instruments GmbH) instrument. 0.1% (w/v) BSFLP and BSFLPH dispersions were dispensed at the lower end of a dosing syringe (diameter 1.83 mm) in a cubic glass cell filled with sunflower oil (density: 0.918 $\mathrm{g/cm}^3$). IFT was calculated according to Young-Laplace equation and plotted as a function of time (Eq. 5):

$$\Delta P = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (5)$$

Where ΔP (Pa) corresponds to Laplace pressure, γ (mN/m) stands for the interfacial tension and R_1 and R_2 (m) are the principal curvature radii of the pendant drop.

After 90 min, reaching IFT equilibrium, successive amplitude and frequency sweeps were applied. Amplitude variations of the drop surface in percent relative to the surface total surface area of 1 to 5% were conducted with a 0.01 Hz frequency. Frequency sweeps were measured between 0.01 to 0.1 Hz at a 2% amplitude. Dilatational moduli E*, E’ and E’’ were plotted as a function of amplitude and frequency ranges. All measurements were carried in triplicate.

The parameters k and n extracted from power-law model described in following equation were used to characterize the structured interfacial layer [261] (Eq.6):

$$E_d^* = k\omega^n \quad (6)$$

4.4.2.6 Shear interfacial rheology

Shear rheology was investigated using a sensitive stress-controlled rheometer (DHR2, TA Instruments), using double wall ring (DWR) geometry. 0.5% (w/v) BSFLP and BSFLPH dispersions (18 mL) were poured in the plate circular channel of DWR. The annular Pt-Ir ring was positioned at the surface of the dispersion and 18 mL of sunflower oil were gently added above. Prior to further measurements, linear viscoelastic region (LVR) was determined by performing an amplitude sweep in strain % from 0.01% to 100% and at a frequency of 0.2 Hz. Time sweep was then conducted at 20 °C, 0.1 Hz and 0.6% strain for 48 h. All experiments were conducted in duplicate. The resulted shear moduli G’ and G’’ were recorded as a function of time.

4.4.2.7 Emulsion preparation

BSFLP and BSFLPH, 0.2% (w/v) dispersions, were emulsified with 5% of sunflower oil (v/v). First emulsification was applied using Ultra-Turrax (IKA) dispersing device at 16 000 rpm during 3 min during which oil was slowly poured within the first minute resulting in a coarse emulsion. A second emulsification was applied with a high-pressure (12 kpsi) Microfluidizer® homogenizer (Microfluidics). Briefly, coarse emulsion was poured into the inland reservoir and pushed through the processor (Figure 6), this step was repeated 3 times. The resulted emulsion from the outland was recovered.

4.4.2.8 Multiple lights scattering measurements

Emulsion physical stability was measured using Turbiscan Lab Expert (Formulaction) and determined with Turbiscan Stability Index (TSI). TSI cumulates in every position measured (h) the backscattered (BS) and transmitted (T) light within its whole height (H) as defined by the following expression (Eq.7):

$$TSI(t) = \frac{1}{N_h} \sum_{t_i=1}^{t_{max}} \sum_{z_i=z_{min}}^{z_{max}} |BST(t_i, z_i) - BST(t_{i-1}, z_i)| \tag{7}$$

Where t_{max} stands for the point corresponding to the time at which the TSI is calculated, z_{min} and z_{max} are respectively the lower and upper height limits of the sample, $N_h = (z_{max} - z_{min})/\,\Delta h$ is the number of height positions in the selected zone of the scan and BST the considered signal.

20 mL of BSFLP and BSFLPH emulsions were poured in tubes, closed with a lid and gently poured in the device. TSI was measured for 2 days, just after emulsification process, at 20 °C.

4.4.2.9 Droplet size

Droplet size was determined at day 0, day 1 and 2 after emulsification using Mastersizer 2000 (Malvern Panalytical) laser particle size analyzer. Several droplets of the emulsion were poured in a dispersion unit filled with distilled water and stirred at 2 000 rpm. Surface area moment mean diameter D [3,2] (Eq.12) was calculated based on droplet diameter d (nm):

$$\mathrm{D}[3,2] = \frac{\sum d^3}{\sum d^2} \tag{12}$$

4.4.2.10 Statistical analysis

For all statistical analyses, it was applied ANOVA followed by pair comparisons using Tukey's test with a significance level p<0.05 were applied using Statistical Package for the Social Sciences software (SPSS 22.0, SPSS Inc).

4.4.3 Results and discussion

4.4.3.1 Protein concentration

Protein contents of BSFLP and BSFLPH are reported in Table 1. No significant difference was visible between samples. The value for BSFLP was in accordance with those found by [11,24]. Other study involving enzymatic hydrolysis with alcalase showed no significant differences between native and hydrolysate chickpea protein content [262]

4.4.3.2 Surface hydrophobicity for BSFLPH

Protein intrinsic fluorescence in the range of 300-400 nm is due to the luminescence of phenylalanine, tyrosine, and tryptophan, 3 aromatic amino acids (AAA) which are hydrophobic residues [262]. With an excitation wavelength of 280 nm, the emission spectrum between 300 and 400 nm is mostly related to tryptophan as well as a part coming from tyrosine, tyrosine being present in great amount (46.3 mg/g of crude protein) in BSFLP according to [24,263] and tryptophan generally occurring at one third of tyrosine in protein [264]. However, due to the indole side chain, tryptophan is usually the predominant contributor to fluorescence in proteins and presents a great Stokes shift allowing to see its contribution to hydrophobicity of a protein [264]. A redshift of emission wavelength maximum from 354 nm to 362 nm for BSFLPH is noticeable in Figure 1A, suggesting that enzymatic hydrolysis has affected the conformation of BSFLP [265]. An increase in FRI followed the DH where DH 12.5% showed the highest and BSFLP had the lowest fluorescence intensity. This fact can be explained by Trp having a high quantum yield when it is re-located into a more hydrophobic environment such as protein structure core, due to protein aggregation [266–268]. As well as the redshift is associated to the shift of fluorophores to a more hydrophobic environment [269]. These results are in accordance with the values found for $H_{0,}$ depicted in Table 1, showing higher value for BSFLP, 130.4 ± 30.2, whereas 59.4 1 ± 10.6 and 35.4 ± 14.6 for respectively DH4 and DH12.5. The decrease of AAA at the protein surface able to bind with ANS, a fluorescence probe, could be due to the formation of aggregates by hydrophobic interactions, as mentioned with size and ζ-potential (Table 1) and free hydrophobic amino acids generated during hydrolysis [265].

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Table 1- Physicochemical properties of BSFL and BSFLPH. Different letters indicate statistically significant difference ($p<0.05$) within the column.

Sample	Protein content (%)	Size (d.nm)	PdI	ζ-Potential (mV)	H_0
BSFLP	69.40 ± 0.02^a	140 ± 0.7^a	0.4^a	-14.9 ± 0.8^a	130.4 ± 30.2
DH4	66.93 ± 2.08^a	101.8 ± 3.3^b	0.5^a	-23.6 ± 1.7^b	59.4 ± 10.6
DH12.5	65.76 ± 0.23^a	151.7 ± 13.1^a	0.5 ± 0.1^a	-37.8 ± 1.3^c	35.4 ± 14.6

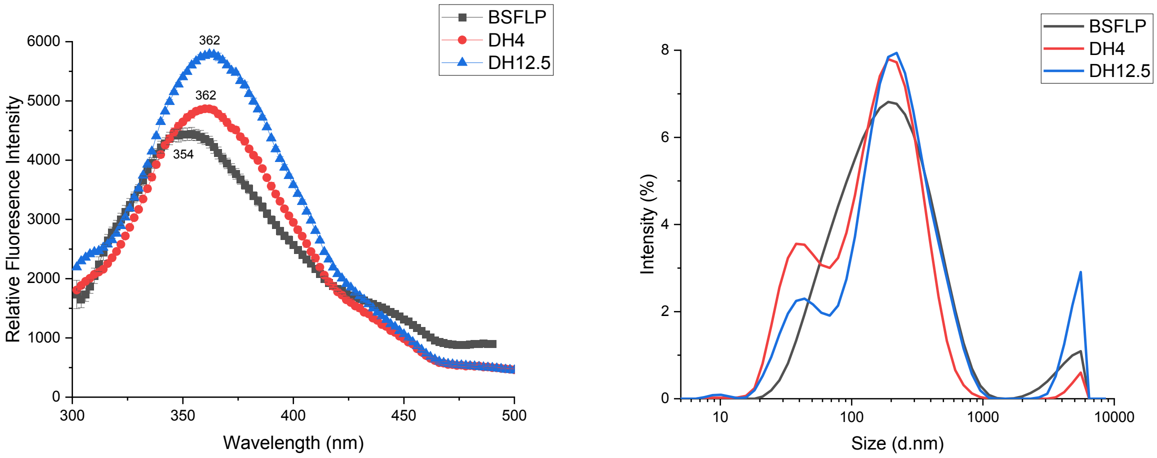

Figure 1- Intrinsic fluorescence spectra (A) and particle size distribution (B) of BSFLP and BSFLPH.

Indeed, alcalase is known to preferentially cleave peptide bonds around hydrophobic residues and thus enhance hydrophobic interactions between resulted peptides [269,270]. Decrease in H_0 with decrease in ζ-potential was also found by [271] with fava bean hydrolysates. Similar results were found by [270] where soy protein hydrolysates above a DH of 3% exhibited drastic lower H_0 than native protein.

4.4.3.4 Particle size by dynamic light scattering (DLS)

BSFLP and BSFPLPH sizes and PdI are depicted in Table 1. All particles were considered as polydisperse systems as their PdI were superior to 0.1 . Protein size was significantly decreased from 140 ± 0.7 to 101.8 ± 3.3 nm with a DH of 4%. In size distribution (Figure 1B), a peak at ~50 nm was noticed only for BSFLPH, showing that hydrolysis led to similar peptide formation for both DH. However, for a DH of 12.5%, the average diameter of particle size was increased,151.7 ± 13.1 nm, and the intensity of the peak at ~5000 nm was also increased when compared to DH4 and BSFLP .This fact could be explained by the higher degree of hydrolysis that caused an initial exposure of more hydrophobic groups, as alcalase tends to break down peptides in the hydrophobic areas [270]. This fact possibly leads to a more hydrophobic interaction and further formation of aggregates, which can also be explained by the higher fluorescence intensity for BSFLPH compared to BSFLP in Figure 1A. The aggregation may come from previously formed peptides at ~50 nm, resulting in the decrease of the peak at ~50 nm for DH12.5 compared to DH4. Hydrophobic interactions may have occurred to reduce protein free energy, and being also further facilitated by temperature treatment during Alcalase activity inhibition [129,264,272] . Indeed, hydrophobic interactions are enhanced when undergoing high temperature whereas weaker at low temperature. Similar results of increasing milk protein size with increase in Alcalase hydrolysis were found by [269].

4.4.3.5 ζ-potential before and after hydrolysis

When a protein is placed in an aqueous solution, the surrounding liquid layer is attached to the protein surface composed of an inner region with ions strongly bounded to the surface and an outer diffuse layer where ions are less firmly attached. This creates a double layer with an electrical potential difference between the inner part and outer part [273]. The ζ-potential corresponds to the electrical potential at the exterior of this diffuse layer. The negative value of each sample (Table 1) was consistent with alkaline pH, above isoelectric point of BSFLP. BSFLP ζ-potential value was found to be -14.9 ± 0.8 mV which is consistent with [11] results. ζ-potential significantly ($p<0.05$) increase with increasing DH, that could be explained by the higher number of carboxylic groups at the surface, this increase was also found for chickpea protein hydrolysates [274]. It is established that when ζ -potential is higher than ±30 mV the particle possesses higher stability, this suggests that BSFLP is less stable in solution than the hydrolysates [275].

4.4.3.6 Interfacial properties of BSFLP and BSFLPH

An interfacial tension (IFT) is created between two immiscible liquids, resulting from the intermolecular forces of molecules at the contact area [129]. The IFT between water and sunflower oil was ~24 mN/m and was kept constant over time as there was no emulsifier added in both liquid. IFT as function of time for sunflower oil-water interface stabilized with BSFLP and BSFLPH are shown in Figure 2. A sharp drop of IFT to 10.9 mN/m was noticeable within the first 10 minutes of measurements for BSFLP, also noticed by [26]. BSFLP greater decreased IFT which can be attributed to its surface hydrophobicity and less charged protein, allowing a denser surface coverage[57]. This step was followed by a quasi-equilibrium state for BSFLP. BSFLH both created an instant decrease but reduced less the IFT and took a longer time to reach the quasi-equilibrium. DH4 decreased IFT to a higher extent compared to DH12.5, that could be due to higher surface hydrophobicity and possibility favorable amino acid disposition to better interact with both phases.[276] showed same patterns for whey protein and two whey hydrolysates with almost twice lower surface hydrophobicity. Indeed, more contact points with the interface were created why native whey protein compared to hydrolysates, causing a readily orientation of proper groups to associated phase enhancing the decrease in IFT for native whey. In the same study, it was also reported that IFT was mostly determined by surface hydrophobicity rather than by decrease in size [276].

4.4.3.7 Dilatational rheology

Dilatational storage modulus E’ and loss modulus E’’ are displayed as function of amplitude for BSFLP and BSFLPH interfacial layers in (Figure 3A). Amplitude sweep characterizes the strength and the linear response of the film at the interface in response to the amplitude of deformation[277]. For all samples, storage modulus E’ was higher than loss modulus E’’, characterizing the dominant elastic behavior of the interfacial layer with BSFLP and BSFLPH. For all samples, E* was independent from the amplitude applied, according to [277] it indicates that all BSFLP and BSFLPH forms easily stretchable films. While DH12.5 was exhibiting fewer reduction in IFT, it seemed to form a stronger interfacial layer noticeable by the highest E*, that could be due to protein conformational rearrangement obtained after 90 min during the static analysis [277] In addition it can be explained by ther greater particle size of DH 12.5%, as the larger size increases the amount of network points, which in turn increases the elasticity [278].

In Figure 3B, E’ and E’’ as function of frequency are reported for BSFLP and BSFLPH interfacial layers. As reported for amplitude sweep, all sample exhibited a dominant viscoelastic behavior characterized by greater values of E’. Frequency sweep was conducted in linear conditions at an amplitude of 2.5% according to the previous applied amplitude sweep. An increase of E' was visible when increasing frequency for BSFLPH whereas E' of BSFLP remained constant. DH 12.5% and DH 4% exhibited the highest complex surface dilatational moduli (E*), compared to BSFLP. This means that BSFLP might have higher resistance against deformation.

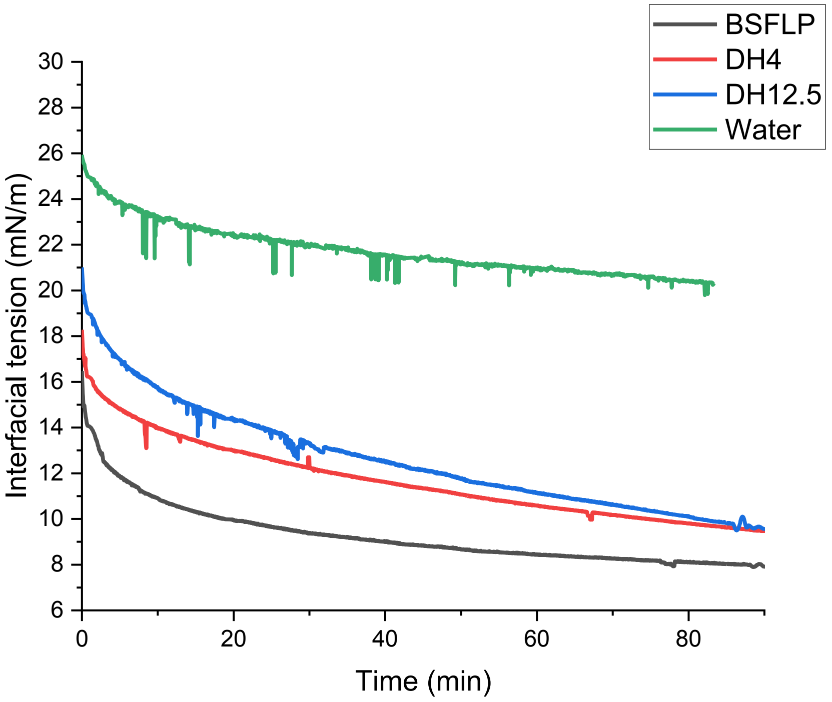

Figure 2- Interfacial tension of BSFLP and BSFLPH at sunflower O/W interface as a function of time

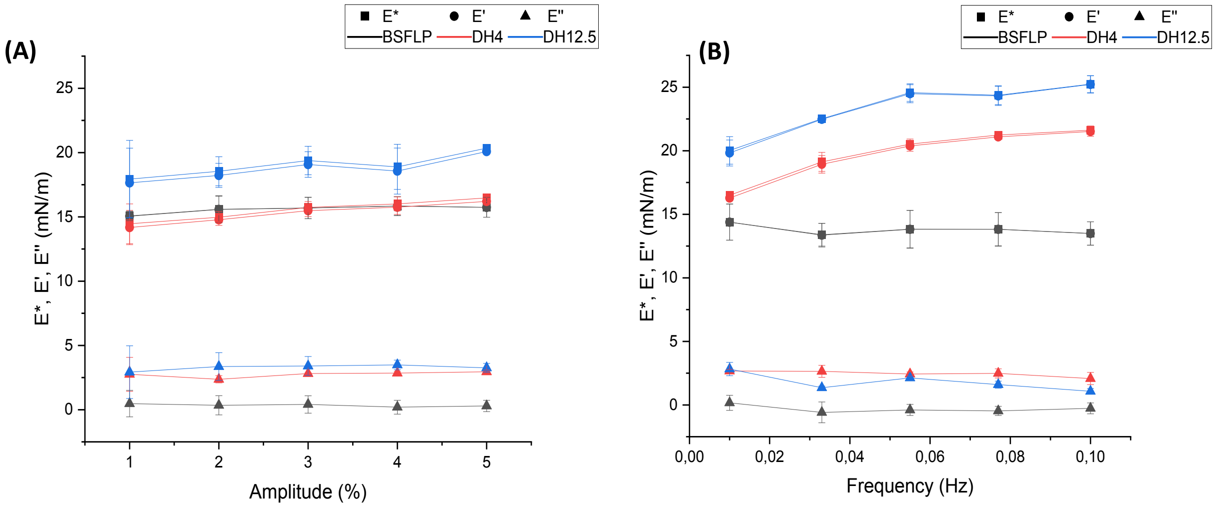

Figure 3- Amplitude (A) and frequency (B) sweeps in dilatational rheology for BSFLP and BSFLPH

Interfacial layers could be characterized by the parameters k and n obtained from a power-law model [261]. Slopes of E* values, n, depicted in Table 2 from dilatational frequency sweep indicates which phenomena contributes to explain this frequency dependency. Indeed, for BSFLP, the slope reported was <0.1, indicating that the sample was less frequency-dependent and that in-plane rearrangements are mainly responsible for the elastic behavior [261]. BSFLPH slopes were slightly higher, ≥0.1, but not significantly higher than BSFLP which indicates that all interfaces stabilized by BSFLP, DH4 and DH 12.5 exhibit mostly elastic solid-like behavior[279]. K gives a measurement of the interfacial strength, as K values for DH4 and DH12.5 were higher with respectively 28.84 and 32.076 mN/m.s compared to BSFLP with a value of 19.399 mN/m.s, it showed that interfacial bonds strength increased as the DH increased. Similar result was also noticed by another study, previously reported in the literature using hydrolyzed pea protein[279]. This result also corroborates with stronger interfacial layer, highest E*, in the amplitude sweep test for DH 12.5%.

Table 1- Dilatational parameters from frequency sweep for BSFLP and BSFLPH

Sample	k (Mn/m.s)	n
BSFLP	19.399	-7.10-4
DH4	28.84	0.12
DH12.5	32.076	0.10

4.4.3.8 Shear Interfacial rheology

Regarding linear viscoelastic region of samples, determined with an amplitude sweep, a strain of 0.6% was chosen to proceed time sweep in order to keep an intact microstructure of proteins (data not shown). Time sweep was conducted under linear conditions within an aging time of the interface of 48h in order to evaluate the time dependency of BSFLP and BSFLPH to form an interfacial structure.

Elastic and viscous interfacial layer traits are reported through respectively storage (G') and Loss (G'') modulus as a function of time in Figure 4. The resulted G' reflects the strength of the interfacial layer [279]. BSFLP results (Figure 4.A) indicated an elastic behavior from the beginning of the measurement without gel transition as no crossover point was noticeable between G' and G''[280]. This instant elastic trait could be attributed to the surface hydrophobicity of BSFLP that enables intermolecular interactions and less repulsive behavior regarding lower absolute ζ-potential value for BSFLP compared to BSFLPH[281]. Indeed, according to[282], while electrostatic repulsion are preferred in emulsion to prevent destabilization, lack or few electrostatic repulsion permits to enhance protein-protein interactions at the interface (i.e. via hydrophobic interactions), in turn leading to stronger interfacial films.

Also, the structured interfacial layer promoting elastic behavior formed by BSFLP is consistent with results from dilatational measurements. There is a lag-time, corresponding to diffusion of protein from the bulk to the interface, for DH4 and DH12.5 **(Figure 4.B-C)** to form a viscoelastic film. Indeed, G' becomes higher than G'' only after a crossover point at ~20 and ~25 h for respectively DH12.5 and DH4. The lower time required for gel transition for DH12.5 could be due to the greater size of DH12.5 particles.

More BSFLP absorbed at the interface and then less space to unfold contrary to BSFLPH, so less improvement of G', whereas BSFLPH unfold and developed an elastic layer.

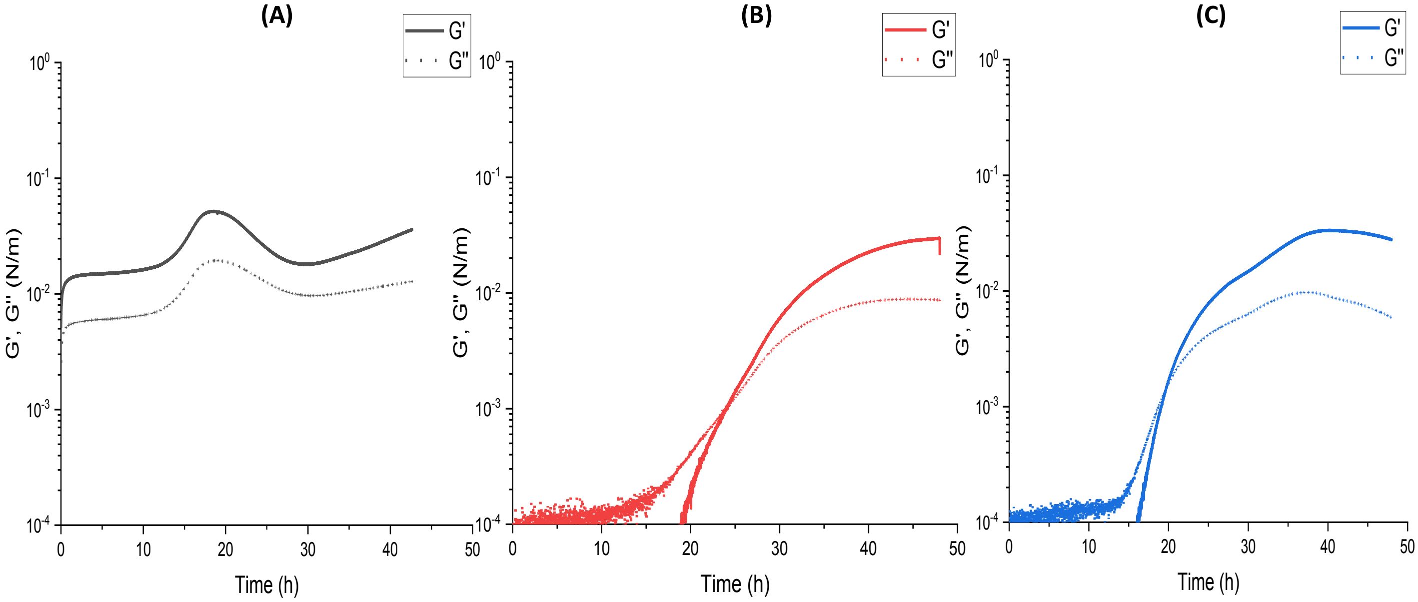

Figure 4- Time sweep in shear rheology for BSFLP (A), DH4 (B) and (C) DH12.5

Strong intermolecular interactions (hydrogen bonds, electrostatic and hydrophobic interactions) leads to high viscoelastic trait and a cohesive layer whereas flexible proteins create a loosely structure [283]. Another work reported the same difference in evolution of interfacial layer between native and hydrolysate soy proteins, elastic trait was dominant since the beginning for the non-hydrolyzed protein sample and a crossover was noticed for the hydrolyzed ones after some time[280].

Even though results from both shear and dilatational rheology can be seen as complementary in their meanings, intermolecular interactions were accompanied by additional effects specific to area changes and systems such as either protein concentration or rheology parameters.

4.4.3.9 Emulsion physical stability

Turbiscan stability index (TSI) is displayed as function of time in Figure 5.Turbiscan analysis is a non-destructive method allowing the detection of physical and colloidal destabilization phenomena that undergo emulsions including coalescence, clarification, flocculation or creaming that are not visible with the naked eyes in first moments after emulsification[284]. When TSI value is up to 3, stability destabilization has already begun and may be visible; droplet size variation as well as creaming could be noticeable into the sample. For all emulsions stabilised with either BSFLP or BSLPH, TSI values exceed 3 after 48 h of measurement. However, BSFPLH emulsions TSI curves are lower than BSFLP, indicating a more stable system within 48 h, DH4 creates the most stable emulsion according to its lower TSI value. Moreover, emulsions made of BSFLPH keep a TSI value under 3 for longer time, indeed DH4 and DH12.5 only reach a value of 3 after ~19 h and ~23 h respectively, whereas with BSFLP the value is reached after ~10 h. [235] reported that BSFLP hydrolyzed by Alcalase at a DH of 18.4% decreased emulsion stability compared to native protein.[285] indicated a negative effect of emulsifying capacity and increasing extent of the hydrolysis, highlighting that limited enzymatic hydrolysis is preferred to produce stable emulsions. This conclusion was also notified by [271] that suggested also that moderate hydrolysis of fava bean (DH of 4%) was enhancing emulsion stability by the formation of suitable peptides with flexibility size, hydrophobicity. Nevertheless, it could have also been expected that BSFLP was better stabilizer as it has been found that large native proteins could enhance long-time emulsion stability thanks to their ability to form stronger interfacial film when compared to highly hydrolysed samples that reduce less interfacial tension[286].

4.4.3.10 Droplet size of BSFLP and BSFLPH emulsions

Emulsion droplet size mean stabilized with BSFLP and BSFLPH at day 0, 1 and 2 were reported in Figure 6A, these values were expressed by the surface weight mean. Smaller droplets were formed with BSFLPH, they have an average size significantly similar of 0.16 nm compared to BSFLP with an average of 0.27 nm. Droplet sizes of emulsions stabilized with BSFLP increased slowly until day 2, comparatively to DH4, whereas those with DH12.5 almost triplicate its size at day 2. It is consistent with TSI results. Indeed, TSI curves of emulsions stabilized by BSFLP and DH4 both showed a similar smooth decreased in stability over time, while DH12.5 showed a sharper instability curve after around 35 h of storage, and this matched with the greater increase in droplet size.

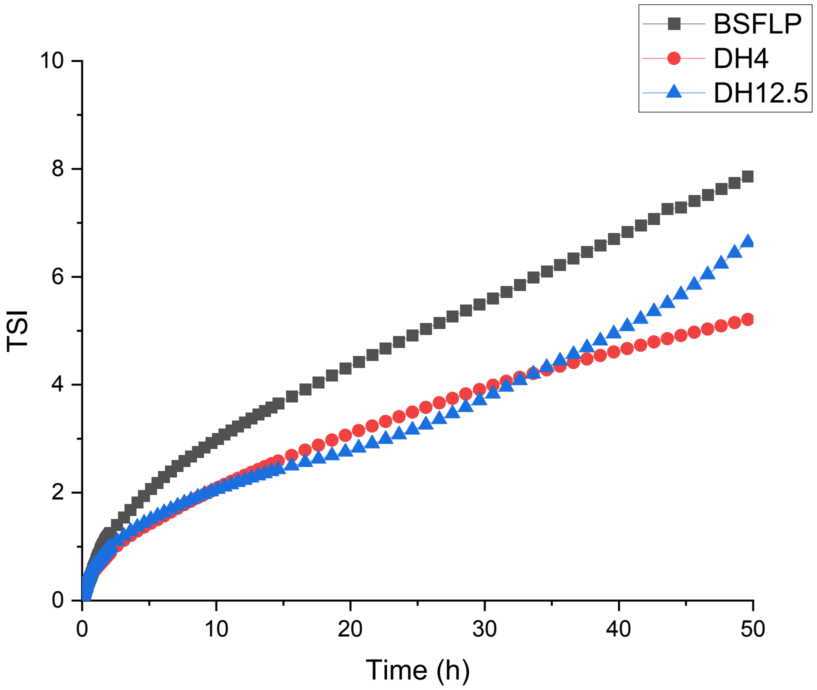

Figure 5- Turbiscan stability index as a function of time for BSFLP and BSFLPH emulsions

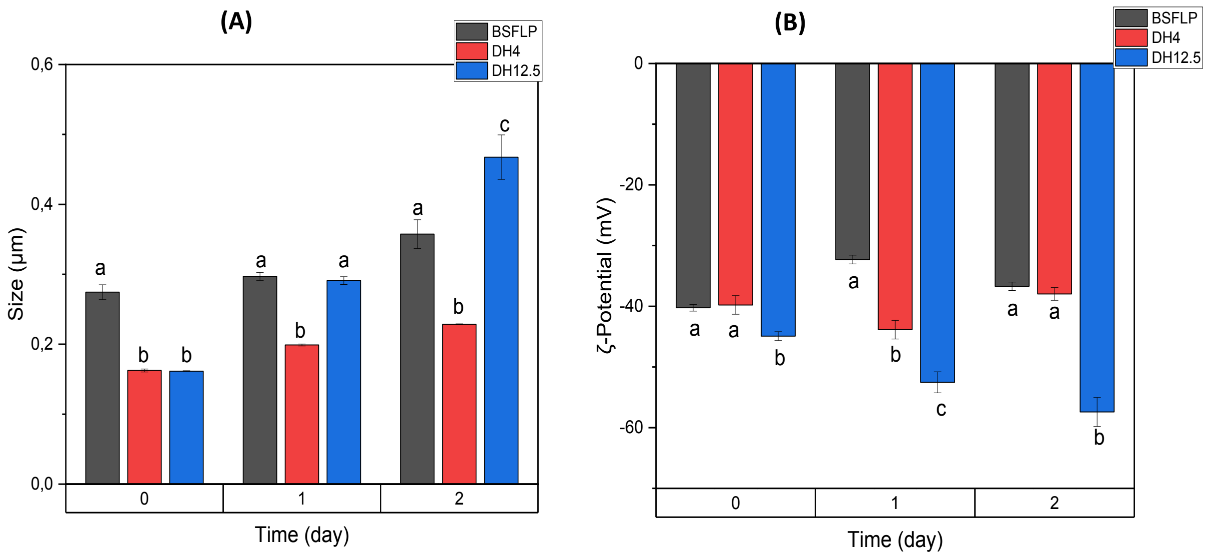

Figure 6- BSFLP and BSFLPH emulsion droplet sizes (A) and zeta potential (B) at day 0,1 and 2. Different letter indicates a significant difference ($p<0.05$) between samples for the same day.

The results are consistent with results found for native and hydrolysate soy proteins that exhibited similar patterns as BSFLP and BSLPH in time sweep of their interfacial layers through shear rheology [280]. This could be attributed to the flexibility of hydrolysates, whereas BSFLP requires time to unfold and rearrange when high shear is applied during emulsification process [280].

4.4.3.11 ζ-potential of emulsions stabilized by BSFLP and BSFLPH

ζ-potential of emulsions stabilized by BSFLP or BSFLPH at day 0, 1 and 2 are shown in Figure 6B. In all cases, absolute ζ-potential values were higher than 30 mV from day 0 to day 2, avoiding van der Waals, hydrophobic interactions and hydrogen bonding helping to avoid flocculation and coalescence [275]. [24] found similar value for BSFLP at -39 mV at day 1. However, at day 1 and 2, an increase in absolute value of ζ-potential was noticeable for DH12.5, that goes against the increase of droplet size noticed before. Through storage, oxidative products from sunflower oil could have led to destabilization and thus higher absolute value of ζ-potential[24]. [235] reported some antioxidant properties of native and hydrolyzed BSFLP. This same study suggested that hydrophobicity plays a role in the scavenging of radicals and that could explained why

DH12.5 was not further able to act against oxidative products as its surface hydrophobicity was the lowest, whereas BSFLP and DH4 could have facilitated the scavenging of free radicals [235].

5-GENERAL CONCLUSION AND FUTURE PERSPECTIVES

5.1 Review Conclusion

Edible insects represent a promising alternative protein source for the world's growing population, and they fulfill all requirements to achieve the main Sustainable Development Goals (SDG) of WHO. Insect cultivation and its inclusion into the mainstream food culture represents a more sustainable and efficient food system. Besides the nutritional factor, the insect proteins have revealed great technological application. It was shown that, in order to obtain protein extracts with great functional and nutritional quality, insects need to be fully studied since their early life stage, providing an appropriate feed and fasting treatment before processing. All other parameters such as the defatting process, drying step, and extraction method, proved to strongly affect protein functionality and therefore, need to be completely comprehended. We emphasize that factors such as substrate, location and use of organic wastes all have a significant influence on the overall sustainability of insect farming. Besides, each insect life stage might represent a completely different protein profile and then show a different result in terms of colloidal properties. In addition, further research should shed light on consumer willingness to pay for animal products from animals fed with insects and also products made out of insects and whether the overall acceptability, in general and from a sensory point of view, will be more interesting compared to conventional products.

5.2 First chapter conclusion

BSFL protein extract revealed a higher protein content (61.1%) compared to insect flour (37.3%). BSFL protein extract and flour were physiochemically characterized. FTIR was applied to identify the main amide bands of insect protein extract and to correlate them to the secondary structure of the proteins. All five amide bands were identified in the BSFL protein extract and the region corresponding to β-sheet structure was also addressed as dominant in amide I band. DSC was applied to verify the glass transition (Tg), unfolding, and solid-melting temperature of the samples. These results give information about the sample's thermal stability, mainly for the protein extract, that might be suitable for food formulation. Also, the techno-functional property, foam stability, showed improved stability after 15 min of heating at 85 °C. This result was explained by

DLS and ζ-potential data, where particle size seems to be increased under heating and ζ-potential decreased under the same treatment. The treatment might provide more protein aggregation and thus a more stable viscoelastic film on the air-water interface of bubbles. Moreover, higher optical density was observed at temperatures 75 and 85 °C when compared to the untreated sample. The data give more information on protein stability and physicochemical characterization which could make the techno-functionalities of BSFL protein extract more interesting to explore beyond their nutritional value. This study indicates that BSFL can be utilized to prepare protein-rich formulations to be further used in the production of food and feed. The protein composition of BSFL revealed an interesting foam capacity and stability which under temperature treatment could perform greater results. Besides temperature, other parameters such as, pH, salt concentration, method of protein extraction and fat removal can be further investigated to better understand how they can improve the foam properties of BSFL proteins. The data showed that BSFL proteins can be a good candidate, to be used as foaming agents, in order to gradually reduce the use of proteins from non-sustainable source. The techno-functionality, sustainability and nutritional value herein reported for BSFL preparation can add value to the product and help improving consumer acceptance, which is an important step towards the development of sustainable products. Further studies must be considered in the colloidal and molecular field, to explore the air/oil-water interfacial properties of these proteins, and how they might migrate to and stabilize the foam and emulsion structure.

5.3 Second chapter conclusion

In our study, we investigate the gelling properties of BSFL protein after ultrasound treatment. The results indicated that untreated samples displayed smaller size, lower turbidity and water holding capacity, reduced surface hydrophobicity as well as lower gel strength. Higher particle size and ζ-potential, increase in turbidity, rise in surface hydrophobicity till an ultrasound treatment of 15 min was observed. The ultrasound treated gels had a higher water holding capacity as compared to untreated gel. The gel strength obtained for the ultrasound treatment after 15 min was also found to be greater than the untreated gels. The microstructure of the gel after 15 min of treatment was found to be the most compact, containing the lowest pore size. Ultrasound treatment induced modifications on physicochemical and functional properties of BSFL protein, which are closely related to molecular changes, mainly increase in the exposure of hydrophobic groups and particle size variation. In conclusion, ultrasound treatment could be applied as a green technology to modify the techno functional properties of BSFL protein and can be further explored for other sustainable protein sources.

5.4 Third chapter conclusion

Emulsions stabilized with BSFL-OH showed the most promising physical stability, which was confirmed by TSI value and the hydrodynamic diameter. Modifications on the secondary structure could be noticed by FTIR after pre-treatment with OH alone and the combination of ultrasound and OH. This indicated that the treatment the influenced the techno-functional properties of proteins. The improved capacity of BSFL-OH to stabilize O–W emulsion was also confirmed by the lowest IFT reported in the pendant drop test. This is the first time that a BSFL sample was treated with OH in order to increase protein content and improve emulsifying properties. Therefore, further research is necessary to investigate how these treatments can work on the functionality of BSFL proteins. For the oxidative stability, BSFL, BSFL-OH and BSFL-UOH emulsions showed higher oxidation when compared to CAS. Peroxide value had an increasing pattern over time when compared to CAS reference emulsion, and the highest oxidation was reported for BSFL-UOH samples, which means that OH and ultrasound treatment in the specific method herein reported did not improve oxidative stability. As oxidation was already occurring for BSFL protein concentrate, antioxidants might be recommended during protein extraction. However, other parameters, such as using the same techniques as pre-treatment, can be applied in order to investigate how physical and oxidative stability can be improved. For further studies, the combination of BSFL protein and surfactants, such as phosphatidylcholine and lecithin, can improve the efficiency in protecting emulsified lipids against oxidation. In addition, the protein concentration and hydrolysis of BSFL protein might play an important role in lowering the interfacial tension and improve the interfacial stability of O–W emulsions.

5.5 Fourth chapter conclusion

This study has shown that enzymatic hydrolysis with alcalase was able to alter physicochemical properties of BSFLP. Initial interactions between hydrophobic residues right after Alcalase hydrolysis of DH 12.5% could have led to further protein aggregation which ended up burying most of the hydrophobic groups. A similar process also might have happened for DH4 but less expressive, as the sample showed smaller particle size. Regarding interfacial properties, both BSFLPH and BSFLP demonstrated surfactant properties as they decreased interfacial tension and created a viscoelastic protective film. BSFLPH were able to form flexible films taking more time to exhibit elastic behavior as shown by shear and dilatational rheology whereas a more structured one was noticed for BSFLP. These changes in structure and behavior of BSFLP allowed to create more stable oil-in-water emulsions when BSFLPH were used as surfactant, data shown by lower TSI values within 2 days. The characterization of these emulsion droplet sizes and ζ -potential was in accordance with the physical stability of the emulsions where DH4% had the best physical stability and smallest droplet size.

It can be concluded that limited enzymatic hydrolysis upon BSFLP could be a clue to enhance their emulsifying properties and can be further investigated to be used as an ingredient in food formulation. Higher DH, 12.5%, had the biggest particle size as it was suggested that the small peptides were able to aggregate more easily. This resulted in a higher interfacial strength with k value of 32.076 mN/m.s and the highest E* which characterizes a stronger interfacial layer in the amplitude sweep for dilatational rheology.

5.6 Future Perspectives

As mentioned above, edible insects remain underutilized in the animal feed industry, as a food product and as an alternative food ingredient; however, with the rapid development of intensive industrial insect farming, their potential use is expected to increase. Specifically, there is still a lot to investigate about BSFLP. Indeed, BSFLP composition and structure seems to depend on many parameters such as insect feed, rearing and protein extraction protocol. Other than enzymatic hydrolysis, different treatments can be performed over BSFLP in other to investigate possible improved functionalities. As a consequence, Increased knowledge of its protein composition and fully characterization could target the best functionality to be further implemented in food formulations. Considering the gelling property of BSFLP, more studies should be focused on that as this was the first time this colloidal property was described for *Hermetia illucens*. *in vitro* and *in vivo* studies regarding the emergent insect proteins will be the next step to fully understand protein safety, digestibility, and allergenicity. In addition, the interaction of these proteins with the gut microbiota needs to be addressed. The study might give a better answer if insect protein-based products will provide benefits for human nutrition and the possible risks. As a relatively new research line, the exploration of insect protein's functionalities can find a variety of applicability from cosmetics to food, considering their gelling, emulsifying, and foaming agent. For future research lines the combination of insect proteins with plant-based and/or dairy proteins would be a new exploratory level that could improve colloidal properties for food application. As well as the usage of alternative and sustainable treatments such as enzymatic hydrolysis, ohmic heating, pulsed electric field and ultrasound methods. These methods have recently called more attention when applied to protein extraction and structure modification to improve protein's colloidal properties. Those treatments can also help with peptides bioactivity as some insect peptides have reported antioxidant , antimicrobial and antihypertensive capacity. Insects represent an enormous biodiversity with a huge biomass, around 95% of animal kingdom are insects, which brings up a wide range of peptides with possible bioactivity and also the chance to combine proteins from different insects looking for optimized colloidal properties. the large-scale production of insect bioactive peptides presents a promising biotechnology business. However, to achieve such demanding goals, a multidisciplinary approach is highly necessary.

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