

Extraction, Quantification, and Comparative Analysis of Black Soldier Fly Larvae Proteins

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Black Soldier Fly Larvae Protein Extraction

Abstract

Agricultural research has revealed that insects can be a source of macronutrients used to supplement diets and feed the growing world population. Larvae of black soldier flies (*hermetia illucens*) have become a focus of research because of their rich macronutrient profile and ability to convert organic waste into their own biomass. Fractionation of the nutrients for their value-added applications is an important task for research and development. In this experiment, black soldier fly larvae (BSFL) underwent pre-treatments of heat drying, freeze drying, or used as fresh biomass before lipids, proteins, carbohydrates, and minerals were extracted and quantified via several analytical techniques. Freeze drying was determined to be the best pre-treatment for protein extraction, and it extracted 48.79% of the protein that was in the initial freeze-dried BSFL biomass. Moreover, albumin, globulin, and glutelin protein fractions (with different solubility characteristics) extracted from freeze-dried BSFL were extracted in higher protein concentrations when compared to heat-dried and fresh pre-treatments. The freeze-dried and heat-dried BSFL pre-treatment groups contained the largest quantities of chitin; the freeze-dried BSFL contained the highest quantities of chitin in the albumin (2.56% chitin) and globulin (2.75% chitin) protein fractions, and the heat-dried BSFL contained the largest quantities of chitin in the prolamin (1.81% chitin) and glutelin (2.40% chitin) protein fractions. These results provide evidence that macronutrients from BSFL can be extracted and utilized in the food industry to fortify feeds and supplement diets. Furthermore, the comparison of the three pre-treatments provided evidence that could be used to establish an industry standard BSFL pre-treatment prior to macronutrient extraction.

1. Introduction and justification

The world population is rapidly growing, and by 2050, the global population is projected to be 10 billion (Queiroz et al., 2021). In order to feed the growing population, farmers and agricultural researchers are looking for innovative ideas and strategies to produce food more efficiently. Alternative protein sources are a focus of research, as increasing livestock numbers is unrealistic and more costly due to requiring large amounts of land, feed, and time to mature. Insects have become a focus of research, specifically black soldier flies. These flies consume organic waste and agricultural byproducts, utilizing waste to grow, and thus, they are great for sustainable agriculture. One study reported that black soldier flies can reduce the weight of wet waste by 50-80%, noting that the flies' waste to biomass conversion rate is 20% (Mertenat et al., 2019). Black soldier flies are already used in the livestock and pet industries as feed (Bessa et al., 2020). These flies possess several macronutrients that can be utilized to enrich products in the food industry; these macronutrients include chitin, lipids, and proteins. It is unlikely that western society will eat black soldier fly larvae whole due to food neophobia, so researchers have focused on trying to isolate and extract protein from BSFL (Bessa et al., 2020).

Though there have been many studies about protein extraction and chemical composition of BSFL there is not currently an industry standard pre-treatment, i.e. drying method, for BSFL prior to macronutrient extraction. Several pre-treatment methods have been used in literature that produce varying impacts on protein extraction. Furthermore, there is no standard procedure for extracting proteins from insects, specifically BSFL, to achieve high extraction yields and content; each method produces varying levels of extraction success. It is hypothesized that the variation of the pre-treatment method of BSFL will impact the percentage of protein extracted. Therefore, the

purpose of this research project was to compare the effect of the different BSFL pre-treatments on protein extraction, providing information that will be used to scale-up BSFL macronutrient extraction for application in the feed industry. **The objective** for this research project is to determine how the heating pre-treatment affect types of protein present which will provide information to further maximize protein extraction.

2. Literature Review

There are several methods that are utilized in the food industry to extract protein from insects. A study by Yi et al. (2013) extracted protein from five samples of different insect species by freezing samples with liquid nitrogen. These samples were mixed with demineralized water and ascorbic acid, blended and filtered, and centrifuged at 15,000g and 4°C. The supernatant and pellet from the centrifugation were freeze-dried to analyze the protein content of each sample.

Another study discussed four different protein extraction methods that can be employed to extract insect proteins. The first extraction method was an alkali extraction in which the pH of the extraction solution was basic and “far from the isoelectric point which disrupts the cell wall and increases protein solubility” (Rahman et al., 2023). Previous research has shown that alkali extraction increases the solubility of proteins, as “the solubility of insect protein is higher (74-95%) in an alkaline environment (pH 9-11) than an acidic environment” (Bußler et al., 2016; Zhao et al., 2016). The second method of extraction was an acid extraction (pH<4) to solubilize protein below the protein’s isoelectric point. The third method of extraction was a salt extraction method. Salt (< 0.15M) can be added to protein meal in a method called salting-in, leading to an increase in protein solubility (Rahman et al., 2023). Salting-out can also be performed, adding salt to a solution to cause protein aggregation and precipitation. When salting extraction methods were

paired with alkaline and acid extraction methods, salting-in assisted alkaline extraction and salting-out assisted acid precipitation yielded 39% protein extraction. The final method of extraction was an enzyme-assisted extraction in which four proteolytic enzymes were used to treat insect prepupae before hydrolysis occurred (Rahman et al., 2023).

Overview of Black Soldier Fly Larvae protein extraction

Black soldier fly larvae proteins were reported to be extracted in several ways in literature. One study utilized three different extraction protocols for BSFL proteins. The first protocol was a one-step extraction on finely ground prepupae (frozen and used immediately) which were mixed with NaOH, centrifuged (4000 rpm for 15 minutes), and precipitated (TCA solution in acetone). The precipitate was incubated overnight (-20°C), centrifuged (4000 rpm for 30 minutes), washed with acetone (twice), and oven dried (90°C for 2 hours) (Caligiani et al., 2018). This first protocol produced an extraction efficacy of 96%.

The second protocol was an osborne fractionation method procedure with modifications. A BSFL sample was defatted, ground, frozen, and mixed with - sodium ascorbate, EDTA, and tris-HCl for 1 hour at 4°C (Caligiani et al., 2018). This solution was also centrifuged (4000 rpm, 4°C, 20 minutes), and the pellet was mixed with NaCl, sodium ascorbate, EDTA and tris-HCl to continue extraction. This solution was mixed and centrifuged under the previously outlined conditions. The pellet was mixed with ascorbic acid in EtOH (1 hour at 4°C), and this solution was centrifuged under the previously outlined conditions. The pellet was mixed with NaOH and ascorbic acid (1 hour at 4°C) and centrifuged under the previously outlined conditions. This protocol extracted

albumin, globulin, prolamin, and glutelin protein fractions, and the second method produced an extraction efficacy of 90%.

The third protocol employed enzymes for sample hydrolysis, using “*Bacillus licheniformis* protease (EC 3.4.21.62), pepsin from porcine gastric mucosa (EC 3.4.23.1), papain from papaya latex (EC 3.4.22.2) and pancreatin from porcine pancreas” (Caligiani et al., 2018). The third method results showed that “*B. licheniformis* protease was the enzyme with the best hydrolytic activity, with $67 \pm 5\%$ of the total protein nitrogen released in the supernatants” (Caligiani et al., 2018).

There are several methods reported in literature which can improve both the percentage of protein extracted and protein purity. First, alkali extraction of proteins under basic conditions via addition of NaOH was shown to produce the highest yield of protein, recovering 96% of proteins found in larvae (Caligiani et al., 2018). While the percent recovery was very high, there were negative effects present in the study, as a degree of hydrolysis was calculated at 15% for the extracted proteins. This means that the alkaline conditions of the extraction lead to breakdown of the extracted proteins. In this study, the BSFL used in the experiments were prepupae frozen at -20°C . Another study reported similar findings, noting that extraction of proteins through alkaline shifting (pH rapidly adjusted to 10.5, 11, 11.5, 12, and 12.5) improved extraction rates by “135-1080%” which was relative to the control (pH 9) (Pan et al., 2023). In this study, the BSFL used in the experiments were microwave-dried (20 kW, 60°C , and 10 min). As alkaline shifting occurred, the extraction rate of BSFL proteins reached a statistically significant maximum value of 44.35% when pH was 12.5 (Pan et al., 2023). Furthermore, the highest calculated zeta potential of the study

occurred at pH of 12.5. This indicates that the proteins were the most stable at pH 12.5, possessing the highest electrostatic repulsion which increased protein purity due to lower aggregation rates. In addition, the solubility of the BSFL proteins was at its highest value (52.20%) when the pH was at 12.5, reinforcing the idea that the extracted protein quality was the highest at the most basic conditions (Pan et al., 2023).

Another factor that was found to affect rates of protein extraction was the preservation method of BSFL. A study used BSFL that were conventionally dried (70°C for 48 hours), frozen (-80°C for 24 hours), dried via microwave (450 W for 20 minutes), scalded in boiling water (5 minutes), blanched (5 minutes), or underwent microwave assisted subcritical treatment (900 W, 120°C, 5 bar, 5 minutes) (Ravi et al., 2020). The study found that conventional drying of BSFL produced “the highest crude protein content of all devitalization techniques examined in the study” (Ravi et al., 2020). Although extraction seemed to be the best with conventionally dried BSFL, these larvae had the lowest solubility of all the groups, indicating that these proteins had the lowest purity. Other data from the study suggested that the highest protein quality at all pH levels came from frozen BSFL that were defatted. In fact, the rate of recovery of frozen BSFL proteins was 64% at a basic pH of 12, suggesting that frozen BSFL might provide the best mix of high-quality extraction and protein purity (Ravi et al., 2020). This study did suggest that there is a portion of BSFL proteins that are insoluble and are left in the larvae after extraction, limiting protein recovery. The study ultimately confirmed that freezing the BSFL at -80°C was the best technique to maximize protein quality, solubility, and degree of hydrolysis (14% after 24 hours) (Ravi et al., 2020).

Finally, a study focused on the effects of solvents used to extract lipids from BSFL. In this study, the BSFL that were used in experiments were freeze-dried, milled (<1mm), and stored at -18°C. This study determined that the best solvent to use for isolation of BSFL lipids was 2-methyloxolane (2-MeO) when compared to n-hexane solvent (Ravi et al., 2019). In addition, 2-MeO is beneficial because it is a solvent that could be used in the food industry due to biological basis from “lignocellulosic biomass” (Ravi et al., 2019). When compared with n-hexane, 2.5% more extracted protein was soluble in water when extracted by 2-MeO. This indicates a higher purity protein. The lipid recovery by 2-MeO was higher than n-hexanes, and the crude protein content was 3.05% higher as well (Ravi et al., 2019).

This background information provides justification for future protein extraction optimization experiments once the current study on how drying method impacts types of proteins and their extractability is completed.

3. Materials and Methods

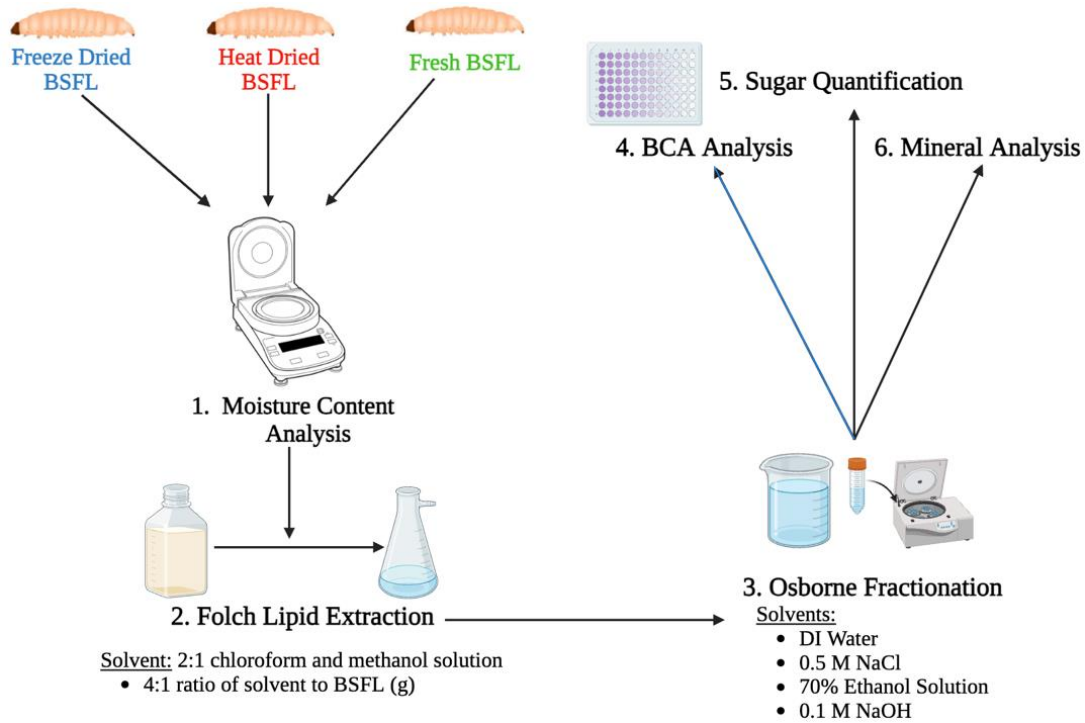


Figure 1. Flow diagram of BSFL protein extraction and quantification

Preparation of BSFL

The BSFL used in this experiment were acquired from EnviroFlight by the Wang Lipid and Protein Innovation lab. The larvae arrived at the lab whole, and the larvae were divided into three different groups which received three different pre-treatments to study the effect of larvae pretreatments on protein extraction behavior. The different pre-treatments were as follows: larvae were frozen in a -80°C freezer for 24 hours (fresh), freeze-dried, and a microwave oven dried. Next, 100 grams of BSFL from each pre-treatment group were weighed in individual 2000 mL beakers and grinded into a fine powder with a mortar and pestle.

Lipid Extraction

To extract the lipids from the ground BSFL, a folch lipid extraction procedure was performed using 2:1 chloroform methanol solution as a solvent. A small sample (<1 g) of BSFL from each pre-treatment group was individually placed in a moisture content analyzer to ensure that an 8:4:3 of ratio of chloroform:methanol:water was kept during folch lipid extraction. Next, 100 g of ground BSFL was mixed with a 400 mL solution of chloroform and methanol (2:1) for 30 minutes. The ratio between the lipid extraction solvent and ground BSFL must be 4:1. After mixing, the solution was poured through a Büchner funnel and vacuum filtration apparatus lined with 2.5 µm pore filter paper to separate the ground BSFL pellet from the extracted lipid and chloroform methanol solution. The ground BSFL was mixed with chloroform methanol solution twice and vacuum filtered two more times. After the third vacuum filtration, the BSFL pellet was placed in a fume hood overnight to allow excess chloroform and methanol to evaporate from the pellet. This procedure was repeated for the two additional BSFL pre-treatment groups. The solution containing the soluble lipid and chloroform methanol solution was rotary evaporated to separate the chloroform methanol solvent from the soluble lipids as well.

Protein Characterization via Osborne Fractionation

After lipids were extracted from the ground BSFL, osborne fractionation was performed to extract proteins from the ground pellet. Osborne fractionation extracts proteins according to solubility, and four different solvents were utilized to extract four protein fractions. Deionized water (DI water) was utilized to extract albumin, 0.5 M NaCl solution was utilized to extract globulin, 70% ethanol solution was utilized to extract prolamin, and 0.1 M NaOH solution was utilized to extract glutelin protein fractions.

To begin, 10 g of lipid extracted, heat-dried BSFL pellet was mixed with 100 mL of DI water. This solution was mixed at 20°C for 1 hour, and after, the solution was poured into centrifuge tubes and centrifuged for 10 minutes at 20°C and 15,000 g's. The supernatant from each of the centrifuge tubes was collected in a 400 mL beaker, and the previous steps were repeated twice. The collected albumin supernatant was freeze-dried to remove water from the samples.

Next, the BSFL pellet from the previous step was mixed with 100 mL of 0.5 M NaCl solution at 20°C for 1 hour. The solution was centrifuged under the previously outlined conditions, the supernatant was collected, and the previous step was repeated twice. To remove salt from the globulin supernatant, dialysis was performed. The globulin supernatant was poured into Thermo Scientific SnakeSkin 3.5K MWCO dialysis tubing, and dialysis tubing was placed in tubs of DI water. Dialysis allowed salt to be removed from the supernatant via passive diffusion. The dialysis procedure occurred over four days, and the DI water in the tubs was re-filled at least twice a day. After dialysis was completed, the collected globulin supernatant was freeze-dried to remove water from the samples.

Next, the BSFL pellet was mixed with 100 mL of DI water for 30 minutes and centrifuged under the previously listed conditions to remove salt from the pellet. The supernatant was discarded, and the previous step was repeated. Next, the pellet was mixed with 100 mL of 70% ethanol solution for 1 hour and centrifuged under the previously outlined conditions. The supernatant was collected, and the previous step was repeated twice. The collected prolamins supernatant was rotary evaporated to remove the ethanol from the protein fractions before the supernatant was freeze-dried.

Finally, the pellet from the previous step was mixed with 100 mL of 0.1 M NaOH solution at 20°C for 1 hour. The solution was centrifuged under the previously outlined conditions, the supernatant was collected, and the previous step was repeated twice. After all the glutelin supernatant was collected, dialysis was performed under the previously outlined conditions to remove salt from the supernatant. After dialysis was completed, the glutelin supernatant and the BSFL pellet were freeze-dried. The entire osborne fractionation procedure was repeated with the freeze-dried and fresh pre-treatment BSFL, and each BSFL pre-treatment group had three replicates.

Mass Balance

After osborne fractionation, mass balance was calculated to determine the percent mass of each extracted protein fraction from the initial 10 g of BSFL (per replicate). Before all supernatants were freeze-dried, the centrifuge tubes were weighed, and the empty mass was recorded on the outside of the tube. After all samples were freeze-dried, the mass of each centrifuge tube containing supernatant or pellet was recorded, and the final mass was subtracted from the initial mass to determine the mass of the supernatant or pellet. The sum of the mass of each protein fraction was calculated along with the standard deviation and mass percent ($\frac{\text{protein fraction sum}}{10 \text{ g of BSFL}}$) for each replicate of all three pre-treatment groups. The average mass percent and standard deviation of all four protein fractions was calculated for all replicates and treatment groups as well.

Protein Quantification

Once proteins were extracted from BSFL, they were quantified to determine the concentration of the extracted protein fractions. This experiment utilized a BCA (bicinchoninic acid) assay procedure to quantify the protein extracted from BSFL. A Pierce BCA Protein Assay Kit with

Dilution-Free BSA Protein Standards from Thermo Scientific was used during the procedure. To begin this procedure, six standard solutions were made with bovine serum albumin (BSA) to create the standard curve. The concentrations of the standards were 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, and 0 µg/mL. Once all six standards were created, 25 µL of each standard was pipetted in triplicate into a well on 96 well plate. Next, approximately 1 mg of each protein fraction and pellet sample from all pre-treatment groups were added to a microcentrifuge tube and solubilized with incubation solution (0.1 M NaOH with 5% sds), making sample solutions at a concentration of 1 mg/mL. The solutions were vortexed for 30 min and incubated for 24 hrs at 37°C. After incubation, 25 µL of each sample was pipetted in triplicate into a well on a 96 well plate. Next, the BCA working reagent was created. The BCA working agent is a solution that is created by mixing 50 parts of reagent A with 1 part of reagent B. To create the necessary volume, the following equation was used:

$$(\# \text{ of standards} + \# \text{ of unknown}) * 3 \text{ replicates} * 205 \text{ mL}$$

Once the BCA working agent solution was created, 200 µL of the working reagent was added to each filled well in the 96 well plate. The well plate was analyzed by a CAMBREX EL_x 808 reader with 490 nm light, revealing the absorbance of each protein fraction in the well plate. After data was collected, a BCA standard curve was created using excel, and the concentration of the protein fractions was calculated.

Chitin Quantification

BSFL contain carbohydrates in the form of chitin. To quantify the chitin content of each protein fraction, 6 mg samples of each fraction were added to graduated screw-cap tubes. The samples were digested through the addition of 3 mL of 6 N HCl, and all samples were placed in an oven

for four hours at 100°C. The samples were removed from the oven to cool, and once at room temperature, 2 mL of 6 N HCl was added to the samples. Next, 200 µL of each sample was transferred to screw-cap tubes, each sample was dehydrated at 45-50°C under vacuum, and the dried samples were dissolved through addition of 1 mL of DI water. Subsequently, six D-glucosamine hydrochloride standard solutions were created at concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, and 0 µg/mL. Concurrently, 1 mL of 0.05 M, NaNO₂ and 0.05 M KHSO₄ were added to all samples. All samples were shaken for 15 minutes at 22°C, and 1 mL of .125 M NH₄SO₃NH₂ was added to dilute all samples. After the dilution, the samples were shaken for an additional 5 minutes. Again, the samples and the standard solutions were diluted by the addition of 0.05 M 3-methylbenzothiazolone hydrazine hydrochloride solution, and the samples and standards were heated by boiling water (100°C) for 3 minutes. After 3 minutes, the samples and standards were removed and allowed to cool to room temperature where 1 mL of 0.05 M FeCl₃ solution was added to all samples and standards. The standards and samples were allowed to sit for 30 minutes. After the samples had sat, samples were transferred into a glass cuvette and analyzed by a VWR UV-3100 PC Spectrophotometer which recorded the absorbance of each solution at 650 nm wavelength.

Mineral Quantification

To begin quantification, 50 mg of each protein fraction from each pre-treatment was added to graduated screw-cap tubes. Next, 2.5 mL of 70% nitric acid (HNO₃), was added to each graduated tube, and 1 mL of DI water was added to each test tube. All test tubes were capped and placed in an oven at 80°C overnight. After the acid digestion finished, extra DI water was added, increasing the volume of each graduated tube to 10 mL. All digested samples were later analyzed by

inductively coupled plasma optical emission spectrophotometer (ICP-OES) to determine the total mineral content. Specifically, the quantity of magnesium (Mg), calcium (Ca), copper (Cu), iron (Fe), phosphorus (P), zinc (Zn), potassium (K), and sulfur (S) was analyzed in mg/Kg.

Statistical Analysis Methods

All values included in Tables 1-6 are mean values, and Tables 1, 2, and 4-6 include standard deviation around the mean. Figures 2-7A-C display mean values as well. All figures were made using Microsoft Excel software version 16.97, and all calculations were performed using Microsoft Excel software version 16.97.

4. Results and Discussion

Mass Balance

The results of BSFL mass balance after osborne fractionation are shown in Table 1. In all three pre-treatments, albumin was the protein fraction extracted in the highest quantity and freeze fried BSFL had the highest quantity of albumin, calculated to be 32.26%. Globulin and prolamin proteins were the two protein fractions extracted in the lowest quantity, with globulin ranging from 2.16-2.77% and prolamin ranging from 2.11-3.69% across the three pre-treatment groups. Glutelin was the protein fraction extracted in a median quantity, with extraction ranging between 7.29-12.36%.

When comparing the mass percent of the pellets between the three pre-treatments, the mass percent of the heat-dried pellet is the highest. This indicates that the heat-dried pellet has the largest mass of insoluble proteins due to chitin-protein cross linking. Cross linking (sclerotization) is also

partially responsible for the weights of the freeze-dried and fresh BSFL pellets as well. Furthermore, there is variation between albumin protein percent mass in the three treatment groups. This is likely due to the differences in pre-treatment conditions, as the freeze-dried BSFL was the easiest to grind via mortar and pestle. In contrast, the fresh BSFL was the hardest pre-treatment to grind, resulting in a majority of BSFL exoskeletons remaining whole after grinding via mortar and pestle. These whole, cross-linked BSFL exoskeletons contained “chitin filaments arranged within a protein matrix”, reducing the quantity of albumin protein fraction available for extraction in fresh BSFL (Andersen, 2010). Finally, the mass percent of prolamin and glutelin fractions extracted from heat-dried BSFL is lower than the respective percents of the freeze-dried and fresh pre-treatments. These low mass percents might have occurred due to denaturation of BSFL proteins caused by the heat drying pre-treatment, as Mintah et al. (2020) reported a decrease in extracted protein yield from BSFL after increasing temperature of extraction.

The percent recovery for all three pre-treatments is under 100%, and several factors could be responsible for the unaccounted mass of each BSFL pre-treatment. Sclerotization, cross linking of chitin and protein which hardens insect exoskeletons, could create insoluble protein fractions contained in insect exoskeletons (Ravi et al., 2020). Furthermore, errors during sample transfer between beakers and centrifuge tubes, and passive diffusion during dialysis could have contributed to a loss of protein fractions as well.

Table 1. Mass balance (%) of BSFL after osborne fractionation

Treatment	Heat Dried	Freeze Dried	Fresh
Albumin	27.18 ± 1.95	32.26 ± 0.26	19.13 ± 1.09
Globulin	2.3 ± 0.54	2.16 ± 0.27	2.77 ± 0.22
Prolamin	2.11 ± 0.02	2.93 ± 0.93	3.69 ± 0.25
Glutelin	7.29 ± 0.29	11.44 ± 1.59	12.36 ± 1.33
Pellet	41.9 ± 1.10	31.17 ± 0.84	33.43 ± 3.14
Recovery	80.78	79.96	71.38

Protein Content Analysis

To further analyze the extracted protein fractions from each BSFL pre-treatment, the concentration of each extracted protein fraction was analyzed via BCA assay, and the results are shown in Figure 2. All three BSFL pre-treatment groups had residual protein left in the pellet, further providing evidence that there is insoluble protein in the BSFL. The insoluble proteins could have cross-bonded to magnesium and calcium cations; the length of extraction employed during osborne fractionation might not have been adequate to extract all protein from the ground BSFL pre-treatments as well. When comparing the residual protein left in pellet of each pre-treatment, the heat-dried BSFL had the highest amount of residual protein in the pellet. Although the freeze-dried BSFL pre-treatment yielded the largest quantity of protein extracted, this treatment group had the second highest quantity of residual protein contained in the pellet, determined to be 17%. The fresh BSFL pre-treatment contained 1.28% residual protein in the pellet, revealing that the fresh BSFL pre-treatment is the most efficient pre-treatment for protein extraction.

In the heat-dried pre-treatment group, the concentration of protein in extracted fractions increased through all four fractions, ranging from 26.61 % concentration in albumin to 84.34% concentration in glutelin. In the freeze-dried pre-treatment, the concentration of albumin and globulin fractions

increased from 52.42 % in albumin to 63.76% in globulin. The concentration of prolamin was the lowest at 36.81%, and the concentration of glutelin was the highest at 92.42%. The observed concentrations of all four protein fractions from the fresh pre-treatment group followed the same trend as the freeze-dried protein fractions. The concentration of protein increased from 35.30% in the albumin fraction to 49.61% in the globulin fraction. Again, the concentration of prolamin was the lowest at 15.91%, and the concentration of glutelin was the highest at 84.36%. The results reveal that the freeze-dried albumin, globulin, and glutelin protein fractions contained the highest concentration of protein when compared to the heat-dried and fresh samples.

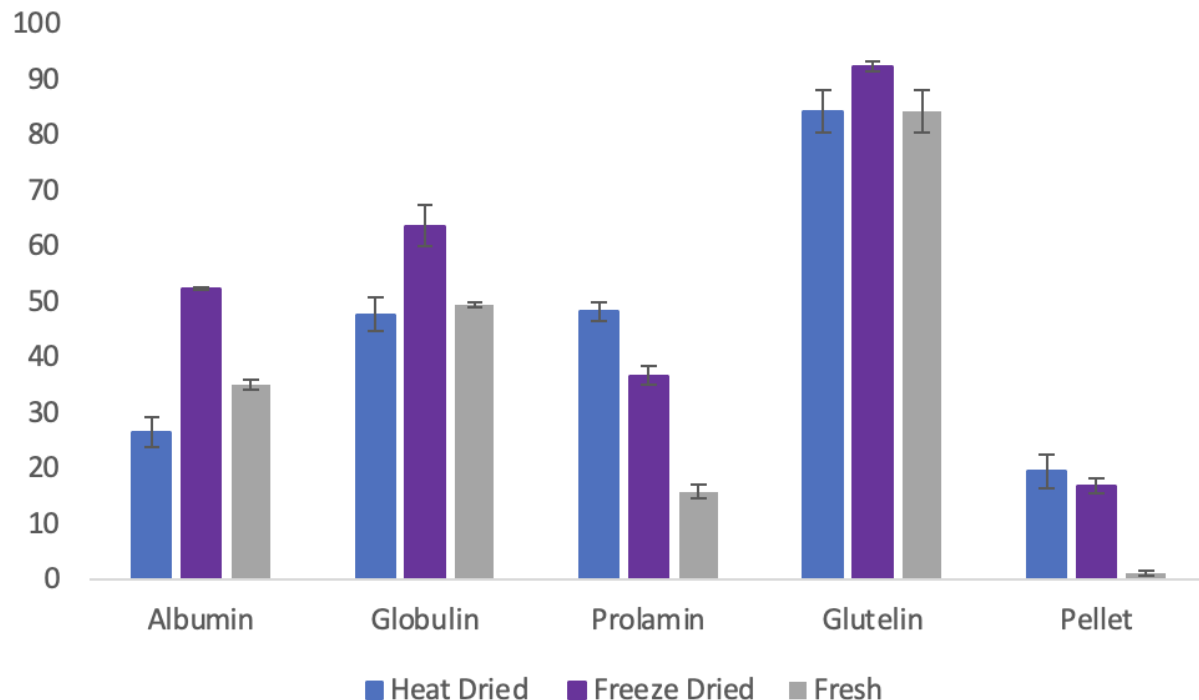


Figure 2. Fractionated protein content (%) of BSFL pre-treatments after Osborne fractionation

Protein Distribution

The protein distribution of each BSFL pre-treatment was also calculated, and the results are shown in Figure 3A-C. Albumin and glutelin made up between 56.43% and 87.78% of total protein

extracted in all three BSFL pre-treatment groups. Albumin was 30.50% of the total extracted protein in the heat-dried pre-treatment, and glutelin was 25.93% of total extracted protein in the heat-dried pre-treatment. In the freeze-dried pre-treatment, albumin was 47.99% of total protein extracted, and glutelin was 30.00% of total protein extracted. Finally, in the fresh pre-treatment, albumin was 34.44% of total protein extracted, and glutelin was 53.34% of total protein extracted.

Globulin and prolamin fractions were the lowest portion of total protein extracted in all three BSFL pre-treatments. Globulin ranged between 3.91-7.03% of total protein extracted, and prolamin ranged between 3.00-4.30% of total protein extracted. These results are supported by other research, as a study reported that albumin and glutelin were the protein fractions extracted in the highest quantities and globulin and prolamin were the protein fractions extracted in the lowest quantities from BSF (Caligiani et al., 2018).

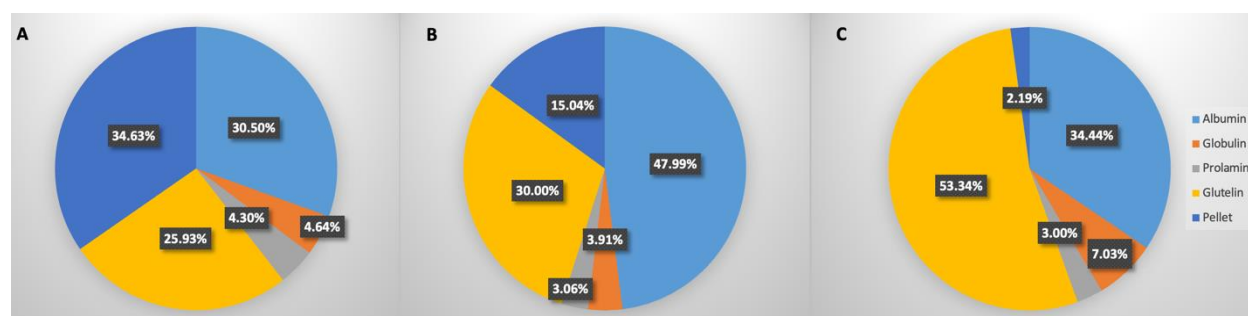


Figure 3A-C. Fractionated protein distribution heat-dried, freeze-dried, and fresh pre-treatments

Chitin Content

Carbohydrate analysis was performed in order to quantify the chitin content present in the protein fractions and pellet of each BSFL pre-treatment. The results of chitin quantification are shown in

Table 2. The defatted samples and pellet samples from all three BSFL pre-treatments contained the higher concentrations of chitin when compared to all four protein fractions. Concentrations of chitin in defatted and unfractionated samples ranged from 2.75% in fresh BSFL to 4.99% in freeze-dried BSFL. Concentrations of chitin in fractionated pellet samples ranged from 3.44-9.54% between the three pre-treatments. Fresh BSFL pellet contained the highest chitin content of 9.54%, and this is likely due to the composition of the fresh pellet. The fresh BSFL was more difficult to grind when compared to the two dried pre-treatments, resulting in a majority of BSFL exoskeletons remaining whole after grinding via mortar and pestle. These exoskeletons remained in the pellet after osborne fractionation, increasing the chitin content of the fresh pellet. In heat-dried samples, glutelin protein fractions contained the highest chitin content of 2.40%, and prolamin protein fractions contained the lowest chitin content of 1.81%. In freeze-dried samples, globulin protein fractions contained the highest chitin content of 2.75%, and prolamin protein samples contained the lowest chitin content of 1.63%. Similarly, in fresh samples, globulin protein fractions contained the highest chitin content of 2.62%, and prolamin protein fractions contained the lowest chitin content of 0.90%. When comparing our results to previous studies, Leyva-Gutiérrez et al. (2022) reported that the chitin content of defatted and deproteinated BSFL was 3.6% and 4.0% during two trials. These values align with the chitin content of the defatted and unfractionated samples for all three pre-treatments, as chitin content ranged between 2.75-4.99%. The results show that the heat-dried and freeze-dried pre-treatments were the most chitin rich pre-treatment groups. Freeze-dried albumin and globulin fractions had the highest chitin concentrations and heat-dried prolamin and glutelin fractions had the highest chitin concentrations when compared between pre-treatments.

Table 2. Chitin content (%) of defatted and fractionated BSFL proteins

Treatment	Heat Dried	Freeze Dried	Fresh
Defatted Sample	4.27	4.99	2.75
Albumin	2.20 ± 0.12	2.56 ± 0.02	2.01 ± 0.14
Globulin	1.93 ± 0.11	2.75 ± 0.25	2.62 ± 0.50
Prolamin	1.81 ± 0.13	1.63 ± 0.33	0.90 ± 0.13
Glutelin	2.40 ± 0.14	2.36 ± 0.29	2.37 ± 0.10
Pellet	3.44 ± 0.11	6.90 ± 1.26	9.54 ± 0.44

Chitin Distribution

The chitin distribution of fractionated and pre-treated BSFL was also calculated to determine the percent of chitin that each protein fraction and pellet sample contained. The results of chitin distribution are shown in Figures 4A-C. The chitin distributions of all three pre-treatments reveal that the defatted, fractionated pellets contain between 49.87-74.76% of total chitin from each BSFL sample. Additionally, albumin contained the next largest portion of total chitin, with albumin containing 32.99% of the total heat-dried chitin, 24.65% of the total freeze-dried chitin, and 16.34% of the total fresh chitin. Glutelin contained the median value of chitin for all three samples, with glutelin containing 12.73% of the total heat-dried chitin, 8.05% of the total freeze-dried chitin, and 6.82% of the total fresh chitin. Globulin and prolamin protein fractions contained the lowest chitin values, with globulin values ranging between 1.42-1.94% and prolamin values ranging between 0.66-2.47%.

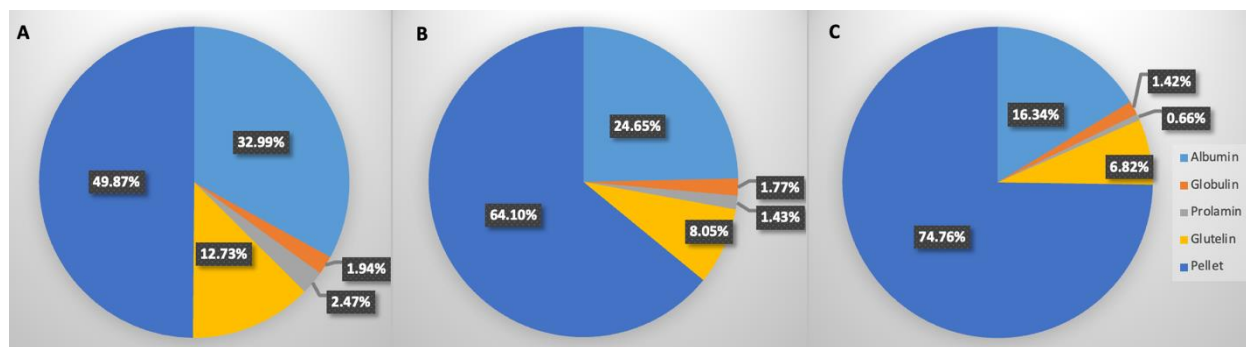


Figure 4A-C. Fractionated chitin distribution heat-dried, freeze-dried, and fresh pre-treatments

Magnesium and Calcium Content

The mineral content of magnesium (Mg) and calcium (Ca) for all three BSFL pre-treatments is shown in Figures 5 and 6 below. The contents of these two minerals are important due to their ability to bond with proteins. Magnesium and calcium are alkaline earth metals, meaning that they are cations and possess low ionization energy, low effective nuclear charge, and large atomic radii. These properties make these metal ions reactive, allowing them to bond to insect proteins to strengthen exoskeletons or cuticular structures (Degtyar et al., 2014). The defatted and unfractionated heat-dried samples had the highest concentration of magnesium and calcium when compared to the other pre-treatments, containing 4,677.6 ppm pf magnesium and 35,307 ppm of calcium. Furthermore, for all three pre-treatments, the concentration of magnesium decreased exponentially between albumin, globulin, prolamin, and glutelin protein fractions. The concentration of calcium in the heat-dried protein fractions also followed this trend. When comparing the concentration of magnesium and calcium in protein fractions between pre-treatments, the fresh samples had the highest concentrations of magnesium and calcium contained in the globulin, prolamin, and glutelin protein fractions. The pellet of the freeze-dried pre-treatment

had the highest magnesium and calcium content in the pellet as well, containing 4,544.6 ppm of magnesium and 52,267 ppm of calcium.

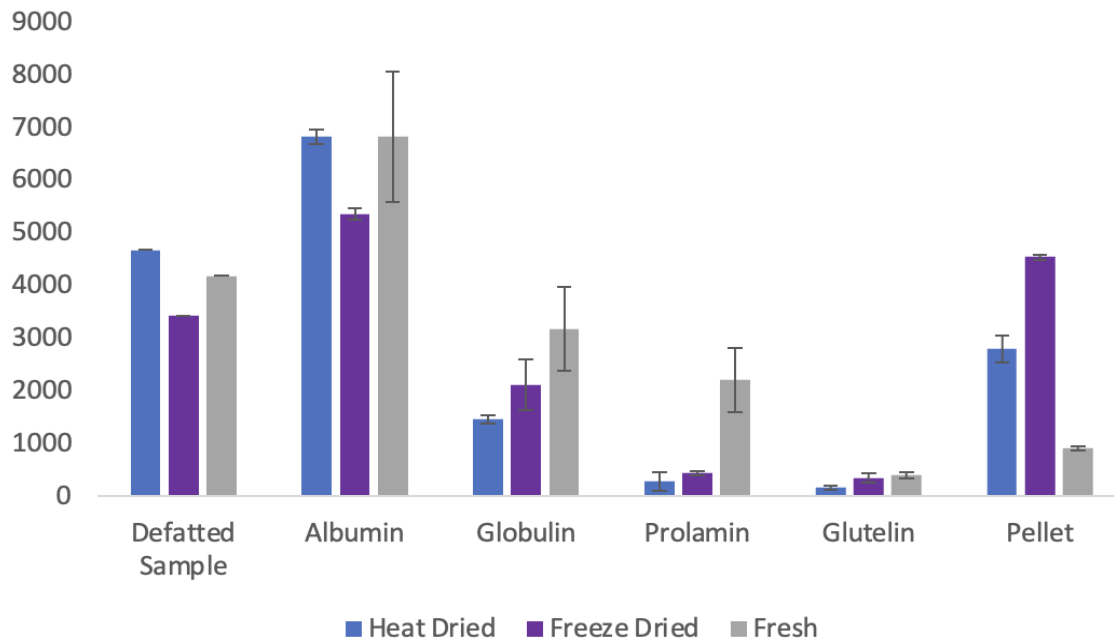


Figure 5. Fractionated magnesium content (ppm) of pre-treated BSFL

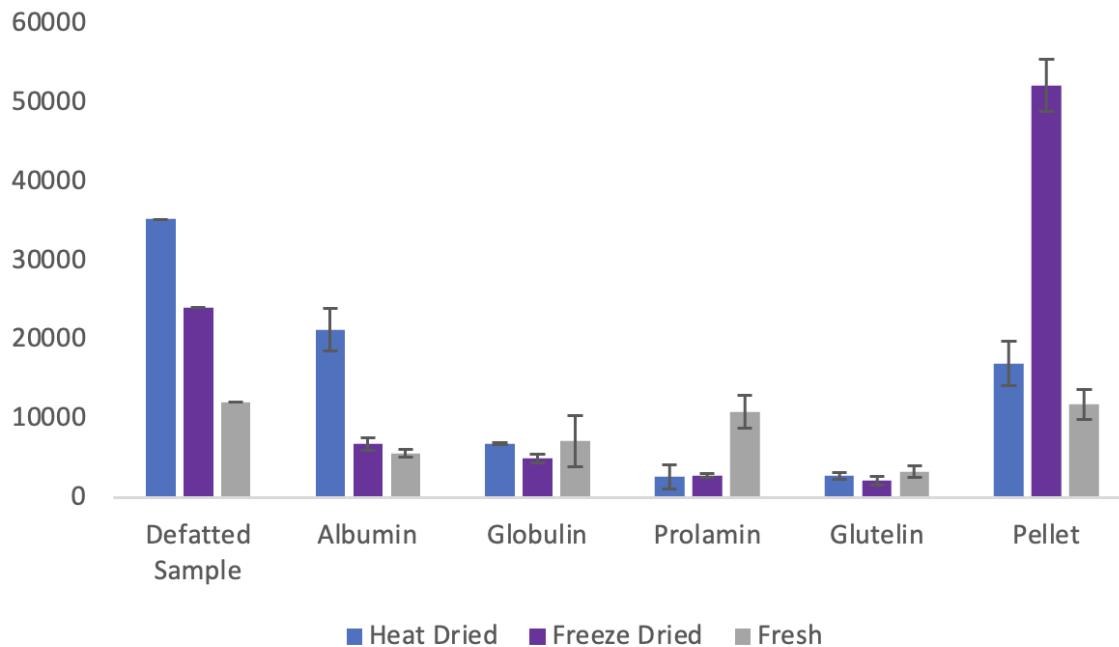


Figure 6. Fractionated calcium content (ppm) of pre-treated BSFL

Magnesium and Calcium Mineral Distribution

The magnesium and calcium distributions are shown in Table 3. For all three pre-treatments, the percentage of magnesium contained in protein fractions was highest in albumin. The pellet of the freeze-dried pre-treatment contained the highest percentages of magnesium and calcium, containing 58.9% of total magnesium and 91.4% of total calcium. It is possible that the insoluble proteins contained in the freeze-dried BSFL pellet cross-bonded to some of the magnesium and calcium ions, increasing the residual magnesium and calcium contained in the freeze-dried pellet.

Table 3. Magnesium and calcium distribution of defatted and fractionated BSFL

	Mg			Ca		
	Heat Dried	Freeze Dried	Fresh	Heat Dried	Freeze Dried	Fresh
Albumin	83.2	34.9	70.7	72	6	30
Globulin	4.2	5.1	17.2	5.5	1.6	20.2
Prolamin	0.5	0.5	6.8	1.3	0.5	17.3
Glutelin	0.3	0.6	1.2	1.3	0.5	5.1
Pellet	11.8	58.9	4.1	19.9	91.4	27.5

Additional Mineral Content

Additional mineral content for all three pre-treatments is contained in Tables 4-6. Copper, iron, phosphorus, zinc, potassium, and sulfur are all minerals necessary in human diets. Phosphorus was the mineral contained in the highest quantity in all BSFL pre-treatments.

Table 4. Mineral content (mg/Kg) of heat dried BSFL pre-treatment

	Cu	Fe	P	Zn	K	S
Defatted Sample	23.1	723.5	11180	156.8	17825	6513
Albumin	26.9 ± 3.5	499.5 ± 320.3	16878 ± 1040	107.5 ± 19.9	56592 ± 4713	4304 ± 331
Globulin	30.8 ± 5.0	539.3 ± 266.7	12718 ± 1131	112.9 ± 23.4	882 ± 122	2669 ± 210
Prolamin	28.1 ± 9.6	375.7 ± 204.1	9407 ± 867	80.0 ± 9.4	1114 ± 283	1491 ± 470
Glutelin	25.2 ± 2.2	198.3 ± 50.0	4393 ± 361	86.0 ± 12.8	737 ± 127	6214 ± 330
Pellet	19.9 ± 2.8	446.1 ± 140.4	10206 ± 1361	251.7 ± 11.4	618 ± 48	8251 ± 607

Table 5. Mineral content (mg/Kg) of freeze dried BSFL pre-treatment

	Cu	Fe	P	Zn	K	S
Defatted Sample	20.7	788	8760.5	137.9	15048	4721
Albumin	15.2 ± 0.6	168.2 ± 21.6	6726 ± 363	93.2 ± 12.4	2538 ± 4306	2944 ± 492
Globulin	42.2 ± 2.7	337.9 ± 53.6	4035 ± 513	173.9 ± 9.8	958 ± 65	4175 ± 360
Prolamin	32.3 ± 2.4	619.5 ± 327.6	3021 ± 593	85.7 ± 10.5	990 ± 55	725 ± 80
Glutelin	34.4 ± 2.1	656.3 ± 321.4	3795 ± 442	141.4 ± 10.9	569 ± 17	2945 ± 597
Pellet	17.5 ± 3.3	990.3 ± 297	25589 ± 1449	421.7 ± 2.9	588 ± 21	6485 ± 127

Table 6. Mineral content (mg/Kg) of fresh BSFL pre-treatment

	Cu	Fe	P	Zn	K	S
Defatted Sample	25.3	430.5	10365	146.2	3363	2132
Albumin	65.9 ± 20.3	882.2 ± 763.1	15034 ± 1289	129.1 ± 33	21722 ± 12328	2971 ± 1468
Globulin	89.0 ± 13.7	802.8 ± 441.3	13003 ± 2372	192.1 ± 26.7	639 ± 42	4185 ± 1958
Prolamin	19.1 ± 7.3	825.9 ± 397.1	977 ± 119	61.3 ± 5.3	859 ± 39	780 ± 154
Glutelin	36.7 ± 6.8	756.5 ± 47.6	6766 ± 1239	192.1 ± 20.4	466 ± 35	4224 ± 1307
Pellet	17.3 ± 8.4	720.5 ± 414.7	6953 ± 110	207.8 ± 28.9	451 ± 67	3108 ± 464

Additional Mineral Distribution

Additional mineral distribution for iron, phosphorus, zinc, potassium, and sulfur is shown in figure 7A-C. The pellet of each pre-treatment contains a minimum of 33.30% of the total minerals and a maximum of 62.96% of total minerals. Albumin protein fractions contain the highest percentage of total minerals when comparing the four protein fractions of each pre-treatment. Albumin contains 62.40% of total minerals in heat-dried samples, 32.7% of total minerals in freeze-dried samples, and 48.20% of total minerals in fresh samples. Moreover, glutelin contains the second

highest percentage of total minerals when comparing the four protein fractions of each pre-treatment.

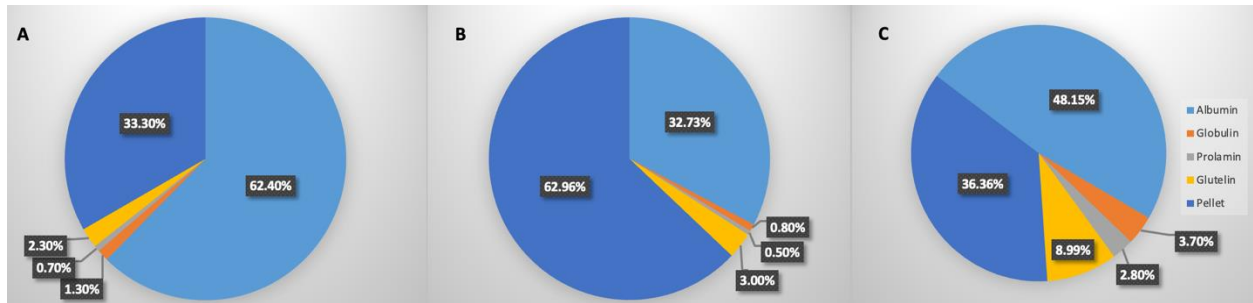


Figure 7A-C. Fractionated total mineral distribution of heat-dried, freeze-dried, and fresh pre-treatment

5. Conclusion

Black soldier fly larvae utilized in this study were heat-dried, freeze-dried, and frozen at -80°C (fresh) to determine the effect of pre-treatments on protein extraction and carbohydrate and mineral content. Freeze drying was the most effective pre-treatment for maximizing BSFL protein recovery, and the extracted albumin, globulin, prolamin, and glutelin protein fractions were 48.79% of the initial freeze-dried BSFL sample mass. Defatted, unfractionated samples of freeze-dried BSFL contained the highest chitin content of 4.99%, and the albumin and globulin protein fractions contained the largest concentrations of chitin when compared between the other pre-treatments. Although the freeze-dried BSFL yielded high protein quantity and chitin quantity, the pellet contained the highest percentage of total minerals after both mineral quantification tests when compared to the heat-dried and fresh pre-treatments. This suggests that the freeze drying is not the best pre-treatment for prioritizing mineral quantity. The results of this study support the use of BSFL as an alternative protein source. Furthermore, the data provides evidence that freeze drying was the most effective pre-treatment for protein extraction due to decreased metal ion binding and denaturation of proteins.

Although freeze drying was the most effective pre-treatment, it is unrealistic to utilize this pre-treatment commercially due to cost and difficulties increasing the scale of the pre-treatment. Future research on BSFL protein extraction should focus on optimizing heat-dried BSFL extraction procedures to increase protein yield and scalability with the goal of establishing an industry standard procedure.

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