2018

Protein and Lipid Characterization of Acheta domesticus, Bombyx mori, and Locusta migratoria Dry Flours

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Protein and Lipid Characterization of *Acheta domesticus*, *Bombyx mori*, and *Locusta migratoria* Dry Flours

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Thesis submitted to the

Davis College of Agriculture, Natural Resources and Design

at West Virginia University

in partial fulfilment of the requirements

for the degree of

Master of Science
in
Nutritional and Food Science

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Division of Animal and Nutritional Sciences

Morgantown, West Virginia
2018

Keywords: Entomophagy, Insects, Nutrition, Food Supply, Protein, Lipid, Amino Acids

ABSTRACT

Protein and Lipid Characterization of *Acheta domesticus*, *Bombyx mori*, and *Locusta migratoria* Dry Flours

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Cricket (*Acheta domesticus)*, silkworm pupae (*Bombyx mori)*, and locust (*Locusta migratoria)* dry flours were obtained to examine the biochemical properties and composition of the flours. This study aimed to characterize the protein and lipid components of three insect species utilizing proximate composition analyses, amino acid composition analysis, protein solubility, SDS-PAGE, fatty acid composition analysis, and thin layer chromatography to determine lipid classes, and lipid extraction efficiency. Kjeldahl determined the cricket, locust, and silkworm flours contained 72.0%, 53.1%, and 71.2% protein, respectively. All proximate composition analyses were significantly ($p<0.05$) different between species. Amino acid composition analysis revealed that the flours contained 3.6-3.9% and 0.90-1.5% of lysine and methionine, respectively. Many countries have cereal grains and legumes as a staple in their diet that contain these limiting amino acids. Essential amino acids were 22% of total amino acids. Protein solubility revealed that the three species studied were most soluble in alkaline environments with highest protein solubility occurring at pH 13 at 66% solubility in silkworm. Lowest solubility occurred in more acidic conditions between pH 4-5. SDS-PAGE revealed five major protein fractions with estimated molecular weights of 27 (cuticle proteins), 41 (arginine kinase), which has been identified as an allergen in shrimp, 42 (actin), 71 (hemocyanin), and 220 (myosin) kDa. Soxhlet extraction determined cricket, silkworm, and locust flours contained 15.4%, 33.3%, and 11.4% lipid, respectively. The only omega-3 fatty acid found in the flours was α-linolenic acid. Silkworm flour contained the most α-linolenic acid at 33.3% of total fatty

acids, followed by locust (13.7%), and cricket (0.6%). TLC resolved lipids for typical lipid classes. The four major lipid classes noted were triacylglycerol, free fatty acid, cholesterol, and phospholipid. Lipid extraction efficiency found that the organic solvents chloroform and methanol had the highest lipid extraction yield in both cricket (69.3%) and locust (93.0%). Methyl-tertiary-butyl ether (MTBE) extracted lipids most efficiently in the silkworm. Proteins and lipids found in insects need to be isolated to further or commercial use.

DEDICATION AND ACKNOWLEDGMENTS

I want to thank the Davis College at West Virginia University for funding my research. I would also like to thank my graduate committee Dr. Jacek Jaczynski, Dr. Kristen Matak, and Dr. Yong-Lak Park for sharing their knowledge with me.

I could not have completed this research without the help of the students and faculty in the Animal and Nutritional Sciences division at the university. I want to specifically thank Derek Warren, Casey Showman, Emily Nadenbousch, and Alleda Rose for their commitment to helping me succeed in the lab. I would like to thank Sarah Beamer for her guidance prior to her retirement as I learned how to navigate the lab, as well as Susan Slider for helping me troubleshoot in the lab and obtain my materials.

Lastly, I would like to thank my family and fiancé for their unending support during my time at West Virginia University

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CHAPTER I

Introduction

The Food and Agriculture Organization (FAO) of the United Nations predicts that the world population will exceed 9 billion by the year 2050 and a food crisis will occur (van Huis et al., 2013). As the population continues to grow and available land mass remains constant, traditional livestock farming may no longer be a viable method for food production. In the United States 51% of American soil alone is used for agriculture (Bigelow, 2016). Entomophagy, the practice of eating insects, is common practice throughout most of the world. However, in Western cultures, it is often seen as exotic or taboo. Insects are viewed in a negative light, yet most edible insects eat fresh plants and wood. In fact, they are more hygienic than crabs and lobsters, which consume carrion, and Western cultures consume heavily (Mitsuhashi, 2010). Producing insect-based processed foods such as insect flours, oils, protein isolates, and protein bars may help facilitate the beginning of incorporating insects into the daily diet of Western cultures (Hamerman, 2015; Mitsuhashi, 2010). These products have the potential to offer a wide array of benefits and nutrients.

Insect caloric and nutrient profiles range widely and are dependent upon developmental stage, species, and diet (van Huis et al., 2013). Protein and lipid content is largely related to feed. The insoluble fiber found in insects is in the form of chitin which makes up their exoskeleton and accounts for approximately 10% of a whole dried insect's weight (van Huis et al., 2013). Chitin is a polysaccharide that is abundant in nature. Chitinase, the enzyme that breaks down the polysaccharide has not been found in Western cultures gastric juice but appears to be present to an extent in the gastric juices of tropical countries that do engage in entomophagy (van Huis et al., 2013). This may suggest either humans lost the enzyme when they stopped practicing

entomophagy, or the enzyme developed over time in cultures heavily consuming insects. A lack of chitinase could potentially lead to gastrointestinal discomfort in the same way that cellulose does. As with all food prepared for human consumption, food safety is a concern.

There are over 2,000 species of insects that have been identified as safe for human consumption throughout the world (Rumpold & Schluter, 2013). Insects harvested from their natural habitat are not advisable to consume due to potential parasitic infection and pesticide contamination (van Huis et al., 2013; Rumpold & Schluter, 2013; Belluco et all., 2013). The most practical way to combat these issues is to rear insects in a controlled and sanitary environment similar to how humans rear livestock for food. Insects are practical to rear for many reasons including their short lifespan and their rapid rate of reproduction (Nadeau, Nadeau, Franklin, & Dunkel, 2014). Small farms rearing insects can be found throughout Thailand and generally have a harvest cycle of 45 days (Nadeau, Nadeau, Franklin, & Dunkel, 2014). This is far shorter than the harvest cycle of traditional livestock which ranges between 4 to 36 months dependent upon the type of animal and cut of meat desired (FAO, n.d.).

There has been some work in the field of entomophagy, but some species have been more commonly explored than others. The silkworm species *B. mori* appears to be the most commonly studied with *A. domesticus* coming in a close second. This work aims to characterize fundamental properties of three insect species to better understand functionality of the insects to be used as food and feed. While the global population rapidly expands, understanding the protein and lipid properties of each species is crucial to development of products that may increase acceptability of insects as a supplemental protein source in Western societies who were previously wary of adopting the practice. Not only can insects serve as a supplemental source of lipid and protein, but they require the use of less land for rearing as well as emit less greenhouse

gasses than traditional livestock. Through education, research, and marketing, insects may truly be the food of the future. Limitations of this study may include the applicability of insect flours throughout the world and their realistic availability to other nations where whole insects are a social norm and affordable. Limitations with the flours we specifically studied were that the samples came from the same vendor and though experiments were run in triplicate, each species came from the same bag.

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CHAPTER II

Review of Literature

Insect Nutrient Profile

Insect caloric and nutrient profiles range widely dependent upon developmental stage, species, and diet, but most edible insects are considered a good source of energy, amino acids, monounsaturated and polyunsaturated fats, copper, selenium, magnesium, riboflavin, zinc, and folic acid (van Huis et al., 2013).

The fiber found in insects is in the form of chitin (van Huis et al., 2013). The exoskeleton may also help protect against parasitic infection which is a potential risk when harvesting insects from their natural habitat (van Huis et al., 2013). Chitin is a polysaccharide that is abundant in nature. Interestingly, chitinase, the enzyme that hydrolyzes chitin, has not been found in Western cultures gastric juices, but appears to be prevalent in the gastric juices of cultures that do traditionally consume insects (van Huis et al., 2013). This may suggest either humans lost the enzyme when they stopped practicing entomophagy, or the enzyme developed over time in cultures heavily consuming insects. A lack of chitinase could potentially lead to gastrointestinal discomfort in the same way that cellulose does.

Insects Allergenic Components

The food allergy hypersensitivity type I is an allergic reaction mediated by immunoglobin E (IgE) (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul, 2015). Edible crustaceans are known to highly allergenic which is of concern when discussing entomophagy. Two major potential allergens in insects are arginine kinase and hemocyanin.

Arginine kinase (AK) has been identified to be a major allergen in shrimp as well as several insects. AK is a transferase enzyme that aids in energy metabolism in invertebrates. AK has been specifically identified in the silk worm species *B. mori* in the literature citing AK to be an IgE binding protein (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul, 2015). Hemocyanin, the oxygen transport protein in the phyla Arthropoda, has been identified through the literature as an allergen (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul, 2015). Shrimp are a common allergen and often the circulatory system is not removed before preparing the shrimp for consumption (Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011). This can cause a high level of hemocyanin to be in the shrimp which can trigger an allergenic immune response. This could potentially occur during insect processing as well and have the same risk. As insect consumption increases it is important to provide allergen labeling on packaging of all insect-based food products and extracts.

Insects as a Source of High Quality Protein

Developing countries have limited access to complete proteins, due to cereal grains and legumes accounting for the bulk of their diet. Notably, cereal grains contain the limiting amino acid lysine and legumes contain the limiting amino acid methionine. Insects are an ample source of supplemental protein in regions that practice entomophagy (van Huis et al., 2013). Protein content varies based upon developmental stage, but generally, adults tend to be higher in protein while pupae and larvae tend to be higher in lipids (van Huis et al., 2013).

Protein quality is often scored using a protein digestibility corrected amino acid score (PDCAAS). PDCAAS determines the true digestibility of proteins in food based on essential amino acid requirements for humans. A protein digestibility corrected amino acid score was assigned to several edible proteins in a 2015 study to assess quality of insect meat in comparison

to chicken, lab grown meat, and soy meal-based meats. (Smetana, Mathys, Knoch, & Heinz, 2015). It was found that for 0.3 kg of digestible protein, one would need to consume 0.97 kg of chicken meat, 1.25 kg of lab grown meat, 2.6 kg of insect-based meat, and 1.8 kg of soy meal based meats (Smetana, et al., 2015). These findings suggest that insects may prove useful as supplemental protein in addition to other plant and meat protein sources. In this same study, it was noted that insects did have the lowest environmental impact even though more needed to be farmed to reach equivalent protein levels (Smetana, et al., 2015). Below, a review of the current literature on insect proteins can be found.

Characterization of Insect Protein

Kjeldahl method is most commonly used to determine crude protein based on nitrogen content of the sample. A wide array of insect species have been examined at varying developmental stages throughout the literature. Tomotake, Katagirl, and Yamato (2010) studied *B. mori* pupae and found it to contain 55.6% protein. They reported that the top three amino acids present in *B. mori* were glutamate, aspartate, and proline containing 95 mg/g, 91 mg/g, and 70 mg/g, respectively. These accounted for 11.1%, 10.7 %, and 8.2% of total glutamate, aspartate, and proline, respectively. Yi, et al. (2013) worked with *A. domesticus* and reported phenylalanine, the essential amino acid, and tyrosine, the conditionally essential amino acid, totaled 92 mg/g, while the nonessential amino acid present in the highest amount was glutamic acid at 110mg/g. *A. domesticus* was reported to contain 21.5% crude protein based on live weight, not a dry weight, basis (Yi, et al., 2013). The crude protein content found by Yi, et al. was far lower than that reported by Nakagaki, Sunde, and Defoliart (1987) who reported *A. domesticus* contained 62.0% crude protein on a dry weight basis. Literature on *Locusta migratoria* (Orthoptera: Acrididae) was limited, however, Clarkson, Mirosa, and Birch (2018)

analyzed *L. migratoria* oil and found it to contain 50.8% protein. This study did not look into amino acid composition, they conducted proximate analyses and then focused on lipid components of the species. To further characterize proteins, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is often utilized.

SDS-PAGE is an analytical method in biochemistry utilized to separate proteins by their molecular mass. This common process allows for clear visualization of protein bands. Yi, et al. (2013) investigated 5 insect species, *Tenebrio molitor* (Coleoptera: Tenebrionidae), *Zophobas morio* (Coleoptera: Tenebrionidae), *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), *Acheta domesticus* (Orthoptera: Gryllidae), and *Blaptica dubia* (Blattodea: Blaberidae) to characterize their protein fractions. The study suspended the ground insects in demineralized water that contained ascorbic acid. The mixture was then run through a stainless-steel filter sieve and the filtrates and residue were collected. The samples were centrifuged at 15,000 x g for 30 minutes at 4°C. The pellet and supernatant were harvested and used independently for protein characterization. For SDS-PAGE preparation the samples were dissolved in 20 mM EDTA pH 8.0 buffer with the protein concentration of 7 mg/mL. After electrophoresis was complete, a range of protein bands <95 kDa were noted in the supernatant sample and a range of bands <200 kDa were noted in the pellet sample for all five insect species. In *A. domesticus,* bands appearing between 14-32 kDa were hypothesized to be cuticle proteins. Cuticle proteins are structural materials that make up the exoskeleton of insects that interact with chitin filaments (Andersen, Hojrup, & Roepstorff, 1995). The bands in *T. molitor* ranging from 32-95 kDa were determined to likely be enzymes or other proteins such as, melanization-inhibiting protein (43 kDa), β-glycosidase (59 kDa), trypsin-like proteases (59 kDa), and melanization-engaging types of protein (65 kDa). These bands found in *T. molitor* were also prevalent in *A. domesticus*. Similar

findings were noted by Buβler, Rumpold, Jander, Rawel, and Schluter (2016) who also looked at *T. molitor* as well as *Hermetia illucens* (Diptera: Stratiomyidae). They also identified bands ranging from 14-32 kDA as cuticle proteins and proteins ranging from 32-95 kDA as assorted enzymes and proteases.

Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul (2015) worked with *Gryllus bimaculatas* (Orthoptera: Gryllidae), a field cricket species and made note of bands at ~40 kDa and ~67-~77 kDa and identified them as arginine kinase and hemocyanin, respectively. Liu, et al (2009) found similar results when working with *B. mori* specifically to identify major allergens in the species. Liu, et al. (2009) reported arginine kinase, a transferase enzyme involved in energy metabolism to mobilize muscle, to be a major allergen in insects that weighs approximately 40 kDa. In another study, Hall, Jones, O'Haire, and Liceaga (2017) studied protein hydrolysates of the tropical banded crickets, *Gryllodes sigillatus* (Orthoptera: Gryllidae). They report bands below 212 to 14.4 kDA in their unhydrolyzed cricket protein. They did not identify what proteins each band may have been. While SDS-PAGE identifies proteins by mass, protein solubility looks at the solubilizing behavior of a protein when exposed to alkaline and acidic environments.

Protein solubility is an important measure in food science to better understand how to isolate compounds and apply them for use in products. Protein solubility also affects other functional properties of the sample such as emulsifying ability, foaming, and gelation (Purschke, Meinlschmidt, Horn, Rieder, & Jager, 2017). Several studies examined protein solubility in different insect species with all reporting an increase in solubility as conditions became more alkaline and a decrease in solubility as conditions became more acidic. Gresiana, Marpaung, Sutanto, and Si (2015) worked with the *Gryllus mitratus* (Orthoptera: Gryllidae) protein isolate

that was homogenized with distilled water. The mixture was pH adjusted and allowed to incubate at room temperature for 2 hours. They found that solubility of the protein was highest at pH 8 and lowest at pH 5. Results were given in mg/mL protein solubilized. At pH 5, only 2.43 mg/mL of protein was solubilized compared to 3.69 mg/mL of protein that was solubilized at pH 8. These findings were similar to the findings of Purschke, Meinlschmidt, Horn, Rieder, and Jager (2017) who worked with the migratory locust, *L. migratoria*. They report the minimum solubility at pH 5 (10%) and the highest solubility at pH 9 (22%), which follows the apparent trend that solubility increases as a solution becomes more alkaline. Proteins are more soluble in alkaline conditions due to a release of small peptide fragments that are associated with an increase in amino and carboxyl groups interacting with water molecules (Purschke, Meinlschmidt, Horn, Rieder, & Jager, 2017).

Zhao, Vasquez-Gutierrez, Johansson, Landberg, and Langton (2016) worked with yellow mealworms, *T. molitor*, examining the solubility of the sample at pH 3, 4, 5, 7, and 9. They report the highest solubility at pH 9 (74%) and lowest solubility between pH 3-4. The percent solubilized protein in this study far surpasses the solubility at pH 8-9 in the previous two studies mentioned above. Hall, Jones, O'Haire, & Liceaga (2017) worked with the tropical banded cricket, *G. sigillatus,* protein hydrolysates and report lowest solubility at pH 3. Low solubility in acidic environments can be explained by carboxyl groups shifting towards unionized forms that in turn reduce the peptide affinity for water molecules (Hall, Jones, O'Haire, & Liceaga, 2017). In the control group, which used the *G. sigillatus,* tropical banded cricket protein unhydrolyzed, highest solubility was reached at pH 10 (~25%). When the samples were subjected to hydrolysis, solubility greatly increased with the highest solubility occurring at pH 10 (92%). It is apparent

from this study that making the proteins more available via hydrolysis greatly impacts their ability to solubilize in a solution.

Insects as a Source of High Quality Lipid

Throughout the developing world, many people have limited access to high quality lipids due to cereal grains accounting for the bulk of their diet. It is not uncommon to consume insects throughout most of the world and these insects may prove to be an ample source of lipids in these regions (van Huis et al., 2013). Lipid content varies based upon developmental stage, but generally, pupae and larvae tend to be higher in lipids than their adult counterparts (van Huis et al., 2013). Insects contain varying amounts of fatty acids that frequently include, α-linolenic and linoleic acid, the two essential fatty acids for humans (van Huis et al., 2013). Insect lipids may prove to benefit countries where omega-3 fatty acid sources are limited. Below is a review of the current literature on insect lipids.

Characterization of Insect Lipid

Several studies have reported both the lipid content of various insect species as well as the fatty acid composition of various species. Tomotake, Katagirl, and Yamato (2010) found *B. mori* pupae to contain 32.2% lipid composed of 28.8% saturated fatty acids, 27.7% monounsaturated fatty acids, and 43.6% polyunsaturated fatty acids. The fatty acid content was determined by analysis of fatty acid methyl esters (FAMEs) and gas chromatography. The predominant fatty acids in silkworm were linolenic acid (36.3%) and oleic acid (26.0%). Clarkson, Mirosa, and Birch (2018) analyzed crude *L. migratoria* oil and report findings of linolenic acid (15.6%) and oleic acid (37.0%). Clarkson, Mirosa, and Birch (2018) continued to report that locust contained palmitic acid (27.3%) and stearic acid (7.2%) as well. They report that locust contained 34.9% lipid composed of 37.2% saturated fatty acids, 38.5%

monounsaturated fatty acids, and 24.6% polyunsaturated fatty acids. They reported the ratio of saturated to unsaturated fatty acids in the oil were equal to 0.58 while the omega-3 to omega-6 fatty acid ratio was similar at 0.55. These findings are relevant in human nutrition as it is well understood that long chain polyunsaturated fatty acids, like α-linolenic acid, have cardioprotective effects (Fleming & Kris-Etherton, 2014). Ekpo, Onigbinde, and Asia (2009) found that in their study of *Macrotermes bellicosus* (Isoptera: Termitidae), *Imbrasia belina* (Lepidoptera: Saturniidae) larvae, *Oryctes rhinoceros* (Coleoptera: Scarabaeoidea) larvae, and *Rhynchophorus pheonicis* (Coleoptera: Curculionidae) larvae the major fatty acids present in the larvae are palmitic and oleic acid, while the major fatty acids in mature adult insects are palmitic and linoleic. Beyond identification of types of fatty acids and the saturation of fatty acids, lipid classes can also be useful to identify in insects.

Thin layer chromatography (TLC) separates lipid classes by polarity across a silica plate. Polar lipids remain closer to the origin of their placement, while nonpolar lipids run to the upper end of the plate (Tzompa-Sosa, Yi, van Valenberg, van Boekel, & Lakemond, 2014). In a 2014 study analyzing insect lipid profiles, it was reported that triacylglycerols appear to make up the bulk of the lipid classes in most insect species, including *A. domesticus* (Tzompa-Sosa, Yi, van Valenberg, van Boekel, & Lakemond, 2014). This study used three extraction methods, Folch (1957), Soxhlet (1995), and an aqueous extraction detailed in Yi et al. (2013) to analyze four insect species, *T. molitor, Alphitobius diaperinus* (Coleoptera: Tenebrionidae), *A. domesticus*, and *Blaptica dubia* (Blattodea: Blaberidae). They found that unsurprisingly, both Folch and Soxhlet using organic solvents extracted the highest quantity of lipid, while the aqueous extraction extracted the least lipid. With the prepared lipid extracts, 5 µL of sample was spotted on a silica plate and the plate was then placed in a development chamber with the solvent

hexane/diethyl ether/acetic acid (70:30:1, v/v). Upon analysis of the TLC plate with *A. domesticus*, it is noted that the organic solvent extractions, Folch and Soxhlet, were able to extract a wider range of lipid classes than the aqueous extraction. With the aqueous extraction, the main lipids extracted were triacylglycerols, cholesterol esters, and carotenoids. The researchers suspect that the reason for those three lipid classes being present in the aqueous extraction while the aqueous extraction showed minimal free fatty acids, monoacylglycerol, and contained no phospholipids, is directly related to those missing classes being polar and staying in the aqueous phase of the initial extraction due to the presence of water. The organic solvent extractions allowed for more lipid classes to be extracted due to the presence of polar and nonpolar components of the organic solvents. The total lipid content varied between solvents with 1.6-7.8% from aqueous extraction, 6.0-12.7% using Soxhlet extraction, and 7.5-12.9% using Folch. Organic solvent choice is crucial to extract as much lipid as possible. Chloroform is a nonpolar solvent that easily extracts nonpolar lipids such as triacylglycerols and free fatty acids, while methanol is a polar substance that easily extracts phospholipids and cholesterol (Hara & Radin, 1978). Chloroform and methanol are the solvents that Folch (1957) used and is used in the above study by Tzompa-Sosa, Yi, van Valenberg, van Boekel, & Lakemond (2014). Other solvents popularly used for lipid extraction include, hexane, isopropanol, and methyl tertiary-butyl ether (MTBE). Hexane is an efficient solvent for lipids with low polarity, such as triacylglycerols and free fatty acids, while isopropanol extracts polar lipids such as phospholipids and cholesterol (Hara & Radin, 1978). When choosing solvents to use for lipid extraction it is crucial to understand the polarity of both the solvents, and the lipids that will be extracted.

Summary

The aim of this literature review was to investigate previous research in the field of entomophagy that characterizes insects destined for human or animal feed. There is not extensive research on the topic as it appears to be a topic that has just begun to gain interest in the last two decades. It can be concluded from the available literature that insects are a promising source of protein overall and contain a wide variety of essential and nonessential amino acids. They are also a promising source of lipids, including the essential fatty acids α-linolenic and linoleic acid. There are over 2,000 species of insects deemed safe for human consumption, yet most studies have only studied *A. domesticus, B. mori,* and *T. molitor*. More research is needed to further characterize edible insects including characterizing extracted proteins and lipids for direct human consumption.

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Chapter III

Characterization of *Acheta domesticus*, *Bombyx mori*, and *Locusta migratoria* flours for potential future isolation of protein destined for food products and feed

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Abstract

Acheta domesticus (Orthoptera: Gryllidae), *Bombyx mori* (Lepidoptera: Bombycidae), and *Locusta migratoria* (Orthoptera: Acrididae) flours were obtained to examine the biochemical properties and composition of the flours on a dry weight basis. This study aimed to characterize the protein components of three insect flours utilizing proximate composition analyses, amino acid composition analysis, protein solubility testing, and SDS-PAGE. Kjeldahl determined *A. domesticus*, *B. mori*, and *L. migratoria* contained 72.0%, 53.1%, and 71.2% protein, respectively. All proximate composition analyses were significantly ($p<0.05$) different between species. Amino acid composition analysis revealed that the flours contained 3.64-3.90% and 0.90-1.49% of lysine and methionine, two limiting amino acids, respectively. Essential amino acids were 22% of total amino acids. Protein solubility revealed that the insect flours were most soluble in alkaline environments with peak protein solubility occurring at pH 13 at 66% solubility in silkworm. Lowest solubility occurred in more acidic conditions between pH 4-5. SDS-PAGE revealed five major protein fractions with estimated molecular weights of 27 (cuticle proteins), 41 (arginine kinase), 42 (actin), 71 (hemocyanin), and 220 (myosin) kDa. By characterizing fundamental protein properties in these three insect flours, a foundation has been set to further isolate proteins for food and feed.

Key words: entomophagy; protein characterization; nutrition; insects

Introduction

The Food and Agriculture Organization (FAO) of the United Nations predicts that the world population will exceed 9 billion by the year 2050 which may lead to a global food crisis (van Huis et al., 2013). As the population rapidly expands and available land mass remains constant, traditional livestock farming alone may no longer be a viable and sustainable method for food production. There have been at least 2,000 species of insects identified as safe for human consumption (Rumpold & Schluter, 2013). Isolating insect nutrients to use in food products may aid in normalizing consumption of insects in cultures that have labeled the practice as taboo (Hamerman, 2015; Mitsuhashi, 2010). Insects contain crucial nutrients that can be overlooked in some cultures solely because of societal norms.

One crucial macronutrient insects contain is protein. Insect protein includes both essential and nonessential amino acids (van Huis et al., 2013). While there are many species of insects deemed safe for consumption, some insects that are safe are not palatable (Parry, 2011). By isolating proteins, food products can use insects that are otherwise overlooked. With the rapidly expanding population, the demand for alternate more sustainable protein sources grows. Meat based diets require more energy, land, and water than plant-based diets (Pimentel & Pimentel, 2003). This heavy usage of resources makes meat-dominated diets unsustainable in the growing world (Pimentel & Pimentel, 2003). Insects use less land than livestock but may still provide the nutritional benefit of complete proteins that livestock have offered humans for years (Chen, 2015).

Traditional livestock such as beef, pork, and chicken are primary sources of protein across the globe, but in some developing countries, the only protein accessible is from plant sources that are incomplete proteins. Incomplete proteins lack the nine essential amino acids and

in turn, the amino acids in the food source are not readily utilized. Lack of complete proteins and lack of protein can lead to protein energy malnutrition (PEM). PEM is common throughout the developing world, particularly India, and is a major health problem that often affects young children during the most crucial time of growth (Bhutia, 2014). PEM can lead to lifelong developmental disabilities and weaken immunity (Bhutia, 2014). Insects, which are accepted in most non-Western cultures, may be a solution to help populations facing PEM to increase their protein intake. Insects and insect protein isolates can supplement diets throughout the world, and, when paired with plant proteins, a meal containing complete proteins can be made. While it is unrealistic to believe that entomophagy could one day become the base of diets throughout the world, utilizing insect and insect extracts to supplement current protein sources is not so far out of reach. By further studying the nutritional properties of insects, specifically protein content, new discoveries can be made to help minimize the instance PEM.

There has been some work in the fields of entomology and food science, but the major insects explored are crickets, locusts, silkworms, mealworms, and beetles. By characterizing insect flours, future research can isolate protein from insects to further understand how to incorporate insects into the every day diet across the globe.

Materials and methods

Obtaining insect flours:

Three insect species were chosen due to their commercial availability. The flours came from a company located in Thailand. The species studied were *Acheta domesticus* (Orthoptera: Gryllidae), cricket, *Locusta migratoria* (Orthoptera: Acrididae), locust, and *Bombyx mori* pupae (Lepidoptera: Bombycidae), silkworm. The samples arrived in a powdered form in an air tight container. The crickets were reared in captivity and fed a diet of mixed grains and vegetables.

The silkworm pupae were reared on mulberry leaves and the locusts were reared on various grasses and vegetables. Upon arrival, the flours were placed in a -80°C freezer until used. All experiments were run in triplicate.

Proximate Composition:

For the determination of moisture content, 1.5 g of a sample was placed on an aluminum dish (Fisher Scientific) and spread evenly across the dish. The moisture content was determined by the oven-drying method (90 °C for 24 hours) (AOAC, 1995). Ash content was obtained by ashing a sample in a muffle furnace at 550 °C for 24 hours using method 942.05 (AOAC, 1995) and expressed as percent (dry basis). Total lipid content was determined according to the Soxhlet extraction using method 920.39 (AOAC, 1995). The sample size was 2 g and extraction with petroleum ether was performed for 16 hours at a drip rate of 10 mL/min. Total lipid content was determined on a gravimetric basis and expressed as percent (dry basis). Crude protein was determined by Kjeldahl assay method 984.13 (AOAC, 1995) and expressed as percent (dry basis). All the results were reported as the mean value of 3 replicates.

Amino Acid Analysis:

A full amino acid profile analysis was conducted according to AOAC method 982.30 E by utilizing high-performance-liquid-chromatography (1995) by the Agricultural Experiment Station Chemical Laboratories, University of Missouri-Columbia.

Protein Solubility:

A total of 2.0 grams of each powder was weighed out and placed in a 250 mL glass beaker. A Teflon coated stir bar and 20 mL of distilled deionized water (ddH_2O) was placed in each beaker. The beakers were placed on a room temperature stir plate and allowed to homogenize for 15 minutes. An Oakton pH 11 series meter was used and calibrated with the

company provided calibration solutions. Initial pH was recorded after the 15 minute period and was then adjusted using hydrochloric acid and sodium hydroxide. Once adjusted to the desired pH, the sample was then homogenized on a stir plate for 15 minutes. Upon completion, 5 mL of the solution was removed via pipette and placed into a plastic centrifuge tube. The samples were placed in the centrifuge at 21°C and spun at 10,000 x g for 10 minutes.

Upon centrifugation completion the tubes were removed and 5 µL of the supernatant was placed into a well on a 96 well plate. 250 µL of Bradford's reagent was added to each well and the plate was placed on an orbital shaker and allowed to develop for 5 minutes. The plate was placed into a plate reader spectrophotometer and absorbance was measured at a wavelength of 595 nm. Bovine serum albumin was used to create a standard curve to interpret the absorbance data from the insect samples. Bovine serum albumin at concentrations of 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2 mg/mL was used (Bio-Rad, Hercules, CA, USA). This curve was completed in triplicate. This curve allowed us to use absorbance data to determine percent solubility.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

The insect flour samples as is were weighed out and placed in 1.5 mL plastic tubes. Each sample was dissolved in 1,000 µL of 20 mM Tris/HCl, 2 mM EDTA pH 8.0 buffer that was freshly prepared. The concentration of the sample was 8 mg/mL. After homogenization was complete via vortex, 5 µL of each sample was transferred to a new labeled tube followed by adding 4.75 µL of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) to each sample tube. The tubes were vortexed for 10 seconds before adding 0.25 µL of β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) to each tube under a well-ventilated hood. The tubes were vortexed again for 10 seconds and placed in a plastic bag that was then submerged in a 100°C hot water bath for 5 minutes to allow protein denaturation. During the hot water bath, the Ready Gel® was loaded

into the electrophoresis chamber and 1x Tris/glycine/SDS buffer was poured into the chamber. Upon removal from the hot water bath the tubes were vortexed for 10 seconds. 10 well Ready Gels® containing 4-20% Tris-HCl (Bio-Rad, Hercules, CA, USA) were used to run the samples. The standard protein used to determine weight was bovine serum albumin ranging from 10-250 kD. 5 µL of the bovine serum albumin was loaded into the first well of the gel. 10 µL of each sample was loaded to each of their respective wells. Three wells were assigned for each of the three types of samples to run in triplicate on one gel. After the gel was fully loaded electrophoresis was run for 50 minutes at 200 V.

Upon electrophoresis completion the gel was removed from the chamber and the gel case and rinsed in deionized double distilled water for 5 minutes while on an orbital shaker. This was repeated three times with fresh water each time. Following the three rinses the gel was placed in 50 mL of Bio-Safe™ colloidal Coomassie Blue G-250 (Bio-Rad, Hercules, CA, USA) stain and shaken for 60 minutes. Due to the use of the Bio-Safe™ stain, the gel could be destained with distilled deionized water (ddH_2O) instead of using a mix of acetic acid and methanol as is necessary in traditional Coomassie destaining. The gel destained in distilled deionized water (ddH_2O) for 30 minutes on an orbital shaker.

Statistical analysis

JMP Pro 12 software (JMP®, Version Pro 12, SAS Institute Inc., Cary, NC, Copyright©2015) was used for all statistical analyses. A two tailed t-test comparing an average concentration to a mean concentration of the same nutrient from a different insect species was determined utilizing significance with a p-value of ≤ 0.05. ANOVA was not used for data analysis, nor were means separated, due to limited variance in the samples (lack of biological replicates), related to using samples from the same vendor and same bag (available technical

replicates). All sample data is presented as a mean of triplicate data and the standard deviation among three trials. When analyzing amino acid and protein solubility data, after significance was determined, the Benjamini-Hochberg (B-H) correction was used to correct for type I error due the high number of t-tests in those two data sets. The B-H correction ranked the p-values in ascending order and then the quantity (i/m)Q was calculated with i representing the individual p-value's rank, m representing the total number of tests, and Q the false discovery rate which was set to 10% (Benjamini & Hochberg, 1995). Then the original p-value was compared to the B-H value and all of the original p=values smaller than (i/m)Q, in the sorted lsit of p-values, were considered significant. All statistical analyses are noted in each table using superscript letters to denote significance after the B-H adjustment.

Results and discussion

Proximate Composition:

The proximate composition of the dried cricket, locust, and silkworm flours is shown in **Table 1.** Significant differences ($p<0.05$) were found between each of the three species in all proximate composition analyses. The percent moisture was found to be 4.7%, 0.5%, and 1.8% in cricket, silkworm, and locust, respectively. The percent protein on a dry weight basis in cricket, silkworm, and locust contained 72%, 53.1%, and 71.2%, respectively. There has been some discussion that a significant amount of nitrogen measured with Kjeldahl comes from the structural polysaccharide chitin leading to an overestimation of insect's true protein content, however, a 2007 study examined this concerned and concluded that chitin represents a small portion of the insect's nitrogen content and the traditional measure of nitrogen x 6.25 reasonably estimates crude insect protein (Finke, 2007).

Percent lipid on a dry weight basis determined via Soxhlet extraction was found to be 15.4%, 33.3%, and 11.4% in cricket, silkworm, and locust, respectively. In a 2011 study (Longvah, Mangthya, & Ramlu), *Samia ricinii* (Lepidoptera: Saturniidae), another silkworm species, pupae were found to contain 54.2% protein and 26.2% lipid on a dry weight basis which is comparable to our findings for *B. mori*. Findings from Buβler, Rumpold, Jander, Rawel, and Schluter (2016) report mealworm (*Tenebrio molitor*) larvae contained 53.8% crude protein on a dry weight basis. This is comparable to our findings for the silkworm pupae. Ash content is the residue remaining from a sample after heating removes the water, lipid, and protein. Percent ash in cricket, silkworm, and locust was found to contain 4.4%, 2.6%, and 3.3% ash, respectively. *A. domesticus* was found to contain 5.4% ash which is comparable to our findings of 4.4% (Finke, DeFoliart, & Benevenga, 1989). Conducting proximate analyses is critical to build a foundation to further isolate compounds.

Amino Acid Composition:

Amino acid findings can be found in **Table** 2 presented in mg/g. The flours contained 3.64-3.90% and 0.90-1.49% of lysine and methionine, respectively. Lysine is a limiting amino acid found in cereal grains and methionine is a limiting amino acid found in legumes. These two limiting amino acids are relevant to note because cereal grains are the basis of standard diets throughout the world, specifically in countries with limited protein sources already. Essential amino acids were 22% of total amino acids. Histidine, threonine, and valine in mg/g in cricket was found to be 15.2, 25.4, and 38.4, respectively. These findings are similar to those reported in Rumpold and Schluter (2013), who report the same species of cricket contained a range of 22.7-23.4, 31.1-36.1, and 48.4-52.2 mg/g, respectively. Nakagaki, Sunde, and Defoliart (1987) reported that *A. domesticus* contains 35 mg/g threonine and 22 mg/g phenylalanine which is

comparable to our finding of 25.4 mg/g and 23.4 mg/g, respectively. The remainder of the amino acid analysis from Nakagaki, Sunde, and Defoliart are present in higher amounts than ours which could potentially be explained by the crickets being reared on standard broiler chick starter mash that is high in protein. In our study, cricket was reared on mixed grains and vegetables, not chicken feed. It is well known that diet greatly influences the nutritional makeup of insects, along with developmental stage (van Huis et al., 2013). Similarly, higher amino acid content findings for cricket were reported by Finke, DeFoliart, and Benevenga (1989) which is related to the feed.

Tomotake, Katagirl, and Yamato (2010) reported *B. mori* containing higher values for all amino acids when compared with our findings. The amino acids reported in this study that are most similar to our results are those findings for glycine, histidine, and isoleucine. Tomotake, Katagirl, and Yamato (2010) found that *B. mori* pupae contained 36 mg/g, 27 mg/g, and 34 mg/g of glycine, histidine, and isoleucine, respectively. We found silkworm pupae to contain 23.5 mg/g for both glycine and isoleucine and 16.9 mg/g of histidine. The largest noted difference in our findings for silkworm when compared to the above study, is with the amino acid proline with our findings being 19.6 mg/g compared to 70 mg/g found in the 2010 study.

Protein Solubility:

Protein solubility is a critical functional property to identify when developing food and feed. The solubility of a protein can aid in protein isolation and extraction while also influencing other functional properties important in food production such as foaming and emulsifying capacity (Hall, Jones, O'Haire, & Liceaga, 2017). Throughout the literature it has been recorded that insect protein solubility is generally low and chitin can interfere with solubility. Solubility results are listed in **Table 3** and **Figure 1**. Solubility results consistently favored alkaline

environments over acidic environments with the lowest solubility at pH of 4-5 and highest solubility at pH 12-13 between the three species. This trend is similar to findings of Hall, Jones, O'Haire, and Liceaga (2017) who studied *Gryllodes sigillatus,* tropical banded crickets. While the cricket species is different, and the study used cricket protein hydrolysates versus whole cricket like we used, they found that solubility generally increased in alkaline conditions and report the lowest solubility at pH 3. These findings align with our observation of lowest solubility occurring at pH 4-5 and highest solubility between pH 12-13. Cricket protein hydrolysates like used in the previous study had higher solubility findings when compared to unhydrolyzed proteins due to the hydrolysis opening the protein and enhancing protein hydration.

Zhao et al. (2016) worked with yellow meal worm larvae (*Tenebrio molitor*) and they too reported an increase in solubility as the solution becomes more alkaline. They reported highest solubility as 80% at pH 9. Our findings at pH 9 for cricket, silkworm, and locust were 25.4%, 20.1%, and 21.5%, respectively. Proteins become less soluble in acidic environments due to carboxyl groups shifting to unionized forms which in turn reduces affinity for water molecules (Hall, Jones, O'Haire, & Liceaga, 2017). Proteins are more soluble in alkaline environments due to the release of small peptide fragments associated with an increase in ionizable groups such as amino and carboxyl groups which interact with water molecules and solubilize proteins more efficiently (Purschke, Meinlschmidt, Horn, Rieder, & Jager, 2017).

The highest percent solubility recorded for cricket was 45.5% at a pH 12. The highest solubility findings for locust and silkworm were recorded at pH 13 at 47.6% and 66%, respectively. Lowest recorded solubility for cricket was recorded at a pH 4 at 7.3% solubility. The lowest solubility findings for both locust and silkworm occurred at a pH 5 and were

recorded at 7.7% and 7.4%, respectively. At pH 13, significant differences were found when silkworm was compared to both cricket and locust, but interestingly, no significant difference was noted when comparing cricket and locust This may relate to the developmental stage of cricket and locust as well as their shared order, Orthoptera.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE resolved five major protein fractions with estimated molecular weights of 27, 41, 42, 71, and 220 kDa (**Figure 2**). The protein fraction at 27 kDa was notable in silkworm while it does not appear to be present in cricket or locust. The protein fraction estimated to be 40 kDa is present in all three species. The fraction at 71 kDa is present only in locust and silkworm, while the fraction at 215 kDa is present only in locust.

According to Andersen, Hojrup, and Roepstorff (1995), cuticle proteins weigh between 14-32 kDa. We suspect the well resolved band in silkworm estimated at ~27 kDa is from cuticle proteins. Cuticle proteins are structural materials that make up the exoskeleton of insects that interact with chitin filaments (Andersen, Hojrup, & Roepstorff, 1995). Cuticle proteins vary based upon developmental stage and interact with chitin to aid in exoskeleton development (InterPro, n.d.). Given that these proteins vary based upon developmental stage, it is not surprising that these proteins appear to only be in the silkworm pupae flour. Developmental stage greatly influences the proteins present in insect species, which has been cited throughout the literature. Yi, et al. (2013) characterized *Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus,* and *Blaptica dubia* and found five major protein fractions at <14 kDa, 14-32 kDa, 32-95 kDa, and >95 kDa. They also examined protein found in both the supernatant and pellet portions formed during sample preparation. This is significant because both *T. molitor* and *B. mori* are in the larva state which we know greatly impacts nutritional

composition of the insect (van Huis et al., 2013). The protein fraction we report at approximately 41 kDa is present in all three of the insect species and is also noted in the above research in the pellet sample in all five species. We suspect this protein is the enzyme arginine kinase which is present in insects and crustaceans (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul, 2015). Arginine kinase is a transferase enzyme that has been identified as an allergen in mollusks and arthropods. Arginine kinase was noted by Liu et al. (2009) to be present in silkworm, which corresponds with our findings for the species. They state the molecular weight of the enzyme to be ~40 kDa. There is another light band visible at ~42 kDa identified as actin. Actin and myosin are muscle proteins. We suspect that the band visible around ~220 kDa is the protein myosin. Actin and myosin are two groups of muscle proteins that bind to allow muscle contraction (Cooper, 2000). Dragonflies and mayflies are the only two insects that have flight muscles attached directly to their wings, most insects fly through a process known as indirect flight (Vickerson, 2012). While we identified actin and myosin, it should be noted that some insect flight muscles exceed 400 kDa which we were not able to measure.

Hemocyanin, which is an oxygen transport protein, is found in several crustaceans is estimated to weigh ~65-77 kDa (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phriyangkul, 2015). Hemocyanin is also found in mollusks and arthropods (van Holde, Miller, & Decker , 2001). In these phyla, the oxygen binding site involves copper, not iron like in hemoglobin transport in other species. Because this protein is found in all mollusks and arthropods it is known that each insect species studied also contains the protein. This may be why all three species visible protein fraction bands around 65-77 kDa. Hemocyanin has been noted to be an allergen as well (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, &

Phriyangkul, 2015). There is not extensive literature to our knowledge documenting insect protein electrophoresis, but it appears that what is available, does align with our findings.

Conclusion

This study characterized fundamental protein properties of insect flours; thus, laying a foundation to develop protein isolation. Insect proteins may become a basis to develop food products destined for direct human consumption. Extracting and isolating protein to potentially use for direct human consumption may be able to supplement diets lacking essential amino acids. Insects are an underutilized source of protein that appear to be sustainable. By identifying proteins through use of SDS-PAGE and measuring protein solubility, extraction and isolation of proteins can begin. Understanding the lipid classes (TLC), fatty acids composition, and lipid extraction efficiency will also allow for oils to be extracted and incorporated into diets across the globe.

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Figures and Tables

Table 1. Proximate composition (% dry weight basis) of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust).

Species	% Moisture	% Protein	% Fat	% Ash
Cricket	4.72 ± 0.07^{a}	72.0 ± 0.33^{a}	15.40 ± 1.06^{b}	4.41 ± 0.08^{a}
Locust	1.84 ± 0.01^{b}	71.20 ± 0.07^{b}	11.42 ± 1.11^{c}	3.33 ± 0.03^{b}
Silkworm	0.47 ± 0.08^{c}	53.07 ± 0.10^{c}	33.3 ± 16^{a}	2.59 ± 0.06^{c}

Data are given as means ± SD (n = 3). Mean values within columns with different letters indicate significant differences ($p < 0.05$).

Table 2. Amino acid composition (mg/g) of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust) with adult DRI reference values[1] for EAA (mg/kg/day).

Amino Acid mg/g	Cricket	Locust	Silkworm	Adult DRI[1] mg/kg/day
Alanine	58.9 ± 0.20^{a}	75.6 ± 0.70^{a}	24.6 ± 0.30^{a}	-
Arginine	40.4 ± 0.60^{a}	38.4 ± 0.70^{a}	27.6 ± 0.20^{b}	-
Aspartic acid	56.6 ± 0.30^{a}	47.4 ± 0.20^{c}	52.2 ± 0.50^{b}	-
Glutamic Acid	64.8 ± 0.30^{a}	62.0 ± 0.30^{b}	49.9 ± 0.70^{c}	-
Glycine	35.0 ± 0.70^{b}	39.4 ± 0.30^{a}	23.5 ± 0.60^{c}	-
Histidine*	15.2 ± 0.50^{a}	15.6 ± 0.00^{a}	16.9 ± 0.30^{b}	8-12
Isoleucine*	29.1 ± 0.10^{a}	29.2 ± 0.10^{a}	23.5 ± 0.10^{b}	10
Leucine*	48.3 ± 0.20^{a}	50.4 ± 0.20^{a}	37.0 ± 0.10^{c}	14
Lysine*	39.0 ± 0.30^{a}	36.4 ± 0.20^{b}	36.8 ± 0.40^{b}	12
Methionine*	11.0 ± 0.10^{b}	9.00 ± 0.10^{c}	14.9 ± 0.20^{a}	cys+met 13

Phenylalanine*	23.4±0.20[b]	20.3±0.10[c]	26.8±0.60[a]	phe+tyr 14
Proline	35.4±0.40[b]	43.1±0.40[a]	19.6±0.30[c]	-
Serine	28.7±0.60[a]	22.2±0.10[b]	19.8±0.20[c]	-
Threonine*	25.4±0.20[a]	23.3±0.10[b]	22.7±0.20[c]	7
Tryptophan*	6.80±0.20[b]	5.20±0.20[c]	9.00±0.20[a]	3.5
Tyrosine	31.8±0.70[b]	36.3±0.90[a]	31.9±0.40[b]	-
Valine*	38.4±0.90[b]	41.8±0.30[a]	21.3±0.10[b]	10

*Denotes EAAs, DRI values provided only for EAAs
[1]Krause's Food and the Nutrition Care Process, 2012
Data are given as means ± SD (n = 3). Mean values within rows with different letters indicate significant differences ($p<0.05$).

Table 3. Protein solubility of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust) flours as is in deionized double distilled water pH adjusted to pH1-13 with HCl and NaOH.

pH	Cricket %	Locust %	Silkworm %
1	14.09 ± 5.72[a]	10.39 ± 7.82[a]	14.89 ± 6.09[a]
2	23.47 ± 6.11[a,b]	25.54 ± 0.90[b]	31.70 ± 1.62[a]
3	17.51 ± 1.42[a]	13.93 ± 9.24[a]	16.41 ± 5.61[a]
4	7.26 ± 2.00[b]	11.85 ± 0.56[a]	16.17 ± 2.54[a]
5	8.07 ± 1.35[a]	7.71 ± 0.76[a]	7.42 ± 2.73[a]
6	16.27 ± 2.28[a]	12.66 ± 4.00[a]	12.72 ± 9.60[a]
7	17.74 ± 4.37[a]	17.24 ± 2.06[a]	10.41 ± 2.92[a]
8	21.98 ± 9.11[a]	19.15 ± 0.22[b]	17.68 ± 2.10[a,b]
9	25.37 ± 6.18[a]	21.45 ± 1.47[b]	20.10 ± 2.95[a,b]
10	29.28 ± 7.06[a]	19.70 ± 4.28[a]	28.57 ± 4.86[a]
11	36.28 ± 3.20[a,b]	31.12 ± 3.42[b]	37.24 ± 8.12[a]
12	45.53 ± 4.42[b]	42.28 ± 4.68[a,b]	58.49 ± 7.80[a]
13	45.01 ±1.04[b]	47.60 ± 3.62[b]	66.00 ± 3.32[a]

Data are given as means ± SD (n = 3). Mean values within rows with different letters indicate significant differences ($p< 0.05$).

Figure 1. Protein solubility of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust) flours pH adjusted pH 1-13.

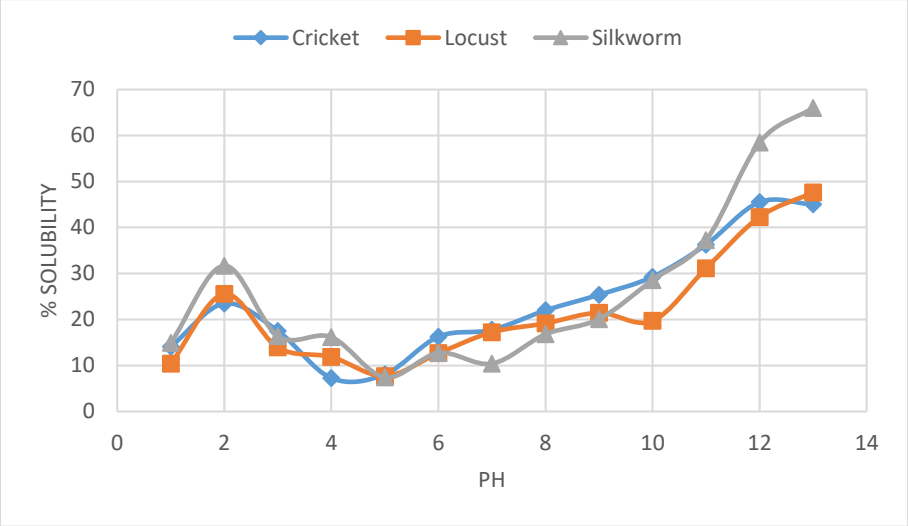
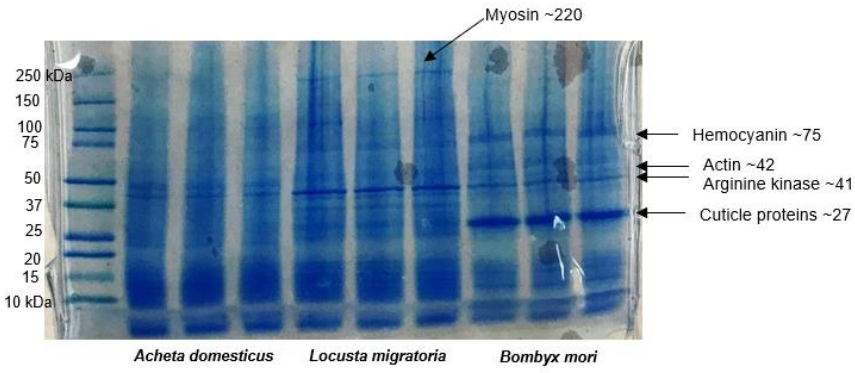


Figure 2. SDS-PAGE 4-20% Tris-HCl gel identification of five major protein fractions using literature in *Acheta domesticus*, *Locusta migratoria*, and *Bombyx mori* flours as is with protein concentrated at 8 mg/mL with a BSA 10-250 kDa reference protein.



Chapter IV

Characterization of *Acheta domesticus*, *Bombyx mori*, and *Locusta migratoria* flours for potential future isolation and extraction of oils destined for food products and feed

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Abstract

Acheta domesticus (Orthoptera: Gryllidae), *Bombyx mori* (Lepidoptera: Bombycidae), and *Locusta migratoria* (Orthoptera: Acrididae) were ordered from a single vendor to examine the biochemical properties and composition of the flours. Insect flours made of dried and ground insect were chosen as they may be more palatable to Western cultures. This study aimed to characterize the lipid components of three insect species utilizing proximate composition analyses, fatty acid composition analysis, thin layer chromatography to determine lipid classes, and lipid extraction efficiency. Soxhlet extraction determined *A. domesticus* (cricket), *B. mori* (silkworm), and *L. migratoria* (locust) contained 15.4%, 33.3%, and 11.4% lipid, respectively. All proximate composition analyses were significantly ($p<0.05$) different between species. Fatty acid composition analysis revealed the only omega-3 fatty acid found in the flours was α-linolenic acid. Silkworm flour contained highest α-linolenic acid at 33.3% of total fatty acids, followed by locust (13.7%), and cricket (0.6%). TLC resolved the four major lipid classes, triacylglycerol, free fatty acid, cholesterol, and phospholipid. Lipid extraction efficiency found that the organic solvents chloroform and methanol had the highest lipid extraction yield in both cricket (69.3%) and locust (93.0%). Silkworm had the highest lipid extraction efficiency when using the solvent methyl tertiary-butyl ether (59.7%) comparatively. More work needs to be done to continue learning more about how the extracted oils behave.

Key words: lipids; insects; entomology; entomophagy; nutrition; proximate composition; lipid extraction

Introduction

The Food and Agriculture Organization (FAO) of the United Nations predicts that the world population will exceed 9 billion by the year 2050 and a food crisis will occur because of this rapid expansion (van Huis et al., 2013). As the population grows and available land mass remains constant, traditional livestock farming may no longer be a viable and sustainable method for food production. There is suggestion that extracted insect oils may help facilitate incorporating insects into the daily diet of Western cultures (Hamerman, 2015; Mitsuhashi, 2010). These isolated oils could offer an easy way to increase monounsaturated and polyunsaturated cardioprotective fatty acids.

Traditional livestock, such as beef and pork, are known to contain high amounts of saturated fat, depending on the cut and quality, while seafood is known to contain unsaturated omega-3 fatty acids, the cardioprotective fats. Over time, high saturated fat intake can increase low density lipoprotein (LDL) cholesterol and high LDL cholesterol is correlated with fatty deposits in the arteries (Mahan, Escott-Stump, & Raymond, 2012).

When looking at developing landlocked countries who have limited access to seafood and primarily raise livestock for food, insects may offer a sustainable and affordable supplemental source of healthy omega-3 fatty acids otherwise limited in their diet. Insects contain varying amounts of fatty acids that frequently include, α-linolenic and linoleic acid, the two essential fatty acids for humans (van Huis et al., 2013). It is well understood that developmental stage plays a key role in lipid content with larvae and pupae containing higher amounts of lipid than their adult counterparts (Ramos-Bueno, Gonzalez-Fernandez, Sanchez-Muros-Lozano, & Garica-Barroso, 2016). Insect lipids are not only important for human consumption and foodstuffs, but also offer other resources such as biodiesel and fatty acid soaps

that can be used in cosmetics (Ramos-Bueno, Gonzalez-Fernandez, Sanchez-Muros-Lozano, & Garica-Barroso, 2016).

There has been some work characterizing insect extracted lipids, however, the current literature focuses more on protein. This work will characterize lipid by determining the percent lipid and fatty acid profile of each species on an as is basis (flour). This research will also extract lipids from each species and use that extracted lipid to identify lipid classes with TLC as well as identify which organic solvents will extract most efficiently. With this information, insect lipids can be extracted and used not only in food and feed, but as a supplement as well.

Methods

Obtaining the flours:

Three insect species were chosen due to their commercial availability and cost. All flours came from the same vendor. The species studied were *Acheta domesticus* (Orthoptera: Gryllidae), cricket, *Locusta migratoria* (Orthoptera: Acrididae), locust, and *Bombyx mori* pupae (Lepidoptera: Bombycidae), silkworm. The flours were a dry ground powder that arrived in an air tight bag. The crickets were reared in captivity and fed a diet of mixed grains and vegetables. The silkworm pupae were reared on mulberry leaves and the locusts were reared on various grasses and vegetables. Upon arrival, the flours were placed in a -80°C freezer until used. All experiments were run in triplicate.

Proximate Analyses:

Moisture content was determined by placing 1.5 g of a sample in an aluminum dish (Fisher Scientific) with the flour spread evenly across the dish. The moisture content was determined by the oven-drying method (90 °C for 24 hours) (AOAC, 1995). Ash content was obtained by ashing a sample in a muffle furnace at 550 °C for 24 hours using method 942.05 (AOAC, 1995).

Lipid content was determined via Soxhlet extraction using method 920.39 (AOAC, 1995). The sample size was 2 g and extraction with petroleum ether was performed for 16 hours at a drip rate of 10 mL/min. Total lipid content was determined on a gravimetric basis and expressed as percent (dry basis). Crude protein was determined by Kjeldahl assay method 984.13 (AOAC, 1995) and expressed as percent (dry basis). All the results were reported as the mean value of 3 replicates.

Fatty Acid Analysis:

Fatty acid profile analysis was conducted according to AOAC methods (1995) of analyzing fatty acid methyl esters (FAMES) with gas chromatography by the Agricultural Experiment Station Chemical Laboratories, University of Missouri-Columbia.

Thin layer chromatography (TLC):

Lipid samples were prepared for TLC plating using the Bligh and Dryer (1959) extraction method as follows. 0.5 g of cricket powder, 0.5 g locust powder, and 0.25 g silkworm powder were weighed out and placed into their respective 35mL Teflon-lined screw-capped Pyrex glass centrifuge test tube. Less silkworm powder was used due to its high fat content as determined by proximate composition via Soxhlet (see **Table 1**). Each of the three samples was extracted and run in triplicate. 5 mL of Trizma/EDTA was added to each tube. The tube was then vortexed for 60 seconds. After vortex, 20 mL of CMA (2:1:0.015) was added to each tube and was vortexed for 60 seconds in two 30 second intervals. The tubes then incubated at room temperature (~21°C) for 10 minutes. After the incubation period, the tubes were loaded into a centrifuge and spun at 10,000 x g for 10 minutes at 10°C. A second 35mL Teflon lined screw cap glass centrifuge test tube for each sample was placed into a test tube rack with a glass funnel in each. A 90 mm Whatman filter paper was placed into each filter and rinsed three times with

Chloroform:Methanol (C:M) (2:1) to assure a clean funnel and filter paper. After centrifugation, the lipids in each sample separated and were carefully removed using a Pasteur pipet and ran through their corresponding funnel and filter paper. This was repeated for all the samples. After the lipids were extracted via Pasteur pipet, 10 mL of CM (4:1) was added to each tube before being vortexed for 60 seconds and then placed back into the centrifuge at 10, 000 x g for 10 minutes at 10°C. After centrifugation, the remaining lipids in the samples separated again and were extracted with a Pasteur pipet and ran through their corresponding funnel and filter paper. After completing funnel use, the filter paper and funnel were rinsed directly into the sample tube with CM (2:1). Tubes were placed in a heating rack at 60°C and blown down with nitrogen gas. After completion of drying, nitrogen gas filled the tube, to avoid oxidation in storage, and the tube was then capped and stored at -20°C.

Silica gel plates were used for chromatography. The plates were heated at 100°C for 15 minutes before use and then allowed to cool at ambient temperature for 5 minutes. Upon cooling, the plate was marked with graphite pencil 2.5 cm from the bottom and a straight horizontal line was drawn with a pencil. Along the line "lanes" of equal width were made and marked for each standard as well as each sample in triplicate. Standards used were phospholipids, cholesterol, oleic acid, and mono-di-triglycerides. The standards and the insect samples were concentrated at 5µL/mL. 20 µL of each standard and sample was placed in each lane horizontally by making a line of each sample using four 5 µL spots. The spots dried in a ventilated hood for 5 minutes. While the TLC plate dried, 200 mL of solvent made from hexane: diethyl ether: acetic acid (78:20:2, v/v) was poured into the TLC development chamber. The lid was placed on the chamber and allowed to sit for 15 minutes to saturate the air in the chamber with the solvent. The spotted TLC plate was placed in the chamber and allowed to develop until the solvent front

reached 2 cm from the top of the plate (~1 hour). The plate dried out of the chamber for 10 minutes and 50% aqueous sulfuric acid was then poured onto each plate. The plate saturated in the 50% aqueous sulfuric acid dried overnight in a ventilated hood. The next day the dry plate was placed into a 100°C drying oven for 40 minutes. Upon removal from the oven the colors and locations of each band was recorded to identify lipid components of the samples. TLC spots were quantified using densitometry. Densitometry assesses the light sensitivity of the lipid classes to determine which class is present in the highest amount. The darker the lipid class, the higher percentage it received.

Lipid Extraction Efficiency:

A one step organic solvent extraction with various organic solvents was used due to traditional methods, such as Folch (1957) and Bligh and Dryer (1959), being time consuming and labor intensive. The one step approach was more concise and left less room for human error than traditional methods.

1 gram of sample was added to a 35mL Teflon-lined screw-capped Pyrex glass centrifuge test tube with 10 mL of organic solvent to have a 1:10 ratio between sample and solvent. The five organic solvents used were hexane, chloroform, C:M (2:1), methyl tertiary-butyl ether (MTBE), and hexane:isopropanol (3:2). The test tube was vortexed for 60 seconds. After vortex, the tube contents were transferred into a glass 250 mL Pyrex beaker containing a Teflon coated magnetic stir bar. The beakers were sealed with Parafilm on the bottom layer and then aluminum foil on the top layer. Two layers were required to keep the organic solvent from deteriorating the Parafilm and evaporating. The beakers containing the samples and stir bars were placed on a stir plate and allowed to homogenize for 15 minutes at room temperature. After homogenization the beaker contents were transferred back into the original Pyrex tube. This tube was then

centrifuged at 900 x g at 10°C for 10 minutes. A second 35mL Teflon lined screw cap glass centrifuge test tube to use for the lipid transfer was weighed. The lipid layer that separated from centrifugation was then transferred with a glass Pasteur pipet over 1-PS filter in a glass funnel and collected into second test tube. The filter was pre-rinsed 3 times with 5 mL 2:1 C:M to remove trace silicone residue on the clean filter. After filter paper was dried from the rinsing the extracted lipids were ran through the filter paper. After the extraction contents filtered and the filter paper was allowed to dry the filter paper was discarded, and the inside and outside of the glass funnel was rinsed with 1 pipette of 2:1 C:M. Samples were blown under nitrogen gas in a 60°C water bath for 60 minutes. Extraction efficiency was determined with the following two equations:

$$\%\ Lipid\ extracted = \frac{wt\ of\ tube\ with\ dried\ down\ sample - wt\ of\ empty\ tube}{initial\ sample\ wt} * 100$$

$$\%\ Lipid\ extraction\ efficiency = \frac{\%\ lipid\ extracted}{\%\ ipid\ determined\ by\ soxhlet} * 100$$

Statistical analysis

JMP Pro 12 software (JMP®, Version Pro 12, SAS Institute Inc., Cary, NC, Copyright©2015) was used for all statistical analyses. A two tailed t-test compared means between species utilizing significance with a p-value of ≤0.05. ANOVA was not used for data analysis, nor were means separated, due to limited variance in the samples (lack of biological replicates), related to using samples from the same vendor and same bag (available technical replicates). All sample data is presented as a mean of triplicate data and the standard deviation among the three trials. All statistical analyses are noted in each table using superscript letters to denote significance.

Results and Discussion

Proximate Analysis:

The proximate composition of the dried cricket, locust, and silkworm flours is shown in **Table 1.** Significant differences ($p<0.05$) were noted between each of the three species in all proximate composition analyses. Cricket, silkworm, and locust contained 4.7%, 0.5%, and 1.8% moisture, respectively. The percent protein on a dry weight basis in cricket, silkworm, and locust contained 72%, 53.1%, and 71.2%, respectively.

Percent lipid on a dry weight basis determined via Soxhlet extraction was found to be 15.4%, 33.3%, and 11.4% in cricket, silkworm, and locust, respectively. In a 2011 study (Longvah, Mangthya, & Ramlu), *Samia ricinii* (Lepidoptera: Saturniidae), another silkworm species, pupae were found to contain 26.2% lipid on a dry weight basis which is comparable to our findings for silkworm (33.3%). Ash content is the residue remaining from a sample after heating at high temperatures removes all organic compounds. Percent ash in cricket, silkworm, and locust was found to contain 4.4%, 2.6%, and 3.3% ash, respectively. *A. domesticus* was found to contain 5.4% ash which is comparable to our findings of 4.4% (Finke, DeFoliart, & Benevenga, 1989). Conducting proximate analyses is critical to build a foundation to further isolate compounds.

Fatty Acid Profile:

The major fatty acids across the three species were palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. **Table 2** contains the fatty acid composition. All three species were significantly different ($p<0.05$) from one another except in the case of oleic acid. Locust and cricket contained 22.4% and 22.0% oleic acid, respectively. This comparison was found to be statistically insignificant ($p>0.05$). The only omega-3 fatty acid found in the flours was α-

linolenic acid. Silkworm flour contained the highest amount of α-linolenic acid at 33.3% of total fatty acids, followed by locust (13.69%), and cricket (0.61%). α-Linolenic acid findings for silkworm were comparable to findings from Tomotake, Katagirl, & Yamato (2010) who reported that α-linolenic acid made up 36.3% of all fatty acids in *B. mori*. Clarkson, Mirosa, and Birch (2018) analyzed crude *L. migratoria* oil and report similar findings for palmitic acid (27.3%), stearic acid (7.2%), and α-linolenic (15.6%). Major saturated fatty acids across out three species were palmitic and stearic which is also comparable to the findings of Tomotake, Katagirl, & Yamato (2010) who identified these two fatty acids as the major saturated fatty acids in *B. mori*. These findings are relevant in human nutrition as it is well understood that long chain polyunsaturated fatty acids, like α-linolenic acid, have cardioprotective effects (Fleming & Kris-Etherton, 2014).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were not detected in any of the species studied. However, it is well understood that that α-linolenic acid serves as a precursor for the synthesis of EPA and DHA, which are very-long-chain omega-3 polyunsaturated fatty acids (Fleming & Kris-Etherton, 2014). Humans are unable to produce omega-3 and omega-6 fatty acids which is why they must be included in the diet (Mahan, Escott-Stump, & Raymond, 2012). Ekpo, Onigbinde, and Asia (2009) found that in their study of *Macrotermes bellicosus, Imbrasia belina* larva, *Oryctes rhinoceros* larvae, and *Rhynchophorus pheonicis* larvae the major fatty acids present in larvae are palmitic and oleic acid, while the major fatty acids in mature adult insects are palmitic and linoleic. The findings, while not representative of the specific species we used, align with our findings for the two adult species cricket and locust and the one pupae, silkworm.

The ratio of saturated/unsaturated fatty acids present across the three species examined was 0.72. In locust we found the saturated/unsaturated fatty acid ratio to be 0.67. Clarkson, Mirosa, and Birch (2018) found a similar saturated/unsaturated fatty acid ratio in *L. migratoria* oil. They reported a ratio of 0.58 in the species. Clarkson, Mirosa, and Birch (2018) report a ratio of 0.55 in *L. migratoria* oil. This difference in the omega-3 and omega-6 fatty acid ratio may be attributed to Clarkson, Mirosa, and Birch (2018) using extracted oils versus flour. Their insects were fed oat grass and ground oats, while our insects were fed various grasses and vegetables.

Thin Layer Chromatography:

TLC resolved lipids for typical lipid classes. The lipid classes separated on the silica TLC plates by polarity. Lipid classes were quantified using densitometry. Polar lipids, such as phospholipids, remain close to their starting location on the plate while nonpolar lipids, such as triacylglycerols, run to the upper end of the plate (Tzompa-Sosa, Yi, van Valenberg, van Boekel, & Lakemond, 2014) Both cricket (**Figure** 1) and locust (**Figure** 2) are visually similar in lipid classes as expected due to their shared order, Orthoptera, as well as their estimated developmental stage. The four major lipid classes in each species were, triacylglycerol, free fatty acids, cholesterol, and phospholipids. Silkworm largely contained triacylglycerols accounting for 74.2% of the four major lipid classes analyzed. On the silica plate, it is notable that the triacylglycerols in the silkworm species are not visually similar to the other two species. This is related to the triacylglycerols accounting for the bulk of lipid classes in that species. Cricket largely contained nonpolar triacylglycerols. These triacylglycerol findings for cricket were not surprising due to findings from Tzompa-Sosa, Yi, van Valenberg, van Boekel, and Lakemond (2014) reporting that triacylglycerols appear to make up the bulk of the lipid classes in the species. Locust largely contained free fatty acids accounting for 41.3% of the four major lipid

classes analyzed while triacylglycerols accounted for 24.7% of major fatty acids in the species. Triacylglycerols were the largest lipid class in both cricket and silkworm. Across all three species, cholesterol when quantified with densitometry, did not account for a large portion of lipid classes. This may differ between orders and developmental stages as well as feed, but in these findings, it is evident that triacylglycerols, free fatty acids, and phospholipids account for most of the lipid classes in the species.

Lipid Extraction Efficiency:

Five organic solvents were used to extract lipids in each of the three species. Extraction efficiency results can be seen in **Table 3**. No significant differences ($p>0.05$) in extraction efficiency were found between cricket and locust regardless of organic solvent used. Both cricket and locust had the highest extraction rate using the one step organic solvent extraction method with C:M as the solvent, with an extraction efficiency of 93.0% for locust. Tzompa-Sosa, Yi, van Valenberg, van Boekel, and Lakemond (2014) report the Folch extraction method, which uses C:M, yielded high lipid extraction rates similar to Soxhlet method, which may explain why the C:M solvent used yielded highest extraction rates in our study. C:M extracted significantly different ($p<0.05$) amount of lipids when comparing silkworm (35.0%) to both cricket (69.3%) and locust (93.0%). The solvent hexane was significantly different ($p<0.05$) when comparing silkworm and locust which had an extraction efficiency of 51.3% and 75.1%, respectively. MTBE is a nonpolar solvent, which is why it had the highest extraction efficiency in silkworm. Nonpolar triacylyglycerols accounted for approximately 74.2% of the four major insect lipid classes in the species. Therefore, MTBE extracted most efficiently in the species containing predominately triacylglycerols. Free fatty acids, cholesterol, and phospholipids were all present in similar amounts to one another in silkworm. Chloroform is a nonpolar solvent that easily

extracts nonpolar lipids such as triacylglycerols and free fatty acids, while methanol is a polar substance that easily extracts phospholipids and cholesterol (Hara & Radin, 1978). Both cricket and locust saw the highest extraction efficiency with the chloroform and methanol solvent. Both species, cricket and locust contained predominantly nonpolar triacylglycerols and free fatty acids as determined by densitometry analysis. Hexane:isopropanol was initially proposed for use by Hara and Radin (1978) as an alternative to the popular solvent mixture, chloroform and methanol, proposed by Folch (1957). The hydrocarbon hexane and the alcohol isopropanol are less toxic than chloroform (Hara & Radin, 1978). Hexane is an efficient solvent for lipids with low polarity, such as triacylglycerols and free fatty acids, while isopropanol extracts polar lipids such as phospholipids and cholesterol (Hara & Radin, 1978). The nonpolar nature of hexane and isopropanol unsurprisingly extracted the second most efficiently in silkworm (54.1%) due to the specie's high triacylglycerol content. Hexane alone extracted the second most efficiently in both cricket (42.7%) and locust (75.1%) unsurprisingly, due to the nonpolar nature of the solvent and both species largely containing triacyglycerols and free fatty acids. When choosing solvents to use for lipid extraction it is crucial to understand the polarity of both the solvents, and the desired lipids to be extracted.

Conclusion

This study characterized fundamental lipid properties of insect flours; thus, laying a foundation to extract oils. Insect lipids may become a basis to develop food products destined for direct human consumption. By extracting and isolating insect lipids, they can be used to supplement diets lacking omega-3 fatty acids. Insects rearing appears to be sustainable currently and has much to offer. While insect lipids may not replace lipids coming from other farmed animals, they may serve as a supplement and a healthy alternative. Understanding lipid classes

(TLC), fatty acids composition, and lipid extraction efficiency will also allow for oils to be extracted and incorporated into diets across the globe.

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Tables and Figures

Table 1. Proximate composition (% dry weight basis) of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust).

Species	% Moisture	% Protein	% Fat	% Ash
Cricket	4.72 ± 0.07^{a}	72.0 ± 0.33^{a}	15.40 ± 1.06^{b}	4.41 ± 0.08^{a}
Locust	1.84 ± 0.01^{b}	71.20 ± 0.07^{b}	11.42 ± 1.11^{c}	3.33 ± 0.03^{b}
Silkworm	0.47 ± 0.08^{c}	53.07 ± 0.10^{c}	33.3 ± 16^{a}	2.59 ± 0.06^{c}

Data are given as means ± SD (n = 3). Mean values within columns with different letters indicate significant differences ($p < 0.05$).

Table 2. Fatty acid composition (w/w%) of *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust) using FAMEs analysis (w/w%) compared to the DRI (g/day) for adult men ages 31-50 years old[1].

Fatty Acid	Cricket	Locust	Silkworm	DRI
Palmitic (16:0)	23.10 ± 0.44^{a}	22.32 ± 0.11^{b}	20.62 ± 0.08^{c}	-
Stearic (18:0)	9.96 ± 0.17^{b}	10.26 ± 0.10^{a}	6.55 ± 0.05^{c}	-
Oleic (C18:1n9c)	22.02 ± 0.35^{b}	22.35 ± 0.14^{b}	30.60 ± 0.06^{a}	-
Linoleic (18:2n6)	35.19 ± 0.26^{a}	23.02 ± 0.57^{b}	5.74 ± 0.03^{c}	17
Linolenic (18:3n3)	0.61 ± 0.01^{c}	13.69 ± 0.69^{b}	33.34 ± 0.08^{a}	1.6

Data are given as means ± SD (n = 3). Mean values within rows with different letters indicate significant differences ($p < 0.05$).

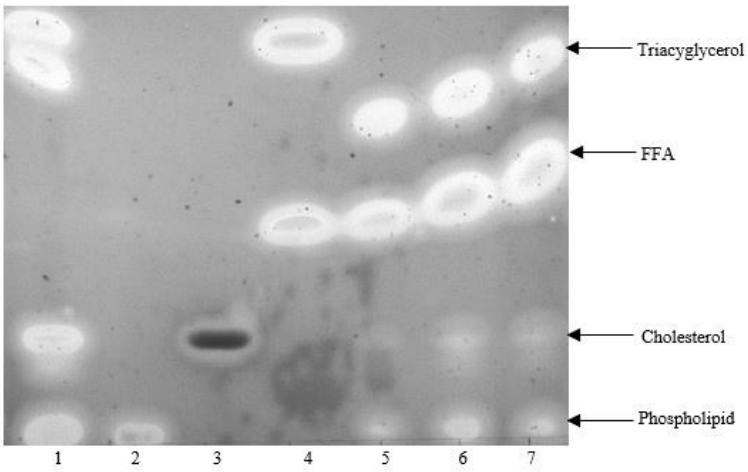
[1] Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (2005)

Table 3. Lipid extraction efficiency in *Acheta domesticus* (cricket), *Bombyx mori* (silkworm), and *Locusta migratoria* (locust) using the five organic solvents, chloroform:methanol (2:1), chloroform, hexane, heaxane:isopropanol (3:2), and methyl-tertiary-butyl ether (MTBE)

Species	C:M	Chloroform	Hexane	Hexane:Isopropanol	MTBE
Cricket	69.3 ± 4.9[a]	37.7 ± 14.4[a]	42.7 ± 14.2[a]	23.6 ± 14.5[a]	27.7 ± 7.6[a,c]
Silkworm	35.0 ± 5.8[b]	42.1 ±14.0[a]	51.3 ± 11.0[b,a]	54.1 ± 15.6[a]	59.7 ± 8.1[a,b]
Locust	93.0 ± 20.4[a]	40.0 ± 4.8[a]	75.1 ± 23.7[a]	18.5 ± 8.2[a]	66.2 ± 53.2[a]

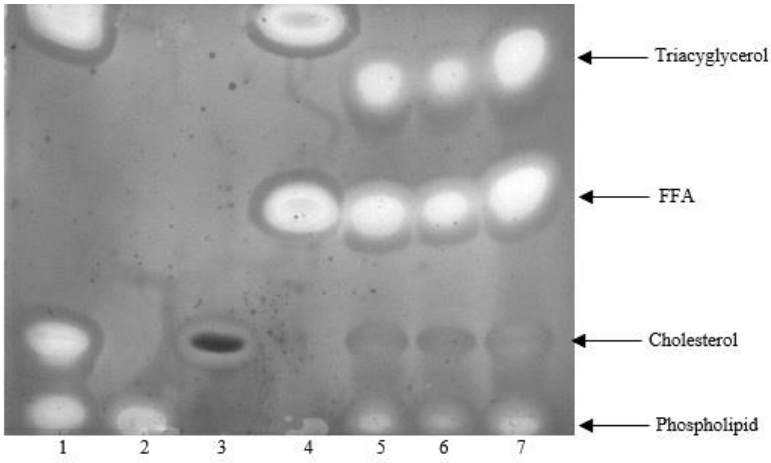
Data are given as means ± SD (n = 3). Mean values within columns with different letters indicate significant differences ($p < 0.05$).

Figure 1. *Acheta domesticus* (cricket) extracted lipid (5 µL/mL) thin layer chromatography plate[1,2]



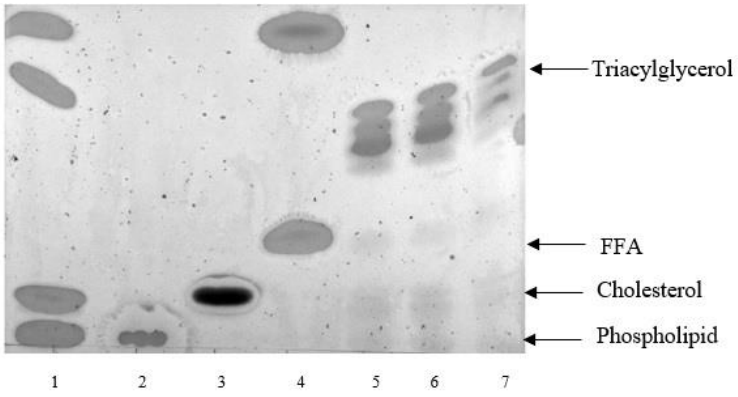
[1]Colums 1-4 are fatty acid standards: mono-di-triacylglycerol, phospholipid, cholesterol, and free fatty acid (FFA), respectively
[2]Colums 5-7 are *A. domesticus*

Figure 2. *Locusta migratoria* (locust) extracted lipid (5 µL/mL) thin layer chromatography plate[1,2]



[1]Colums 1-4 are fatty acid standards: mono-di-triacylglycerol, phospholipid, cholesterol, and free fatty acid (FFA), respectively
[2]Colums 5-7 are *L. migratoria*

Figure 3. *Bombyx mori* (silkworm) extracted lipid (5 µL/mL) thin layer chromatography plate[1,2]



[1]Colums 1-4 are fatty acid standards: mono-di-triacylglycerol, phospholipid, cholesterol, and free fatty acid (FFA), respectively
[2]Colums 5-7 are *B. mori*