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Structural properties of black soldier fly larvae protein concentrates extracted via salting-in and salting-out coupled with ultrafiltration

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ABSTRACT

The black soldier fly (BSF) larvae are one of the promising edible insect species for industrial food production. The aim of this study was to investigate the effect of the salting in/out extraction techniques, coupled with ultrafiltration, on BSF larvae protein. The protein isolates were extracted using alkaline extraction and acid precipitation (AAF), salting-in (AAFSI), salting-out (AAFSO), and salting-in-out (AFFSISO) coupled with ultrafiltration. All samples exhibited significantly ($p < 0.05$) negative zeta potentials, ranging from −9.8 to −26.9 mV. Moreover, no significant differences ($p > 0.05$) were observed in the levels of exposed sulfhydryl groups between AAF and AAFSI proteins (6.8 and 6.9 μM/g, respectively). Fourier transform infrared spectroscopy (FTIR) of protein extracts derived from AAFSO and AAFSISO revealed a significant N–H free stretching vibration in the spectral range of 3500 – 3200 cm^{-1}. The β-sheet and random coil structures were transformed into α-helix and β-turns by salting-out treatments, as demonstrated by circular dichroism, indicating a more compact and organized conformation. AAFSO and AAFSISO, had the highest content of ordered protein structures and showed the largest ΔH values, measured at 141.0 J/g and 137.0 J/g, respectively. This research demonstrates that the integration of salting-out extractions and ultrafiltration techniques holds significant potential for protein extraction, especially as an innovative approach to producing insect proteins with excellent nutritional quality for food-related applications.

1. Introduction

Consumer interest towards consuming more sustainable proteins has led the way towards adopting edible insect proteins for human food applications. Including insects in an unidentifiable form as functional ingredients could reduce the negative perceptions associated with them. This approach would involve combining insect powders or flours with everyday foods, making them more acceptable to consumers (Gmuer et al., 2016; Hartmann & Siegrist, 2017; Schösler et al., 2012; Tan et al., 2015). One such insect source that has gained attention in recent years is the black soldier fly (BSF) larvae. BSF larvae are known for their low environmental impact, as they can be reared on a variety of organic waste materials (van Huis, 2013). In terms of nutrition, BSF larvae are highly rich in both protein (69 %) and lipids (39 %) (Bußler et al., 2016; Mshayisa et al., 2021; Queiroz et al., 2021; Vanqa et al., 2022). There is a significant amount of non-striated muscle and structural proteins found in BSF larvae, which supports the use of these proteins as a substitute for traditional meat and soybean protein sources (Altmann et al., 2018).

The consumption of whole insects is generally considered unacceptable for the majority of populations, although alternative forms of insect consumption are becoming increasingly popular; therefore, extracting insect derivatives is a more viable option for producing ingredients that can be used in processed food products. Previous research has revealed that the protein extracted from BSF larvae can be used in various applications, including animal feed, aquaculture, and human consumption (Ghaly, 2009; Nguyen et al., 2015; Sheppard et al., 2002; Wang et al., 2017). However, the profile, quality and the yield of BSF larva protein isolates depend heavily on the extraction method used. The extraction techniques directly influence the physicochemical and structural properties of proteins.

Protein isolation from insects typically involves four primary steps: pre-treatment, defatting, protein extraction, and protein purification. The first step usually entails blanching and freeze-drying the insect

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larvae before grinding and sieving to produce a fine powder. This fine powder increases the surface area, which promotes better protein-solvent contact during subsequent extraction steps. Some researchers have incorporated a defatting step to enhance insect protein yield at the laboratory scale (Choi et al., 2017; Ghaly, 2009; Nguyen et al., 2015; Zozo et al., 2022). A significant increase in protein content was observed in both *Tenebrio molitor* and *Hermetia illucens* when their respective insect flours were defatted with hexane. Specifically, the protein content of *Tenebrio molitor* increased from 58 ± 1.2 % to 65 ± 0.3 %, while that of *Hermetia illucens* increased from 35 ± 0.2 % to 45 ± 1.4 % (Choi et al., 2017). Numerous techniques have been employed in the isolation of insect proteins. One such method, employed by Yi et al. (2013), involved the use of acidic solubilization to extract protein isolates from the supernatants of five distinct insect species (*Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus* and *Blaptica dubia*), achieving protein yields ranging from 50 to 61 %. In contrast, Chatsuwan et al. (2018) obtained protein isolates from *Patanga succincta* and *Chondracris rosea* with extraction efficiencies of 49 % and 43 %, respectively. According to Purschke et al. (2018), a protein yield of 51.7 % and purity of 82.3 % were achieved by using either 1 M or 4 M NaOH to enhance the solubility of protein in a protein concentrate produced from *Locusta migratoria*. These results demonstrate the potential of using both aqueous and alkaline solubilization methods for insect protein isolate production. Nevertheless, the high pH and temperature required in these methods can result in protein denaturation and loss of functionality, as reported by Makkar et al. (2014).

Ultrafiltration (UF) is a widely used method for purifying proteins from various sources. In one study, tangential ultrafiltration was used to carry out protein fractionation with a membrane molecular weight cut-off of ≤ 300 kDa on algae proteins that had been solubilized at pH 7 and 12 (Ursu et al., 2014). However, the majority of the proteins were found in the retentate, which was attributed to the macromolecular aggregates of algae proteins being larger than the molecular weight cut-off. Membrane filtration has not yet been investigated using insect proteins, as it is necessary to have knowledge of the protein's size and properties. Unfortunately, there is currently limited information available on insect proteins.

A single protein extraction processing method cannot satisfy the needs of the food industry in terms of functionality and product application. Therefore, it is necessary to explore other techniques to complement these processing methods to achieve high extraction efficiency and purity. Salt-based extraction is a well-established technique in the food industry, and this method has been widely used to extract biologically active substances from agricultural products, such as pea protein (Sun & Arntfield, 2011), oleosin (Sun et al., 2022), soy protein (Jiang et al., 2010), and *Tenebrio molitor* protein (Jiang et al., 2021). Salt-based extraction has many attractive advantages over other traditional extraction techniques because it is environmentally friendly, low-cost, has a high polarity, low viscosity, high yield, and is easy to scale up (Deng et al., 2019). This technique can be achieved in two ways: salting-in or salting-out extraction. The salting-in method involves adding salt to the protein solution to increase its solubility and promote protein precipitation. The salting-out method involves adding salt to the protein solution to decrease its solubility and promote protein precipitation and aggregation (Duong-Ly & Gabelli, 2014). The Hofmeister series (Okur et al., 2017) demonstrates that the ionic strength of different salts varies in their capacity to salt in or out proteins.

Subsequently, NaCl is commonly used as a salting-in agent, whereas $(NH_4)_2SO_4$ is utilized to expedite protein salting-out. These techniques that employ salting agents have been shown to be effective in the isolation of various proteins, such as, bovine serum albumin, ovalbumin, cytochrome, and cytochrome from aqueous solutions (Gadalkar et al., 2017; Mune et al., 2014; Okur et al., 2017). Some researchers have reported that an effective increase in the solubility of albumin and globulin present in plant proteins (during the process of alkaline extraction) and enhanced protein extraction efficiency can be achieved using NaCl. Thus, the quantity and quality of endogenous proteins can be simultaneously improved (De Chirico et al., 2020; Gadalkar et al., 2017; Mune et al., 2014). Despite extensive research in the field of protein extraction from BSF larvae, there is currently no published data on the effectiveness of salting in/out techniques coupled with ultrafiltration. It is essential to evaluate the efficiency of these methods and their impact on the quality and yield of BSF larvae protein isolates. Therefore, the aim of this study was to elucidate the impact of salting-in and salting-out extraction coupled with ultrafiltration on the physicochemical, structural, and thermal characteristics of BSF larvae protein isolates.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all chemicals used in the study were of analytical grade. The following chemicals were obtained from Sigma-Aldrich (Johannesburg); hexane, isopropanol (88 %), petroleum ether, o-phthaldialdehyde (OPA), fluorenyl-methylchloroformate (FMOC), nitric acid (HNO_3) (55 %), bovine serum albumin (BAS), 1 M phosphate buffer, 1-anilino-8 naphthalenesulfonate (ANS), Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA (ethylenediaminetetraacetic acid), pH 8 containing 8 M urea), Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid), 2-mercaptoethanol and trichloroacetic acid (TCA) and potassium hydroxide. Sodium chloride and ammonium sulphate were acquired from Science World (Cape Town, South Africa). The prepared solutions and reagents were stored under appropriate conditions to prevent contamination or deterioration, and all solutions were prepared with deionized water (Milli-Q system, Millipore, Gradient model), which has a resistivity of 18.2 MΩ.cm.

2.2. Preparation of the insect flour

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

2.3. Preparation of the protein isolates

Four different methods were used to prepare BSF larvae concentrate (see Table 1). The salt extraction isolates were prepared according to the method of Jiang et al. (2021) with modifications. The alkaline-acid extraction (AAF) was conducted as previously described by Azagoh et al. (2016) with slight modifications. By combining defatted BSF larvae flour with Milli-Q water in a 1:10 (w/v) ratio and adjusting the pH to 10 using 1 M NaOH solution, a suitable mixture was prepared. The slurry was stirred for 30 min (60 °C ± 1.0) and then cooled to room temperature before centrifugation (Z-326 K, Hermle Labortechnik GmbH,

Table 1
Preparation of the black soldier fly larvae protein isolates.

Protein isolation details		
	Abbreviation	**Salt addition**
Alkaline - acid extraction + filtration	**AAF**	None (control sample)
AAF + salting-in	**AAFSI**	1 % NaCl
AAF + salting-out	**AAFSO**	80 % $(NH_4)_2SO_4$
AAF + salting in & salting out	**AAFSISO**	1 % NaCl + 80 % $(NH_4)_2SO_4$

German) at 25 °C, 10,000 × g for 30 min. The pH of the supernatant was adjusted to 4.5 with 1 M HCl, and the suspension was left at 4 °C overnight for protein precipitation. The precipitate was recovered by centrifugation and then resuspended in 250 mL of water and pH adjusted to 7.0 with 1 M NaOH. The dissolved protein isolate was obtained after centrifugation, and a 200 mL solution was subjected to dialysis through ultrafiltration using a 3 kDa nominal molecular mass cut-off membrane in an Amicon ultrafiltration cell (8400, Amicon, Beverly, MA, USA). The dialysate was subsequently lyophilized and preserved at a temperature of −18 °C for subsequent analysis.

The AAFSI process was comparable to the AAF method, except for the inclusion of 1 % NaCl (w/v) in the NaOH solution to improve protein solubility (Yi et al., 2017). The AAFSO method followed the AAF process, with the exception of adding 80 % $(NH_4)_2SO_4$ (w/v) to the supernatant to enhance protein precipitation (Duong-Ly & Gabelli, 2014). The AAFSISO process combined both salting-in and salting-out methods, involving the addition of 1 % NaCl (w/v) to the NaOH solution to facilitate protein solubility and 80 % $(NH_4)_2SO_4$ (w/v) to the supernatant to promote protein precipitation.

2.4. Colour change

The colour of the insect protein isolates was assessed using a Konica Minolta Sensing Americas Inc. spectrophotometer, which is based on the CIE L*a*b* colour space system. The device was calibrated using a white reference plate and zero-calibration process. Powdered samples were uniformly distributed in a 30 mm diameter cuvette for reflectance measurements, which were taken in triplicate at three different locations within the samples. The results were expressed in terms of L* (lightness/brightness), a* (redness/greenness), and b* (yellowness/blueness). The overall colour difference (ΔE) was calculated using the values of ΔL, Δa, and Δb, where ΔE is a quantitative representation of the difference between two colours.

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

2.5. Amino acid composition

A 0.5 g fine powder sample (containing 10 mg nitrogen content) was weighed and placed into a screw-capped test tube. Then, 7 ml of 6 M HCl was added, and the tube was capped and heated for 6 h at 110 °C to carry out hydrolysis. After cooling to room temperature, the sample was dried using a Reacti-Therm™ heating/stirring module at 70 °C under a stream of nitrogen. The remaining residue was quantitatively transferred and made up to volume in 100 mL volumetric flasks with 0.1 M HCl. The resulting solutions were filtered using 0.22 µm pore size cellulose membrane syringe filters (Sigma-Aldrich, Johannesburg, South Africa) and dispensed into high-performance liquid chromatography (HPLC) vials. The hydrolysed samples were derivatised with o-phthaldialdehyde (OPA) and fluorenyl-methylchloroformate (FMOC) and analyzed using a Zorbax Eclipse-AAA column (150 mm × 4.6 mm, i.d., particle size 5 µm; Chemetrix) at 40 °C, with fluorescence detection.

2.6. Crude protein content and yield

The quantity of crude protein was evaluated using the Dumas combustion method (TruSpec-N Leco, St. Joseph, MI, USA) and applying a protein-to-nitrogen conversion factor of 5.6, as recommended by Janssen et al. (2019) for edible insects. The extraction yield (%) was calculated as follows:

$$Extraction\ yield\ (\%) = \frac{Extract}{Sample}\ x100$$

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

The method used to determine the SH values was described by Deng et al. (2019). To perform this assay, 30 mg of BSF larvae was dissolved in 10 mL of Tris-Gly buffer containing 0.086 M Tris, 0.09 M Gly, 4 mM EDTA, and pH 8, as well as 8 M urea. Next, 50 µL of 4 mg/ml Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid) in Tris-Gly buffer was added, and the mixture was incubated at 25 °C for 60 min. Following this, the supernatant's absorbance was measured using a buffer containing Ellman's reagent as a blank after centrifugation at 8000 × g for 5 min. To determine the total SH content, 15 mg of BSF larval protein was dispersed in 10 mL of Tris-Gly buffer, along with 100 µL of 2-mercaptoethanol. The samples were then incubated at 25 °C for 60 min, after which 10 mL of 12 % trichloroacetic acid solution (TCA) was added. The mixture was then centrifuged at 8000 × g for 10 min, and the precipitate was washed with three 10-mL portions of 12 % TCA. The precipitate was then dissolved in Tris-Gly buffer, and 50 µL Ellman's reagent was added to initiate the color reaction. The SH content was calculated based on the absorbance values obtained as follows:

$$SH\left(\frac{uM}{g}\right) = \frac{73.53\ x\ A_{412}\ x\ D}{C}$$

Where A412 was the absorbance at 412 nm, D was the dilution factor, and C was the protein concentration (in mg/mL). The sulfhydryl (SH) content was calculated as the average of the total SH value and the free SH value.

2.8. Surface charge (zeta potential)

The zeta potential of protein isolates was measured based on the method of Deng et al. [37] and Mshayisa et al. (2022). The protein isolate solutions with a concentration of 1 % (w/v) were prepared by thoroughly mixing the protein samples with Milli-Q water. The solutions were filtered through Whatman No. 1 filter paper. The pH of these diluted solutions was adjusted to pH 7 with 0.1 M HCl or 0.1 NaOH. The zeta potential was determined using a Zetasizer Nano Series (Malvern Instruments, Malvern, Worcestershire, UK).

2.9. Fourier transform infrared spectroscopy (FTIR)

The analysis of protein concentrate samples was carried out using FT-IR on a Perkin Elmer instrument that was equipped with a universal attenuated total reflectance (UATR) polarization accessory, as explained by Zozo et al. (2022). Prior to data collection for each sample, a background spectrum was collected. The powdered samples were placed directly on the surface of the ATR crystal after being ground using a mortar and pestle. To collect spectra, 32 scans were co-added with a resolution of 4 cm^{-1} in the range of 400 – 4000 cm^{-1}. To ensure that no residual contribution from previous samples remained, the UATR crystal was cleaned with acetone.

2.10. Circular dichroism (CD)

Protein solutions were formulated with a final concentration of 0.5 mg/mL in 100 mM phosphate buffer with pH 7.0. A Jasco J-600

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

2.11. X-ray diffraction (XRD)

An X-ray diffraction (XRD) pattern of the isolated protein was obtained using a diffractometer, specifically the Panalytical X'pert PRO model (Almelo, Netherlands), which employed copper K radiation (Å = 1.54056). The samples were scanned at a rate of 3.2° per minute from 5° to 40° (2θ) with a step size of 0.013°. The measurement of crystallinity was performed by the X'pert HighScore plus software. Crystalline index (CrI) values of the proteins were calculated using the formula below.

$$CrI_{110} = \frac{I_{110} - I_{am}}{I_{am}}\ x100$$

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

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

2.12. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The distribution of molecular weight of soluble proteins derived from BSF larvae was determined using SDS-PAGE in a mini protean tetrae-lectrophoresis cell system sourced from Bio-Rad (United States) and 4 – 20 % Mini-PROTEAN TGX Precast Gels from Bio-Rad (United States). Samples weighing 50 mg were dissolved in 1 mL of Laemmli buffer containing 62.5 mM Tris–HCl (pH 6.8), 5 % 2-mercaptoethanol, 2 % SDS, 0.01 % bromophenol blue, and 20 % glycerol. The samples were then subjected to agitation for 2 min and an ultrasonic bath for 10 min before being heated at 100 °C for an additional 10 min. Following this, the samples were centrifuged at 10,000 g for 2 min, and 10 µL of the resulting solution was applied to the gel. The electrophoresis was carried out at a voltage of 180 V. The gels were stained with Coomassie Brilliant blue G250 (Bio-Rad, United States). The Sigma Pre-Stained Protein Marker was employed to identify each protein fraction.

2.13. Thermal properties (TGA and DSC)

The thermal properties of freeze-dried protein concentrates were evaluated using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Both analyses required the weighing of approximately 4.0 to 6.0 mg of the sample into an aluminum pan. An empty aluminum pan that had been sealed was utilized as a reference. The DSC profiles of the samples were assessed using a thermal analyzer, specifically the Q2000 model manufactured by Perkin Elmer (Singapore). The pans were insulated and heated from 30 °C to 180 °C at a rate of 20 °C per minute. The thermal stability of the protein concentrates was assessed using a TGA system (TGA-7, Perkin Elmer, Singapore). The heating rate was set at 20 °C/min, and nitrogen was used as the carrier gas at a flow rate of 50 mL/min. The temperature range was set from 30 to 650 °C for the analysis.

2.14. Data analysis

All data were subjected to multivariate analysis of variance (MANOVA). The Duncan multiple comparison post hoc test was employed to assess significant differences ($p < 0.05$) between individual means. The statistical analyses were conducted using SPSS 29.0 for Windows®, with a confidence level of $p < 0.05$ established for determining significance.

3. Results and discussion

3.1. Colour change

Determination of colour in food or novel ingredients is useful due to the association between colour, taste and flavour perceptions in humans. The colour measurements for the proteins extracted from the BSF larvae using different techniques are displayed in Table 2. There is a significant difference ($p < 0.05$) in the colour attributes in terms of lightness (L*), redness (a*), yellowness (b*) and the colour difference (ΔE). AAFSISO isolates were characterised by significantly ($p < 0.05$) higher lightness (higher L* value), lower redness (a*) and yellowness (b*) than the AAF isolates. The L* value of AAF was significantly ($p < 0.05$) lower when compared to other samples, and these findings are similar to those reported by Bußler et al. (2016) and Mshayisa et al. (2022).

Generally, BSF larvae flour is darker in appearance, and the resulting protein is much lighter, especially when using the AAFSISO extraction. In this study, ΔE denotes the overall colour variation between the control sample and samples containing added salt. The parameter serves as an indicator of color quality and is typically used to assess the visibility of a colour to consumers. A value below 1.5 is considered to be not visible, while a value above 6.0 is generally considered to be visible to consumers (Wibowo et al., 2015). This study found that the range of ΔE was between 11.6 and 21.4, indicating that colour differences were perceptible at a glance, meaning that the changes in colour could be easily distinguished without the need for a closer examination. Due to the addition of salts, the redness (a*) and yellowness (b*) were significantly ($p < 0.05$) decreased; similar results were obtained by Wu et al. (2022), who studied the effect of salt addition on textural and quality properties in the chicken-surimi products.

The use of alkaline conditions during the extraction of AAF proteins can result in the co-extraction of phenolics from BSF larvae protein, producing protein isolates with a dark green to brown colour. As explained by Janssen et al. (2019), phenolic compounds tend to undergo enzymatic and non-enzymatic oxidation when exposed to alkaline conditions, resulting in the formation of quinones. These quinones can subsequently bind to proteins, leading to a darker colour (Xu & Diosady, 2002). However, the presence of salts can reduce phenolic oxidation and ionic interactions, resulting in a lighter-coloured protein powder (Hadnađev et al., 2018). Treatments that eliminate phenolic compounds result in the production of lighter-colored protein isolates (Xu & Diosady, 2002). The results of this study indicate that AAFSISO produces protein powders that are lighter in colour and may be more desirable for food products where a lighter appearance is preferred, such as dairy alternatives or foams and emulsions.

3.2. Amino acid composition and protein quality analysis

The essential and non-essential amino acid content of BSF larvae extracted with various methods and FAO (Food and Agricultural Organisation) protein intake recommendations for adults are displayed in Table 3. The method of extraction had a significant ($p < 0.05$) influence on the crude protein yields of the isolates. For instance, the basic AAF extraction resulted in a protein yield of 15.9 %, which increased to 19.5 % when the salting-in-assisted treatment was used. When alkali

Table 2
Effect of extraction on the colour attributes of black soldier fly larvae protein isolates.



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

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

solutions were utilized, the solubility of proteins was directly proportional to the ionic strength at lower concentrations of ions. This led to a greater amount of protein salting-in. Additionally, treatments that involved salting out resulted in a significant increase in crude protein yields, which is consistent with previous findings that higher concentrations of $(NH_4)_2SO_4$ result in increased protein yields (Duong-Ly & Gabelli, 2014). However, no significant ($p > 0.05$) differences in crude protein yields were observed between AAFSI and AAFSISO extractions, which yielded 36.1 % and 44.5 %, respectively (Table 3). Moreover, these protein yields were in line with those previously reported for BSF larvae (ranging from 26.4 % to 59.9 %), which could be attributed to variations in process parameters such as pH, temperature, heating duration, NaCl and $(NH_4)_2SO_4$ concentrations, and the liquid-to-solid ratio (Lam et al., 2018).

The amino acid composition of a protein not only plays a role in determining its structure but also has broader implications. The protein extracts in this study contained all essential amino acids (EAA) except for tryptophan and cysteine, which were not measured. Tryptophan is susceptible to degradation under acidic hydrolysis conditions, and severe conditions result in its destruction, which can lead to inaccurate measurements. Similarly, cysteine's reactivity and sensitivity to oxidation are attributed to its thiol (-SH) group. During standard acidic hydrolysis, cysteine may undergo oxidative changes, resulting in inaccurate measurements. Table 3 provides a comprehensive examination of the amino acid composition, which encompasses essential amino acids, such as threonine, methionine, valine, histidine, lysine, leucine, phenylalanine, and isoleucine. It is evident that the techniques used to extract and isolate the proteins had a substantial influence on the amino acid profiles, as discernible variations were detected in the amino acid compositions of the diverse protein isolates. In quantitative terms, the total sum of all the essential amino acids was 322.0 mg/g for AAF, 301.0 mg/g for AAFSI, 286.3 mg/g for AAFSO, and 281.8 mg/g for AAFSISO. These values meet the adult dietary requirements established by the World Health Organization, which specify a minimum of 277 mg/g. However, it's worth noting that the sulfur-containing amino acid methionine fell short of the recommended levels (FAO/WHO/UNU, 1985) in the AAFSI isolate. The AAF method likely preserves the native structure and composition of proteins, resulting in a moderate yield and a balanced amino acid profile. The AAFSISO isolate displayed lower values for essential amino acids like histidine, threonine, methionine, and valine when compared to other extracts. Therefore, the amino acid profile of protein not only determines the protein structure but also is of great importance for BSF larvae protein to be considered as a supplement in food products or a novel functional ingredient. The salting-out treatments use high concentrations of salts to precipitate proteins, which significantly alter the amino acid composition by preferentially isolating certain proteins. The different ionic strengths and pH levels used can also impact the solubility and stability of specific amino acids.

However, except for methionine, the AAFSI isolate exhibited higher levels of all essential amino acids in comparison to the daily requirements of the FAO adult, which makes the extraction method noteworthy from a nutritional perspective. It's worth noting that Spranghers et al. (2017) have previously demonstrated that the concentration of each amino acid may vary depending on the diet of BSF larvae. Here, we highlight that amino acid content can also differ after protein extraction and prior processing steps, such as the method used for killing the larvae (Bessa et al., 2020). This technique enhances protein solubility at lower ion concentrations, leading to higher protein yields. However, it may cause slight alterations in the amino acid profile due to the specific ionic interactions involved. Mintah et al. (2020) also documented variations in amino acid content resulting from different protein extraction techniques for BSF larvae. When comparing BSF larvae protein extract to other protein sources such as meat and chicken, it emerges as a sustainable alternative due to its superior values for certain essential amino acids (EAAs). In regions where lysine is a dietary concern, particularly in developing countries where it is often found in low quantities (Bessa et al., 2020), BSF larvae protein extraction can provide a convenient means to meet the recommended concentration of lysine (45.0 mg/g) when used for food fortification purposes.

Furthermore, the AAFSI protein extracts exhibited lower levels of methionine, possibly due to reduced S-S bonds or total SH groups (Chen et al., 2018). If these protein solutions were concentrated and converted into powder form, the resulting extracted protein would meet the requirements for human consumption. As shown in Table 3, the extraction method significantly ($p < 0.05$) affects the total essential amino acid content, with AAF having the highest value, followed by AAFSI and AAFSO. AAFSI demonstrated a higher content of tyrosine, serine, alanine, arginine, and proline. The use of NaCl and $(NH_4)_2SO_4$ can lead to selective precipitation of proteins, influencing the amino acid composition of the final extract. These findings suggest that salting-in and salting-out extraction methods have the potential to enhance the bioactive properties of BSF larvae proteins, making them valuable as

Table 3
Amino acid (AA) composition (mg/g of crude protein) of the Black Soldier Fly larvae protein extracts, compared with the adult daily requirements.

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

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

functional ingredients in various food processing applications. The structural, compositional, and functional changes of a dietary protein macromolecule are reflected in its zeta potential (Barba et al., 2019). A zeta potential of around ±30.0 mV is typically considered a sign of stability, while values approaching zero indicate instability and the potential for hydrophobic-hydrophobic interactions, which can cause protein aggregation (Casanova et al., 2020; Subaşı et al., 2020). A higher absolute zeta potential results in stronger electrostatic repulsion and a greater separation distance between particles in a suspension, reducing aggregation and flocculation driven by Van der Waals interactions (Thaiphanit et al., 2016). Various external factors, including temperature, ionic strength, and pH, can also influence the zeta potential of proteins (Deng et al., 2019). In this study, all BSF larvae isolates at pH 7 exhibited negative zeta potentials, ranging from −9.8 to −26.9 mV. As indicated in Table 3, the zeta potential values for AAF and AAFSI (- 9.8 mV and −15.0 mV, respectively) were significantly ($p < 0.05$) lower than those for the salting-out methods, with AAFSO at −24.1 mV and AAFSISO at −26.9 mV. The AAFSO extraction, characterized by a more negative zeta potential (−24.1 mV) compared to AAFSI (−15.0 mV), indicates that proteins extracted using this method maintain their intrinsic electrostatic properties with minimal ionic influence, resulting in moderate stability and higher electrostatic repulsion. The combined salting-in and salting-out method (AAFSISO), yielding a zeta potential of −26.9 mV, suggests a synergistic influence, potentially resulting in a net reduction in the electrostatic repulsion among protein particles, which could be due to a balance between ionic strength and protein interactions. The greater the zeta potential of a suspension, the more likely it is that particles will repel one another, which in turn makes them less prone to flocculation (Azagoh et al., 2016). These findings suggest that the proteins treated with the salting-out method possessed more electrostatic repulsion, likely contributing to their increased solubility. Considering these zeta potential results in the context of utilizing the protein extract as a novel ingredient in food products, the observed anionic charge implies potential applications in formulations where colloidal stability is paramount. The repulsive forces associated with the negative zeta potential may mitigate issues such as particle aggregation, flocculation, and sedimentation in food matrices such as emulsions, dairy alternatives and beverages.

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

Proteins contain sulfhydryl (SH) groups and disulfide bonds (SS), which contribute to their structural stability and rigidity (Deng et al., 2019; Hu et al., 2021). However, processing methods, such as heating and high pressure, can lead to the destruction of sulfhydryl content, which can negatively impact protein functionality in food applications (Lin et al., 2020). The content of free SH and disulfide bonds is depicted in Fig. 1.

In this research, there were no substantial differences ($p > 0.05$) in the levels of exposed SH groups between the AAF and AAFSI proteins (which were 6.8 and 6.9 μM/g, respectively), despite the fact that these levels were higher than those of the salting-out proteins (as shown in Fig. 1). Proteins that have a greater number of exposed SH groups, such as AAF and AAFSI, are more inclined to aggregate or self-associate, which can lead to a decrease in solubility. This phenomenon has been previously reported in the literature (Deng et al., 2019). These results are in line with the analysis of zeta potential explained in Section 3.2.

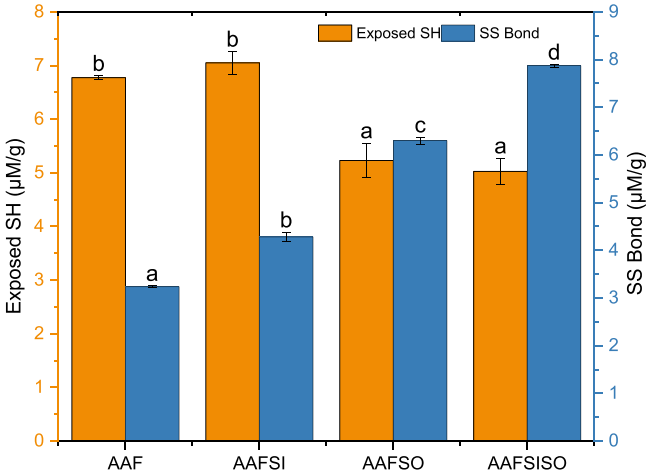


Fig. 1. Free sulfhydryl (SH) and disulfide (SS) bond contents in protein isolates from black soldier fly larvae subjected to various treatments: AAF (alkaline-acid extraction), AAFSI (salting-in technique), AAFSO (salting-out technique), and AAFSISO (salting-in and out technique). Values are presented as mean ± standard deviation, with means having different superscripts indicating significant differences ($p < 0.05$).

The disulfide bonds (SS) content in the salting-out protein samples (AAFSO and AAFSISO) was found to be significantly ($p > 0.05$) higher than that of the AAF and AAFSI proteins. The SS content in the salting-out samples was 6.3 and 7.9 μM/g, whereas the AAF and AAFSI proteins exhibited SS contents of 3.2 and 4.3 μM/g, respectively. The results of this study are in line with those reported by Jiang et al. (2021) on the extraction of proteins from *T. molitor* proteins. The high SS bonds are due to the high salt concentrations, which can promote protein unfolding due to the salting-out effect. This unfolding exposes more cysteine residues (the building blocks of SS bonds) within the protein. Therefore, further indicates that the presence of SS bonds in a protein enhances its thermal stability by stabilizing its folded conformations and reducing conformational entropy.

The disulfide bond (SS) content in the salting-out protein samples (AAFSO and AAFSISO) was significantly ($p > 0.05$) higher than that of the AAF and AAFSI proteins. The SS content in the salting-out samples was 6.3 and 7.9 μM/g, whereas the AAF and AAFSI proteins exhibited SS contents of 3.2 and 4.3 μM/g, respectively. This increase in SS content due to the salting-out effect is consistent with findings from Jiang et al. (2021) on T. molitor protein extraction. The higher salt concentrations used in the salting-out method promote protein unfolding, which exposes more cysteine residues that can form disulfide bonds. This confirms that SS bonds contribute to enhanced thermal stability by stabilizing protein conformations and reducing conformational entropy.

The FTIR results (Fig. 2) further support these findings, particularly the 600 cm^{-1} peak observed in the salting-out protein samples, which is associated with disulfide bond vibrations. This aligns with previous research indicating that proteins with higher SS content tend to be more thermally stable (Zhang et al., 2019). Disulfide bonds, being covalent in nature, enhance protein stability by reinforcing peptide chains, leading to increased structural rigidity. Du et al. (2014) demonstrated that disulfide bonds play a crucial role in maintaining protein conformation, reducing entropy, and improving thermal stability. Moreover, the molecular weight and SS content of proteins significantly influence emulsion stabilization. Proteins obtained via salting-out were found to be more thermally stable and structurally robust, making them suitable for food applications requiring high thermal stability.

Overall, this study highlights that salting-out extraction methods enhance the structural stability and thermal properties of BSF larvae proteins due to their higher disulfide bond content. These findings are essential for optimizing protein extraction techniques to obtain BSF larvae protein isolates with desirable functional properties for novel food ingredient applications.

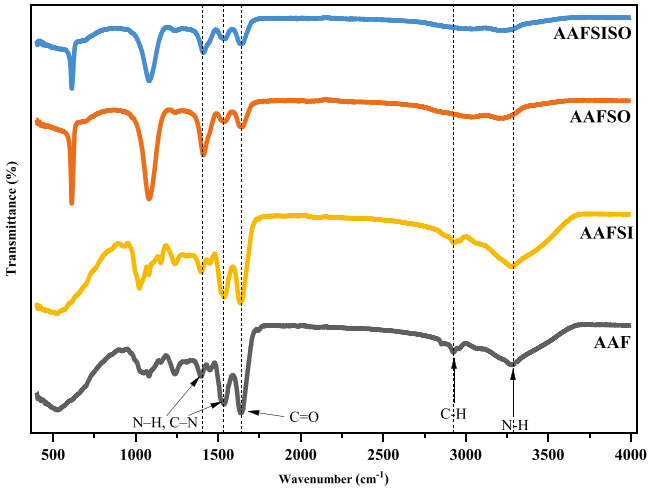


Fig. 2. Fourier Transform Infrared (FTIR) spectroscopy spectrum of black soldier fly larvae protein isolates obtained through different treatments: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) - blue.

3.4. Fourier transform infrared spectroscopy (FTIR)

An FT-IR analysis was performed to verify the structural modification resulting from the extraction techniques applied (Fig. 2). This technique provides significant information about the molecular composition, functional groups, and secondary structure of proteins, offering valuable insights into these aspects. Infrared spectra of the BSF larvae isolates are categorized into five distinct regions, each corresponding to the Amide group, which originates from the presence of peptide bonds between amino acids (as shown in Table 4).

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

Subsequently, the Amide I and II bands stand out as the most prominent and characteristic vibrational bands originating from the protein backbone in FTIR spectra (as discussed by Kong and Yu (2007). The Amide I band is typically found in the range of 1700 – 1600 cm^{-1}, attributed to the stretching vibrations of the carbonyl group C═O within the peptide linkage. It is pertinent to note that the frequency of the Amide I band is intricately linked to the secondary structural characteristics of proteins (Lozano-Vazquez et al., 2015). The results depicted in Fig. 2 show a prominent peak at 1634 cm^{-1} for AAF and AAFSI, while the salting-out extraction displays a lower intensity. These findings align with the amino acid content results reported in Section 3.2, clearly illustrating the impact of extraction methods on protein structure. According to Kong and Yu (2007), the region spanning 1640–1620 cm^{-1} has been attributed to the presence of a β-sheet structure. The salting-out (AAFSO and AAFSISO) protein concentrate FT-IR spectra were largely comparable. However, variations were observed in a few characteristic peaks and intensities (Fig. 2), suggesting minor differences in structure, amino acids, and protein function groups.

Additionally, in the FTIR spectra (Fig. 2), prominent peaks are observed around 600 cm^{-1} and 1100 cm^{-1}, particularly for AAFSISO and AAFSO. The peak at 600 cm^{-1} is likely associated with disulfide (SS) bond vibrations, which align with findings from previous studies (Jiang et al., 2021; Zhang et al., 2019). Disulfide bonds play a crucial role in protein structure by enhancing stability and rigidity, particularly in proteins obtained through the salting-out technique, where higher salt concentrations promote the formation of SS bonds by facilitating cysteine oxidation. The higher intensity of this peak in AAFSISO and AAFSO correlates well with the increased SS content discussed in Section 3.3, further supporting the notion that salting-out extraction enhances disulfide bond formation, leading to improved thermal stability.

Meanwhile, the peak at 1100 cm^{-1} may correspond to C–O stretching or C—N vibrations, which are often linked to carbohydrate or peptide

Table 4
Characteristic infrared band frequencies and their corresponding functional groups in BSF larvae protein isolates.

Classification	Approximate frequency (cm^{-1})	Functional group and vibration type
Amine *A*	3274	N—H stretching
Amine *B*	2927	C—H stretching
amide *I*	1665	C═O stretching
amide *II*	1558	N—H bending, C–N stretching
amide *III*	1320	N—H bending, C—N stretching

structures (Kong & Yu, 2007). The presence of this peak suggests potential interactions between proteins and other macromolecules or modifications due to the extraction process. These structural changes could impact the protein's functional properties, such as solubility and emulsification, which should be explored further in future studies.

The FTIR analysis suggests that the salting-out extraction methods (AAFSO and AAFSISO) might disrupt the protein's secondary structure, leading to lower peak intensities in the Amide I and II regions. The preservation of structural integrity is better with AAF and AAFSI methods, making them more suitable for applications where maintaining protein structure is critical.

3.5. Circular dichroism (CD) analysis

When analysing the structure of proteins and estimating the proportions of secondary structural components, CD offers useful information that is complementary to FTIR spectroscopy. The secondary structure of the proteins in phosphate buffer, as analysed by CD spectroscopy, is shown in Fig. 3. The presence of α-helical structures is indicated by negative peaks at 204 and 234 nm, while the presence of β-sheets is signalled by a negative peak at 218 nm and a positive peak at 198 nm. A positive peak at 206 nm suggests the presence of β-turns, whereas a negative peak at 224 nm and a positive peak within the 210 – 230 nm range indicate the existence of unordered structural elements (Malik & Saini, 2018).

The CD spectra of the AAF proteins were systematically compared with those acquired subsequent to salting-assisted treatments. The employment of salt treatments exerted a discernible influence on the secondary structures, eliciting alterations within the 194 – 240 nm spectral range. Initially, the basic AAF protein exhibited 20.7 % β-sheet, 19.0 % β-turn, and 59.3 % unordered structures. Salting-out treatments have been shown to transform β-sheet and random coil structures into α-helices and β-turns, suggesting a more compact and ordered conformation. The influence of salt is particularly significant when characterizing protein-folding intermediates, with sulfate ions (SO_4^{2-}) typically stabilizing native protein conformations (Crevenna et al., 2012). In contrast, the AAFSI procedure resulted in an increased β-sheet content compared to that of the basic AAF protein, as depicted in Fig. 3. This observation suggested a slight rise in the unfolded conformation for this particular group (Ijarotimi et al., 2018). Additionally, Jiang et al. (2021) reported that random coil structures transform after salting-out treatment into α-helix and β-structure, indicating a more compact and orderly conformation in *Tenebrio Molitor*. Their report highlights the significance of salt effects in characterising protein folding intermediates and how salt ions can stabilise the native conformation of proteins.

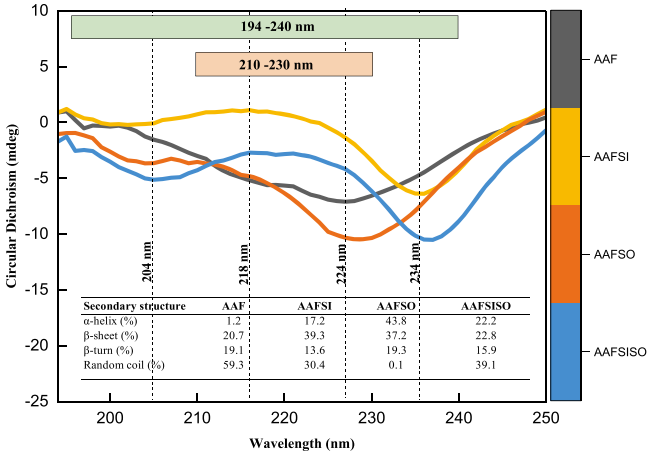


Fig. 3. Effect of different extraction methods on the structural properties of black soldier fly larvae proteins: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) - blue. This includes circular dichroism spectra and secondary structure compositions.

3.6. Powder X-ray diffraction

X-ray diffraction analysis was conducted to investigate the variations in the crystal structure of the protein extracts from BSF larvae (Fig. 3). Weak peaks were detected for AAF and AAFSI at 2θ positions of approximately 9° and 20°. In contrast, AAFSO and AAFSISO displayed several broad and intense peaks at around 16.5°, 19.0°, 23.0°, 28.8°, 29.6°, 34.1°, 35.9°, and 39.3°. The calculation of the crystallinity index, by comparing the counts at 19° to 13°, yielded average crystallinity values of 81.0 % for AAFSO and 82.2 % for AAFSISO. This is similar to the degree of crystallinity observed in commercial protein (casein), which was found to be 87.6 (Tan et al., 2021). These results suggest that the BSF larvae samples possess a more rigid crystalline structure, likely contributing to their stability (Sagheer et al., 2009).

In line with the earlier findings of the amino acid analysis (Section 3.2), the addition of salt significantly impacted the crystal structure of the protein isolates. The diffraction intensity increased progressively with the addition of ammonium sulfate compared to the addition of other salts. This implies that the treatment with ammonium salt enhanced the size of the protein isolate crystals. Joshi et al. (2011) reported that proteins with a higher percentage of crystallinity exhibited higher diffraction intensity and vice versa in lentil protein isolates. This indicates that a higher degree of crystalline structure corresponds to a greater diffraction intensity. While these results indicate that BSF larvae protein generally has an ordered structure, it is still more amorphous compared to protein extracted from shrimp (Kaya et al., 2015). The findings indicate the presence of multiple polymorphic forms after salting-out treatments, with AFSISO exhibiting additional peaks. These outcomes are favorable, as distinct polymorphic forms can possess divergent properties that may influence their biological function, stability, solubility, and interactions with other molecules. This diversity in protein usage provides numerous opportunities for the food industry. To the best of our knowledge, this is the first study to evaluate the effect of the salting in/out extraction techniques coupled with ultrafiltration on BSF larvae protein. Therefore, it adds to the limited body of evidence in this field by offering a thorough understanding of the crystal structure of the BSF larvae protein through the use of X-ray diffraction analysis.

3.7. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The alterations in the distribution of molar masses in AAF, AAFSI, AAFSO, and AAFSISO were confirmed using SDS-PAGE with protein staining. In Fig. 4, the marker image from the same gel was included alongside the protein extract line to facilitate band identification. The SDS-PAGE profiles in Fig. 4 revealed noticeable differences in the breadth of electrophoretic bands, indicating distinct molecular weight distributions in BSF larvae extracted using different methods. Previously, Ravi and colleagues (Ravi et al., 2020) reported a similar protein distribution using various product processing techniques, including conventional drying, freezing methods, microwave drying, scalding, and blanching of BSF. The study found that lower protein bands were associated with the cleavage of disulfide bonds under reducing conditions, resulting in protein denaturation and further breakdown into peptides. According to research conducted by Rabani et al. (2019), structural and muscular proteins were found to be highly prevalent, exhibiting stronger bands in the molecular weight range of 75 to 50 kDa in insect samples. This range has been previously linked to enzymes, muscle proteins, and exoskeleton proteins in studies on *Hermetia. illucens* and *Tenebrio molitor* (Ravi et al., 2020; Yi et al., 2013). Proteins with low molecular weights, specifically those under 31 kDa, may also be glutelin proteins from the actin family, with Troponin C being the most abundant, as reported by

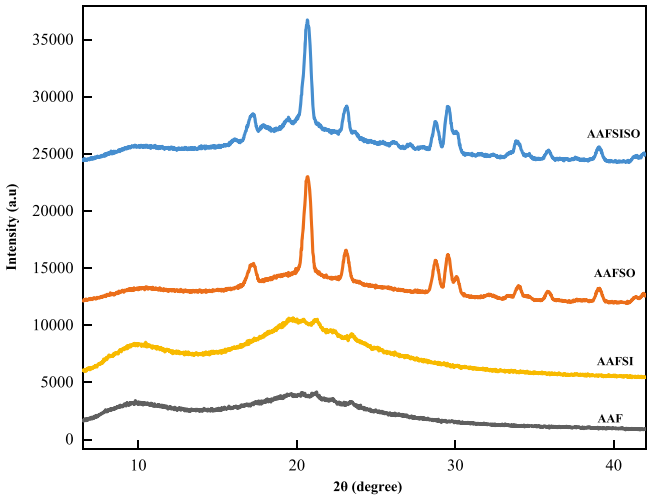


Fig. 4. X-ray diffraction patterns of black soldier fly larvae protein isolates extracted using different treatments: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) – blue.

Leni et al. (2019). Hexamerin F, a storage protein commonly found in insect larvae (Chen et al., 2018), was identified by Leni et al. (2019) as the primary protein in the bands ranging from 116 kDa to 66 kDa. The methods applied to BSF larvae samples, including protein extraction, defatting, drying, and killing, are known to result in different protein profiles, emphasizing the need for consideration in further analyses (Ravi et al., 2020, 2019). The presence of a darker region at the top of the gel in the protein extract area may indicate that certain proteins did not migrate fully through the gel due to aggregation, resulting in an increase in size. The use of gels with larger pores could potentially aid in the separation and identification of such proteins in future studies. Based on the analysis of BSF larvae protein extract, it is suggested that insects hold promise as a source for fortification and technological investigations (Altmann et al., 2018; Latunde-Dada et al., 2016; Rabani et al., 2019).

The AAF exhibited a distinctive electrophoretic pattern with no apparent changes in the bands. Analysis through SDS-PAGE revealed that AAFSI had few components of low molecular weight proteins (<15 kDa), whereas AAFSI displayed more pronounced bands in the range of 80 to 20 kDa. Vinayashree and Vasu (2021) reported a similar outcome for salt-extracted (0.5 mol/L NaCl) pumpkin protein, showing a lighter band at 110–20 kDa and a darker band at <10 kDa. Literature on *Moringa oleifera* indicates molecular weights of coagulating proteins at 26.5 kDa and 30 kDa, but these two distinct bands were not observed in this study. Conversely, AAFSISO exhibited stronger band near 32 kDa suggesting potential variations in raw materials or protein extraction methods. AAF and AAFSO displayed intense staining in the range of <75 kDa, indicating a higher content of high molecular weight proteins exceeding 20 kDa. It is noteworthy that the SDS-PAGE pattern of AAFSISO differed significantly from the control and AAFSI. Original protein components (6500–66,400) disappeared, and larger molecular weight polymers formed. The molar mass distribution of proteins showed a visibly stained broadband near the top of the SDS-PAGE gel during electrophoresis. This observation in SDS-PAGE was further supported by DSC analysis, as discussed below. Consequently, the concentration of ammonium sulfate extraction favoured the presence of high molecular weight proteins in BSF larvae.

3.8. Thermal properties (DSC and TGA)

The alterations in the tertiary and quaternary structures of food proteins also result in changes to their functional properties. It is crucial to explore such structural modifications because they are closely associated with the functional properties of the protein in food. DSC can directly measure the heat changes in the sample at different temperatures and provide valuable information about the thermal stability of the sample. Fig. 5A and B display the TGA and DSC curves for the BSF larvae isolates, respectively. Both TGA and DSC were employed to evaluate the impact of salting-assisted extraction processes on the thermal stability of the isolates. The TGA analyses revealed three distinct thermal processes: (I) an initial dehydration phase occurring between 50 and 100 °C, (II) a rapid decomposition phase in the range of 200 – 300 °C, involving the breakdown of non-covalent bonds, including intermolecular and intramolecular hydrogen bonds, hydrophobic interactions and electrostatic. (III) A slower degradation phase spanning 300 – 600 °C. This phase also encompasses the degradation of covalent bonds at higher temperatures, as noted by Qu et al. (2018). The application of salting-in treatments resulted in a greater reduction in mass during the initial stage, which suggests a higher degree of water association in these groups. Furthermore, during the second thermal stage, the degradation temperatures for these groups increased, suggesting the presence of more thermally stable structures. Despite the salting-out group exhibiting significantly higher residual weights compared to the non-salting-out group by the end of the second stage, this outcome contradicted the findings reported by Jiang et al. (2021). In the third stage, weight losses became more apparent, indicating that the thermal stability resulting from the salting-out treatments contributed to an overall improvement in the thermal stability of the proteins (as shown in Fig. 6A). One possible explanation for this phenomenon could be the presence of higher levels of disulfide (SS) bonding in the salting-out group, which helped maintain folded conformations and reduced conformational entropy, thereby enhancing thermal stability. This suggests that the effect of salting-out could further enhance the stability of BSF larvae proteins compared to salting-in.

DSC analysis provides comprehensive insights into the qualitative and quantitative thermal properties of solid materials, including parameters such as melting and degradation temperatures and, specifically, the heat-induced denaturation of proteins. The thermal denaturation temperature (Td) is typically used as an indicator of a protein's thermal stability, while enthalpy (ΔH) represents the extent of the ordered structure within a protein. The DSC thermogram revealed two significant endothermic transitions: around 40 – 60 °C for AAFSO and 70 °C for AAF and AAFSI (Fig. 5B). AAFSO exhibited an endothermic peak at approximately 35 °C, which may be attributed to the

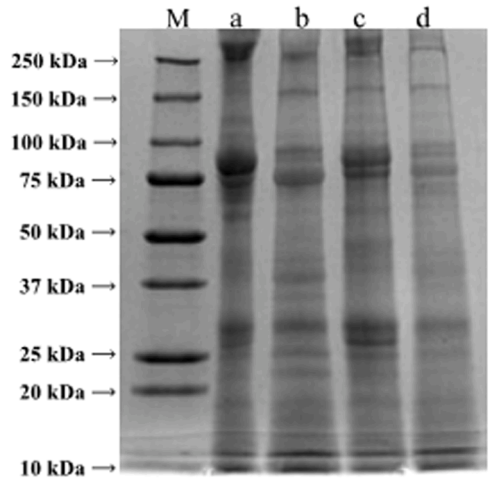


Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of black soldier fly larvae proteins obtained through different treatments: (a) AAF (alkaline-acid extraction), (b) AAFSI (salting-in technique), (c) AAFSO (salting-out technique), and (d) AAFSISO (salting-in and out technique). M represents the molecular weight marker.

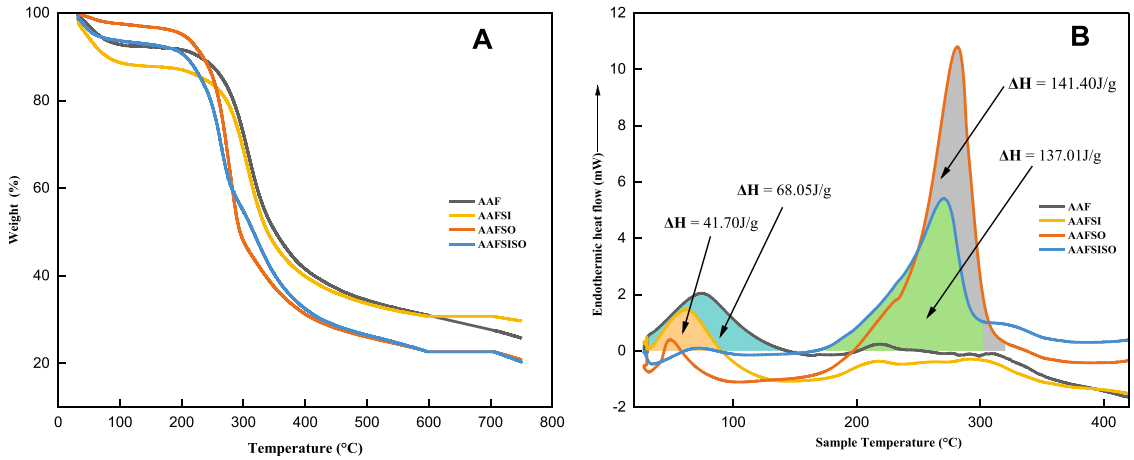


Fig. 6. Effects of salting-in/out on (A) thermogravimetric analysis (TGA) and (B) differential scanning calorimetry (DSC) of black soldier fly larvae protein isolates obtained through different treatments: AAF (alkaline-acid extraction) - gray, AAFSI (salting-in technique) - yellow, AAFSO (salting-out technique) – orange, and AAFSISO (salting-in and out technique) – blue.

formation of new intermolecular bonds during the initial heating process, as discussed by Fitzsimons et al. (2007). As depicted in Fig. 6B, AAFSISO exhibited a higher enthalpy value compared to the other insect proteins, and this elevated enthalpy value is indicative of a greater content of ordered protein structures, as noted by Chandrapala et al. (2011). Conversely, AAF displayed the lowest ΔH value at 68 J/g, suggesting that AAF had the poorest thermal stability, which aligns with the results regarding its ordered structure. AAFSO and AAFSISO, on the other hand, had the highest content of ordered protein structures and exhibited the largest ΔH values, measured at 141 J/g and 137 J/g, respectively. Furthermore, these high ΔH values can be attributed to the disruption of hydrogen bonds and covalent bonds within the protein structure, such as deamidation, desulfuration, or decarboxylation as discussed by Sobral et al. (2011).

4. Conclusions

The study employed a comprehensive range of analytical techniques to investigate various properties of these proteins, including their composition, structural characteristics and thermal stability. The protein extraction methods, particularly salting-out assisted treatments, significantly altered the properties of BSF larvae proteins. These methods enhanced protein yields and changes in amino acid composition, making them more suitable for potential food applications. Notably, the proteins obtained through salting-out exhibited higher thermal stability, as evidenced by TGA and DSC analyses. This improved thermal stability is attributed to a higher level of disulfide (SS) bonding, which contributes to maintaining folded conformations and reducing conformational entropy. The FTIR analysis provided insights into the molecular composition and structural elements of the proteins, indicating the presence of β-sheet structures and various functional groups. The zeta potential measurements also suggested increased electrostatic repulsion, contributing to greater protein solubility in the salting-out-treated samples. Overall, the study highlights the potential of BSF larvae proteins as a sustainable and nutritionally valuable protein source, particularly in regions where lysine deficiency is a concern.

The insights gained from this research can be valuable for developing innovative food processing techniques and functional ingredients and addressing nutritional challenges in various populations. This research demonstrates that the integration of salting-out extractions and ultrafiltration techniques holds significant potential for protein extraction, especially as an innovative approach to producing insect proteins with excellent nutritional quality for food-related applications. Further work is required to obtain a more in-depth understating of the effect of the salting-out extraction methods on the functional and rheological properties of these proteins in diverse food matrices, coupled with sensory assessments and consumer acceptability.

Data availability

The data employed is not available. The data that has been used is confidential.

Ethical statement – studies in human and animals

This study focuses on the extraction methods for black soldier fly larvae, so ethical clearance from the Ethical Research Committee of Cape Peninsula University of Technology was not required.

CRediT authorship contribution statement

B. Zozo: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **V.V. Mshayisa:** Writing – review & editing, Formal analysis, Conceptualization. **M.M. Wicht:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **J. van Wyk:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

Altmann, B., Neumann, C., Velten, S., Liebert, F., & Mörlein, D. (2018). Meat quality derived from high inclusion of a micro-alga or insect meal as an alternative protein source in poultry diets: A pilot study. *Foods,, 7*(3), 34. https://doi.org/10.3390/foods7030034

Azagoh, C., Ducept, F., Garcia, R., Rakotozafy, L., Cuvelier, M.-E., Keller, S., Lewandowski, R., & Mezdour, S. (2016). Extraction and physicochemical characterization of *tenebrio molitor* proteins. *Food Research International, 88*, 24–31. https://doi.org/10.1016/j.foodres.2016.06.010

Barba, A. A., Bochicchio, S., Dalmoro, A., Caccavo, D., Cascone, S., & Lamberti, G. (2019). Polymeric and lipid-based systems for controlled drug release: An

engineering point of view. *Nanomaterials for drug delivery and therapy* (pp. 267–304). Elsevier. https://doi.org/10.1016/B978-0-12-816505-8.00013-8

Bessa, L. W., Pieterse, E., Marais, J., & Hoffman, L. C. (2020). Why for feed and not for human consumption? The black soldier fly larvae. *Comprehensive Reviews in Food Science and Food Safety, 19*(5), 2747–2763. https://doi.org/10.1111/1541-4337.12609

Bußler, S., Rumpold, B. A., Jander, E., Rawel, H. M., & Schlüter, O. K. (2016). Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. *Heliyon, 2* (12). https://doi.org/10.1016/j.heliyon.2016.e00218, 1–24.

Casanova, F., Mohammadifar, M. A., Jahromi, M., Petersen, H. O., Sloth, J. J., Eybye, K. L., Kobbelgaard, S., Jakobsen, G., & Jessen, F. (2020). Physico-chemical, structural and techno-functional properties of gelatin from saithe (Pollachius virens) skin. *International Journal of Biological Macromolecules, 156*, 918–927. https://doi.org/10.1016/J.IJBIOMAC.2020.04.047

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics Sonochemistry, 18*(5), 951–957. https://doi.org/10.1016/J.ULTSONCH.2010.12.016

Chatsuwan, N., Nalinanon, S., Puechkamut, Y., Lamsal, B. P., & Pinsirodom, P. (2018). Characteristics, functional properties, and antioxidant activities of water-soluble proteins extracted from grasshoppers, Patanga succincta and Chondracris roseapbrunner. *Journal of Chemistry*, 1–11. https://doi.org/10.1155/2018/6528312, 2018.

Chen, J., Mu, T., Zhang, M., Goffin, D., Sun, H., Ma, M., Liu, X., & Zhang, D. (2018). Structure, physicochemical, and functional properties of protein isolates and major fractions from cumin (Cuminum cyminum) seeds. *International Journal of Food Properties, 21*(1), 685–701. https://doi.org/10.1080/10942912.2018.1454467

Choi, Y. S., Kim, T. K., Choi, H. D., Park, J. D., Sung, J. M., Jeon, K. H., Paik, H. D., & Kim, Y. B. (2017). Optimization of replacing pork meat with yellow worm (*Tenebrio molitor* L.) for frankfurters. *Korean Journal for Food Science of Animal Resources, 37* (5), 617–625. https://doi.org/10.5851/KOSFA.2017.37.5.617

Crevenna, A. H., Naredi-Rainer, N., Lamb, D. C., Wedlich-Söldner, R., & Dzubiella, J. (2012). Effects of Hofmeister Ions on the α-helical structure of proteins. *Biophysical Journal, 102*(4), 907–915. https://doi.org/10.1016/j.bpj.2012.01.035

De Chirico, S., di Bari, V., Romero Guzmán, M. J., Nikiforidis, C. V., Foster, T., & Gray, D. (2020). Assessment of rapeseed oil body (oleosome) lipolytic activity as an effective predictor of emulsion purity and stability. *Food Chemistry, 316*, Article 126355. https://doi.org/10.1016/j.foodchem.2020.126355

Deng, Y., Huang, L., Zhang, C., Xie, P., Cheng, J., Wang, X., & Li, S. (2019). Physicochemical and functional properties of Chinese quince seed protein isolate. *Food Chemistry, 283*, 539–548. https://doi.org/10.1016/j.foodchem.2019.01.083

Du, S., Jiang, H., Yu, X., & Jane, J. (2014). Physicochemical and functional properties of whole legume flour. *LWT - Food Science and Technology, 55*(1), 308–313. https://doi.org/10.1016/j.lwt.2013.06.001

Duong-Ly, K. C., & Gabelli, S. B. (2014). Salting out of proteins using ammonium sulfate precipitation. In *Methods in enzymology, 541* pp. 85–94). Academic Press Inc. https://doi.org/10.1016/B978-0-12-420119-4.00007-0

Fitzsimons, S. M., Mulvihill, D. M., & Morris, E. R. (2007). Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocolloids, 21*(4), 638–644. https://doi.org/10.1016/j.foodhyd.2006.07.007

Gadalkar, S. M., Gogate, P. R., & Rathod, V. K. (2017). Recovery of proteins from Rice mill industry waste (Rice Bran) using alkaline or NaCl-assisted alkaline extraction processes. *Journal of Food Process Engineering, 40*(3). https://doi.org/10.1111/jfpe.12430

Ghaly, A. E. (2009). The black cutworm as a potential human food. *American Journal of Biochemistry and Biotechnology, 5*(4), 210–220. https://doi.org/10.3844/AJBBSP.2009.210.220

Gmuer, A., Nuessli Guth, J., Hartmann, C., & Siegrist, M. (2016). Effects of the degree of processing of insect ingredients in snacks on expected emotional experiences and willingness to eat. *Food Quality and Preference, 54*, 117–127. https://doi.org/10.1016/J.FOODQUAL.2016.07.003

Hadnađev, M., Dapčević-Hadnađev, T., Lazaridou, A., Moschakis, T., Michaelidou, A.-M., Popović, S., & Biliaderis, C. G. (2018). Hempseed meal protein isolates prepared by different isolation techniques. Part I. physicochemical properties. *Food Hydrocolloids, 79*, 526–533. https://doi.org/10.1016/j.foodhyd.2017.12.015

Hartmann, C., & Siegrist, M. (2017). Consumer perception and behaviour regarding sustainable protein consumption: A systematic review. *Trends in Food Science & Technology, 61*, 11–25. https://doi.org/10.1016/J.TIFS.2016.12.006

Hu, Y., Cheng, K., He, L., Zhang, X., Jiang, B., Jiang, L., Li, C., Wang, G., Yang, Y., & Liu, M. (2021). NMR-based methods for protein analysis. *Analytical Chemistry, 93*(4), 1866–1879. https://doi.org/10.1021/acs.analchem.0c03830

Ijarotimi, O. S., Malomo, S. A., Fagbemi, T. N., Osundahunsi, O. F., & Aluko, R. E. (2018). Structural and functional properties of Buchholzia coriacea seed flour and protein concentrate at different pH and protein concentrations. *Food Hydrocolloids, 74*, 275–288. https://doi.org/10.1016/j.foodhyd.2017.08.018

Janssen, R. H., Canelli, G., Sanders, M. G., Bakx, E. J., Lakemond, C. M. M., Fogliano, V., & Vincken, J. P. (2019). Iron-polyphenol complexes cause blackening upon grinding *Hermetia illucens* (black soldier fly) larvae. *Scientific Reports, 9*(1), 1–11. https://doi.org/10.1038/s41598-019-38923-x

Jiang, J., Xiong, Y. L., & Chen, J. (2010). pH shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of Agricultural and Food Chemistry, 58*(13), 8035–8042. https://doi.org/10.1021/JF101045B

Jiang, Y., Zhu, Y., Zheng, Y., Liu, Z., Zhong, Y., Deng, Y., & Zhao, Y. (2021). Effects of salting-in/out-assisted extractions on structural, physicochemical and functional properties of *tenebrio molitor* larvae protein isolates. *Food Chemistry, 338*, Article 128158. https://doi.org/10.1016/j.foodchem.2020.128158

Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. F., & Kasapis, S. (2011). Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. *Food Chemistry, 129*(4), 1513–1522. https://doi.org/10.1016/J.FOODCHEM.2011.05.131

Kaya, M., Mujtaba, M., Bulut, E., Akyuz, B., Zelencova, L., & Sofi, K. (2015). Fluctuation in physicochemical properties of chitins extracted from different body parts of honeybee. *Carbohydrate Polymers, 132*, 9–16. https://doi.org/10.1016/J.CARBPOL.2015.06.008

Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica, 39*(8), 549–559. https://doi.org/10.1111/j.1745-7270.2007.00320.x

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: Structure, extraction, and functionality. In *Food reviews international, 34* pp. 126–147). Taylor and Francis Inc. https://doi.org/10.1080/87559129.2016.1242135

Latunde-Dada, G. O., Yang, W., & Vera Aviles, M. (2016). In vitro iron availability from insects and sirloin beef. *Journal of Agricultural and Food Chemistry, 64*(44), 8420–8424. https://doi.org/10.1021/ACS.JAFC.6B03286/ASSET/IMAGES/LARGE/JF-2016-032869_0003.JPEG

Leni, G., Caligiani, A., & Sforza, S. (2019). Killing method affects the browning and the quality of the protein fraction of Black Soldier Fly (*Hermetia illucens*) prepupae: A metabolomics and proteomic insight. *Food Research International, 115*(May), 116–125. https://doi.org/10.1016/j.foodres.2018.08.021

Lin, N., Liu, B., Liu, Z., & Qi, T. (2020). Effects of different drying methods on the structures and functional properties of phosphorylated Antarctic krill protein. *Journal of Food Science, 85*(11), 3690–3699. https://doi.org/10.1111/1750-3841.15503

Lozano-Vazquez, G., Lobato-Calleros, C., Escalona-Buendia, H., Chavez, G., Alvarez-Ramirez, J., & Vernon-Carter, E. J. (2015). Effect of the weight ratio of alginate-modified tapioca starch on the physicochemical properties and release kinetics of chlorogenic acid containing beads. *Food Hydrocolloids, 48*, 301–311. https://doi.org/10.1016/j.foodhyd.2015.02.032

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology, 197*, 1–33. https://doi.org/10.1016/J.ANIFEEDSCI.2014.07.008

Malik, M. A., & Saini, C. S. (2018). Rheological and structural properties of protein isolates extracted from dephenolized sunflower meal: Effect of high intensity ultrasound. *Food Hydrocolloids, 81*, 229–241. https://doi.org/10.1016/J.FOODHYD.2018.02.052

Mintah, B. K., He, R., Agyekum, A. A., Dabbour, M., Golly, M. K., & Ma, H. (2020). Edible insect protein for food applications: Extraction, composition, and functional properties. *Journal of Food Process Engineering, 43*(4), 1–12. https://doi.org/10.1111/jfpe.13362

Mshayisa, V. V., Van Wyk, J., & Zozo, B. (2022). Nutritional, techno-functional and structural properties of black soldier fly (*Hermetia illucens*) larvae flours and protein concentrates. *Foods (Basel, Switzerland), 11*(5), 724. https://doi.org/10.3390/foods11050724

Mshayisa, V. V., Van Wyk, J., Zozo, B., & Rodríguez, S. D. (2021). Structural properties of native and conjugated black soldier fly (*Hermetia illucens*) larvae protein via Maillard reaction and classification by SIMCA. *Heliyon, 7*(6), e07242. https://doi.org/10.1016/j.heliyon.2021.e07242

Mune, M. A., Minka, S. R., & Mbome, I. L. (2014). Optimising functional properties during preparation of cowpea protein concentrate. *Food Chemistry, 154*, 32–37. https://doi.org/10.1016/J.FOODCHEM.2013.12.108

Nguyen, T. T. X., Tomberlin, J. K., & Vanlaerhoven, S. (2015). Ability of black soldier fly (Diptera: Stratiomyidae) larvae to recycle food waste. *Environmental Entomology, 44* (2), 406–410.

Okur, H. I., Hladílková, J., Rembert, K. B., Cho, Y., Heyda, J., Dzubiella, J., Cremer, P. S., & Jungwirth, P. (2017). Beyond the Hofmeister Series: Ion-specific effects on proteins and their biological functions. *Journal of Physical Chemistry B, 121*(9), 1997–2014. https://doi.org/10.1021/acs.jpcb.6b10797

Ozel, B., Aydin, O., Grunin, L., & Oztop, M. H. (2018). Physico-chemical changes of composite whey protein hydrogels in simulated gastric fluid conditions. *Journal of Agricultural and Food Chemistry, 66*(36), 9542–9555. https://doi.org/10.1021/acs.jafc.8b02829

Purschke, B., Brüggen, H., Scheibelberger, R., & Jäger, H. (2018). Effect of pre-treatment and drying method on physico-chemical properties and dry fractionation behaviour of mealworm larvae (*Tenebrio molitor* L.). *European Food Research and Technology, 244*(2), 269–280. https://doi.org/10.1007/s00217-017-2953-8

Qu, W., Zhang, X., Han, X., Wang, Z., He, R., & Ma, H. (2018). Structure and functional characteristics of rapeseed protein isolate-dextran conjugates. *Food Hydrocolloids, 82*, 329–337. https://doi.org/10.1016/j.foodhyd.2018.03.039

Queiroz, L. S., Regnard, M., Jessen, F., Mohammadifar, M. A., Sloth, J. J., Petersen, H. O., Ajalloueian, F., Brouzes, C. M. C., Fraihi, W., Fallquist, H., de Carvalho, A. F., & Casanova, F. (2021). Physico-chemical and colloidal properties of protein extracted from black soldier fly (*Hermetia illucens*) larvae. *International Journal of Biological Macromolecules, 186*, 714–723. https://doi.org/10.1016/j.ijbiomac.2021.07.081

Rabani, V., Cheatsazan, H., & Davani, S. (2019). Proteomics and lipidomics of black soldier fly (Diptera: Stratiomyidae) and blow fly (Diptera: Calliphoridae) larvae. *Journal of Insect Science, 19*(3). https://doi.org/10.1093/jisesa/iez050

Ravi, H., Degrou, A., Costil, J., Trespeuch, C., Chemat, F., & Abert Vian, M. (2020). Effect of devitalization techniques on the lipid, protein, antioxidant, and chitin fractions of

black soldier fly (*Hermetia illucens*) larvae. *European Food Research and Technology, 246*, 2549–2568. https://doi.org/10.1007/s00217-020-03596-8

Ravi, H. K., Vian, M. A., Tao, Y., Degrou, A., Costil, J., Trespeuch, C., & Chemat, F. (2019). Alternative solvents for lipid extraction and their effect on protein quality in black soldier fly (*Hermetia illucens*) larvae. *Journal of Cleaner Production, 238*, Article 117861. https://doi.org/10.1016/j.jclepro.2019.117861

Sagheer, F. A. A., Al-Sughayer, M. A., Muslim, S., & Elsabee, M. Z. (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydrate Polymers, 77*(2), 410–419. https://doi.org/10.1016/j.carbpol.2009.01.032

Schösler, H., Boer, J.de, & Boersema, J. J (2012). Can we cut out the meat of the dish? Constructing consumer-oriented pathways towards meat substitution. *Appetite, 58* (1), 39–47. https://doi.org/10.1016/j.appet.2011.09.009

Sheppard, D. C., Tomberlin, J. K., Joyce, J. A., Kiser, B. C., & Sumner, S. M. (2002). Rearing methods for the black soldier fly (Diptera: Stratiomyidae): Table 1. *Journal of Medical Entomology, 39*(4), 695–698. https://doi.org/10.1603/0022-2585-39.4.695

Sobral, P. A., Palazolo, G. G., & Wagner, J. R. (2011). Effect of water content on thermal behavior of freeze-dried soy whey and their isolated proteins. *Journal of Agricultural and Food Chemistry, 59*(8), 3950–3956. https://doi.org/10.1021/JF104918M

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P., & De Smet, S. (2017). Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *Journal of the Science of Food and Agriculture, 97*(8), 2594–2600. https://doi.org/10.1002/JSFA.8081

Subaşı, B. G., Casanova, F., Capanoglu, E., Ajalloueian, F., Sloth, J. J., & Mohammadifar, M. A. (2020). Protein extracts from de-oiled sunflower cake: Structural, physico-chemical and functional properties after removal of phenolics. *Food Bioscience, 38*, Article 100749. https://doi.org/10.1016/j.fbio.2020.100749

Sun, X. D., & Arntfield, S. D. (2011). Gelation properties of salt-extracted pea protein isolate catalyzed by microbial transglutaminase cross-linking. *Food Hydrocolloids, 25* (1), 25–31. https://doi.org/10.1016/J.FOODHYD.2010.05.002

Sun, Y., Zhong, M., Wu, L., Huang, Y., Li, Y., & Qi, B. (2022). Effects of ultrasound-assisted salt (NaCl) extraction method on the structural and functional properties of Oleosin. *Food Chemistry, 372*, Article 131238. https://doi.org/10.1016/j.foodchem.2021.131238. August 2021.

Tan, H. S. G., Fischer, A. R. H., Tinchan, P., Stieger, M., Steenbekkers, L. P. A., & van Trijp, H. C. M (2015). Insects as food: Exploring cultural exposure and individual experience as determinants of acceptance. *Food Quality and Preference, 42*, 78–89. https://doi.org/10.1016/J.FOODQUAL.2015.01.013

Tan, Y. N., Chin, Y. L., & Chen, W. N. (2021). Comparison of sustainable lipid and protein removal methods for the isolation of insect chitin from black soldier fly exoskeleton. *ACS Food Science and Technology, 1*(4), 698–706. https://doi.org/10.1021/acsfoodscitech.0c00104

Thaiphanit, S., Schleining, G., & Anprung, P. (2016). Effects of coconut (Cocos nucifera L.) protein hydrolysates obtained from enzymatic hydrolysis on the stability and rheological properties of oil-in-water emulsions. *Food Hydrocolloids, 60*, 252–264. https://doi.org/10.1016/J.FOODHYD.2016.03.035

Ursu, A.-V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation and functional properties of proteins from the microalgae Chlorella vulgaris. *Bioresource Technology, 157*, 134–139. https://doi.org/10.1016/j.biortech.2014.01.071

van Huis, A. (2013). Potential of insects as food and feed in assuring food security. *Annual Review of Entomology,, 58*(1), 563–583. https://doi.org/10.1146/annurev-ento-120811-153704

Vanqa, N., Mshayisa, V. V., & Basitere, M. (2022). Proximate, physicochemical, techno-functional and antioxidant properties of three edible insect (*Gonimbrasia belina, Hermetia illucens* and *Macrotermes subhylanus*) flours. *Foods (Basel, Switzerland), 11* (7), 976. https://doi.org/10.3390/foods11070976

Vinayashree, S., & Vasu, P. (2021). Biochemical, nutritional and functional properties of protein isolate and fractions from pumpkin (Cucurbita moschata var. Kashi Harit) seeds. *Food Chemistry, 340*, 128177. https://doi.org/10.1016/j.foodchem.2020.128177

Wang, C., Qian, L., Wang, W., Wang, T., Deng, Z., Yang, F., Xiong, J., & Feng, W. (2017). Exploring the potential of lipids from black soldier fly: New paradigm for biodiesel production (I). *Renewable Energy, 111*, 749–756. https://doi.org/10.1016/j.renene.2017.04.063

Wibowo, S., Vervoort, L., Tomic, J., Santiago, J. S., Lemmens, L., Panozzo, A., Grauwet, T., Hendrickx, M., & Van Loey, A. (2015). Colour and carotenoid changes of pasteurised orange juice during storage. *Food Chemistry, 171*, 330–340. https://doi.org/10.1016/j.foodchem.2014.09.007

Wu, Y. H. S., Lin, Y. L., Wang, S. Y., Lin, D., Chen, J. W., & Chen, Y. C. (2022). Effects of washing step and salt-addition levels on textural and quality properties in the chicken-surimi products. *Poultry Science, 101*(6), Article 101885. https://doi.org/10.1016/J.PSJ.2022.101885

Xu, L., & Diosady, L. L. (2002). Removal of phenolic compounds in the production of high-quality canola protein isolates. *Food Research International, 35*, 23–30. www.elsevier.com/locate/foodres.

Yi, L., Lakemond, C. M. M., Sagis, L. M. C., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2013). Extraction and characterisation of protein fractions from five insect species. *Food Chemistry, 141*(4), 3341–3348. https://doi.org/10.1016/J.FOODCHEM.2013.05.115

Yi, L., Van Boekel, M. A. J. S., & Lakemond, C. M. M. (2017). Extracting *tenebrio molitor* protein while preventing browning: Effect of pH and NaCl on protein yield. *Journal of Insects as Food and Feed, 3*(1), 21–31. https://doi.org/10.3920/JIFF2016.0015

Zhang, Y., Zhou, X., Zhong, J., Tan, L., & Liu, C. (2019). Effect of pH on emulsification performance of a new functional protein from jackfruit seeds. *Food Hydrocolloids, 93*, 325–334. https://doi.org/10.1016/J.FOODHYD.2019.02.032

Zozo, B., Wicht, M. M., Mshayisa, V. V., & van Wyk, J. (2022). Characterisation of black soldier fly larva protein before and after conjugation by the Maillard reaction. *Journal of Insects as Food and Feed, 8*(2), 169–183. https://doi.org/10.3920/JIFF2021.0017