COMPARATIVE PROTEIN DETERMINATION IN INSECTS

Internship Report

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Abstract

With the increase of the world population, there is a race in research and development to obtained more sustainable alternative sources of protein. Insects have attracted attention because of their ability to effectively convert feed into biomass, have a high growth and development rate, require less land and water to grow, emit less greenhouse gas, and are able to convert wasted food to biomass, among other reasons. Many scholars suggest that insects have high nutritional values that could enhance human diet. However, the biochemical structure of insects is characteristically different from common sources of protein. Insects contain chitin which is an amino polysaccharide that contains nitrogen, and this affects protein concentration determination methods that rely on measuring nitrogen atoms in a sample. It is therefore important to examine the methods used to extract insect protein and methods used to determine insect protein concentration.

In this study, protein extraction methods or buffers were assessed. These included Radioimmunoprecipitation assay buffer (RIPA buffer), sodium dodecyl sulphate (SDS) buffer, SDS lysis buffer, urea extraction buffer, urea extraction buffer without thiourea, and a defatting step followed by urea extraction buffer without thiourea. Protein concentration determination methods assessed were Bradford, Bicinchoninic acid (BCA) and Lowry protein assays. Gel electrophoresis was performed to visualize protein molecules extracted by the different extraction buffers. From the gel images, it was observed that urea extraction buffer extracted more proteins from insect samples compared to other protein extraction buffers. Protein concentration of samples extracted using urea buffer could not be determined by Lowry and BCA assay due to the presence of thiourea which is a strong reducing agent that causes interference with both methods. However, this did not cause interference with Bradford assay. It was concluded that Bradford protein assay was better for determining protein concentration for samples extracted using urea extraction buffer.

There was a significant difference between protein concentrations of insects at different development stages. There was also a significant difference between protein concentration of insects fed on different diets. These, among other findings in this study could be important for further research.

1. Introduction

There is a steady increase in human population globally which has raised concerns of food security to cater for this demand. It has been anticipated that it will require approximately double the amount of the current food production to feed the whole population by 2050 (Dobermann et al., 2017; Kim et al., 2019). Consequently, there is an increase in the demand of sustainable sources of food to ensure environmental protection while meeting the human nutritional requirements. Particularly protein sources are given priority mostly because conventional animal farming is considered unsustainable due to high greenhouse gas emissions, vast amount of water and land use among other factors (Tuhumury, 2021; van Huis & Oonincx, 2017). This has led to many research and developments of alternatives to meat (including sea foods) and animal products like dairy products (Lima et al., 2022). Insects have lately been considered as alternative protein options by many scholars, companies, and organisations. Insects have attracted attention due to their nutritional values and potential to be a great source of fat (Riekkinen et al., 2022), several minerals (Meyer-Rochow et al., 2021), protein (Liceaga, 2022), and fiber (Meyer-Rochow et al., 2021) among others. Furthermore, insects are associated with many other advantages like taking a shorter time to grow and develop, less greenhouse gas emissions involved in rearing them, high conversion factor of feed to biomass, require less water and land to grow among others. Insects can convert biowaste to body mass. This could also solve the problem of food wastage as the Food and Agriculture Organization (FAO) reports that one third of food is lost or wasted, that is about 1.3 billion tons of wasted food every year (Gustavsson et al., n.d.).

Over 2000 species of edible insect are consumed worldwide mostly in Africa, Asia, and South American countries while it has not been popular in Europe and North American countries until the 20th century (Ordoñez-Araque et al., 2022). Insects are referred to as novel foods because they have not been consumed to a significant degree by humans in the European Union (EU) before 15 May 1997 ("Izvedbena Uredba Komisije (EU) 2017/2470," 2017). The European Food Safety Authority (EFSA) which is the agency of the EU that provides independent scientific advice already gave a go-ahead for some insects and insect products to be sold on the EU market. These include yellow mealworm (*Tenebrio molitor*) (Regulation (EU) 2022/169), migratory locust (*Locusta migratoria*) (Regulation (EU) 2021/1975), house cricket (*Acheta domesticus*) (Regulation (EU) 2015/2283; Turck et al., 2021; Regulation (EU) 2023/5) and many more insects are in review for authorization.

Insect biochemistry is characteristically different from the common sources of protein and one of the reasons is presence of chitin which forms the exoskeleton on all insects (Nikahd et al., 2020). Chitin is the most abundant amino polysaccharide occurring in nature (Elieh-Ali-Komi et al., 2016). It contains a lot of nitrogen, and this interferes with protein determination methods that rely on measuring nitrogen atoms in a sample to determine protein concentration. This therefore makes determining protein concentration from insects more complicated and sometimes inaccurate. There are other methods that can be used to determine protein concentration without relying on the number of nitrogen atoms. Some of the methods include Bradford protein assay based on the binding of protein molecules to Coomassie dye(Bradford, 1976), Lowry protein assay based on the reaction between protein and Folin-Ciocalteu reagent (Lowry et al., 1951) and Bicinchoninic acid (BCA) protein assay based on the reduction of copper by protein molecules forming a purple colour by BCA (Walker, 1994).

Aims

In this study, we assessed the most efficient protein extraction method and ascertained the most efficient protein concentration determination method relevant for measuring insect protein concentration as well as other samples. We compared the effect of feeding on the protein concentration of different insects and compared protein concentration of specific insects at different development stages. Furthermore, we visualized the distribution of protein molecules of the samples using gel electrophoresis.

2. Materials and Methods

2.1 Samples

House cricket (*A. domesticus*) samples (fed on vegetables like cucumber, carrots, and cabbage) were purchased from Fressnapf in Kulmbach, Germany. Other samples were provided by Mr. Finn Richter. The samples included; fish, shrimps, chicken breast, turkey breast, pork from neck region, pork from stomach region, pork fillet, fresh peas, carrots, spinach, adult grasshopper (*L. migratoria*), nymph grasshopper (*L. migratoria*), stick insect (*P. hispanica*), yellow mealworm (*T. molitor*) larva fed on wheat flour, yellow mealworm (*T. molitor*) larva fed on Oats and whole grain, yellow mealworm (*T. molitor*) larva fed on side-stream diet (Side-stream diet included vegetables like salads), yellow mealworm (*T. molitor*) pupa fed on Oats and whole grain, yellow mealworm (*T. molitor*) pupa fed on side-stream diet, yellow mealworm (*T. molitor*) adult fed on wheat flour, yellow mealworm (*T. molitor*) adult fed on Oats and whole grain, buffalo worms (*A. diaperinus*) larva fed on side stream diet, buffalo worms (*A. diaperinus*) larva with no special feeding (feeds included vegetables and fruit peelings) and cockchafer/maybug (*M. melolontha*) larvae.

2.2 Sample powders

For the samples to be analysed, sample powders were made from samples using a mortar and pestle after shock freezing with liquid nitrogen at around -180°C. Sample tissues were placed in a mortar. Liquid nitrogen (-180°C) was then added just to cover all the sample tissues. A pestle was then used to carefully grind all the sample tissues to make a powder. Using a spatula, the powder was then transferred to sterile Eppendorf tubes that were already pre-weighed and the weight was recorded. The sample powders were kept on dry ice and if not used immediately, they were kept at -20°C.

2.3 Preparation of protein extraction buffers

2.3.1 RIPA lysis buffer: Radioimmunoprecipitation assay buffer (RIPA buffer) was prepared as follows; 50mM Tris-HCl (1.513g), 150mM NaCl (2.192g), 1mM EDTA (73g), 0.25%(w/v) Na-Deoxycholate (625mg), 10% Triton X-100 (2.5ml) and pH was adjusted to 7.2. All reagents were dissolved and solution filled to 250ml using double distilled (dd) water.

2.3.2 SDS Lysis buffer: Sodium dodecyl sulphate (SDS) lysis buffer was prepared as follows; 2mM EDTA (0.0146g), 10mM Tris-HCl (0.03028g), 1%SDS (2.5ml) and pH was adjusted to 8.1. All reagents were dissolved and solution filled to 25ml using dd water.

2.3.3 SDS buffer: Sodium dodecyl sulphate (SDS) was prepared as follows; 100mM Tris-HCl (0.30285g), 4% SDS (1ml), 50mM Dithiothreitol (0.19g) and pH adjusted to 7.6. All reagents were dissolved and solution filled to 25ml using dd water.

2.3.4 Urea extraction buffer: Urea extraction buffer was prepared as follows; 20mM Tris-HCl (0.06057g), 8M Urea (12.012g), 2M Thiourea (3.806g), 50mM Dithiothreitol (0.19291g)

and pH adjusted to 8.5. All reagents were dissolved and solution filled to 25ml using dd water.

2.3.5 Urea extraction buffer without thiourea: Urea extraction buffer without thiourea was prepared as follows; 20mM Tris-HCl (0.06057g), 8M Urea (12.012g), 50mM Dithiothreitol (0.1929g) and pH adjusted to 8.5. All reagents were dissolved and solution filled to 25ml using dd water.

2.4 Preparation of the Bovine Serum Albumin (BSA) standard

The predetermined protein, Bovine serum albumin (BSA) was used as a standard. 20 mg of the BSA stock standard was dissolved in 20 ml of double distilled water making a stock concentration of 1 mg/ml. Serial dilutions of 0, 0.025, 0.05, 0.075, 0.10, 0.125, 0.15, 0.175 and 0.20 mg/ml concentrations were made to obtain a standard curve.

2.5 Measurement of absorbance spectrum

Among other quality control checks, an absorbance spectrum of spinach was assessed to rule out interference by the green colour due to chlorophyll. Two sets of four dilutions (1:10, 1:25, 1:50 and 1:100) were prepared from which one set was reacted with Bradford protein assay and another set reacted with double distilled water. 20µl of each dilution was reacted with 200µl of either Bradford protein assay or double distilled water. Absorbance was measured using CLARIOstar® Plus machine set to measure between wavelength of 320nm – 800nm.

2.6 Protein extraction

Approximately 20 mg of the sample powder was weighed in a 1.5 ml Eppendorf tube. The sample powder was homogenized with the extraction buffer at 100 mg/ml (e.g., 20 mg of sample powder in 200 µl of the extraction buffer). The mixture was vortexed (about 30sec – 1 min) and all the tissue parts were spun down (2 minutes at 14,000 rpm). The samples were then sonicated for 10 minutes in ultrasonic bath. After sonication, the samples were shaken on the thermoshaker at 600 rpm for 20 minutes at room temperature. The samples were then centrifuged at 14,000 rpm at 4ºC for 10 minutes. The supernatant was transferred in a new labelled 1.5 ml Eppendorf tube and was used for further analysis. The pallet was discarded. The samples were then analysed immediately, and if not, they were kept at -20ºC.

2.7 Protein extraction after defatting

Approximately 20 mg of sample powder was weighed in a 1.5 ml Eppendorf tube. This was then mixed with 200 µL n-pentane (a ratio of 1mg of sample: 10 µL of n-pentane was used). This was then mixed on a thermoshaker for 15 minutes. The mixture was then centrifuged for 5 minutes at 14,000 rpm and the supernatant was discarded. This step was repeated twice. After the last step, the pellet was air-dried for 20 minutes. Protein extraction was then done as described in 2.6, using the urea buffer without thiourea.

2.8 Preparation of protein determination assay

2.8.1 Bradford protein assay

Prepared according to the procedure described by Bradford (Bradford, 1976).

2.8.2 Bicinchoninic acid (BCA) protein assay

Prepared according to the procedure described by J.M Walker (Walker, 1994).

2.8.3 Lowry protein assay

Prepared according to the procedure described by Lowry (Lowry et al., 1951)

2.9 Protein measurement

The CLARIOstar® Plus high-performance monochromator multimode microplate reader was used to measure absorbance of the samples and standard.

2.10 Data analysis

The collected data was analysed using Microsoft 365 Excel and MARS Data Analysis Software by BMG Labtech GmbH.

3. Results and Discussion

3.1 Comparison between protein extraction methods/ buffers

Fish, mealworm, grasshopper, and spinach samples were analysed using different extraction buffers and the protein concentration was determined using Bradford protein assay (figure 1; Appendix Table 2). These samples were selected to have a representation from all the samples. It was observed that the protein concentration was highest across samples extracted using urea buffer, followed by urea buffer without thiourea, SDS lysis buffer, RIPA buffer, a defatting step followed by urea buffer without thiourea and finally SDS buffer. However, it has been documented that SDS interferes with Bradford protein assay (Rabilloud, 2018).

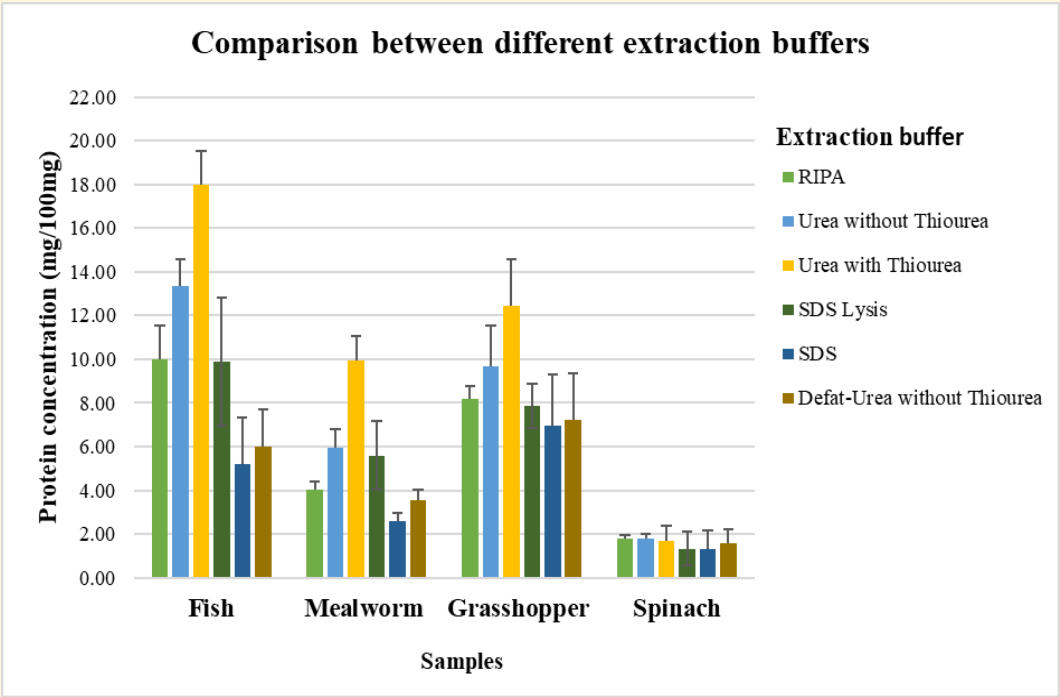


Figure 1. A graphical representation of protein concentration of fish, mealworm, grasshopper, and spinach samples using different protein extraction buffers and determined using Bradford protein assay.

Urea extraction buffer showed better protein extraction because urea is a chaotropic agent, which effectively causes protein denaturation by breaking hydrogen bonds, non-covalent and ionic links between amino acid residues (Stumpe & Grubmüller, 2007). The thiourea is effective at breaking hydrophobic interactions thus leading to a high solubility of membrane proteins. The DTT in the urea buffer promotes the re-oxidation of di-sulphide bonds hence preventing protein loss by aggregation or precipitation.

3.2 Comparison between protein determination methods

Different protein determination methods i.e., Bradfords, Lowry and BCA protein assay were assessed in this study (figure 2&3; Appendix Table 3&4). It was generally observed that Lowry and BCA methods showed higher protein concentration in all the samples compared to Bradford assay.

It was also observed that Lowry and BCA protein assays were prone to many interferences as already reported by (Rabilloud, 2018). For example, reducing substances like thiourea greatly interfere with protein concentration measurement (Krieg et al., 2005). In this study, there was no detectable values of protein concentration for urea extraction buffer that contained thiourea measured using Lowry or BCA assay. It is also important to note that SDS interferes with Bradford (Rabilloud, 2018) but not Lowry and BCA assay. SDS buffer shows more protein concentration than SDS lysis buffer in all samples when measured using lowry and BCA, yet it is a reverse when measured with Bradford assay. It was also observed that BCA showed the highest protein concentration in fish, mealworm, and grasshopper unlike spinach sample where lowry assay showed the highest protein concentration.

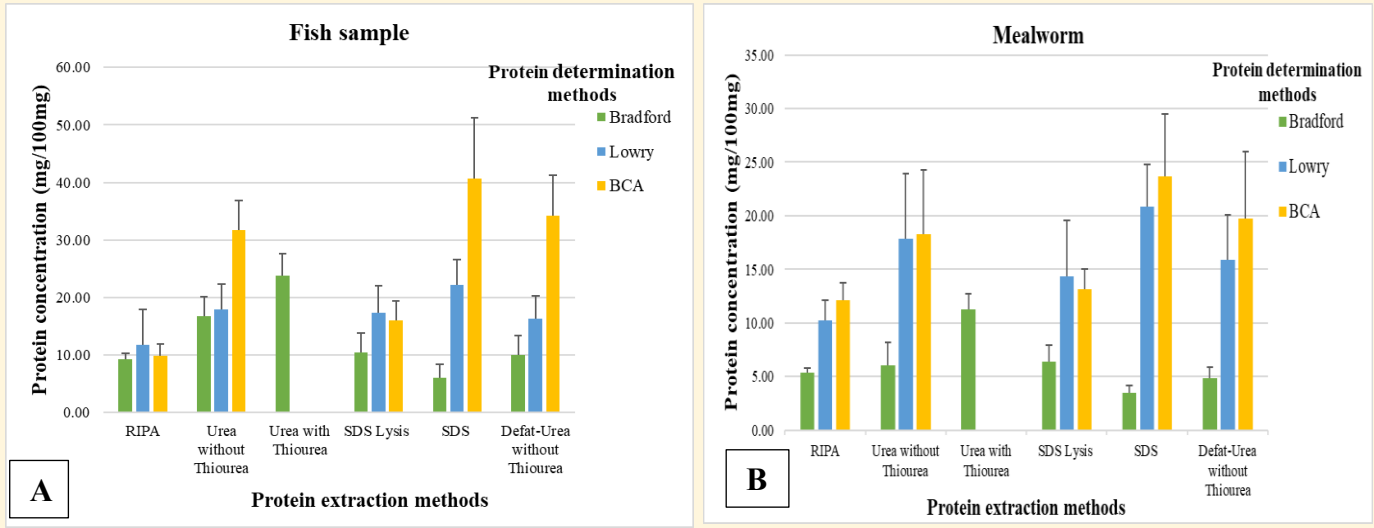


Figure 2. A graphical representation of protein concentration by Bradford, Lowry and BCA assays using different protein extraction methods for fish sample (A) and mealworm sample (B).

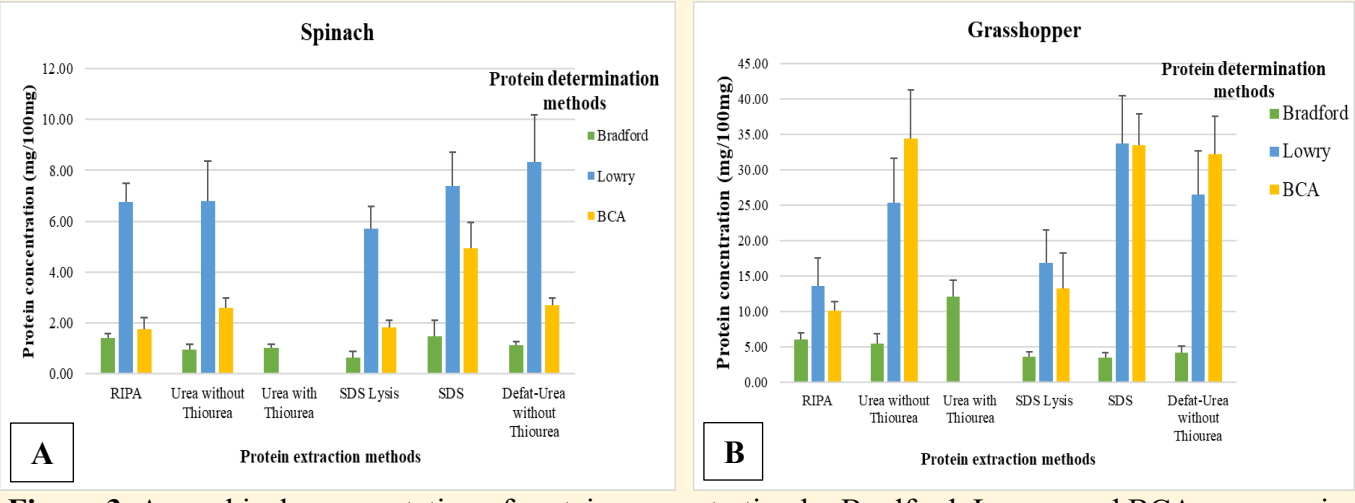


Figure 3. A graphical representation of protein concentration by Bradford, Lowry, and BCA assays using different protein extraction methods for spinach sample (A) and grasshopper sample (B).

3.3 Absorbance spectrum

Absorbance measurement was done between wavelength of 320nm to 800nm. From figure 4, it was observed that there was high absorbance gradually descending at 320nm, this is because it is close to 280nm which is the absorbance range of protein, specifically, the two aromatic amino acids tryptophan and tyrosine. There was also some absorbance between the wavelength of 400nm and 500nm, this is due to carotenoids and chlorophyll that have an absorbance within this wavelength and these are present in spinach. There was also absorbance between 600nm and 700nm, this is due to chlorophyll that also exhibits absorbance between the wavelength of 600nm and 700nm and this is abundant in spinach.

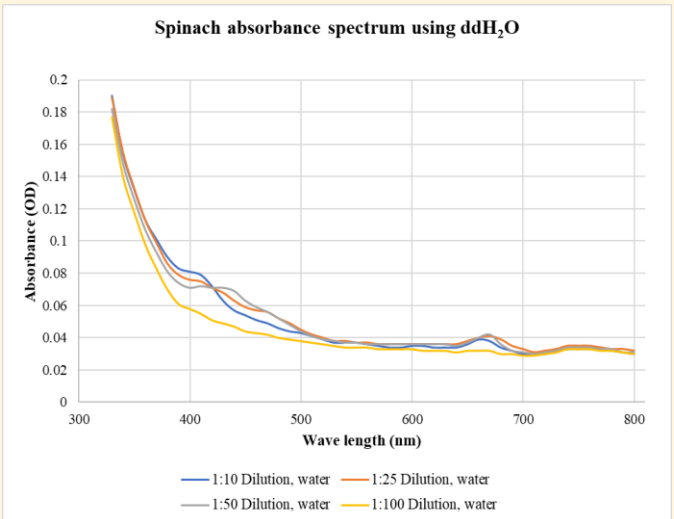


Figure 4. Absorbance spectrum of spinach sample with dilutions of 1:10, 1:25, 1:50 and 1:100 from which 20µl was mixed with 200µl of double distilled water and measured between the wavelength of 320nm and 800nm.

Reacting with Bradford assay generally increased absorbance of the samples (figure 5) this is due to the colour formation by the assay. It was observed that a sample dilution of 1:10 had the highest absorbance followed by 1:25 then 1:50 and 1:100. This is because the lower the dilution the higher the protein concentration detected around the wavelength of 595nm. General protein detection using Bradford assay was observed around the wavelength of 550nm to 720nm.

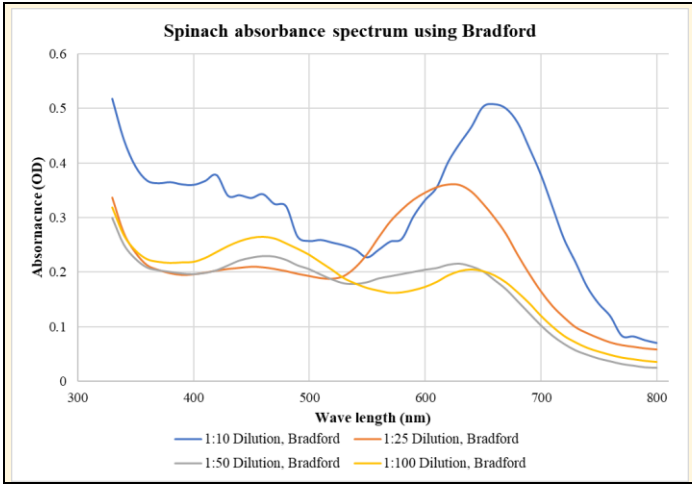


Figure 5. Absorbance spectrum of spinach sample with dilutions of 1:10, 1:25, 1:50 and 1:100 from which 20µl was mixed with 200µl of Bradford protein assay and measured between the wavelength of 320nm and 800nm.

Figure 6 shows a comparison between the absorbance spectrum of spinach using double distilled water and using Bradford assay. It was observed that there was no interference of the

green colour (by chlorophyll) around the wavelength of 650nm and 700nm with the Bradford assay detection around the wavelength of 595nm.

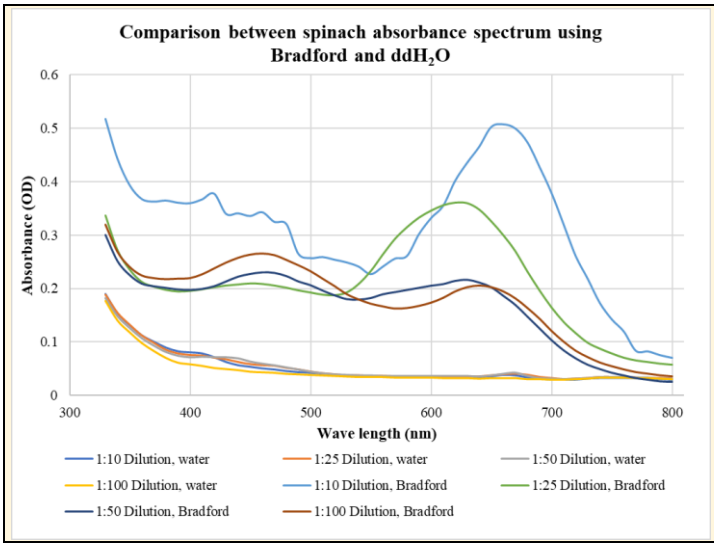


Figure 6. A comparison between absorbance spectrum of spinach sample using Bradford assay and using double distilled water.

3.4 Gel electrophoresis

SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) is a method used to separate protein with molecular masses between 5 and 250 kDa. Here, protein molecules are solely separated depending on their molecular weight. This method was used in this study to visualize the distribution of protein molecules from the different samples.

3.4.1 Comparison between protein extraction buffers using mealworm sample.

SDS-PAGE image of mealworm sample extracted with different buffers is shown in figure 7. Assessment is done at similar concentration (25µg) and similar volume (4µl). It was observed that RIPA extraction buffer (A) extracted protein mainly between 100 and 55kDa, the other protein bands were faint and between 55 and 15kDa. The urea extraction buffer (B) without thiourea extracted protein molecule mostly below 35 kDa. The SDS lysis buffer (C) showed faint bands between 130 and 100kDa, there were several clear bands between 100 and 55 kDa and below 25kDa, the bands were not completely distinct due to protein fragmentation by SDS lysis buffer. The SDS protein extraction buffer (D) showed a very clear protein band at 55kDa and most of the bands were below 35kDa although not so clear and below 15kDa the bands were not distinct due to protein fragmentation by the SDS buffer. Samples A-D were measured using sample concentration (25µg). Urea protein extraction buffer (E) overall showed more protein bands compared to other protein extraction buffers with the biggest band at 40kDa but with several clear band at different molecular weights. Using similar sample volume (4µl) for samples extracted using RIPA buffer (F), urea buffer without thiourea (G), SDS lysis buffer (H) and SDS buffer (I) did not change the protein distribution pattern of these extraction buffers.

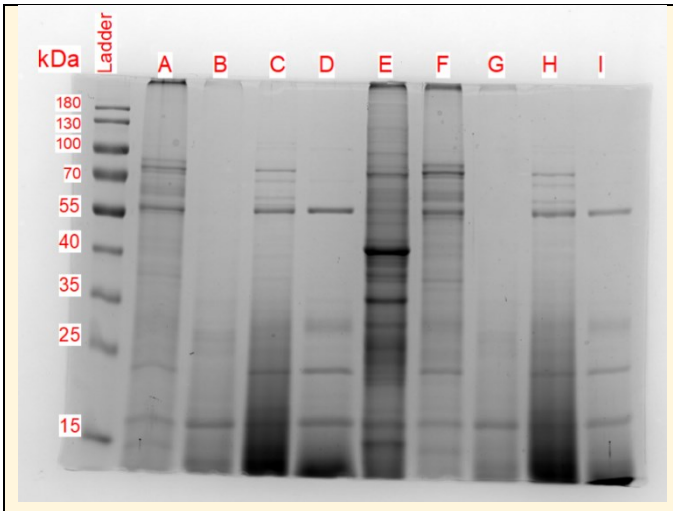


Figure 7. SDS-PAGE image of mealworm sample comparing different protein extraction buffers using same sample concentrations (25µg) and same sample volume (4µl) using 12% acrylamide. *(From left to right:* Mealworm protein extracted using RIPA buffer 25µg(**A**), Mealworm protein extracted using Urea buffer without thiourea 25µg (**B**), mealworm protein extracted using SDS lysis buffer 25µg (**C**), mealworm protein extracted using SDS buffer 25µg (**D**), mealworm protein extracted using urea buffer containing thiourea 4µl (**E**), mealworm protein extracted using RIPA buffer 4µl (**F**), mealworm protein extracted using urea buffer without thiourea 4µl (**G**), mealworm protein extracted using SDS lysis buffer 4µl (**H**), mealworm protein extracted using SDS buffer 4µl (**I**).*)*

Since some of the protein bands were fragmented by some protein extraction buffers and could not be visualized by using 12% acrylamide gel, a 15% acrylamide gel was prepared to observe fragmented protein molecules or very low molecular weight protein molecules (figure 8). The protein distribution pattern was observed was similar as that described for 12% acrylamide gel. However, it was clearly observed that SDS and SDS lysis buffers caused a lot of protein fragmentation followed by urea extraction buffer without thiourea.

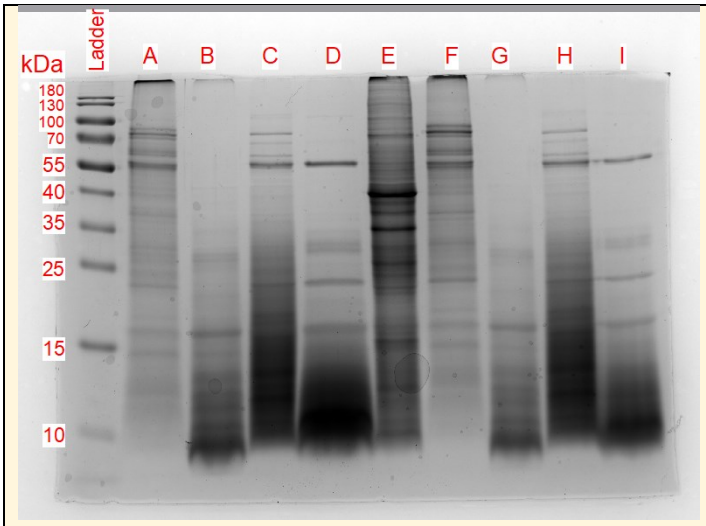


Figure 8. SDS-PAGE image of mealworm sample comparing different protein extraction buffers using same sample concentrations (25µg) and same sample volume (4µl) using 15% acrylamide. (From left to right: Mealworm protein extracted using RIPA buffer 25µg(**A**), Mealworm protein extracted using Urea buffer without thiourea 25µg (**B**), mealworm protein extracted using SDS lysis buffer 25µg (**C**), mealworm protein extracted using SDS buffer 25µg (**D**), mealworm protein extracted using urea buffer containing thiourea 4µl (**E**), mealworm protein extracted using RIPA buffer 4µl (**F**), mealworm protein extracted using urea buffer without thiourea 4µl (**G**), mealworm protein extracted using SDS lysis buffer 4µl (**H**), mealworm protein extracted using SDS buffer 4µl (**I**).)

3.4.2 Comparison between urea buffer and urea buffer without thiourea.

To analyse the urea buffer protocols with and without thiourea, SDS-PAGE of fish, mealworm, grasshopper, and spinach samples were compared by loading similar concentration of 20 µg (Figure 9).

It was observed that urea buffer with or without thiourea extracted relatively similar protein molecules in fish (A&F) and spinach (D&I) samples. While this was not the case for mealworm and grasshopper samples. It was observed that urea buffer extracted more protein in mealworm and grasshopper samples showing clear bands unlike urea buffer without thiourea. Urea extraction buffer showed better protein extraction as described in section 3.1.

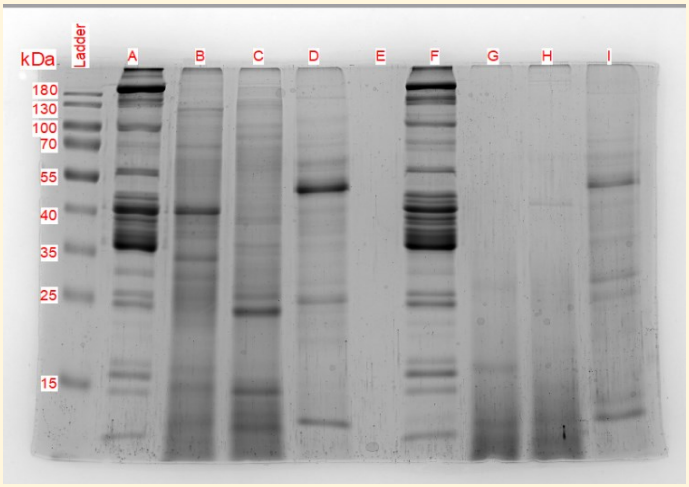


Figure 9. Gel image of 12% acrylamide showing fish protein extracted using urea buffer with thiourea (**A**), mealworm protein extracted using urea buffer with thiourea (**B**), grasshopper protein extracted using urea buffer with thiourea (**C**), spinach protein extracted using urea buffer with thiourea (**D**), an empty well to separate the two sections (**E**), fish protein extracted using urea buffer without thiourea (**F**), mealworm protein extracted using urea buffer without thiourea (**G**), grasshopper protein extracted using urea buffer without thiourea (**H**) and spinach protein extracted using urea buffer without thiourea (**I**). A similar concentration of 20µg was used in each sample well.

To rule out possibilities of protein fragmentation, a 15% acrylamide gel was performed (figure 10). It was observed that urea extraction buffer did not cause protein fragmentation in all the samples. It was also observed that urea buffer without thiourea did not cause fragmentation in fish and spinach samples. However, this caused protein fragmentation in the mealworm and grasshopper samples.

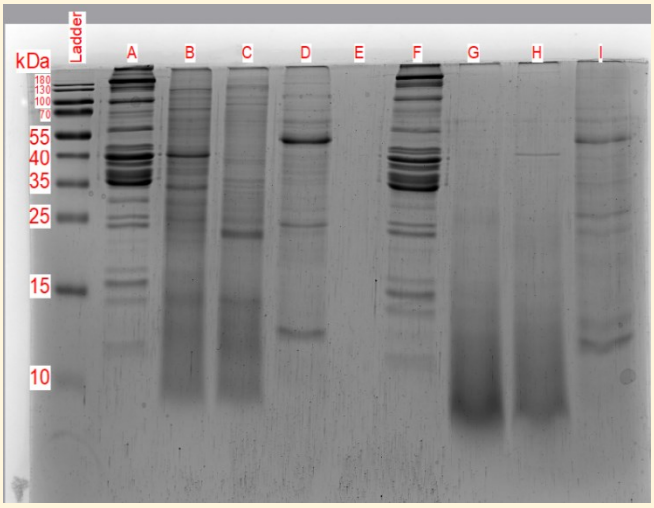


Figure 10. Gel image of 15% acrylamide showing fish protein extracted using urea buffer with thiourea (**A**), mealworm protein extracted using urea buffer with thiourea (**B**), grasshopper protein extracted using urea buffer with thiourea (**C**), spinach protein extracted using urea buffer with thiourea (**D**), an empty well to separate the two sections (**E**), fish protein extracted using urea buffer without thiourea (**F**), mealworm protein extracted using urea buffer without thiourea (**G**), grasshopper protein extracted using urea buffer without thiourea (**H**) and spinach protein extracted using urea buffer without thiourea (**I**). A similar concentration of 20µg was used in each sample well.

3.5 Protein concentration comparison between samples

After determining that urea protein extraction buffer and Bradford protein assay yielded more reliable results of protein concentration, all other samples were analysed using this extraction method and protein concentration determination method.

3.5.1 Protein comparison between house cricket, grasshopper, and stick insects.

Analysis of house cricket (*A. domesticus*) (figure 11D), adult grasshopper (*L. migratoria*) (figure 11B), grasshopper nymph (*L. migratoria*) (figure 11C) and stick insect (*P. hispanica*) showed protein concentration of 11.69±1.31mg/100mg, 12.86±1.52mg/100mg, 4.31±0.59mg/100mg, and 9.33±1.04mg/100mg respectively (Figure 11A; Appendix Table 5). Adult grasshoppers contain more protein compared to nymph grasshopper because the adult stage is the more active stage for insects that undergo incomplete metamorphosis. At this stage they actively feed, reproduce and locomote and this require energy from food and consequently proteins.

It was also observed that protein concentration of house cricket (11.69±1.31mg/100mg) was far less that reported in many previous studies for example 71.7±0.5mg/100mg (71.7±0.5%) reported by (Udomsil et al., 2019). This is due to the use of nitrogen-based methods to determine protein concentration and these methods are interfered by chitin which is abundant in crickets as well as all other insects.

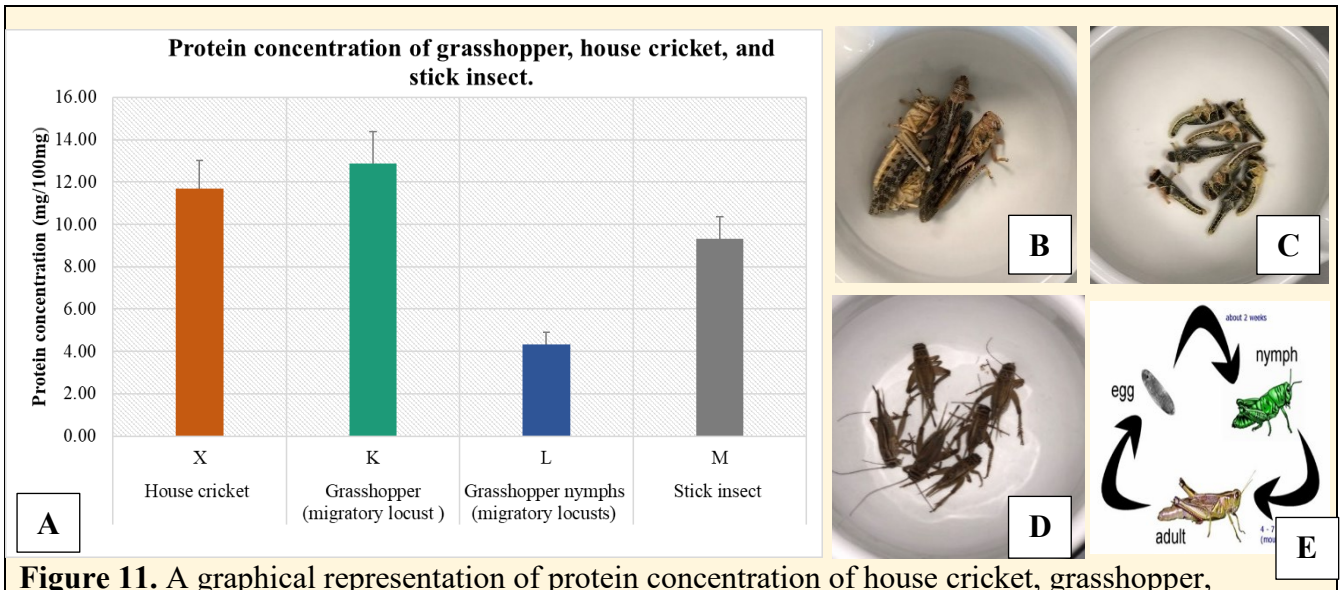


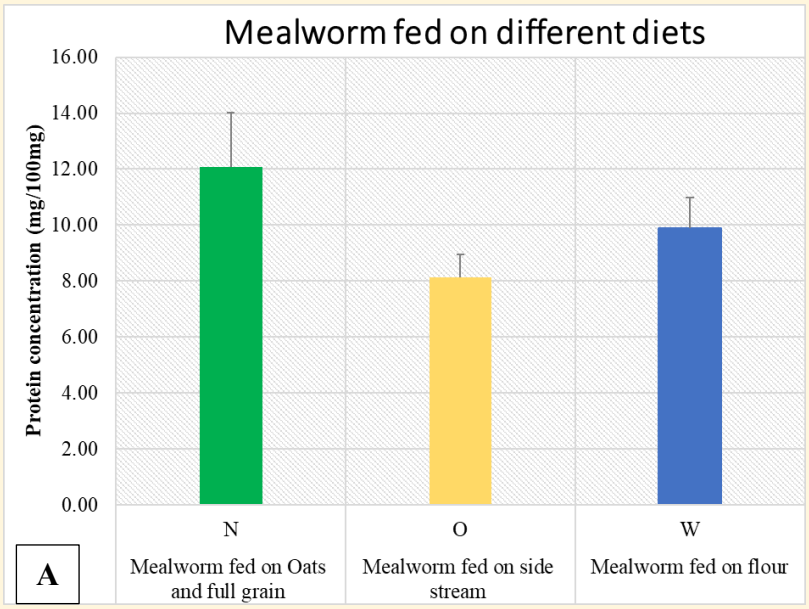
Figure 11. A graphical representation of protein concentration of house cricket, grasshopper, grasshopper nymph, and stick insect (**A**). Image of grasshopper samples (**B**), image of grasshopper nymph samples (**C**), image of house cricket samples (**D**) and scheme of their life cycle (**E**).

3.5.2 Protein comparison between mealworms fed on different diets

The larvae stage of mealworm is the most active stage where they feed as much as possible to store nutrients in preparation for the pupa stage where they are basically dormant.

Feeding mealworm larvae on Oats and full grain yielded more protein concentration (12.07±1.95mg/100mg) compared to those fed on side stream diet (8.13±0.80mg/100mg) and those fed on flour (9.87±1.12mg/100mg) (figure 12 A; Appendix Table 7). This implies that mealworm larvae can effectively convert feeds into protein by utilizing different protein synthesis mechanisms.

Although side streams feeding may not have significantly increased protein concentration in mealworm larvae, it would be relevant in yielding vitamins and minerals. This could be a relevant area for further investigation. Figures 12 B and 12 C show mealworm larvae and mealworm larvae powder.



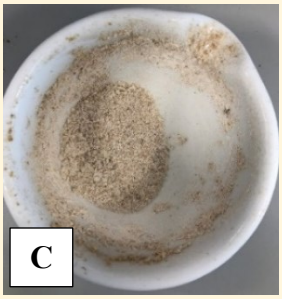
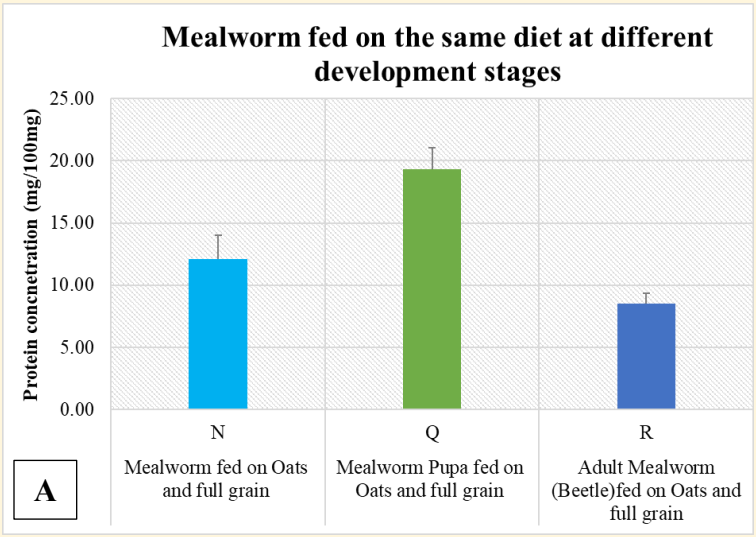


Figure 12. A graphical representation of protein concentration of mealworm fed on different diets (**A**). An image of mealworm samples (**B**) and image of mealworm powder obtained using liquid nitrogen and a mortar and pestle (**C**).

3.5.3 Protein comparison of mealworm at different development stages

Mealworms undergo a complete metamorphosis in their life cycle. The stages of development include egg, larva, pupa, and adult (figure 13B). The time it between these stages depends on the temperature and availability of food. Samples analysed were fed on the same diet in all development stages.

It was observed that mealworm pupa from larvae fed on Oats and full grain contained more protein concentration (19.29±1.74mg/100mg) compared to adult mealworms (8.53±0.83mg/100mg) and those at larvae stage (12.07±1.95mg/100mg) (figure 13A; Appendix Table 8). This is biologically relevant since adult mealworm don't require a lot of protein to survive and can easily obtain necessary nutrients unlike pupa that are in a dormant stage and require stored nutrients from the larvae stage. Mealworm larvae actively store nutrients to be utilized at the pupa stage hence their protein concentration can be affected by diet. However, there is no significant difference in protein concentration on mealworm pupa from larvae fed on oats and full grain (19.29±1.74mg/100mg) and those from larvae fed on side stream diet (21.05±1.09mg/100mg). This is probably because mealworm require a certain protein threshold to develop from one stage to another. Therefore, regardless of the feeding, development from one stage to another will only happen if this threshold is obtained.



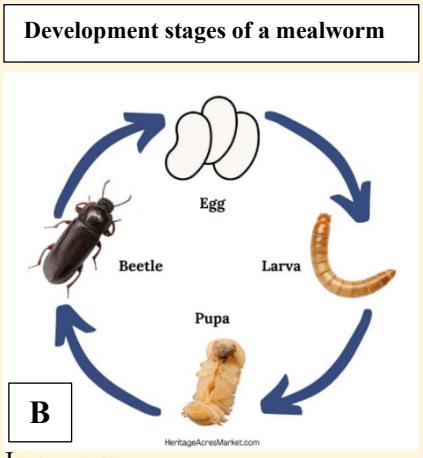


Image source: https://www.heritageacresmarket.com/mealworm-farm/

Figure 13. A graphical representation of protein concentration of mealworm at different development stages (**A**). The life cycle of a mealworm (**B**).

3.5.4 Protein comparison between buffalo worms fed on different diets

As in mealworm larvae, it was observed that protein concentration of buffalo worm larvae (figure 14B) is also greatly affected by diet. Buffalo worm larvae fed on side stream contained higher protein concentration (10.11±1.56mg/100mg) compared to those that had no special feeding (6.69±0.51mg/100mg) (figure 14A; Appendix Table 6). Figure 14C shows buffalo worm powder that was used for analysis.

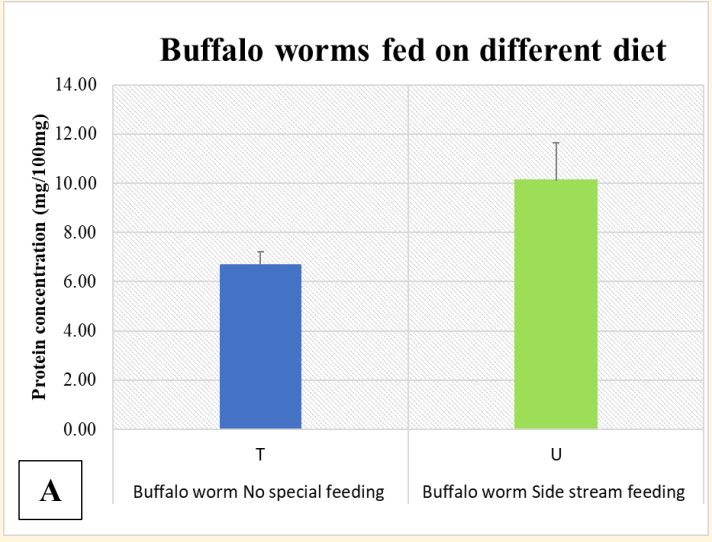


Figure 14. A graphical representation of protein concentration of buffalo worms fed on different diets (**A**). An image of buffalo worm samples (**B**) and image of buffalo worm powder obtained using liquid nitrogen and a mortar and pestle (**C**).

3.5.5 Protein comparison of all the insect samples available in this study.

From samples analysed in this study, it was observed that mealworm pupa from larvae fed on side stream yielded the most amount of protein (21.05±1.09mg/100mg) although this was not significantly different from mealworm pupa from larvae fed on full grain (19.29±1.74mg/100mg), cockchafer larvae yielded the least amount protein (1.53±0.35mg/100mg) and other samples had protein concentration in between (figure 15) and (table 1).

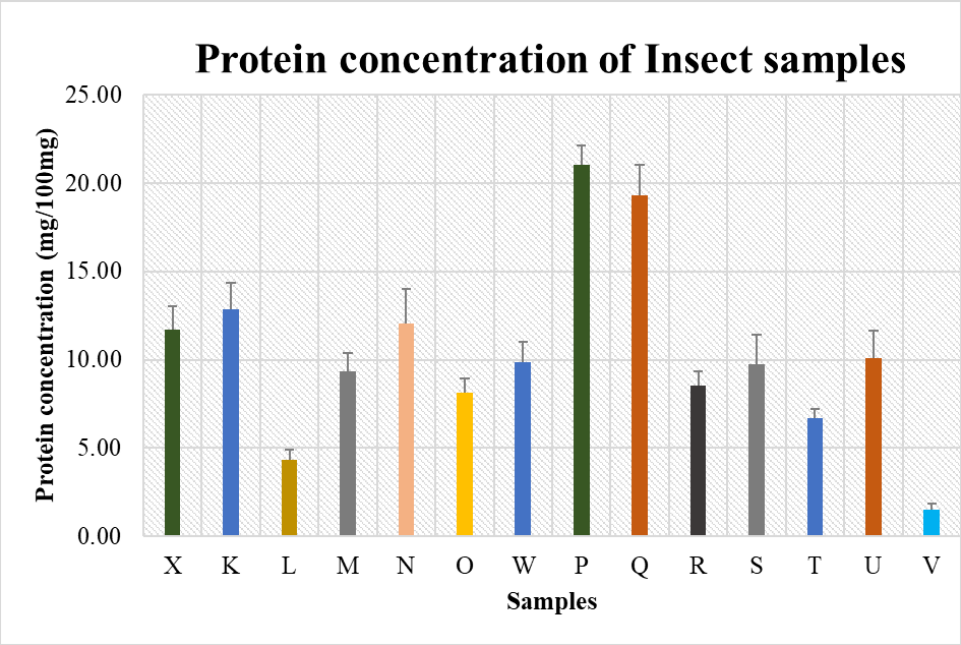


Figure 15. A graphical representation of protein concentration of all insect samples that were available for analysis.

Table 1. Protein concentration of all insect samples that were available for analysis

Sample ID	Sample	Protein concentration (mg/100mg)
X	House cricket	11.69±1.31
K	Grasshopper (migratory locust)	12.86±1.52
L	Grasshopper nymphs (migratory locusts)	4.31±0.59
M	Stick insect	9.33±1.04
N	Mealworm fed on Oats and full grain	12.07±1.95
O	Mealworm fed on side stream	8.13±0.80
W	Mealworm fed on flour	9.87±1.12
P	Mealworm Pupa fed on side stream diet	21.05±1.09
Q	Mealworm Pupa fed on full grain	19.29±1.74
R	Adult Mealworm (Beetle)fed on Oats	8.53±0.83
S	Adult Mealworm (Beetle) fed on flour	9.75±1.66
T	Buffalo worms, No special feeding	6.69±0.51
U	Buffalo worm Side stream feeding	10.11±1.56
V	Maikäfer larvea(Cockchafer)	1.53±0.35

3.5.6 Comparison of protein concentration of common protein sources.

Protein concentration from common protein sources including fish (20.84±2.12mg/100mg), shrimps (11.49±1.17mg/100mg), chicken breast (20.18±1.81), turkey breast (26.44±0.87mg/100mg), pork from neck region (23.27±1.47mg/100mg), pork form stomach

region (21.03±1.63mg/100mg) and pork fillet (30.20±2.34mg/100mg) were also analysed using urea extraction buffer and Bradford protein determination assay (figure 16; Appendix Table 9).

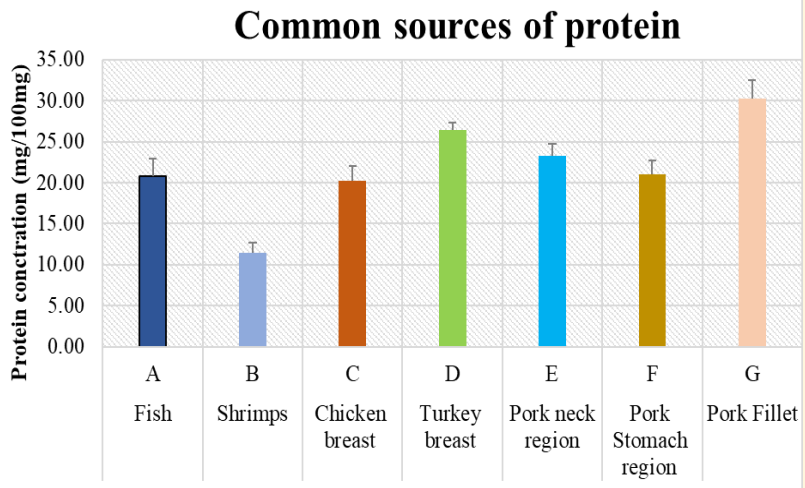


Figure 16. A graphical representation of protein concentration of common sources of protein.

3.5.7 Protein concentration from plant samples used in this study.

Protein concentration from plant samples including fresh peas (7.53±1.81mg/100mg), carrots (1.24±0.03mg/100mg) and spinach (1.88±0.05mg/100mg) were also analysed using urea extraction buffer and Bradford protein determination assay (figure 17; Appendix Table 10).

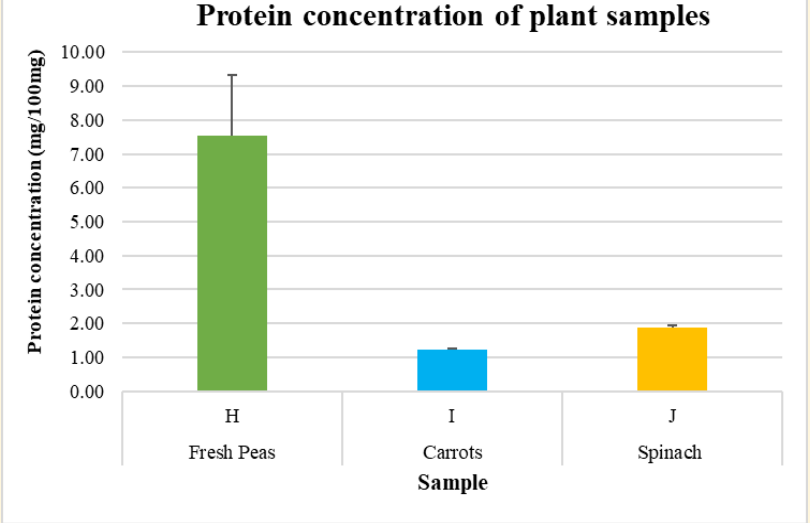


Figure 17. A graphical representation of protein concentration of plant-based samples.

4. Conclusion

This preliminary study demonstrates that different types of insects contain protein that would enhance human diet. From this study however, it can be concluded that the protein content of insect in many previous studies has been over estimated. This is due to the use of protein concentration determination methods that rely on nitrogen atoms. These methods work well for samples whose only source of nitrogen is protein. However, this is not the case for insects because they have chitin which also contains nitrogen. Nevertheless, this does not mean that insect do not contain protein at all. Insects contain a good amount of protein as shown in this study. For example, mealworm larvae contain more protein (ranging between 8.13mg/100mg – 12.07mg/100mg depending on the feeding) than fresh peas (7.53±1.81mg/100mg) which is a common source of protein (figure 17; Appendix Table 10).

Therefore, to meet the daily protein requirement for humans i.e., 0.8g of protein per kg body weight (Gaytán-González et al., 2020) implying that the average 70kg man should eat approximately 56g of protein per day and the average 60 kg woman should eat approximately 48g of protein per day. If, for example, the only source of protein are mealworm larvae fed on oats and full grain, the required amount would approximately be 463.96g for an average man and 397.68g for an average woman to meet the daily nutritional requirement for protein.

Future research areas could be in product development to make insect products more appealing and functionally usable as food products for consumers. Other studies could be focused on aspects like microbiology, pharmacology and toxicology, several nutritional aspects among others on mice fed with insect diets.

Insect development stages: Insects can be classified into two categories depending on the development stages they go through in their life cycle. These are (a) complete metamorphosis, this involves eggs, larvae, pupa, and adult (figure 13B) and (b) incomplete metamorphosis this involves eggs, nymphs, and adults (figure 11E). From this study, it can be concluded that there is more protein concentration at the pupa stage for insects that undergo complete metamorphosis regardless of the type of feeding while the adult stage contains more protein concentration for insects that undergo incomplete metamorphosis.

Protein content in insects at the larvae stage is significantly affected by the type of diet they feed on; for example, mealworms yielded more protein when fed on oats and full grain (12.07±1.95mg/100mg) compared to those fed on side stream diet (8.13±0.80mg/100mg) and those fed on flour (9.87±1.12mg/100mg). Further investigation could be carried out to determine the quality of protein and other nutritional significances of these different diets.

Insect protein extraction and determination: It can also be concluded that urea protein extraction duffer extracts more protein from insect samples, and this causes no interference with Bradford protein assay which showed more reliable protein concentration for both insects and other samples.

Best visualization of insect protein on the agarose gel can still be achieved with 12% acrylamide when samples are extracted using urea extraction buffer. 15% acrylamide gel could be used to show protein fragments for samples extracted with SDS lysis buffer, SDS buffer and urea extraction buffer without thiourea.

5. Appendix

Table 2. Protein concentration of fish, mealworm, grasshopper, and spinach samples using different protein extraction buffers and determined using Bradford assay.

	Protein concentration (mg/100mg)			
	Fish	**Mealworm**	**Grasshopper**	**Spinach**
Extraction Buffer				
RIPA	9.33 ±0.96	5.38 ±0.39	5.97 ±0.97	1.40 ±0.17
Urea without Thiourea	16.71 ±3.44	6.03 ±2.13	5.40 ±1.42	0.92 ±0.23
Urea with Thiourea	23.81 ±3.82	11.27 ±1.48	12.01 ±2.37	1.01 ±0.14
SDS Lysis	10.43 ±3.33	6.36±1.62	3.56 ±0.75	0.61 ±0.27
SDS	6.07 ±2.36	3.48 ±0.68	3.46 ±0.66	1.47 ±0.61
Defat-Urea without Thiourea	10.00 ±3.33	4.87 ±0.98	4.11 ±0.98	1.12 ±0.12

** Results reported as mean ±SD

Table 3. Protein concentration of fish and mealworm samples using different protein extraction methods and using different protein determination methods i.e., Bradford, Lowry, and BCA protein assays.

	Protein concentration (mg/100mg)					
	Fish			**Mealworm**		
	Bradford	**Lowry**	**BCA**	**Bradford**	**Lowry**	**BCA**
Extraction Buffer						
RIPA	9.33±0.96	11.71±6.25	9.84±2.02	5.38±0.39	10.26±1.91	12.15±1.63
Urea without Thiourea	16.71±3.44	17.96±4.34	31.70±5.14	6.03±2.13	17.84±6.12	18.29±5.99
Urea with Thiourea	23.81±3.82	ND	ND	11.27±1.48	ND	ND
SDS Lysis	10.43±3.33	17.40±4.67	15.99±3.45	6.36±1.62	14.32±5.24	13.15±1.88
SDS	6.07±2.36	22.12±4.46	40.62±10.59	3.48±0.68	20.89±3.88	23.65±5.81
Defat-Urea without Thiourea	10.00±3.33	16.39±3.87	34.18±7.07	4.87±0.98	15.89±4.22	19.72±6.27

** Results reported as mean ±SD
**ND – Not detected

Table 4. Protein concentration of grasshopper and spinach samples using different protein extraction methods and using different protein determination methods i.e., Bradford, Lowry, and BCA protein assays.

	Protein concentration (mg/100mg)					
	Grasshopper			**Spinach**		
	Bradford	**Lowry**	**BCA**	**Bradford**	**Lowry**	**BCA**
Extraction Buffer						
RIPA	5.97±0.97	13.61±3.88	10.11±1.28	1.40±0.17	6.75±0.72	1.75±0.46
Urea without Thiourea	5.40±1.42	25.39±6.24	34.41±6.93	0.92±0.23	6.80±1.57	2.60±0.37
Urea with Thiourea	12.01±2.37	ND	ND	1.01±0.14	ND	ND
SDS Lysis	3.56±0.75	16.86±4.61	13.17±5.04	0.61±0.27	5.69±0.88	1.83±0.26
SDS	3.46±0.66	33.67±6.82	33.45±4.49	1.47±0.61	7.39±1.33	4.94±1.01
Defat-Urea without Thiourea	4.11±0.94	26.49±6.24	32.26±5.31	1.13±0.12	8.33±1.86	2.67±0.28

** Results reported as mean ±SD
** ND – Not detected

Table 5. Protein concentration of house cricket, grasshopper, grasshopper nymphs, and stick insect.

Sample	Sample ID	Protein concentration (mg/100mg)
House cricket	X	11.69±1.31
Grasshopper (migratory locust)	K	12.86±1.52
Grasshopper nymphs (migratory locusts)	L	4.31±0.59
Stick insect	M	9.33±1.04

** Results reported as mean ±SD

Table 6. Protein concentration of buffalo worms with no special feeding and those fed on side stream diet.

Sample	Sample ID	Protein concentration (mg/100mg)
Buffalo worm with no special feeding	T	6.69±0.51
Buffalo worm Side stream feeding	U	10.11±1.56

** Results reported as mean ±SD

Table 7. Protein concentration of mealworm fed on different diets

Sample	Sample ID	Protein concentration (mg/100mg)
Mealworm fed on Oats and full grain	N	12.07±1.95
Mealworm fed on side stream	O	8.13±0.80
Mealworm fed on flour	W	9.87±1.12

** Results reported as mean ±SD

Table 8. Protein concentration of mealworm at different development stage fed on same diet.

Sample	Sample ID	Protein concentration (mg/100mg)
Mealworm fed on Oats and full grain	N	12.07±1.95
Mealworm Pupa fed on full grain	Q	19.29±1.74
Adult Mealworm (Beetle)fed on Oats	R	8.53±0.83

** Results reported as mean ±SD

Table 9. Protein concentration of common sources of protein.

Sample	Sample ID	Protein concentration (mg/100mg)
Fish	A	20.84±2.12
Shrimps	B	11.49±1.17
Chicken breast	C	20.18±1.81
Turkey breast	D	26.44±0.87
Pork neck region	E	23.27±1.47
Pork Stomach region	F	21.03±1.63
Pork Fillet	G	30.20±2.34

** Results reported as mean ±SD

Table 10. Protein concentration of plant-based samples.

Sample	Sample ID	Protein concentration (mg/100mg)
Fresh Peas	H	7.53±1.81
Carrots	I	1.24±0.03
Spinach	J	1.88±0.05

** Results reported as mean ±SD

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