A Study on the Potential of Insect Protein and Lipid as a Food Source

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Thesis

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CHAPTER 1

GENERAL INTRODUCTION

1 INTRODUCTION

With an increase in the world population, an increased consumer demand for protein, and a large amount of available agricultural land occupied, the availability of sustainably produced meat is considered to be a serious challenge for the future. Global population doubled from 2.7 to 6 billion between 1950 and 2000. In the meantime, meat production increased with a factor five from 45 to 233 billion kg per year. In 2006 FAO estimated that in 2050 the world population has reached up to 9 billion and meat consumption up to 410 billion kg per year (Aiking, 2011; Boland et al., 2013). Animal proteins are of higher nutritional quality than plant proteins (Sant'Ana et al., 2011). In developed countries, the main protein sources consist of cereals (29.1%) and meat (26.4%) (USDA, 1993), while in developing counties mainly cereals (58.8%) are consumed. The reason is that animal proteins are generally more expensive (Friedman, 1996).

The exploration of alternative protein sources is urgently required to meet the growing food demand. Generally, the main protein sources are meat, fish, milk and legumes (*e.g.* soybeans, and broad beans) in foods. Beside conventional protein sources, novel protein sources (like insects, algae, and duckweed) are proposed in recent research (van der Spiegel, Noordam, & van der Fels-Klerx, 2013) as an alternative for animal-derived proteins to enter the European feed and food market.

1.1 Insects as a source of food

The number of insect species accounts for approximately 80% of the species within the animal kingdom. Entomophagy means the habit of eating insects as food (Gahukar, 2011). Traditionally, there are 113 countries where "Entomophagy" is common practice, and in these countries more than 2,000 insect species [1] are consumed. Most of these countries are situated in Africa, Asia and Latin America (Rumpold & Schluter, 2013; van Huis, 2013). As a food source, insects are nutritious, rich in protein and they provide minerals and vitamins.

[1] http://www.wageningenur.nl/en/Expertise-Services/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm, according to the list of edible insect species in the world updated by Yde Jongema

Insect production is a potential agricultural business because insects have a high nutritional value and their rearing has a low environmental impact (Oonincx et al., 2010; van Huis, 2013). In fact, insects are already used as a natural ingredient for human food applications. For example, the red colorant carmine (E120) used in yogurt is an extract of the female cochineal insect (Verkerk et al., 2007). In most developed countries people dislike eating insects due to their "dirty" and "scary" image (Chen, Feng, & Chen, 2009). For consumer acceptance, protein extracts from insects for further use in food products may be particularly relevant for countries that have no history of consuming insects, such as Europe and North America (Del Valle, Mena, & Bourges, 1982). Insects have a long history of being eaten in other parts of world. In Africa, about 250 edible insect species are known (van Huis, 2003). Banjo, Lawal, & Songonuga (2006) reported that fourteen edible species *e.g.* termites (*Macrotermes spp.*) and grasshoppers (*Ruspolia nitidula*) are collected and investigated in terms of nutritional value, among others in Uganda. Also, Ghaly (2009) investigated that *Anthoaera zambezina* (saturniid caterpillars; 56.7% protein in dry matter) and *Gonimbrasia belina* (mopane caterpillar; 48% protein in dry matter) were studied for food use in Zambia. In China, around 178 species of edible insects are utilized as health food and Chinese traditional medicine. There common edible insects are larvae of honeybees, silkworms, and houseflies (Chen, Feng, & Chen, 2009). In Mexico, Ramos-Elorduy et al., (1997) analysed over 68 indigenous species of edible insects and reported the protein contents for the following orders: 34-72% for Lepidoptera, 52-77% for Orthoptera, and 20-69% for Coleoptera. These values were expressed in g/100g dry weight.

Whole insects are normally consumed as egg, larvae, pupae or adult depending on the insect order (Table1.1). The main orders of edible insects are Lepidoptera, Coleoptera, Orthoptera, Diptera, Hymenoptera, and Isoptera, accounting for 80% of the edible species known.

Table 1.1: Commonly eaten insect species

Order	English name	Consumption stage
Lepidoptera	Butterflies and moths	Larvae
Coleoptera	Beetles	Larvae
Orthoptera	Locusts, crickets, cockroaches, and grasshoppers	Adults
Diptera	Flies	Larvae
Hymenoptera	Ants, bees, and wasps	Adults/larvae
Isoptera	Termites	Adults

Insect farming holds promise in terms of a sustainable form of agriculture. Several benefits on cultivating edible insects for food consumption have recently been reported and investigated: insects have a high feed conversion efficiency compared with conventional livestock. van Huis (2013) stated that the feed conversion of House cricket (*Acheta domesticus*), as an example, can be calculated to be twice as efficient as that of chickens, almost 4 times more efficient than for pigs and over 12 times more than for cattle. Cultivating insects for protein has less environmental impact than cattle ranching, due to less production of greenhouse gas and NH_3 emissions (Oonincx et al., 2010). In a life cycle assessment, the production of one kg of edible protein from mealworms resulted in less greenhouse gas emissions, and required much less land than from chicken, pork or beef (Oonincx & de Boer, 2012). Besides, high yield and low environmental impact, insect feeds can be obtained from a wider range of plants than that of conventional livestock, such as cattle or swine (Durst & Shono, 2010).

1.2 The Yellow mealworm (*Tenebrio molitor*)

Nowadays, the majority of edible insects are collected in the wild. However, safety and quality monitoring for rearing and harvesting insects are needed to ensure food or feed safety (Rumpold & Schlüter, 2013). Therefore, candidate species for insect rearing under

controlled conditions are chosen due to factors including their size, safety, reproductive and survival potential, nutritional benefits. Potential for storage, and marketability, were also mentioned as reasons to select candidate insect species (Schabel, 2010). Due to availability in the Netherlands and their frequent use as animal feed, several mealworm species (Coleoptera: Tenebrionidae) were selected for this PhD study, in particular the Yellow mealworm (*Tenebrio molitor*), but also the Lesser mealworm (*Alphitobius dioperinus*) and the Superworm (*Zophobas morio*). The feed of *T. molitor* mainly consists of wheat, wheat bran, oats, soy, rye, corn, carrot and beer yeast. The species has four distinct life stages, which are egg, larva, pupa and adult, illustrated in Figure 1.1. The larval stage takes normally around 6 - 8 months at optimal conditions, but can be up to 2 years depending on temperature, humidity, feed and water availability. During the larval stage, *T. molitor* has a length of 2.0-2.5 cm, and mainly keeps eating and growing in order to save up energy for the next transformation. The colour of the larva changes from white to brown over time (Ghaly & Alkoaik, 2009). Next to that, the larva grows a hard exoskeleton quickly for body support and protection.

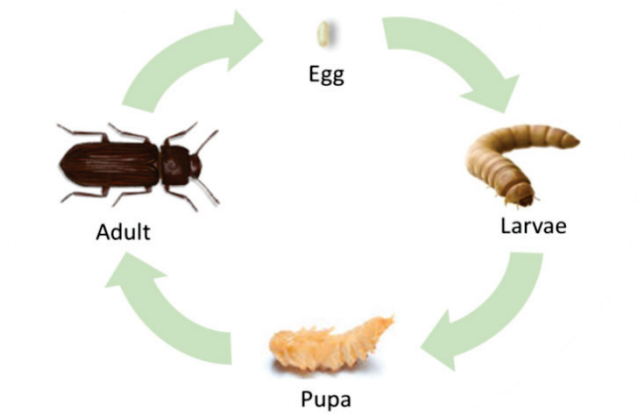

Figure 1.1: The life cycle of *T. molitor*

1.3 Proteins

Amino acids are building blocks for proteins in the human body. Furthermore, proteins contribute to flavour of food and colour formation in processing and food storage. Next to

this, proteins are important for physical properties of food with regard to texture, emulsification, foam and gel formation. Using crude protein extracts has its limitations for food applications. These limitations include undesirable colour or taste, and weak gel forming ability (Belitz, Grosch, & Schieberle, 2004). Therefore, further purification of proteins from crude protein extract could be used to enhance the physical and sensory properties of foods. A variety of protein extraction and separation techniques is used in food production. These separation techniques are widely applied and make use of the biochemical properties of food proteins , *e.g.* protein solubility, size, charge, or adsorption characteristics (Smith & Nielsen, 2010).

1.4 Protein quality of *T. molitor*

According to Jones, Cooper, & Harding (1972); Martin, Rivers, & Cowgill (1976); Pennino, Dierenfeld, & Behler (1991); Barker, Fitzpatrick, & Dierenfeld (1998); Finke (2002); Ghaly & Alkoaik (2009), the proximate crude protein content of *T. molitor* ranges from 48 to 66% on a dry basis (about 19 - 26% on a fresh basis), while other components include lipids, carbohydrates, fibre and ash. The crude protein content of *T. molitor* is comparable, or even higher than that of conventional meat sources that have protein content of about 15 to 22% on a fresh basis. The chemical composition or nutritional value of intact edible insects has been reported in many articles (*e.g.* Finke, 2002; Ramos-Elorduy et al., 2002) before, often from an entomological and zoo-biology science point of view; still very little information from a food science point of view is available.

In order to evaluate insect protein quality, amino acid composition of *T. molitor* was determined by Jones, Cooper, & Harding (1972); Finke (2002); Ramos-Elorduy et al. (2002), and their results are compared in Table 1.2. According to those studies, the sum of essential amino acids (EAA) of *T. molitor* was between 387 and 488 mg/g of crude protein, which is higher than the essential amino acids of daily requirements (277 mg/g crude protein) for human. Differences between essential amino acids were explained by differences in feed composition (Ramos-Elorduy et al., 2002).

Table 1.2: Amino acid profile of *T. molitor* in comparisons from various literature sources

Amino acids (mg/g total protein content)	Jones et al., 1972	Ramos-Elorduy et al., 2002	Finke, 2002
Histidine	39	29	32
Isoleucine	45	40	51
leucine	81	69	108
lysine	50	47	55
Methionine + Cystine	21	21	22
Phenylalanine + Tyrosine	124	77	110
Threonine	48	36	42
Tryptophan	-	9	8
Valine	61	59	60
Sum of EAA	469	387	488
Alanine	68	42	84
Arginine	41	77	53
Asparagine	61	72	-
Aspartic acid	-	-	83
Glutamic acid	112	110	115
Glycine	42	52	56
Proline	76	-	71
Serine	121	43	52
Sum of total AA	990	783	1000

The nutritional value of a food protein is evaluated not only in terms of amino acid composition, but also in protein digestibility. No information is available on protein digestibility of *T. molitor* proteins. However, few studies gave an indication that some insect species might give poor digestibility, for instance, termites, locusts, and grasshoppers due to their hard exoskeletons containing chitin (consisting of nitrogen). Most of these studies measured protein digestibility by using nitrogen determination. This could result in inaccurate estimation of protein content determination because of the presence of non-protein nitrogen (Bukkens, 1997; Dufour, 1987).

1.5 Insect lipid

Several studies (Hanson et al., 1985; Yang, Siriamornpun, & Li, 2006; Womeni et al., 2009) showed that insects and their larvae are potential sources of lipids. Since lipids have several physiological and biochemical functions in insects, it is not surprising that they are rich in fat.

Bukken (1997) reported that the total fat content for Lepidoptera (*e.g.* caterpillar) ranges from 8 to 59 g/100 g dry weight; the total fat content for Coleoptera (*e.g.* palm weevil) ranges from 8 to 42 g/100 g dry weight. However, Orthoptera (*e.g.* cricket) had a relatively lower level of lipids ranging from 10 to 20 g/100g dry weight. As mentioned by Pennino, Dierenfeld, & Behler (1991), Barker, Fitzpatrick, & Dierenfeld (1998), and Finke (2002), the crude lipid content of *T. molitor* ranged between 23 and 43 g/100 g dry matter. The crude lipid content of the Lesser mealworm (*A. diaperinus*) is around 20 g/100 g dry matter, and that of the House cricket (*A. domesticus*) is found to be from about 13 to 23 g/100g dry matter according to studies by Barker et al. (1998); Iverson, Lang, & Cooper (2001). No literature is available for the Dubia cockroach, *Blaptica dubia,* probably because it was not considered as edible insect species. Furthermore, Ramos-Elorduy et al. (1997) stated that insects are high in fat in comparison to other protein sources, and thus provide more calories. They found that on average the caloric value for serving 100 g whole insects is 50% higher than that of soybeans; 87% higher than for corn; 63% higher than for beef; 70% higher than for fish, lentils and beans; and 95% higher than for wheat by analysing 97 insect species. No literature exists on characteristics of insect lipids that can be used as an alternative lipid source. Such knowledge is of relevance in view of future expected fractionation of insect lipid.

1.6 AIM AND OUTLINE OF THIS THESIS

The objective of this thesis is to investigate protein quality of insects and their protein fractions in order to gather knowledge for supporting the use of insect proteins for human consumption. The specific objectives were firstly to extract protein fractions from insects by using an aqueous extraction procedure, and to characterize the obtained protein

fractions in terms of chemical and physical properties. Besides elucidating chemical and physical properties, protein digestibility of those fractions was also to be determined. Next to several protein-rich fractions, a lipid fraction was obtained as a by-product from the extraction. The effect on extraction method on chemical properties of lipid fraction was investigated in relation to two extractions (aqueous and Soxhlet) with relation to possible industrial methods and one extraction (Folch) with relation to a commonly used laboratory method.

In this thesis, we first decribed the potential of using insects as a future protein source for food (**chapter 2**). In this chapter, protein characteristics and functionality were determined and evaluated for five insect species, *T. molitor, Z. morio, A. diaperinus, A. domesticus, and B. dubia* industrially rearedin the Netherlands. The specific objectives of this study were to: (a) extract proteins and characterize obtained fractions; (b) evaluate protein purity and yield of the obtained fractions; (c) establish some functional properties of the protein fractions focused on foaming and gelation; (d) study protein quality by analysis on protein content and amino acid composition.

In **chapter 3**, we focussed on *T. molitor* as target protein source, and investigated mainly the effect of pH and NaCl levels on protein yield of water-soluble protein fractions of *T. molitor*, as well as prevention of brown colour formation during protein extraction in order to obtain high protein quality. The specific objectives were: 1) to investigate protein solubility of water-soluble fractions as influenced by pH and salt concentration; 2) to prevent browning reactions to take place during extraction using sodium bisulphite and ascorbic acid; 3) to purify crude protein further with acid precipitation and determine protein yield & purity; 4) to investigate molecular distributions of protein fractions obtained after precipitation.

In **chapter 4**, the objective was: 1) to investigate protein digestibility of the ground whole insect and its fractions (supernatant, pellet and residue) after *in vitro* gastric-duodenal digestion, and 2) to identify which specific proteins were present in the fractions studied.

For protein identification, LC-MS/MS was used. Protein digestibility was determined by o-phthaldialdehyde (OPA) method.

In **Chapter 5,** in view of future expected industrial bio-fractionation of insects, we investigated the influence of extraction methods on chemical characteristics of insect lipids. Lipids from *Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus* and *Blaptica dubia*, reared in the Netherlands, were extracted by two industrial extraction processes (aqueous & Soxhlet) and one laboratory method (Folch extraction). Chemical characterization in terms of fatty acid composition (GC-FID), triacylglycerol profile (GC) and lipid classes (TLC) was performed on all the extracted lipids.

Finally, the final **chapter 6** summarizes the major findings of this thesis, which is followed by a discussion and recommendation for further research.

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CHAPTER 2

EXTRACTION AND CHARACTERIZATION OF PROTEIN FRACTIONS FROM FIVE INSECT SPECIES

Yi, L., Lakemond, C. M. M., Sagis, L. M. C., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2013). Extraction and characterization of protein fractions from five insect species. **Food Chemistry**, 141, 3341-3348**.**

Extraction and characterization of protein fractions from five insect species

ABSTRACT

Tenebrio molitor, *Zophobas morio*, *Alphitobius diaperinus*, *Acheta domesticus* and *Blaptica dubia* were evaluated for their potential as future protein source. Crude protein content ranged from 19 - 22% (Dumas analysis). Essential amino acid levels in all insect species were comparable with soybean proteins, but lower than for casein. After aqueous extraction, next to a fat fraction, a supernatant, pellet, and residue were obtained, containing 17 - 23%, 33 - 39%, 31 - 47% of total protein, respectively. At 3% (w/v), supernatant fractions did not form stable foams and gels at pH 3, 5, 7, and 10, except for gelation for *A. domesticus* at pH 7. At 30% (w/v), gels at pH 7 and pH 10 were formed, but not at pH 3 and pH 5. In conclusion, the insect species studied have potential to be used in foods due to: 1) absolute protein levels; 2) protein quality; 3) ability of forming gels.

KEYWORDS

Insect protein; Protein extraction; Protein characterization; Foaming; Gelation

1 INTRODUCTION

1.1 Insects as a source of food

In most developed countries, human consumption of insects is infrequent or even culturally inappropriate, although its nutritional value is comparable to conventional meat (van Huis, 2013). In many regions and countries of the world, insect form part of the human diet and it is a misconception to believe that this is prompted by starvation (van Huis, 2013). About 1900 insect species are consumed globally as human food in the world (http://www.ent.wur.nl/UK/Edible+insects/Worldwide+species+list/).

With an increase in the world population, increased consumer demand for protein, and the amount of available agricultural land being constrained, the sustainable production of meat will represent a serious challenge for the future. Insects can be considered as an alternative protein source with less environmental impact (van Huis, 2013). Insects can be consumed as a whole. However, they can also be processed in less recognizable forms, which may increase consumer acceptability. Insects are already used as natural food ingredient, *e.g.* the red colorant carmine (E120) used in yogurt is an extract of the female cochineal insect.

1.2 Edible insects

Insects are consumed in different life stages like eggs, larvae, pupae or adults. The main species consumed are in order of importance: beetles (Coleoptera); caterpillars (Lepidoptera); ants bees and wasps (Hymenoptera); grasshoppers and locusts (Orthoptera); true bugs, aphids and leafhoppers (Hemiptera); termites (Isoptera) and flies (Diptera) and some others. Lepidoptera, Coleoptera, and Diptera (including flies) are commonly consumed in the larval stage; while the Orthoptera, Hymenoptera, Hemiptera and Isoptera are mainly consumed in the adult stage.

Cultivating edible insects for food consumption has several advantages: 1) Insects have a high feed conversion efficiency compared with conventional livestock. For example, the feed conversion ratio of house cricket (*Acheta domesticus*) can be calculated twice as efficient as chickens, almost 4 times more efficient than pigs and over 12 times more than

cattle (van Huis, 2013); 2) Cultivating insects for protein has less environmental impact than cattle ranching, due to less production of greenhouse gas and NH_3 emissions (van Huis, 2013); 3) Besides the higher production yield and less environmental impact, insect feeds can be obtained from a wider range of plants than that of conventional livestock, such as cattle or swine (Durst & Shono, 2010). Overall, insect farming can be introduced in terms of a sustainable form of agriculture.

1.3 Proteins of edible insects

As a food source, insects are potentially nutritious, rich in protein and fat, and providing a certain amount of minerals and vitamins. Studies on protein quality, nutritional value, protein content, and the amino acid composition of various insects were performed by (Ladrón de Guevara et al., 1995; Renault et al., 2006; Barker, Fitzpatrick, & Dierenfeld, 1998). The protein content of common edible insects was around 9 - 25% (Finke & Winn, 2004), and the Yellow mealworm beetle larvae (24%) (Ghaly & Alkoaik, 2009), *Zophobas morio* larvae (19%) (Finke, 2002), and *Acheta domesticus* adult (19%) (Finke & Winn, 2004) to compare with conventional meat protein sources (about 15 to 22%) (Ghaly & Alkoaik, 2009). In addition, some insects have not only protein content comparable to meat, but also to plant protein (up to 36.5%).

People may consume insect food more easily when unrecognizable insect protein (extracts) is incorporated in food in comparison to consuming whole insects. Del Valle, Mena, & Bourges (1982) also indicated that extraction of proteins from insects for further use in food products is particularly relevant for countries that do not have the habit of consuming insects, such as Europe and North America.

In this study, there are five insect species selected based on their availability (species reared by companies in the Netherlands): three species of Coleoptera considered edible, including the Yellow mealworm (*Tenebrio molitor*), the Superworm (*Zophobas morio*), the Lesser mealworm (*Alphitobius diaperinus*) and one species of Orthoptera; the House cricket

(*Acheta domesticus*) considered edible and one of the Blattodea; the Dubia cockroach (*Blaptica dubia*) not edible, but can be reared in large numbers and used for animal feed.

1.4 Objective

Although researchers from entomological and zoo-biology science have studied intact edible insects, still very little information from a food science point of view is available on characteristics and functionality of extracted insect proteins.

The aim of this study was to investigate if insects could be used as a future protein source in food. Therefore, insect protein characteristics and functionality were determined and evaluated for each of the five insect species. The specific objectives of this study were to: (a) extract proteins and characterize obtained fractions; (b) evaluate protein purity and yield of the obtained fractions; (c) establish some functional properties of the protein fractions focused on foaming and gelation; (d) study protein quality by analysis on protein content and amino acid composition.

2 MATERIALS AND METHODS

2.1 Insects used

Tenebrio molitor, Z. morio, A. diaperinus, A. domesticus and B. dubia were purchased from the commercial supplier Kreca V.O.F, Ermelo, the Netherlands. *Tenebrio molitor, Z. morio, A. diaperinus* species were supplied in the larvae stage, *A. domesticus and B. dubia* in the adult stage. The feed for *T. molitor*, and *Z. morio* mainly consisted of wheat, wheat bran, oats, soy, rye, corn, carrot and beer yeast. The feed for *A. diaperinus, A. domesticus* and *B. dubia* mainly consisted of carrot and chicken mash obtained from Kreca V.O.F. All insects were sieved to get rid of feed and stored alive at 4 ºC for about one day before processing.

2.2 Analysis of water content, protein, and fat content

All fresh insects were frozen using liquid nitrogen and subsequently ground using a blender (Braun Multiquick 5 (600 Watt), Kronberg, Germany). Frozen ground insects were freeze-dried (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands) to

determine moisture and dry matter content. The freeze-drying process was stopped at a stable sample weight. Next, the freeze-dried insects were used for protein content analysis. Crude protein content was determined by Dumas (Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience, Breda, the Netherlands) using a protein-to-nitrogen conversion factor of 6.25. D-methionine (Sigma, CAS nr. 348-67-4) was used as a standard. Furthermore, fat content was determined after hexane extraction (Biosolve, CAS nr. 110-54-3) in a Soxhlet apparatus for 6 hours. Afterwards, hexane was removed using a Rotary evaporator (R420, Buchi, Switzerland). Defatted insect meal was stored at - 20 ºC. All experiments were performed in two duplications of the same sample.

2.3 Determination of amino acid composition and protein quality

Amino acid composition of freeze-dried insect powder was analysed using ion exchange chromatography following the International standard ISO 13903:2005. Tryptophan was determined by reversed phase C_{18} HPLC using fluorescence detection at 280 nm, according to the procedure described by International standard ISO 13904:2005. The amino acid composition of the five insect species was compared to literature data of soybean protein and casein, representing high quality proteins among vegetable and animal proteins (Sosulski & Imafidon, 1990; Young & Pellett, 1994). Protein quality was evaluated by the essential amino acid index (EAAI), which is based on the content of all essential amino acids compared to a reference protein, being values for human requirements in this case (Smith & Nielsen, 2010). EAAI gives an estimate on the potential of using insects as a protein source for human consumption without correcting for protein digestibility (Equation 1).

$$\text{EAAI} = \sqrt[9]{\left(\frac{\text{mg of lysine in 1 g of test protein}}{\text{mg of lysine in 1 g reference protein}}\right) \times (\text{etc. for the other 8 essential amino acids})}$$

2.4 Protein extraction procedure

For protein extraction, 400 g of N_2-frozen insects was used. After adding 1200 mL demineralized water, that was mixed with 2 g ascorbic acid beforehand, blending for one

minute took place (Braun Multiquick 5 (600 Watt), Kronberg, Germany). Then the obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 500 µm. The filtrates and residues were collected. After centrifugation at 15,000 g for 30 min at 4 ºC, three fractions were obtained from the filtrate: the supernatant, the pellet, and the fat fraction. The residue, the pellet and the supernatant fractions were freeze dried for further analysis. The freeze-dried supernatant and pellet fractions of all insect species studied were characterized in terms of colour, protein content and molecular weight distribution using SDS-PAGE. The extraction procedure was performed in duplicate starting twice with a new insect batch.

2.5 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight distribution of the insect protein fractions. For the detection of the supernatant, pellet and residue fractions, 12.5% acrylamide Phastgels (15 kDa to 250 kDa) and 20% acrylamide Phastgels (2 kDa to 150 kDa) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used. The applied markers were ordered from SigmaMarker (S8445, wide range, molecular weight 6.5 - 200 kDa SigmaMarker). The samples were dissolved in 20 mM Tris/HCl, 2 mM EDTA pH 8.0 buffers with protein concentration of 7 mg/mL and placed in an ultrasonic bath for 10 min. The protein concentration of samples was calculated based on protein content (Dumas) and amount of dry matter. Next, protein solutions were diluted with ratio 1:1 in a sample buffer, containing 20 mM Tris/HCl, 2 mM EDTA pH 8.0 (Across Organics, Cas nr. 6381-92-6), 5% (w/v) SDS (Sigma, Cas nr. 152-21-3), 0.016% (w/v) DTT (DL- Dithiothreitol, Sigma, Cas nr. 3483-12-4), 0.02% Bromophenol Blue (Merck, Cas nr. 115-39-9). Afterwards, the samples were heated at 100 ºC for 5 min and centrifuged for 2 min at 10,000 rpm before applying to the gel.

2.6 Foamability and foam stability

The stability of foam stabilized by insect supernatant protein was determined using foam tubes with a diameter of 2.0 cm, and a glass grid at the bottom (Deak, Murphy & Johnson, 2007). The tubes were filled with 20 mL supernatant solution with a concentration of 3%

w/v, at pH 3, 5, 7, and 10. The solutions were aerated from below with nitrogen gas, at a flow rate of 10.0 mL/min. Some of the samples had insufficient foamability to form stable foam at these concentrations. For those samples with sufficient foamability, the samples were aerated until the foam level reached 30 cm. After stopping the flow of gas, the height of the foam was determined as a function of time. From these curves, the half-time of the foam (the time in which foam height is reduced by 50%) was determined. All tests were performed in duplicate.

2.7 Gel formation

2.7.1 Visual observation of gelation

Insect supernatant solutions were heated in a water bath (86 ± 1 ºC) for 10, 20 and 30 min. The supernatant fractions were dissolved at concentrations of 3% w/v and 30% w/v at pH 3, 5, 7 and 10. Depending on the initial pH, the final pH was adjusted by slowly adding 1 and 5 M HCl/ NaOH solutions. Gel formation was determined through visual observation. If the liquid was not moving upon turning the tube, it was considered a gel. This method was previously used by Beveridge, Jones, & Tung (1984) for albumin gel formation. Experiments were performed in duplicate.

2.7.2 Strain sweeps

Freeze-dried supernatant fractions from five insect species were used for this experiment. Protein solutions were prepared as followed: freeze-dried supernatant fractions were dissolved in demineralised water at a concentration of 15% w/v, stirred for 30 minutes at room temperature and adjusted to pH 7 using 1 M NaOH.

To determine the rheological properties of the supernatant protein solutions and gels made from them, oscillatory strain tests were performed on a stress-controlled rheometer (Physica MCR 501, Anton Paar, Graz, Austria) with stainless steel and titanium CC-10 concentric cylinder geometry (diameter inner cylinder: 9.997 mm; diameter cup: 10.845 mm). After filling the geometry with supernatant solution, all samples were covered with a

thin layer of silicone oil to prevent sample evaporation. Samples were first heated from 20 to 90 °C at a heating rate of 1 °C/min (phase 1), kept at 90 °C for 5 min (phase 2), and cooled to 20 °C at a rate of 3 °C/min (phase 3). During the temperature ramp, the storage modulus G' and loss modulus G" were determined by applying oscillatory deformations with a strain amplitude of 0.005 and a frequency of 0.1 Hz. The point at which G' started to increase and became greater than the background noise, was designated as the gelation temperature (Renkema, Knabben, & van Vliet, 2001).

After formation of the gel, an oscillatory strain sweep was performed on the samples, with strains ranging from 10^{-4} to 10, and a frequency of 0.1 Hz. Strain sweeps were also performed to confirm whether this strain was in the linear response regime. All samples were tested at a supernatant fraction concentration of 15% (protein content of around 8% for five types of insects) w/v. *Tenerio molitor* was also tested at concentrations of 7% (protein content of 4.1%), and 30% (protein content of 16.6%) w/v. Values for G' for this fraction from the linear response regime were plotted against protein concentration C , and the exponent n, in the relation $G' \sim C^n$, was determined using linear regression to obtain information on the structure of the gels. For all fractions also the maximum linear strain, where G' starts to decrease as a function of increasing strain, was determined. This was done by separately fitting the data points in the linear regime and the fully nonlinear regime, and extrapolating both curves to their point of intersection. This method of determining the maximum linear strain is only approximate, but since we are not interested in the absolute value of this strain, but rather in the differences in this strain for the various protein samples, this approximation was considered sufficiently accurate. All tests were performed in duplicate.

3 RESULTS AND DISCUSSION

3.1 Chemical composition of five insect species

The proximate composition of five insect species with regard to moisture, fat, protein was determined on live weight basis (Table 2.1). The moisture content of the five insect species ranged from 60% to 71%, fat content ranged from 3.6% to 16%, and crude protein from 19%

to 22% (including chitin nitrogen). Other components, calculated by difference, ranged from 3.4% to 7.5%.

Table 2.1: Proximate composition of five insect species on live weight basis (mean ± S.D., n=2).

Insects	Moisture (%)	Fat (%)	Crude protein (%) (including chitin nitrogen)	Other components (%) (*e.g.* carbohydrates, minerals and vitamins)
T. molitor	63.5±1.8	9.9±1.0	19.1±1.3	7.5±2.2
A. diaperinus	64.5±1.0	8.5±0.2	20.6±0.1	6.4±1.0
Z. morio	59.9±5.4	16.0±0.7	20.7±0.3	3.4±5.5
A. domesticus	70.8±2.0	3.6±0.4	21.5±0.5	4.1±2.1
B. dubia	67.4±2.1	7.7±0.1	19.3±0.9	5.6±2.3

The proximate composition of *T. molitor* was comparable to the results of (Jones, Cooper, & Harding, 1972; Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002; Ghaly & Alkoaik, 2009). In addition, the crude protein content measured for *A. domesticus* and *Z. morio*, 19.3% and 20.6% respectively, was comparable to the range described in literature, namely 17.3% to 20.5% (Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002). For *A. diaperinus* and *B. dubia*, no crude protein data are available in literature. The measured crude protein contents of the five insect species might be relatively higher than their actual protein content, since amounts of nitrogen are also bound in the exoskeletons as chitin. Barker, Fitzpatrick, & Dierenfeld (1998) reported that 5 - 6% of total nitrogen was measured as chitin-bound nitrogen in *T. molitor*. This would lead to an overestimation in protein content of 1.1 - 1.3% on fresh weight basis. It is a reasonable estimate for true protein content in most insect species. However, no detailed study on this issue is available.

The measured protein content of the tested insect species (around 20%) in this study is comparable with that of beef (18.4%), chicken (22.0%) and fish (18.3%) (Ghaly, 2009b). Further, measured insect protein content was higher than that of lamb (15.4%), pork (14.6%)

(Ghaly, 2009), eggs (13%), and milk (3.5%), but lower in comparison to soy (36.5%) (Young & Pellett, 1994).

3.2 Amino acid composition and protein quality of five insect species

The insect protein quality of the insect species was estimated by the amino acid composition (Table 2.2). The larvae of *A. diaperinus, T. molitor and Z. morio* contained all the essential amino acids in quantities that are necessary for humans (FAO/WHO/UNU, 1985).

Table 2.2: Amino acid pattern of five insect species, casein, soybean protein, recommendation for adult and calculated essential amino acid index of five insect species and casein & soybean protein (FAO/WHO/UNU, 1985) and (Young & Pellett, 1991).

unit (mg/g crude protein)	*A.diaperinus*	*T.molitor*	*Z.morio*	*A.domesticus*	*B. dubia*	Casein	Soybean	1985 (FAO/ WHO/ UNU)
Essential amino acid (EAA)								
Histidine	34	29	31	21	23	32	25	15
Isoleucine	43	43	46	36	31	54	47	30
Leucine	66	73	71	66	56	95	85	59
Lysine	61	54	54	53	43	85	63	45
Methionine + Cysteine	26	26	24	25	23	35	24	22
Phenyl-alanine + tyrosine	120	100	111	92	93	111	97	38
Threonine	39	39	40	35	32	42	38	23
Tryptophan	12	12	14	9	8	14	11	6
Valine	58	61	63	55	52	63	49	39
Sum of EAA	459	437	454	392	361	531	439	277
Non-essential amino acid								
Alanine	66	70	68	81	71			
Arginine	54	54	54	65	46			
Aspartic acid	83	80	82	73	67			
Glutamic acid	123	109	127	110	96			
Glycine	46	50	48	51	53			
Proline	56	66	56	54	48			
Serine	40	44	42	38	34			
Sum of total AA	927	910	931	864	776			
EAAI	**1.65**	**1.60**	**1.66**	**1.39**	**1.28**	**1.93**	**1.56**	**1.00**

Also, the sum of the amount of total essential amino acids (EAA) for *A. diaperinus, T. molitor and Z. morio* was comparable to that of soybean protein, but slightly lower than that of casein as reported by Young & Pellett (1991). Furthermore, the sum of EAA for *A. domesticus* and *B. dubia* was lower than to casein and soybean protein, but EAA were available in quantities that are necessary for human requirement (sum of 277 mg/g crude

protein).The amino acid profiles found for *T. molitor* were similar to the profiles that were reported by Ghaly (2009b); Finke (2002) and Jones, Cooper, & Harding (1972). The amino acid profiles of *Z. morio* reported by Finke (2002) and those of *A. domesticus* reported by DeFoliart & Benevenga (1989) were similar to ours. To our knowledge, no literature is reported on the amino acid profiles for *A. diaperinus* and *B. dubia* before.

The sum of total amount of amino acids (TAA) per g crude protein of *A. diaperinus* (927 mg/g)*, T. molitor* (910 mg/g) *and Z. morio* (931 mg/g) was higher than that in *A. domesticus* (864 mg/g) and *B. dubia* (776 mg/g). The fact that the sum of the total amount of amino acids did not add up to 1000 mg/g crude protein is likely mainly explained by the presence of non-protein nitrogen in the form of chitin. *Acheta domesticus* and *B. dubia* are used in adult form and are known to contain a higher level of chitin as compared to *T. molitor*, *A. diaperinus and Z. morio*.

The calculated essential amino acid index (EAAI) of *A. diaperinus, T. molitor* and *Z. morio* was somewhat higher than that of soybean, but lower than that of casein, also indicating that the quality of the insect protein for these three insect species was comparable to conventional food protein sources. The EAAI of *A. domesticus and B. dubia* was the lowest in comparison to other insects, and lower than the EAAI for casein and soybean. For a more detailed insight in insect protein quality, digestibility data need to be taken into account in future studies, since digestibility is not included as a factor in determining EAAI. Ramos-Elorduy et al. (1997) found that protein digestibility calculated from a vitro study ranged from 76 to 98% for seventy-eight species of edible insects, representing twenty-three insect families in Mexico. Their study indicated that insect proteins might have a high nutritional value.

3.3 Protein distribution in obtained fractions and colour of supernatant fractions

A mass balance was built up based on protein content in residue, pellet and supernatant fractions (Figure 2.1). The amount of protein in fractions was calculated based on protein content determined by Dumas in combination with weight of the fractions (dry matter based). The protein recoveries ranged from 86.5% to 103% (Figure 2.1). The losses did occur

during the extraction procedure, especially for *B. dubia*. The pellet contained 32.6% to 39.4% of total protein and the residue 31.4% to 46.6% of total protein (Figure 2.1). The obtained pellet and residue fractions were higher in protein content than that in the supernatant (17% to 23.1%) for all five types of insects. The amount of proteins in the residue was higher than that in the pellet, except for *Z. morio* (31.4%).

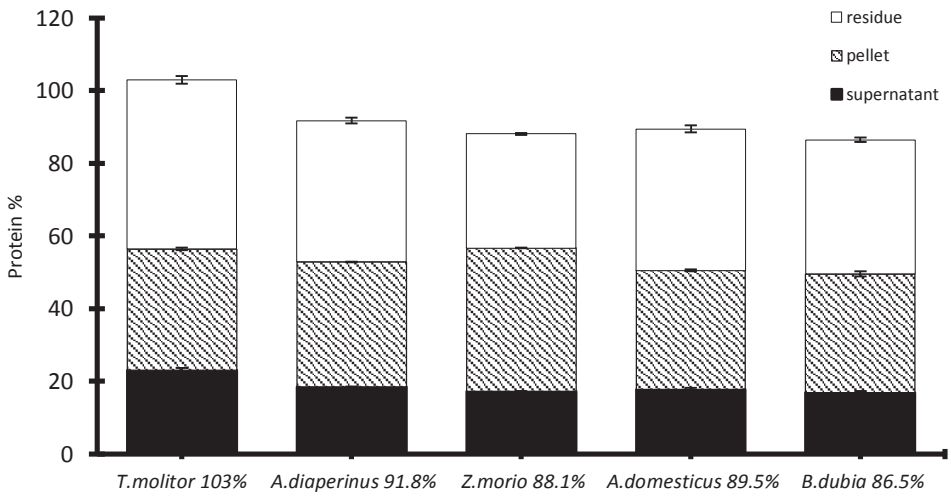


Figure 2.1: Protein content of supernatant, pellet and residue fractions expressed as percentage of total protein and total recovery (n=2).

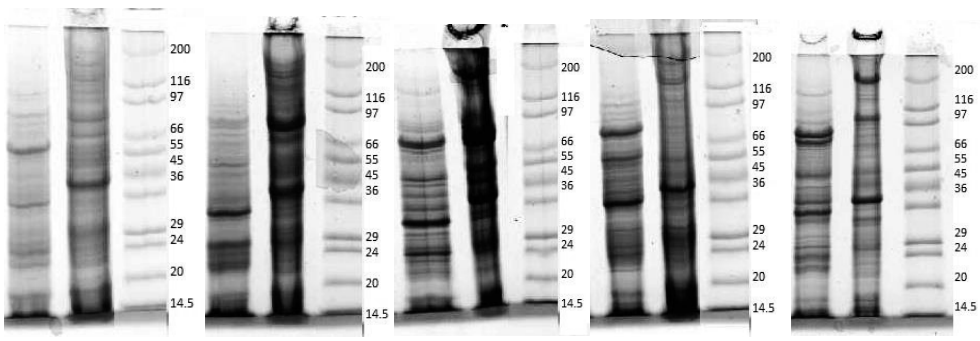
In addition, protein content on dry matter basis of each fraction ranged from 50% to 61% in the supernatant, from 65% to 75% in the pellet, from 58% to 69% in the residue and around 0.1% in the fat fraction. All chitin-bound nitrogen is expected to be present only in pellet and residue fractions, because chitin is insoluble in aqueous solvents (Goycoolea et al., 2000). Except for the presence of chitin-bound nitrogen, there is also uncertainty in the protein-to-nitrogen conversion factor of 6.25 leading to inaccuracy in the absolute protein content reported.

After aqueous extraction, the *B. dubia* had the lightest (light yellow) and the *T. molitor* the darkest colour (dark brown) among all insect supernatant solutions. The colour of *A. diaperinus, Z. morio and A. domesticus* supernatant solutions was comparable. This visual

observation indicated that chemical reactions took place during processing. Preliminary experiments showed that colour formation was most likely due to enzymatic browning reactions. In addition, the colour of residue and pellet fractions was similar to that of the supernatant fractions.

3.4 SDS-PAGE

The reduced SDS-PAGE using 12.5% acrylamide gels results show a range of protein bands of the supernatant fractions < 95 kDa, and that of the pellet fractions < 200 kDa for all five insect species (Figure 2.2). Five major groups of protein bands could be distinguished in Figure 3, namely bands ≤ 14 kDa, 14 - 32 kDa, 32 - 95 kDa and > 95 kDa. Due to insolubility in sample buffer, protein bands of the residue fractions were absent on the gels used in this experiment.



T. molitor *A. diaperinus* *Z. morio* *A. domesticus* *B. dubia*

Figure 2.2. Molecular weight distribution of *T. molitor* protein fractions, determined by SDS-PAGE using 12.5% homogeneous phastgel and (Samples from left to right: supernatant, pellet and marker); marker is ranging from 6.5 kDa to 200 kDa. Mw is molecular weight.

Based on intensity, the bands ≤ 14 kDa were abundant, especially for *T. molitor.* SDS-PAGE analysis using 20% acrylamide gels showed that the band ≤ 14 kDa consisted of a range of protein bands from 6.5 kDa to 14 kDa for all insect species studied (results not shown). For *T. molitor*, the bands ≤ 14 kDa could possibly originate from anti-freeze type of proteins ranging from 8.5 - 13 kDa, including hemolymph proteins having a molecular weight ~12

kDa (Graham et al., 1997; Liou et al., 1999; Graham et al., 2001). For the other insect species studied, no literature is available for specific proteins, not for those ≤ 14 kDa but also not for those > 14 kDa.

Next, the bands observed ranging from 14 to 32 kDa could possibly originate from *T. molitor* cuticle proteins with molecular weights predominantly between 14 and 30 kDa (Andersen et al., 1995), *e.g.* chymotrypsin-like proteinase (24 kDa) (Elpidina et al., 2005).

Then, the bands observed ranging from 32 to 95 kDa in the *T. molitor* supernatant fractions could possibly be linked to enzymes and other proteins, *e.g.* melanization-inhibiting protein (43 kDa), β-glycosidase (59 kDa), trypsin-like proteinases (59 kDa), and melanization-engaging types of protein (85 kDa) (Ferreira et al., 2001; Zhao et al., 2005; Prabhakar et al., 2007; Cho et al., 1999).

Above 95 kDa, no bands were observed in the supernatant fractions of *T. molitor*. Compared to *T. molitor*, the pattern of protein bands from supernatant fractions in *A. diaperinus* and *A. domesticus* was similar, but not identical. For *Z. morio* and *B. dubia,* more bands were found in the range of 30 to 95 kDa.

The observed bands with molecular weight > 95 kDa in the pellet fractions of *T. molitor* possibly originate from vitellogenin-like protein with a molecular weight of 160 kDa (Lee et al., 2000). No subunit structures of the proteins mentioned were found using UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5), so that actual molecular weight reported in literature is similar to apparent molecular weight on gel.

Besides the proteins mentioned before, proteins incorporated in the exoskeleton and muscle proteins are present in the five types of insects and in the fractions obtained. For the adult stage of *A. domesticus* and *B. dubia* muscle proteins include insect flight and leg muscles, which mainly consist of large size proteins, *e.g.* M-line protein, (flight and leg muscle, 400 kDa); kettin (leg muscle isoform, 500 kDa); kettin (flight muscle isoform, 700 kDa) (Bullard & Leonard, 1996; Lakey et al., 1990). For the larval stage of *T. molitor, A.*

diaperinus and *Z. morio* skeletal muscles, which likely consist of large size proteins, are present.

3.5 Protein functionality measurements

Due to the insolubility of the pellet and residue fractions, only the supernatant fraction of the protein was tested for its functionality with respect to foamability, foam stability, and gelation.

3.5.1 Foamability and foam stability

As a reference for the foam stability measurements, albumin from chicken egg white was used at a concentration of 1.5% w/v. The reference sample is a good stabilizer for foam, and was capable of producing foam with a half-time of 17 minutes. *Zophobas morio* formed foam at pH 3, 7 and 10 with a half-time of 6 minutes, *A. domesticus* at pH 3 with a half-time of 4 minutes, and *B. dubia* produced foam at pH 5 with a half-time of 5 minutes. Foams with half-time of < 6 minutes are not considered to be stable foams. All other supernatant fractions had negligible foam ability at a concentration of 3% w/v, at pH 3, 5, 7, and 10. This may be due to the protein concentration in the supernatant fraction solution (around 1.7% w/v) being too low to generate stable foam. The stability of the foam can be influenced by protein structure, protein concentration, and ionic strength. In addition, the stability of the foam can be also influenced by presence of oil. As mentioned by (Lomakina & Mikova, 2006), the effect of oil at levels above 0.5% reduced the volume of egg white foam. In our case, the supernatant fractions obtained from five insect species also contained some amount of oil in concentration of around 0.1%, which may also influence foamability of proteins in supernatant fractions.

3.5.2 Gelation

3.5.2.1 Visual observation of gelation

The visual appearance was determined of gels of five supernatant fraction solutions, with fraction concentrations of 3 and 30% w/v, at pH 3, 5, 7, and 10, after heating for 10 minutes in a water bath at 86 ± 1 °C (Table 2.3).

Table 2.3: Gel formation of supernatant fractions from five insect species (X: no gel formation; A: aggregation; V: viscous fluid; O: gel formation).

Supernatant	pH 3	pH 5	pH 7	pH 10
3%				
T. molitor	X	A	A	X
A. diaperinus	X	A	A	X
Z. morio	X	A	A	X
A. domesticus	X	A	O	X
B. dubia	X	A	A	X
30%				
T. molitor	X	V	O	O
A. diaperinus	X	V	O	O
Z. morio	X	V	O	O
A. domesticus	X	V	O	O
B. dubia	X	V	O	O

A heating time of 20 and 30 minutes was also tested, but no differences were seen in gel formation (not shown). Factors affecting the gel properties in general are pH, protein concentration, and thermal treatment. The protein concentrations selected for gelation are in the range from 0.5 to 25% concentration that are used in general to make gels. At a concentration of 3% w/v, none of the protein fractions showed gel formation, except for *A.*

domesticus at pH 7. At pH 5 and pH 7, for all samples (except *A. domesticus* at pH 7) heating induced the formation of visible large aggregates rather than gel formation.

All 30% w/v supernatant fractions formed a gel at pH 7 and 10, but not at pH 3. At pH 5, very weak gels were formed, that yielded when being turned upside down. In table 3, these samples are designated as "V" (viscous fluid). All samples at pH 7 and 10 were turbid, indicating that the characteristic size of the structures forming the gel was larger than the wavelength of visible light. All gels were already formed after 10 minutes and longer heating times had no influence on the appearance of the gel.

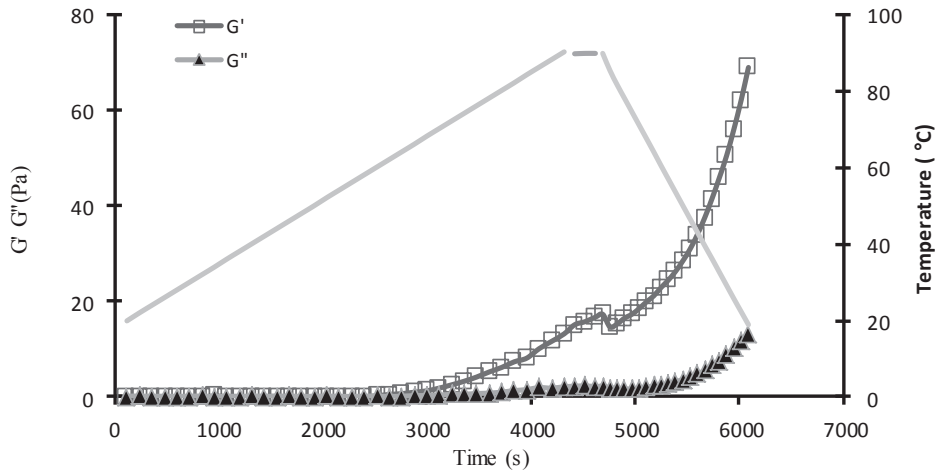
Some insect proteins have an isoelectric point of about 5. For instance, the pI of proteins from silkworm (*Bombyx mori)* and spider (*Nephila edulis)* are 4.37 - 5.05, and 6.47, respectively (Foo et al., 2006). If our protein fractions also have a pI of around pH 5, this may explain why all fractions at this pH formed aggregates at a concentration of 3% w/v, and very weak gels at concentrations of 30% w/v. Close to the pI, the electrostatic interactions between the proteins are very weak, which upon denaturation tends to lead to the formation of dense aggregates. These dense aggregates have a much higher gelling concentration than aggregates formed at a pH above or below the isoelectric point. To form a firm gel at this pH, higher protein concentrations are needed.

Samples at pH 3 and 10 at 3% w/v were more transparent than samples heated at pH 5 and 7. The increased charge on the protein at pH 3 may prevent the proteins from aggregating, since even at 30% w/v these fractions did not form a gel or even a viscous fluid. The decrease in turbidity observed at pH 10 suggests that the aggregates formed at this pH were less dense and/or smaller than the ones formed at pH 5 and 7.

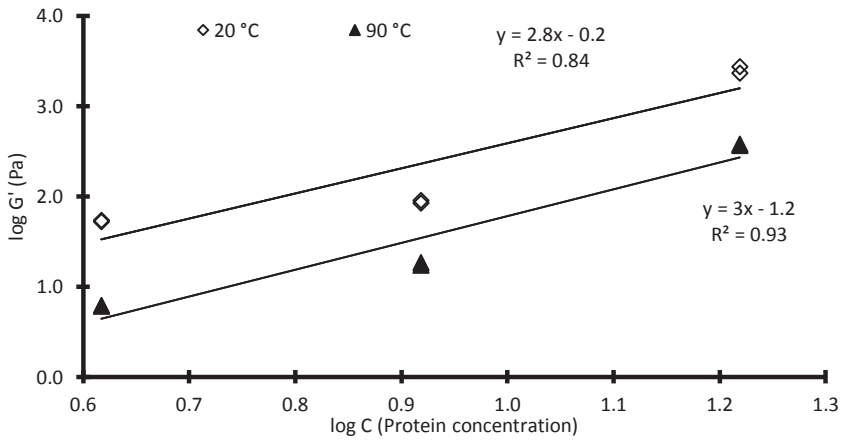
3.5.2.1 Rheological properties of gels

According to the visual observation of gelation, at a pH of 7 and a concentration of 3% w/v a weak gel was formed, and at 30% w/v a strong gel was formed. Therefore, for studying gel strength, fraction concentrations in between these two values (7.5 and 15% w/v) were

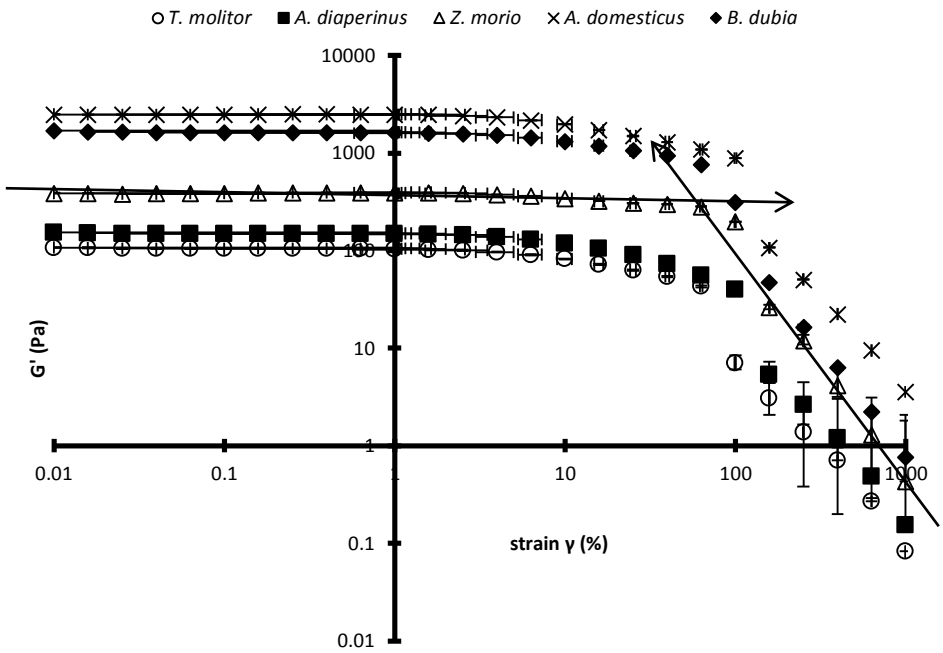
chosen. For all five fractions, we determined the evolution of the storage modulus G' and loss modulus G" during the temperature ramp at a concentration of 15% w/v and a pH of 7.



A.



B.



C.

Figure 2.3: A: Dynamic moduli G' and G'' of *T. molitor* supernatant solution as a function of time. Heating and cooling phases are plotted as a secondary axis. **B**: Plots of the storage modulus G' as a function of protein concentration of *T. molitor* supernatant fractions on a logarithmic scale at pH 7 (heating period 90 °C and cooling period 20 °C). **C**: Storage modulus G' (Pa) as a function of strain γ% for insect supernatant gelation at 20 °C at a supernatant fraction concentration of 15% w/v.

The storage modulus is a measure for the elastic energy stored reversibly in a gel during deformation, and characterizes its stiffness; the loss modulus is a measure for the energy dissipated during deformation as a result of viscous friction. As an example, the results for the mealworm supernatant fraction (the other fractions showed similar results) are provided (Figure 2.3A). G' gradually increased during the heating phase of the ramp. During the second phase, when the temperature was kept constant at 90 °C, G' kept on increasing gradually. This observation showed that the gel structure did not yet reach an equilibrium

state. During the cooling phase, both G' and G" increased sharply. This is typical for gels in which hydrogen bonds are formed between structural elements (Ould Eleya, Ko, & Gunasekaran, 2004). The gelation temperature observed ranged from about 51 °C to 63 °C (*T. molitor* 61.7 ± 1.1°C , *A. diaperinus* 58.2 ± 2.1 °C, *Z. morio* 51.2 ± 1.5 °C, *A. domesticus* 56.2 ± 0.7 °C, *B. dubia* 63.2 ± 0 °C, from which the lowest and the highest temperature were from *Z. morio* and *B. dubia* supernatant fractions respectively (results not shown).

To obtain more information on the gel structure, the value of log G' of *T. molitor* supernatants was determined as a function of log C (concentration) with fraction concentrations of 7.5% w/v, 15% w/v and 30% w/v (corresponding to actual protein concentrations of 4.1%, 8.3% and 16.6%) at 90 °C and 20 °C (Figure 2.3B). Values for G' at 90 °C were taken from end of phase 2 from the ramp, and values at 20 °C were taken from the end of phase 3, which is similar to the procedure of (Ould Eleya, Ko, & Gunasekaran, 2004). The values of the power-law exponent n in the scaling relation $G' \propto C^n$, were used for evaluation of gel structure (Shih et al., 1990). The parameter n had a value equal to 3.0 ± 0.4 at the end of the isothermal stage at 90 ºC, and a value of at 2.8 ± 0.6 from the end of the cooling stage at 20 ºC. These two values are comparable, so there were no significant structural rearrangements in the gel network upon cooling of the samples. An exponent n of about 2.8 is typical for fractal protein gels and points to a fractal dimension d_f which is close to 2 (Ould Eleya, Ko, & Gunasekaran, 2004).

Figure 2.3C shows G' at the end of phase 3 of the temperature ramp as a function of strain, for insect supernatant gels at 20 °C and a concentration of 15% w/v. The value for G' in the linear response regime of *A. domesticus* supernatant gels was around 2500 Pa, which was almost 1.5 times stronger than that of *B. dubia* (around 1600 Pa), 6 times stronger than that of *Z. morio* (around 390 Pa), and 25 times stronger than that of *T. molitor* (around 100 Pa) and *A. diaperinus* (around 140 Pa). In interpreting these results, we must be careful, since the actual protein concentrations in the fractions was lower than 15% w/v, and differed slightly from fraction to fraction. As seen before, the actual protein contents were for *T. molitor* 8.3%; *A. diaperinus* 9.2%; *Z. morio* 7.6%; *A. domesticus* 9.2% and *B. dubia* 7.4%.

Several conclusions can be drawn from these results. Although the *B. dubia* supernatant sample had the lowest actual protein content, it formed the strongest gels among all other three insect species, except *A. domesticus*. Supernatants from *A. diaperinus* and *A. domesticus* had similar protein concentration, but they showed significant differences in gel strength. In addition, supernatants from *B. dubia* and *A. domesticus* those were in the adult stage formed relatively stronger gels than the other three insect species that were in the larvae stage. Apparently, the insect growth stage influences the body protein composition and different species differ in protein type and structure (Wilson, 2010).

All insect gels had a comparable maximum linear strain at supernatant fraction concentration of 15% w/v, with a value of around 50%. An example is shown for *Z. Morio* (Figure 2.3C). The maximum linear strain is of course dependent on heating rate and protein concentration, and it would therefore be interesting to investigate the concentration dependence of this property, since it can provide additional information on the fractal dimension of the gels.

These detailed rheological results show that insect proteins can form gels that have similar properties as those formed from conventional food proteins. It therefore shows that insect proteins have indeed functionalities that are desirable for food application.

4 CONCLUSIONS

Proteins were extracted from five insect species and protein purity and yield of the obtained fractions was evaluated: Around 20% of total protein was found back in the supernatant, the rest of the protein was divided about equally over the residue and the pellet fraction for all five insect species after aqueous extraction. The extraction method is easy and feasible to apply, but the yield of extracted supernatant fractions is relatively low. The purity of measured protein content expressed as percentage of dry matter ranged from 50% to 61% of supernatant fractions, from 65% to 75% of pellet fractions and from 58% to 69% of residue fractions depending on the insect species.

We established some functional properties of the protein fractions, focusing on foaming and gelation: The soluble protein fractions of all five types of insects had poor foaming capacity at pH 3, 5, 7, and 10, but could form gels at a concentration of 30% w/v. At a concentration of 15% w/v at pH 7 and 10, *A. domesticus* supernatant formed the strongest gels among all insect species. The gelation temperature ranged from about 51 °C to 63 °C for all insect species at pH 7. In addition, all insect gels had a comparable maximum linear strain at this concentration, with a value of around 50%.

We studied protein quality of whole insects by analysis of protein content and amino acid composition: The protein content of the five insect species was comparable to conventional meat products in terms of protein quantification. The sum of EAA per g protein for all insect species was comparable with the sum of EAA for soybean protein, lower than that for casein, but higher than that for the daily protein requirement of an adult (FAO/WHO/UNU, 1985). Differences in calculated EAAI were similar.

Although differences are observed in protein content, amino acid composition, protein distribution of the fractions obtained, SDS-PAGE data, foaming and gelation properties, the similarities between the insect species are more apparent than the differences. The fact that gels could be formed for all five insect species, using the soluble fractions obtained by a simple aqueous extraction procedure, is promising in terms of future food applications. More research is needed for developing further extraction and purification procedures, and for more detailed insight in functional properties.

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CHAPTER 3

EXTRACTING *TENEBRIO MOLITOR* PROTEIN WHILE PREVENTING BROWNING: EFFECT OF pH AND NaCl ON PROTEIN YIELD

Yi, L., van Boekel, M. A. J. S, Lakemond, C. M. M. (2014). **Extracting** *Tenebrio molitor* protein while preventing browning: effect of pH and NaCl on protein yield. **PLOS ONE (Submitted)**

Extracting *Tenebrio molitor* protein while preventing browning:
effect of pH and NaCl on protein yield

ABSTRACT

The potential of insects as an alternative meat (protein) source for food applications was investigated by studying the effect of pH and NaCl on the extraction yield of water-soluble proteins from *Tenebrio molitor*, while preventing browning due to polyphenol oxidation. Minimum protein solubility (29.6%) was at pH 4 - 6 and maximum (68.6%) at pH 11. Protein extraction at 0.1 M NaCl, pH 10 gave a recovery of 100%. The observed increase in browning at pH 8-11 corresponded to a lower monomeric phenol content. Sodium bisulphite (studied from 0.5 - 4%) could prevent browning, whereas ascorbic acid (studied in the range 0.01 - 0.04%) could not prevent as strong as sodium bisulphite. After acid precipitation (pH 4) an isolate with a protein content of 74% (yield of 22% of total protein) was obtained. It was observed that proteins from *Tenebrio molitor* behave more or less the same as proteins from meat and fish with respect to aqueous extraction.

KEYWORDS

Insect protein extraction; *Tenebrio molitor;* protein solubility and muscle protein

1 INTRODUCTION

With an increasing world population, in combination with rising meat consumption per capita in developing nations, there is a need to find alternative (meat) protein sources for future food applications. Animal proteins from mammals, fish and shellfish are considered to be the highest quality protein foods for human consumption (Del Valle, Mena, & Bourges, 1982). In many countries of South America and Africa, edible insects are habitually used as animal protein food for human consumption. However, people in the western world have, on average, a strong bias against insects as food, especially when the insects are offered in a recognizable form. Del Valle, Mena, & Bourges (1982) indicated that extraction of proteins from insects for use in food products is particularly relevant for countries that do not have the habit of consuming insects, such as Europe and North America. The common use of the Yellow mealworm (Coleoptera: *Tenebrio molitor*) is as fish bait or as feed for birds or reptiles (Ghaly & Alkoaik, 2009). Yellow mealworms are professionally reared in insect rearing companies in Europe, but also in other parts of the world *e.g.* China (http://www.fao.org/forestry/edibleinsects/65429/en/). The protein content of *T. molitor* ranges from 24.3-27.6% in fresh insects (or 63-69% in dry matter), which is comparable to conventional meat protein sources (15 to 22%) (Finke & Winn, 2004; Ghaly, 2009; Ghaly & Alkoaik, 2009). Ghaly & Alkoaik (2009) and Yi et al. (2013) reported that *T. molitor* contains all the essential amino acids needed for human nutrition. *T. molitor* is currently marketed for human consumption in Europe, *e.g.* in the Netherlands under the name “Bugs Triobolo” (http://www.bugsacademy.nl/).

To obtain protein as a food ingredient, many separation techniques are available on a laboratory scale that are generally based upon differences in protein solubility, size, charge, and biological affinities, *e.g.* salting out, isoelectric precipitation and solvent fractionation (Smith & Nielsen, 2010). For extraction purposes, protein solubility at various pH and ionic strengths can be used to obtain high yield and protein content of proteins. Del Valle, Mena, & Bourges (1982) reported on characteristics and functionalities of extracted protein fractions from Mexican fruit fly larvae (*Anastrepha ludens*) using isoelectric point precipitation. This study is one of the few described in literature on extracting proteins from

insects at various pH values. After aqueous extraction, a protein concentrate was obtained with a protein content of 65% and a yield of 94%. After further purification using isoelectric precipitation, a protein isolate with a protein content of 87% and yield of 85% was obtained. The protein isolate showed minimum solubility of 8% at pH 5 and maximum solubility of 95% at pH 10.

For industrial purposes, next to protein content and yield, the quality of the obtained isolate is important for potential applications in food industry. Yi et al. (2013) reported that brown colour formation took place during aqueous extraction of protein from insects. This visual observation indicated that chemical reactions took place during processing, which was most likely due to enzymatic browning reactions. Enzymatic browning can be prevented, for example, by addition of bisulphite (Golan-Goldhirsh & Whitaker, 1984), or ascorbic acid (Sapers & Ziolkowski, 1987) for inhibiting or inactivating polyphenol oxidase (Goptar et al., 2013; Jiang & Fu, 1998).

Very little information in literature is available on extraction and isolation of extracted *T. molitor* protein. Our previous work presented results on protein extraction using a basic aqueous extraction procedure, in which *T. molitor* was one of the five insect species studied (Yi et al., 2013). This study reported that the water-soluble fraction of *T.molitor* was brown/black coloured and only contained 23% of total protein present. Increasing protein yield would be relevant in view of future industrial application. Therefore, the objective of this study was to investigate factors affecting protein yield of the water-soluble fraction and further purification while preventing brown colour formation during protein isolation. The specific objectives were: 1) to investigate protein solubility of water-soluble fractions as influenced by pH and salt concentration; 2) to prevent browning reactions taking place during extraction using sodium bisulphite and ascorbic acid; 3) to purify crude protein further with acid precipitation; 4) to investigate molecular distributions of protein fractions obtained after precipitation.

2 MATERIALS AND METHODS

2.1 Insect used

Tenebrio molitor in the larval stage was purchased from a commercial supplier (Kreca V.O.F, Ermelo, the Netherlands). The feed for *T. molitor* consisted mainly of wheat, wheat bran, oats, soy, rye, corn, carrot, and beer yeast. The insects were sieved to get rid of feed, and then killed by immersing them into liquid nitrogen before processing.

2.2 Preparation of the water-soluble protein fractions

Extraction procedure

Protein extraction was performed using the procedure of Yi et al. (2013) as a basis. The frozen *T. molitor* was blended in buffer with varying amounts of salt/anti-browning agents in a weight ratio of 1:3 for one min (Braun Multiquick 5 (600 W), Kronberg, Germany). The protein solutions were stirred overnight at 4 °C, to ensure that most proteins were dissolved. Next, the solutions were stirred for one hour at room temperature. Then, the pH was adjusted to the starting pH by addition of 1 M & 5 M HCl (Merck, CAS nr. 7647-01-0)/ NaOH (Merck, CAS nr. 1310-73-2).

The obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 315 µm. The filtrate was collected, and centrifuged at 15,000 g for 30 min, 4 ºC. After removing the lipid from the upper layer, the supernatant (SUP A) was collected, and freeze-dried (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands) for further analysis. The extraction procedure was performed in duplicate, starting twice with a new insect batch.

Varying pH values, addition of NaCl, and concentration of browning agents during extraction

In the extraction procedure, pH and salt concentration were varied to study their effect on protein solubility. For extraction at pH 2 - 6, 10 mM citric acid buffers (Merck, CAS nr. 77-92-9; CAS nr. 6132-04-3) were used. For extraction at pH 7 - 11, 10 mM sodium phosphate buffers (Merk, CAS nr. 13472-35-0; CAS nr. 10025-24-7) were used.

The influence of salt concentration on protein solubility was studied for extracting at pH 8 and 10. Sodium chloride (Sigma, CAS nr. 7647-14-5) was added in concentrations of 0.1 and 0.5 and 1 M.

For preventing brown colour of the soluble fractions, sodium bisulphite (Sigma, CAS nr. 7631-90-5) was tested in concentrations of 0, 1%, 2%, 3%, and 4% , and ascorbic acid (Sigma, CAS nr. 50-81-7) in concentrations of 0.01%, 0.02%, and 0.04%. This concentration of sodium bisulphite was also used in relation to prevention of browning in shrimp, catfish and potato (Otwell, 1992; van Koningsveld et al., 2002; Li et al., 2013). The concentration used for ascorbic acid was studied before in relation to inhibition of browning in cloudy apple juice (Özoğlu & Bayındırlı, 2002).

2.3 Colour

The colour of supernatant solutions with sodium bisulphite (0, 0.5%, 1%, 1.5%, 2%, 3%, and 4%), and ascorbic acid (0.01%, 0.02%, and 0.04%) was determined with a HunterLab spectrophotometer (ColorFlex, Utrecht, the Netherlands) that was calibrated using black/white tiles. L*, a* and b* values were recorded (only L* shown). All experiments were performed in duplicate.

2.4 Determination of protein content

The Dumas method was used to determine crude protein content (Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience, Breda, the Netherlands) using a protein-to-nitrogen conversion factor of 6.25. D-Methionine (Sigma, CAS nr. 348-67-4) was used as a standard. Protein yield was expressed as the percentage of protein obtained relative to the total amount of protein present in the fresh insects.

2.5 Polyphenol quantification

The amount of total phenols was measured by using Folin-Ciocalteu's phenol reagent (Lowry, Rosebrough, Farr, & Randall, 1951). The supernatant (SUP A; 10 mg/mL) was mixed with methanol (Bio solve, CAS nr. 67-56-1) that contained 1.48% HCl (Merck, CAS nr. 7647-

01-0) in a volume ratio of 4:6, and vortexed for 2 min. After centrifugation (14,000 rpm, 2 min), 100 µl of sample added to 2 ml sodium carbonate solution (2% (w/v) was vortexed for 3 min, and incubated with 50 µL of Folin-Ciocalteu reagent for 2 min at room temperature. Absorbance was measured at 765 nm. Bovine serum albumin (Sigma, CAS nr. 9048-46-8) dissolved in 2 mg/mL gallic acid (Sigma, CAS nr. 149-91-7) (in the HCl-Methanol mixture) at a concentration of 1mg/mL was used to check if the HCl concentration was high enough to precipitate the proteins. Total phenol content was expressed as mg gallic acid equivalents/g SUP A. Freeze-dried proteins extracted in the range pH 2 to pH 11 were re-solubilised in demineralized water overnight at 4 °C at a concentration of 50 mg/mL for colour observation. In addition, the total phenol content of freeze-dried protein extracted using sodium bisulphite (0, 0.5%, 1%, 1.5%, 2%, 3%, and 4%) and ascorbic acid (0.01%, 0.02%, and 0.04%) was also determined.

2.6 Acid precipitation

The crude protein extract was brought to pH 4 by slowly adding 1M & 5 M HCl followed by stirring at 4°C overnight. The precipitate was obtained by centrifugation at 15,000 g 4 ^{o}C for 30 min. The extracts were further washed using demineralized water at pH 4 and freeze-dried afterwards.

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight distribution of the insect protein fractions. Supernatant fractions were analysed on 12% Bis/Tris NuPAGE gels (Invitrogen, Carlsbad, USA) by using MES running buffer under reduced and non - reducing conditions. The Mark12™ Unstained Standard (2.5 - 200 kDa) (Invitrogen, Carlsbad, USA) was applied as a reference. The gels were then Coomassie-stained. A standard curve was made by measuring the migration distance of proteins with known molecular weight (Mw standards). Unknown molecular weights were calculated using this standard curve (n=2).

3 RESULTS AND DISCUSSION

3.1 Influence of pH and salt concentration on protein solubility

The pH and salt concentration influenced protein solubility (Figure 3.1A). Protein solubility decreased in the pH range 2 - 4, remained at the same level but was low in the pH range 4 - 6, and gradually increased in the pH range 6 - 11. The highest solubility was found at pH 11, at which around 11.6 g protein was extracted from 100 g fresh *T. molitor* (68.6% of total protein present in *T. molitor*). The lowest amount of protein in supernatant fractions was found at pH 4 - 6 at 5 g per 100 g fresh *T. molitor* (29.6% of total protein present in *T. molitor*). The crude protein content of *T. molitor* was about 16.9 g per 100 g fresh insects for the insect batch used, which was about 2% lower than the value found by Yi et al. (2013). This was because of a 2% higher moisture content.

The amount of protein in the pellet correlated negatively with the amount of protein found in the supernatant fraction over the pH range studied (Figure 3.1A). Maximum levels were found in the pH range 4 - 6 with values of 4 - 5 g protein out of 100 g fresh *T. molitor.* The protein content in the residue was relatively constant over the pH range studied in Figure 3.1A. For pH 2 - 4, values around 3 g/100 g fresh insect were found, and 4 g/100 g fresh insect were found in pH range 5 - 11.

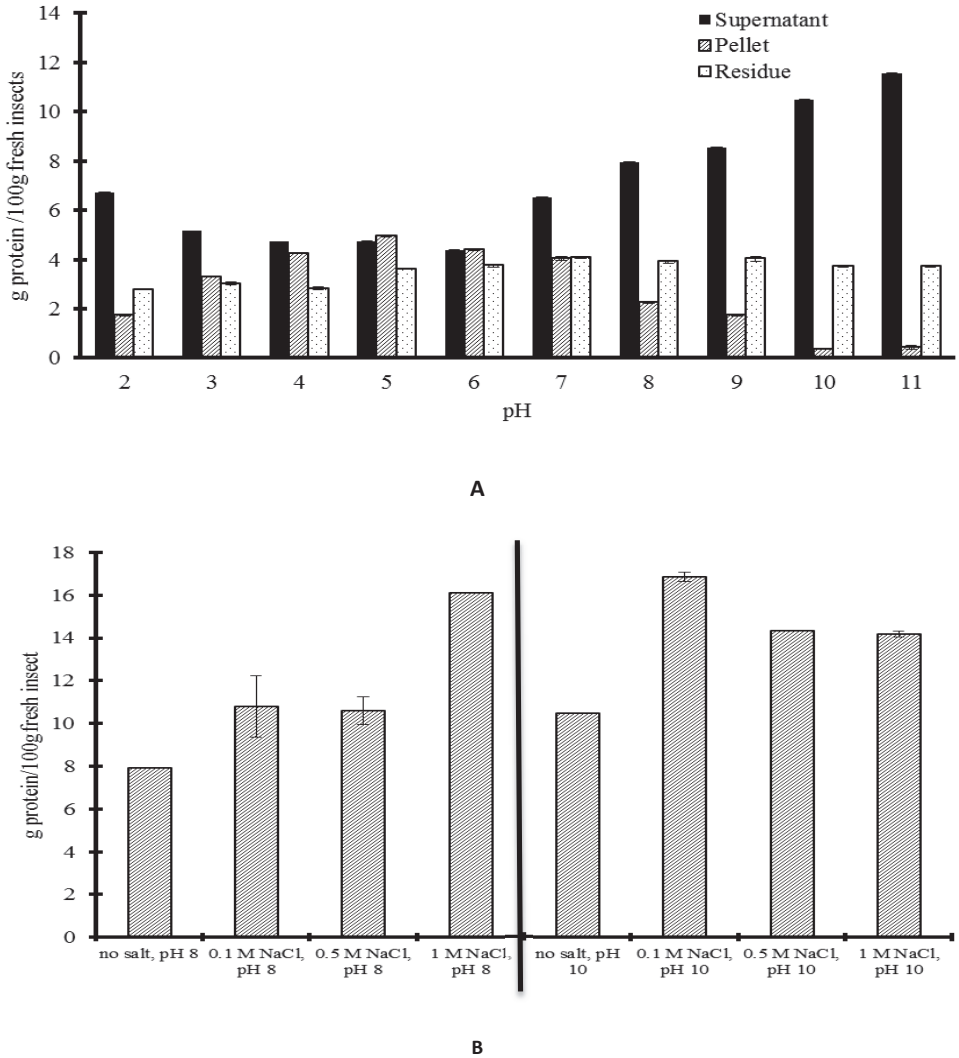


Figure 3.1: **A:** Amount of protein (g/100 g fresh insects) extracted at pH values from 2 to 11 in residue, pellet and supernatant; **B**: Amount of protein obtained by addition of NaCl (0, 0.1, 0.5, and 1 M at pH 8 and pH 10) (n=2).

The highest amount of soluble protein from *T. molitor* could be recovered around pH 11, and the lowest amounts were recovered around pH 4. Similar to our results, maximum overall protein recovery of alkali-aided extraction for Tilapia fish muscle proteins was observed at pH 11 with a recovery level of about 86%, while minimum recovery of 20% was

between pH 5 to 6 (Kristinsson & Ingadottir, 2006). Furthermore, in line with our results, Omana, Xu, Moayedi, & Betti (2010) who investigated protein extraction from chicken dark meat, found protein recovery to be at a maximum (94.21%) after alkali treatment at pH 12. In general, alkaline extraction at high pH (10 - 12) is used to obtain high yields of fish muscle proteins and meat muscle proteins (Hultin & Kelleher, 1999, 2000; Undeland, Kelleher, & Hultin, 2002; Feng et al., 2004; Nolsøe & Undeland, 2009; Kristinsson & Ingadottir, 2006; Kristinsson et al., 2005). Based on high protein solubility and pH of various food ingredients (FDA,http://www.fda.gov/food/foodborneillnesscontaminants/causesofillnessbadbugbook/ucm122561.htm), pH 8 and pH 10 were chosen for further experimentation. The increased solubility at higher pH can be explained due to the formation of charged amino acid residues in the proteins at pH above the isoelectric point, causing more electrostatic repulsion, thereby promoting protein solubility.

Furthermore, in comparison to other meat and fish sources, the amount of protein remaining soluble (29.6%) in the pH range pH 4 - 6 is low in comparison to the data of (Nolsøe, Imer, & Hultin, 2007). They found total protein remaining soluble ranging from 58 - 75% for cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* using an alkaline extraction process (pH 10.8 to pH 5.5). Furthermore, Undeland, Kelleher, & Hultin (2002) found that sarcoplasmic proteins, being 20 - 30% of fish muscle, were soluble in water and in solution with low ionic strength. A large portion of those proteins remained soluble at pI (pH 5.1 to 5.5).

A possible disadvantage of using strong alkaline conditions is amino acid degradation, *e.g.* formation of lysinoalanine (Tibbetts, Verreth, & Lall, 2011), lanthionine (LAN), and histidinoalanine (HAL). LAL formation decreases digestibility and nutritional quality. The formation of LAL is known to occur in alkali-treated proteins at very high pH values (above pH 9 and higher) when combined with elevated temperature (Friedman, 1999). However, heat treatment is not used in this study, and addition of anti-oxidative agents like ascorbic acid and sulphite ions prevent LAL formation to a large extent (Finley & Kohler, 1979). The reason is probably that sulphite ions prevent LAL formation by supressing dehydroalanine formation (Finley & Kohler, 1979; Friedman, 1999).

Furthermore, alkaline conditions at high pH (pH > 10.5) could lead to irreversible protein denaturation, possibly leading to loss of functionality (Betti & Fletcher, 2005). Its relevance depends on the specific future application of such a protein extract. Whether LAL formation or irreversible protein denaturation takes place needs to be investigated.

Figure 3.1B shows the amount of water-soluble protein extracted at pH 8 and pH 10 at sodium chloride concentrations of 0, 0.1, 0.5 and 1 M. At pH 8, the amount of water-soluble protein was 25% higher after addition of 0.1 and 0.5 M NaCl, it increased to 10 g/100 g fresh insects (around 59.2% of total protein present in *T. molitor*). For addition of 1 M NaCl, a value of 16 g/100 g fresh insects was found, which corresponds to 94.7% of total protein present in *T. molitor*.

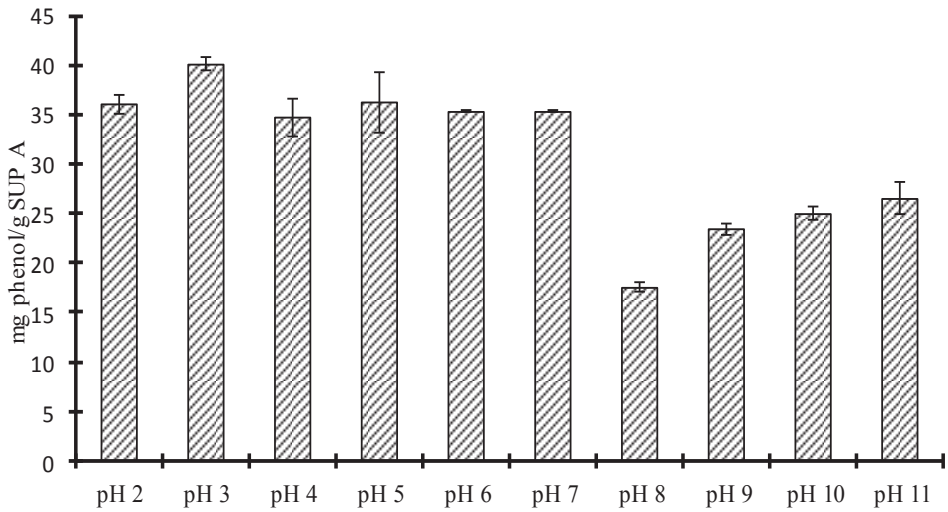
At pH 10, the amount of soluble protein obtained without salt was about 10.5 g/100 g fresh *T. molitor*, which was similar to the amount of protein obtained with 0.1 or 0.5 M NaCl at pH 8. Furthermore, at pH 10, the amount of protein was found highest at 0.1 M NaCl. At this condition, an amount of 16.9 g/100 g fresh *T. molitor* was obtained corresponding to around 100% of total protein present in *T. molitor*.

In accordance with our findings, it is known that besides the effect of pH on protein solubility, ionic strength also affects protein recovery (Zayas, 1997). Myofibrillar proteins obtained from muscle protein solubilize in a salt solution (Zayas, 1997). Therefore, salt is commonly used in extracting meat-derived products (Kristinsson & Hultin, 2003). Due to the presence of muscle proteins in insects (Bullard & Leonard, 1996), we expect that salt-soluble proteins are present in *T. molitor* as well, which could explain increased protein yields at elevated salt concentration. This effect is likely due to the effect of salting in, which enhances protein solubility (Zayas, 1997). Stefansson & Hultin (1994) found that more soluble proteins were obtained at relatively low salt concentration, and Dagher, Hultin, & Liang (2000), who studied myofibrillar protein in fresh cod fillets, found highest solubility (> 90%) at a low NaCl concentration of 0.1 M. Indeed, we observe that protein yield decreases upon further increase of salt concentration, which may be explained as salting-out, inducing

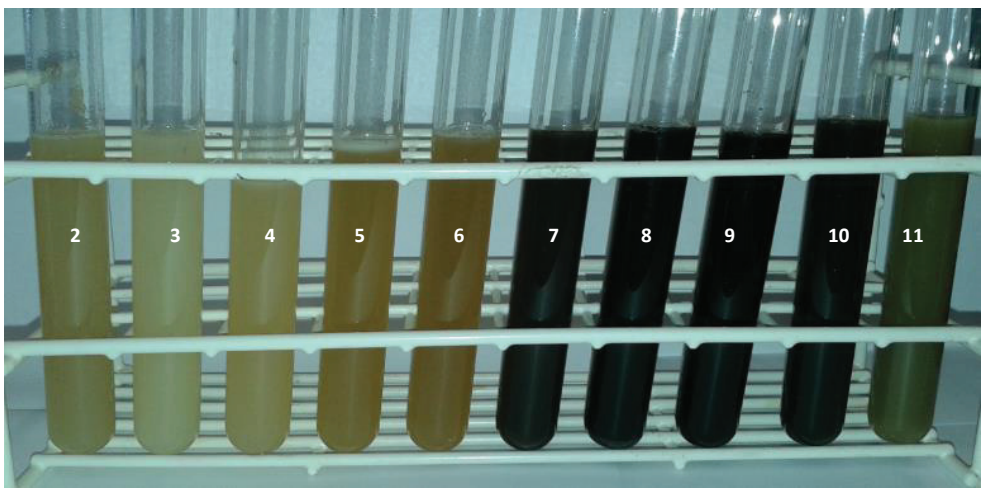
protein aggregation and precipitation at higher salt concentration (Stefansson & Hultin, 1994).

3.2 Influence of pH on total phenol content of supernatant fractions

Total phenol content of supernatant fractions (SUP A) extracted in the range of pH 2 to pH 11 is shown in Figure 3.2A. The absolute amounts of total phenolic compounds were around 36 mg/ g SUP A from pH 2 to pH 7, decreased to about 18 mg/g SUP A at pH 8 and then gradually increased to 27 mg/g SUP A from pH 8 to 11. The trend for brown colour observed over the range pH 2 - 11 is the same as the observed trend of measured phenol content as shown in Figure 3.2B. The decrease in total phenol content corresponds to the formation of dark-coloured compounds as was also found by (Altunkaya & Gökmen, 2008).



A



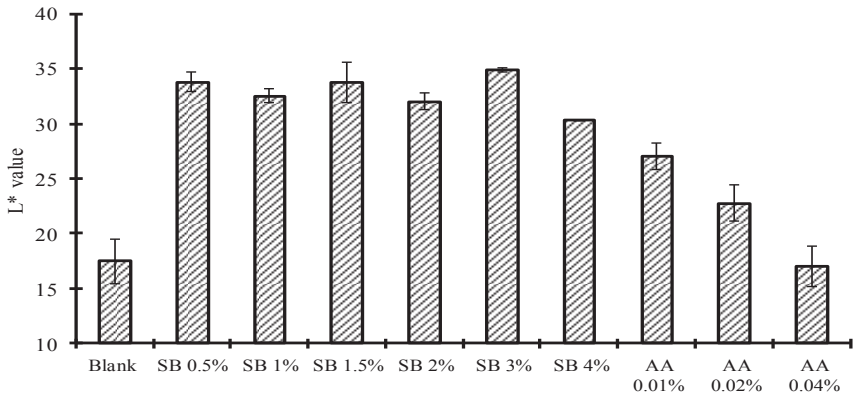
B

Figure 3.2. A: Total phenol content of supernatant fractions extracted from pH 2 to pH 11, expressed as mg gallic acid equivalents/g SUP A; **B**: Colour of protein extracted from pH 2 to pH 11after dissolving freeze-dried protein (50 mg/mL) in demineralized water.

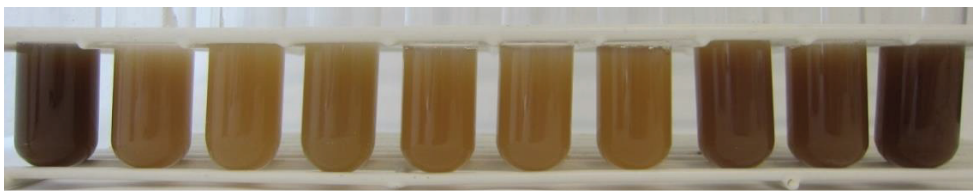
The results obtained on browning and total phenol content are in line with previous findings showing that the colour of protein fractions of *T. molitor* changed to dark brown at pH 6.5 (Yi et al., 2013). The chemical reaction causing this was suggested to be enzymatic browning. Enzymatic browning is known to be a major quality problem in the processing of fruits, vegetables and seafood. In our study, the brown colour was observed in the water-soluble protein fraction shortly after grinding insects in water, and developed over time (results not shown). Insect cuticles contain melanin that give brownish or black colours to the insect in the adult stage (Andersen, 2010). Because cuticular proteins transform to melanino-proteins after enzymatic oxidation (Andersen, 2010). Generally, enzymatic oxidation is explained as follows: enzymes (polyphenol oxidases: PPOs) are responsible for the enzymatic browning by oxidising phenolic compounds. PPO could induce on o-hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones. Subsequently, the rapid polymerization of o-quinones result in dark colour pigments called melanins (Yoruk & Marshall, 2003). The pH stability of PPO enzymes varies widely with plant source but is generally in the range of 4 - 8. For crustaceans this range is even wider, from 3 - 10 (Yoruk & Marshall, 2003; Simpson et al., 2012). Discolouration is also a serious problem in fresh shrimp and other shellfish processing (Gokoglu & Yerlikaya, 2008) Furthermore, the brown colour at pH > 8 could also be caused by phenolic polymerization reactions due to autoxidation (Talcott & Howard, 1999). For example, (Cilliers & Singleton, 1990) found that the oxidation of caffeic acid and formation of brown colour are increasing very rapidly between pH 9 and 11. Besides the general presence of PPO in plant sources, it is also found in shrimp and lobster, but not in common meat and fish sources.

3.3 Preventing browning reaction of supernatant fractions

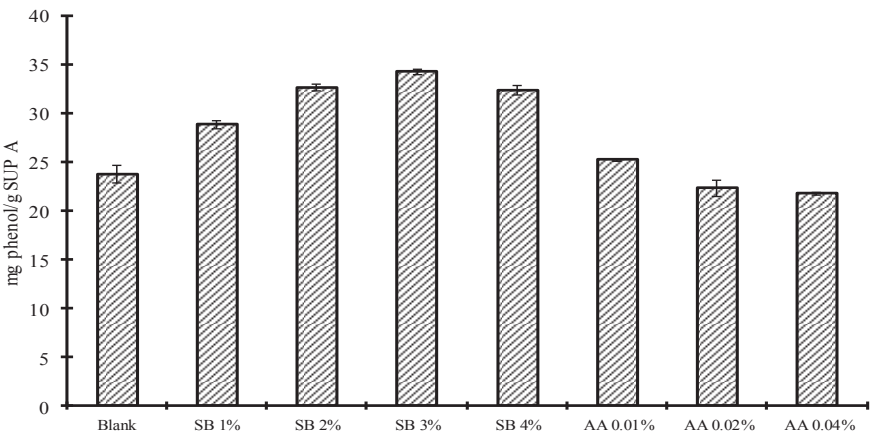
To prevent enzymatic browning, sodium hydrogen sulphite ($NaHSO_3$, also referred to as sodium bisulphite SB) and ascorbic acid are widely used to inhibit browning reaction in food applications. We investigated the effect of anti-browning agents on insect supernatant fractions (Figure 3.3A). The colour of SUP A with sodium bisulphite at concentrations of 0, 0.5%, 1%, 1.5%, 2%, 3% and 4% and ascorbic acid (AA) at 0.01%, 0.02% and 0.04% was expressed as L* values. The L* values of SUP A with 0.5% to 4% SB were found to be around 32, and were higher than that of SUP A without SB (L*= 17). Furthermore, the L* values of SUP A treated with AA 0.01%, 0.02% and 0.04% were decreasing with increasing AA concentration, and were lower than the values found for SB. Thus, AA showed less influence preventing browning than SB. According to visual observation, indeed a higher intensity of the brown colour corresponded to a lower L* value (Figure 3.3B). Total phenol content of supernatant fractions was determined after adding SB (0, 1%, 2%, 3%, and 4%) and AA (0.01%, 0.02% and 0.04%) by using Folin-Ciocalteu's phenol reagent (Figure 3.3C). The phenol content is expressed as mg gallic acid equivalents/g SUP A. Total phenol content of supernatant fractions increased from 24 to 34 mg/g with increasing concentration of sodium bisulphite (0 to 3%) (Figure 3.3C). The maximum value found for total phenol content was approximately 34 mg/g SUP A at SB concentration of 3%. Increasing concentrations of ascorbic acid did not correspond to an increased total phenol content of supernatant fractions. Furthermore, the lowest total phenol content was measured when no anti-browning agent was added, indicating the highest amount of polymerized material, and corresponding to the darkest colour.


L* value
40
35
30
25
20
15
10
Blank
SB 0.5%
SB 1%
SB 1.5%
SB 2%
SB 3%
SB 4%
AA 0.01%
AA 0.02%
AA 0.04%

A


0
SB 0.5%
SB 1%
SB 1.5%
SB 2%
SB 3%
SB 4%
AA 0.01 %
AA 0.02%
AA 0.04%

B


mg phenol/g SUP A
40
35
30
25
20
15
10
5
0
Blank
SB 1%
SB 2%
SB 3%
SB 4%
AA 0.01%
AA 0.02%
AA 0.04%

C

Figure 3.3. A: L* color value of SUP A **B**: Color of SUP A after adding sodium bisulphite (0, 0.5%, 1%, 1.5%, 2%, 3% and 4%), and ascorbic acid (0.01%, 0.02% and 0.04%), and **C**: Total phenol content of supernatant fractions as determined by Folin-Ciocalteu assay expressed as mg gallic acid equivalents/g SUP A.

In order to prevent discoloration, sodium bisulphite and ascorbic acid were used in our study as anti-browning agents based on their wide application in plant, fruit and fresh sea food (Yoruk & Marshall, 2003; Alonso et al., 1999). Alike our study, Rababah, Ereifej, & Howard (2005) also found that addition of ascorbic acid (0.1%) had no effect on concentrations of total phenolic components in fresh fruit (including apple, peach, strawberry). No literature is available on the prevention of browning using sodium bisulphite or ascorbic acid in *T. molitor*. Several mechanisms for inhibiting browning using sodium bisulphite for food application were summarized by Kuijpers et al. (2012): irreversible inhibition of PPO and reduction of o-quinones. As an alternative anti-browning agent, ascorbic acid can inhibit browning by acting as a reducing agent for o-quinones. However, it known that AA can only delay the reaction (Ros, Rodriguez-Lopez, & Garcia-Canovas, 1993). The Folin-Ciocalteu reagent does not only measure phenols, but will react with any reducing substance. Ascorbic acid and sulphite were reported to be important potentially interfering substances on the total polyphenol content determination by the Folin-Ciocalteu method (Singleton et al., 1999). However, as mentioned by Wrolstad (2005), the Folin-Ciocalteu method does not respond to sodium bisulphite as a form of sulphur dioxide alone, but does respond to bisulphite in the presence of phenolic compounds. Stevanato, Fabris, & Momo (2004) mentioned that the value obtained from the Folin-Ciocalteu method was estimated 11% higher for wine with 3 mM sulphite added and 39% higher when 3 mM ascorbic acid was added. However, as mentioned by Singleton et al. (1999), little or no ascorbic acid remains after normal processing in wines, due to its rapid oxidation. In our study, a similar trend was observed when comparing the L* values (Figure 3.3A) to the total polyphenol content (Figure 3.3C). However, the kinetics behind bisulphite interference with Folin-Ciocalteu possibly occurring in *T.molitor* extracts is unknown, and needs further research.

3.4 Acid precipitation at pH 4: effect on yield and purity

The crude proteins of the supernatant obtained with 0.1 M NaCl, 3 % sodium bisulphite at pH 10 were further fractionated by acid precipitation at pH 4. The yield of crude protein extract obtained after extraction with 0.1M NaCl, 3% sodium bisulphite at pH 10 was found at 16.1 g/100 g fresh *T. molitor* (95.3% of total crude proteins) This extract had a protein content of 52.3%. After acid precipitation, the pellet had a protein content of 74%, and the yield of the protein extract was 3.8 g/100 g fresh *T.molitor*, corresponding to 22% of total protein present in *T. molitor*.

Relatively low protein content and yield were found in the protein fraction obtained after precipitation at pH 4. A reason could be the presence of proteases in the midgut lumen of *T. molitor*, which can break down proteins (Applebaum et al., 1964). The major digestive proteinases of Tenebrio molitor, the trypsin-like proteinases were purified and characterized by Tsybina et al. (2005). According to this study, the trypsin-like proteinases were active at alkaline condition corresponding to insect's midgut pH (pH 7.9), and were highly stable from pH 5 to 9.5 with an activity level always above 80%. Therefore, proteins extracted from *T. molitor* in our study were likely digested by active proteases before precipitation, which lead to low protein content & yield. Next to this, the protein content is calculated by using a conversion factor of 6.25 on a basis of protein-nitrogen values of 16%. It might lead to underestimation of the total protein content of *T. molitor* if the conversion factor for insect protein would be higher than 6.25. Hall & Schönfeldt (2013) investigated various meat pieces and mentioned that conversion factors can be calculated as total amino acids (g) divided by total nitrogen (g); they then find factors that are lower than 6. If we do this calculation for insects, we find a factor higher than 7; this seems rather high. As also mentioned by Hall & Schönfeldt (2013), a small change in the amount of nitrogen-containing amino acids can have a substantial impact on calculating protein factors. In the case of insects, there is an additional problem of chitin-bound nitrogen in *T. molitor* that may result in inaccuracy in calculating a protein factor. However, chitin, due to its insoluble nature, is not present in the aqueous fraction when pH precipitation takes place. It is clear that an

accurate estimate of the conversion factor for the true protein content in *T. molitor* requires a research project on its own.

3.5 SDS-PAGE

The results of non-reduced SDS-PAGE using 12% Bis/Tris gels show the protein band patterns of the supernatant fractions extracted from pH 2 to pH 11 (Figure 3.4A). It is apparent that both overall intensity as well as the band pattern changes upon extraction pH. The least total intensity of the protein bands was found at pH 4; several protein bands were visible over a range of around 6 to 75 kDa. For pH 3, prominent bands were found around 12, 26 and 39 kDa, for pH 2 the major bands were observed around 12, 30, 42, and 75 kDa. In addition, a strong wide "smear" was present at pH 2, corresponding to Mw's below 6 kDa.

The band patterns for extraction at pH 5 and 6 were similar to those of pH 4. In comparison to patterns found for pH 4 - 6, an additional band was found around 20 kDa for pH 7 - 10, and a band around 6.6 kDa was present for pH 7-11 (Figure 3.4B). For extraction at pH 10 and 11, also a high molecular weight band was observed above 200 kDa. Patterns obtained after reduced SDS-PAGE (data not shown) were similar to the ones obtained after non-reduced SDS-PAGE. However, the band at 205 kDa was not visible.

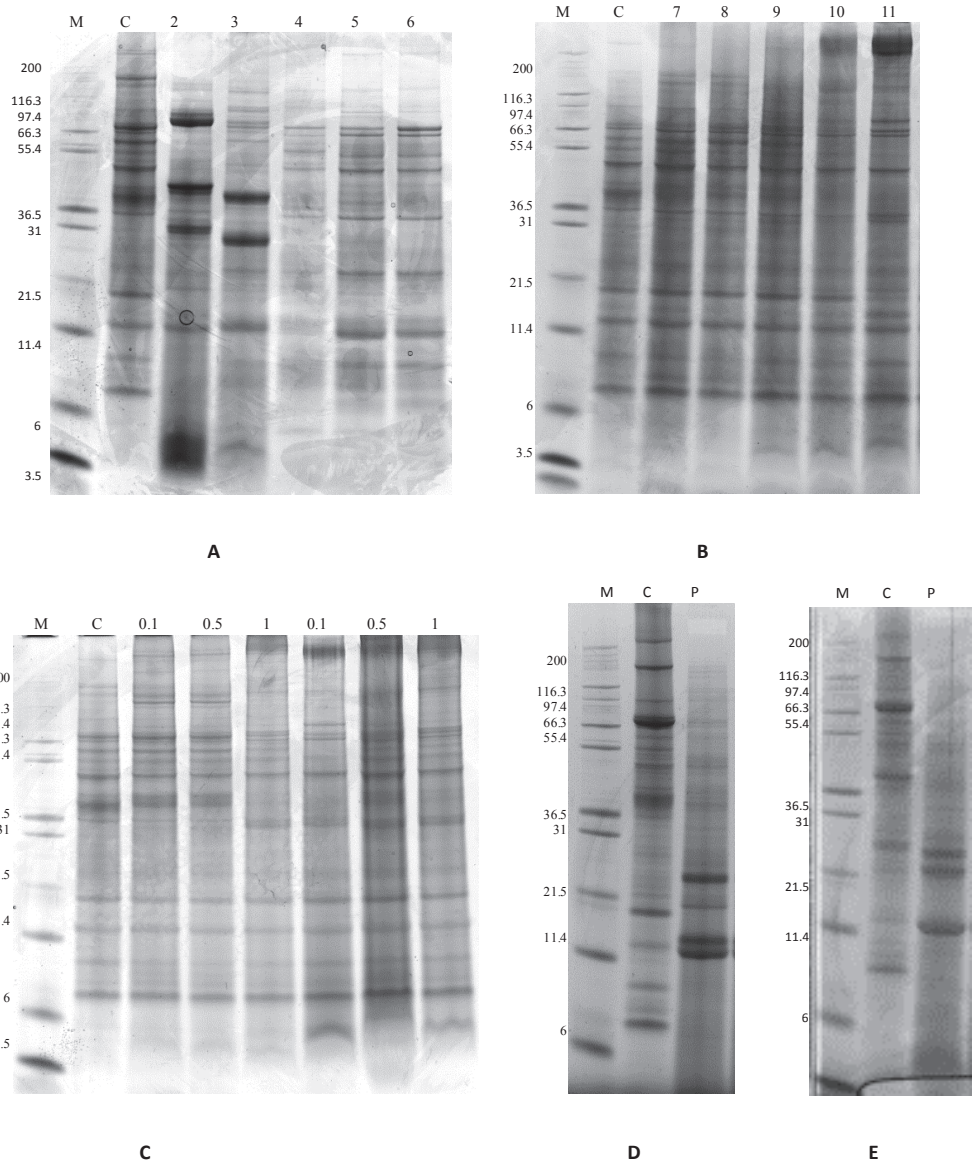


Figure 3.4: **A**: Protein patterns of the supernatant fractions extracted from pH 2 to pH 6 (Non-reducing SDS-PAGE); **B**: protein patterns of the supernatant fractions extracted from pH 7 to pH 11(Non-reducing SDS-PAGE); **C**: protein patterns of the supernatant fractions extracted from both pH 8 and pH 10 in combination with NaCl (0, 0.1, 0.5 and 1 M) (Non-

reducing SDS-PAGE); **D** and **E**: protein patterns of a protein isolate obtained after acid precipitation (reducing SDS-PAGE (**D**) and non-reducing SDS-PAGE (**E**)). M: marker, C: the supernatant extracted from water, control sample, P: protein isolate.

It is known that *T. molitor* contains muscle protein, including myosin, actin and sarcoplasmic Ca binding proteins as analysed by LC-MS/MS (Verhoeckx et al., 2014). It is known that myosin aggregates at pH 3 and pH 4 (Park, 2008), which could explain the low protein band intensity found at pH 4. In addition, Kim et al. (2005) found for rockfish that the main sarcoplasmic proteins consisting of proteins of 43, 40, 17, 11 and 8 kDa, were least solubilized at pH 2 - 4, which may also explain the low amount of protein found at corresponding pH for *T.molitor*. These sarcoplasmic proteins form a major portion of muscle proteins that could be extracted by using alkaline or dilute salt-containing solutions (Nollet & Toldra, 2010; Park, 2008).

The protein band found above 200 kDa for extraction at pH 10 and 11 could correspond to myosin heavy chain (225.4 kDa) or myosin-2 (223 kDa), as was based on the results of Verhoeckx et al. (2014). Besides myosin, actin also is one of the major muscle proteins. Verhoeckx et al. (2014) found *T. molitor* actin at 42.1 kDa, corresponding to the 42 kDa band found at pH 2.

The protein band found at 12 kDa for extraction at pH 2 and 3, also visible in the pH range 5-11, likely corresponds to hemolymph protein, an anti-freeze type of protein, having a molecular weight~12 kDa as was described by Yi et al. (2013). The protein bands present in the pH 2 and 3 extract, ranging from 14 to 32 kDa (Yi et al., 2013), possibly originate from *T. molitor* cuticle proteins (Andersen et al., 1995), *e.g.* chymotrypsin-like proteinase (24 kDa). Another possibility is the presence of myosin light chain (31.3 kDa), as deduced from data Verhoeckx et al. (2014).

Figure 3.4C shows the influence of different NaCl concentrations (0.1, 0.5 and 1 M) on protein band distribution for extraction at both pH 8 and pH 10 using non-reduced SDS-PAGE. Addition of NaCl gave similar band patterns for all three pH 10 extracts. For pH 8, similar band patterns were observed for 0.1 and 0.5M NaCl, but the band pattern for 1M

NaCl was similar to the pH 10 extracts. Three main differences were found. A band around 40 kDa disappeared and one around 31 kDa appeared at pH 8, 1 M NaCl and pH 10, 0.1 M, 0.5 M & 1 M NaCl. Further at these conditions, a band found above 200 kDa appeared that may explain the higher protein recovery found in Figure 3.1B. Lin & Park (1996) indeed reported that myosin heavy chain protein could be extracted by addition of NaCl. Reduced SDS-PAGE data were very similar to non-reduced SDS-PAGE data (results not shown), except for the protein band > 200kDa.

Figure 3.4D shows that the protein extracts obtained after acid precipitation contain main bands of around 12, 15 and 25 kDa, and a minor band at 19 kDa as was determined by using reduced SDS-PAGE. Next to that, Fig. 3.4E illustrates that major bands were observed around 12, 19 and 25 kDa using non-reduced SDS-PAGE. The 12 kDa band probably corresponds to hemolymph proteins.

4 CONCLUSIONS

In conclusion, we could increase soluble protein recovery of *T. molitor* from 23% (when using water) (Yi et al., 2013) to 100% by using high extraction pH in combination with the presence of NaCl. With respect to the effect of pH on protein yield, the highest protein yield of 68.6% was obtained at pH 11, and the lowest protein yield was 29.6% between pH 4 - 6. Furthermore, with respect to the influence of salt on protein yield, we could extract up to 100% of the protein at 0.1 M NaCl, pH 10. The increased solubility corresponded partly to a protein band above 200 kDa, likely caused by the myosin heavy chain protein. Acid precipitation at pH 4 could increase protein content from 52% to 74% (yield was 22.2% of total protein), thereby extracting as one of the main proteins a 12 kDa protein, likely corresponding to hemolymph protein.

The colour of supernatant extracted at alkaline conditions was much darker than that extracted at acidic conditions, corresponding to the trend observed for total phenol content as a function of pH. Sodium bisulphite could effectively prevent browning of water-soluble

protein fraction of *T. molitor*. Those findings support that brown colour formation during extraction is likely linked to enzymatic browning processes, like in plants and crustaceans.

All in all, it is clear that high protein yield of *T. molitor* can be obtained at extreme alkaline condition in combination with the presence of salt, the same as was reported for conventional protein sources*, i.e.* fish muscle proteins and meat muscle proteins (Hultin & Kelleher, 1999, 2000; Nolsøe & Undeland, 2009; Feng et al., 2004; Kristinsson & Ingadottir, 2006; Kristinsson et al., 2005; Undeland et al., 2002). So, the conclusion is that proteins from *T. molitor* show comparable behaviour with conventional fish and meat protein when pH and salt conditions are modified during aqueous extraction.

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CHAPTER 4

PROTEIN IDENTIFICATION AND *IN VITRO* DIGESTION OF FRACTIONS FROM *TENEBRIO MOLITOR*

Yi, L., van Boekel, M. A. J. S., Lakemond, C. M. M. (2014) Protein identification and *in vitro* digestion of fractions from *Tenebrio molitor.* **(To be submitted)**

Protein identification and *In vitro* digestion of fractions from *Tenebrio molitor*

ABSTRACT

Although amino acid composition of *Tenebrio molitor* has been studied before, limited knowledge is available, on which bulk proteins it consists of, and on its digestibility, being a determinant of the nutritional value of protein. The objective of this study was to investigate *in vitro* protein digestibility of whole *Tenebrio molitor* larvae, a water-soluble fraction (supernatant), and water-insoluble fractions (pellet and residue), and to identify which proteins were present in the fractions studied. The digestibility of the supernatant fraction (~80%) was much higher than that of pellet (~50%) and residue (~24%) after *in vitro* gastro-duodenal digestion as was determined using the OPA method. More proteins were digested after pepsin/pancreatin digestion than after only pepsin digestion. The most abundant proteins were hemolymph protein (~12 kDa) & putative allergens (*e.g.* arginine kinase ~30 kDa) in supernatant, and muscle proteins (*e.g.* actin 30-50 kDa) in the pellet fraction as determined from LC-MS/MS & SDS-PAGE. In conclusion, the proteins in the soluble fraction that contained hemolymph proteins were more easily digestible than the insoluble, muscle protein containing fractions.

KEYWORDS

Insect protein; *Tenebrio molitor*; *in vitro* digestion; Protein identification; LC-MS/MS

1 INTRODUCTION

The Yellow mealworm (*Tenebrio molitor*) of the order Coleoptera is currently reared as fish bait or as feed for fish, amphibians, reptiles, turtles, birds, fowls and small mammals kept as house hold pets or in zoos (Ghaly & Alkoaik, 2009). The protein content of the Yellow mealworm ranged from 24.3 - 27.6% in fresh insects (63 - 69% in dry matter), which is comparable to conventional meat protein sources (about 15 to 22%) (Finke & Winn, 2004; Ghaly, 2009; Ghaly & Alkoaik, 2009). In studies on protein quality, Yi et al. (2013) reported that the Yellow mealworm contains all the essential amino acids needed for human nutrition.

However, the nutritional value of a food protein is evaluated not only by its amino acid composition, but also by protein digestibility. Protein digestion in humans generally starts with pepsin cleavage in the stomach, subsequently trypsin and chymotrypsin digestion in the intestinal lumen, and the last step includes cleavage by proteases present on the intestinal surface (Akimov & Bezuglov, 2012). *In vitro* digestion is often used as an approximation for in *vivo* processes (Wickham, Faulks, & Mills, 2009). The major advantage of an *in vitro* method is that the procedure of digestion is relatively simple and rapid in comparison to *in vivo* digestion. However, *in vitro* methods cannot mimic completely real pH and temperature conditions in the digestive system. Furthermore, *in vitro* experiments often give lower protein digestibility values than *in vivo* studies (Butts, Monro, & Moughan, 2012).

During protein digestion and absorption in the human body, protein is broken down to amino acids and peptides by digestive enzymes (Akimov & Bezuglov, 2012). Afterwards, free amino acids and small peptides are absorbed through the gastro-intestinal wall. The extent of protein hydrolysis can be evaluated by measuring the degree of hydrolysis (DH). The DH is defined as the percentage of the total number of peptide bonds in a protein that have been cleaved during hydrolysis (Rutherfurd, 2010). Several methods to measure protein hydrolysis were reviewed by Rutherfurd, 2010: 1) determining the amount of nitrogen released during hydrolysis (after precipitation by acids like trichloroacetic acid) by the Kjeldahl method or a spectrophotometric determination like the biuret reaction; 2)

quantifying the amount of free amino groups released during hydrolysis by formol titration; 3) measuring compounds that react specifically with amino groups such as trinitrobenzenesulphonic acid (TNBS), o-phthaldialdehyde (OPA), ninhydrin (Silvestre et al., 2013); 4) determining the protons released during hydrolysis by titration to calculate the DH (pH stat method Spellman et al. (2003). Nielsen, Petersen, & Dambmann (2001) and Schasteen et al. (2007) stated that prediction of amino acid digestibility of food proteins *in vitro* assays by using o-phthaldialdehyde (OPA) is more rapid and accurate when compared to other methods. However, the reaction between cysteine and OPA reagent is weak and unstable, which could lead to a lower estimation of protein hydrolysis (Spellman et al., 2003).

There is no literature on protein digestibility of *T. molitor* as a whole or on its extracted protein fractions. However, protein digestibility of other edible insects has been reported. Protein digestibility of eri silkworm (*Samia ricinii*) pupae was about 87% determined via the Kjeldahl method using a nitrogen factor of 6.25 (Longvah, Mangthya, & Ramulu, 2011) as tested on rats by *in vivo* digestion. Furthermore, protein digestibility via *in vitro* methods using pepsin - pancreatin was found to be around 91% in fresh termites of the species *Macrotermes subhylanus*, 82 - 86% in the grasshopper *Ruspolia differens*, as determined by TCA-nitrogen content. The values obtained were comparable to the values reported of conventional animal sources (89% for whole beef, 90% for pork, 78% for turkey, and 85% for salmon) (Kinyuru et al., 2010). According to Ramos-Elorduy et al. (1997), protein digestibility of 21 selected types of edible insect species in Mexico was found to be 60 - 98% based on nitrogen content analysed after *in vitro* digestion.

The studies that deal with protein digestibility of insects do not give any information on the types of proteins that are digested. The reason for this is that very limited knowledge exists on which bulk proteins are present in insects (Verhoeckx et al., 2014). Mass spectrometry-based methods can be used for protein identification. Often digestion of proteins into similar sized peptides is performed as a pre-treatment to create peptide fragments, which are easily ionized in the mass spectrometer, so insoluble samples can be analysed. A strength of tandem mass spectrometry is the inherent ability to sequence peptides directly from mixtures (Delahunty & Yates Iii, 2005).

Yi et al. (2013) extracted one water-soluble protein fraction (supernatant) and two water-insoluble protein fractions (pellet and residue) from *T. molitor* using an aqueous extraction method. In that study, the fractions were characterized in terms of protein content & molecular weight by SDS-PAGE. The objective of the present study was to identify proteins using LC-MS/MS and investigate protein digestibility (*in vitro*) of the ground whole insect and its fractions (supernatant, pellet and residue) obtained by aqueous extraction according to Yi et al. (2013).

2 MATERIALS AND METHODS

2.1 Materials

Tenebrio molitor larvae were purchased from a commercial supplier (Kreca V.O.F, Ermelo, the Netherlands). The insects were sieved to get rid of feed, and then killed by immersing them into liquid nitrogen before processing.

2.2 Preparation of tested protein fractions

Frozen insects were ground, freeze-dried and defatted as described by Yi et al. (2013). The proximate composition of *T. molitor* was determined after processing. Defatted *T. molitor* meal of the whole larvae was stored at - 20 ºC.

Water-soluble and water-insoluble protein fractions were obtained by an aqueous extraction according to Yi et al. (2013). Three protein fractions were thus obtained: a supernatant (water-soluble protein fraction), a pellet (water-insoluble protein fraction), and a residue (water-insoluble protein fraction). After freeze-drying all fractions, pellet and residue fractions were defatted by using hexane extraction (Biosolve, CAS nr. 110-54-3) in a Soxhlet apparatus for 6 hours. Subsequently, protein content was determined by Dumas. The proximate composition (including fat and protein content) of water-soluble and water-insoluble protein fractions was determined after the above-mentioned processing. The extraction procedure was performed in duplicate starting twice with a new insect batch.

2.3 Filter-aided sample preparation (FASP)

FASP was used to prepare protein samples from the three protein fractions obtained as described by (Lu et al., 2011; Wisniewski et al., 2009) with some modifications. The pellet fractions were washed twice with water to remove soluble protein in pellet fractions before FASP. Peptide measurements were performed by nanoLC-LTQ-Orbitrap XL-MS/MS (Thermo electron, San Jose, CA, USA) as described by Lu et al. (2011). Results from LC-MS/MS were searched by MaxQuant 1.3.0.5 as described by Cox & Mann (2008), using default settings for the Andromeda search engine (Cox et al. 2011) except that extra variable modifications were set for de-amidation of N and Q.

Two databases of *T. molitor* from UniProt: Universal Protein Resource Knowledge base (UniProt: taxonomy 7067, *T. molitor* (database size: 240) and UniProt: taxonomy 50557, *Insecta* (database size: 1,070,041) were used for protein identification. These databases were used together with a contaminants database that contains sequences of common contaminants as for instance: BSA (P02769, bovin serum albumin precursor), Trypsin (P00760, bovin), Trypsin (P00761, porcin), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human). The "label-free quantification" as well as the "match between runs" (set to 2 minutes) options were enabled. De-amidated peptides were allowed to be used for protein quantification and all other quantification settings were kept default.

Extra filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus 1.3.0.4 module (available at the MaxQuant suite) as described before by Smaczniak et al. (2012). The proteomics result contained peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least two identified peptides of which at least one should be unique and at least one should be unmodified without any reversed hits.

Total non-normalized protein intensities corrected for the number of measurable tryptic peptides (intensity based absolute quantitation (iBAQ)) were, after taking the normal

logarithm, used for further data analysis (Schwanhausser et al., 2011). The key words "myosin, actin, sarcoplasmic, troponin" were used for searching muscle proteins. In addition, family and domain databases (including interPro, Pfam and PRINTS) were used for searching on most relevant proteins to better describe putative uncharacterized proteins.

2.4 *In vitro* digestion of proteins

Gastric - duodenal digestion of protein fractions from *T. molitor* was simulated by using the method of Vreeburg et al. (2012) as a basis. The water-soluble / water-insoluble protein fraction (4.5 g) was suspended in 30 mL Millipore water containing 140 mM sodium chloride (Merck, Cas nr. 7647-14-5) and 5 mM potassium chloride (Merck Cas nr. 7447-40-7), and vortexed 5 minutes for homogenizing the samples. The pH was adjusted to 2 with 1 M HCl (Merck, Cas nr. 7647-01-0). Six gram of the mixture was incubated with 0.667 mL of 40 mg/mL pepsin (Sigma Aldrich, Cas nr. 9001-75-6, 3,200-4,500 units/mg protein) in HCl (0.1 M) during 0, 10, 20, 30, 60, and 120 minutes at 37 °C while shaking. The reaction was stopped by adjusting to pH 5.8 using a solution of 1 M $NaHCO_3$ (Merck, Cas nr. 144-55-8). The mixture was called simulated gastric fluid (SGF). After centrifugation (3200 g, 4 °C for 30 minutes), the supernatant was stored as gastric digestible protein fractions (GDP). The experiment was performed in duplicate.

Subsequently, three gram of SGF was added to 0.95 mL of 4 mg/mL pancreatin from porcine pancreas (Sigma Aldrich Cas nr. 8049-47-6) in 0.1 M $NaHCO_3$, and 0.5 mL of a mixture of 94.6 mg/mL taurocholic acid sodium salthydrate (Sigma Aldrich Cas nr. 345909-26-4) and 83 mg/mL sodium glycodeoxycholate (Sigma Aldrich Cas nr. 16409-34-0) in 0.1 M $NaHCO_3$. The pH was again adjusted to 6.5 with 1 M $NaHCO_3$, and the headspace was flushed with nitrogen gas. Next, the mixture was incubated in a 37°C water bath, while shaking for 2 hours. After centrifugation (3200 g, 4°C for 30 minutes), this supernatant is further referred to as duodenal digestible protein fraction (DDP). The experiment was performed in duplicate.

2.5 Protein digestion quantification

Free α-amino groups were determined after reaction with o-phthaldialdehyde (OPA), following the method of Nielsen et al. (2001). An amount of 200 mL OPA reagent was prepared by using 7.62 g of sodium tetraborate (Boraxdecahydrate) (Sigma Aldrich Cas nr. 1303-96-4) and 200 mg of sodium dodecyl sulphate (SDS) (Sigma Aldrich Cas nr. 151-21-3) in 150 mL deionized water. Besides that, 160 mg OPA was dissolved in 4 mL ethanol (Merck Cas nr. 64-17-5), and 176 mg dithiothreitol (DTT) (Sigma Aldrich Cas nr. 3483-12-3) was added before adjusting the volume to 200 mL. The OPA reagent was freshly made for every experiment. A calibration curve was made using L-leucine (Sigma Aldrich, Cas nr. 61-90-5) ranging from 0.078 mM to 10 mM. Absorbance was measured at 340 nm.

Protein digestion was quantified based on determining the amounts of free NH_2 groups based on Schasteen et al. (2007) with some modifications. The values for digestibility were expressed as the amounts of free NH_2 groups digested from 1 mg protein. Further, initial free NH_2 groups, in which "initial" refers to the undigested sample, was presented separately within all figures. Digestibility values were expressed using Equation 2. "Final" refers to the digested protein fractions, and "acid" to complete hydrolysis in 6 N HCl, 110°C for 24 h.

Equation 2: Digestibility = $[Free\ NH2\ (final)]/[Free\ NH2\ (acid)]$

2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight distribution of the insect protein fractions. Undigested and digested fractions were analysed on 12% Bis/Tris NuPAGE gels (Invitrogen, Carlsbad, USA) using MES running buffer under reducing conditions. The Mark12™ Unstained Standard (2.5 - 200 kDa) (Invitrogen, Carlsbad, USA) was applied as a reference. The gels were then Coomassie-stained. A standard curve was made by measuring the migration distance of proteins with known molecular weight (Mw standards). Unknown molecular weights were calculated using this standard curve.

3 RESULTS

3.1 The proximate composition of protein fractions

The proximate composition of *T. molitor* and its protein fractions with regard to lipid and protein content was determined on a dry matter basis (Table 4.1). The measured crude protein content was 52% in ground *T. molitor*, 57% in supernatant fraction and 69% in both pellet/residue fractions. After defatting the whole *T. molitor* and its water-insoluble fractions (pellet and residue), the measured protein content increased 24% in ground *T. molitor*, 11% and 14% in the pellet and the residue fraction, respectively. Furthermore, the lipid content of ground *T. molitor* was 31% on a dry matter basis. The lipid content of pellet was found 15%, similar to that of residue. No lipid was found in supernatant fractions.

Table 4.1: The proximate composition of ground *T. molitor* and its protein fractions (mean ± S.D., n=2).

	Protein % dry matter (DM)	**Fat % DM**	**Protein %DM after defatting**
T. molitor	52.0±0.9	30.8±0.9	76.5±1.2
Supernatant	56.7±0.8	-	56.7±0.8
Pellet	68.9±1.6	14.5±0.4	80.0±1.6
Residue	69.1±1.6	15.9±1.5	83.1±1.1

3.2 Identification of proteins from the water-soluble and water-insoluble fraction of *T. molitor*

Proteins extracted as water-soluble fraction (supernatant) or as water-insoluble fraction (pellet) of *T. molitor* were identified by LC-MS/MS analysis (as shown in supplementary data). According to *T. molitor* database (7067), proteins only identified in supernatant were: hemolymph protein (~14 kDa), C protein (16.05 kDa), putative serine proteinase (28.18 kDa and 27.97 kDa), putative cathepsin B-like like proteinase (32.65 kDa), chitinase (39.55 kDa), serpin 1 (41.04 kDa), 41 kDa zymogen (41.97 kDa), GNBP1 (51 kDa), 93 kDa serpin (96.34

kDa), E cadherin (179.04 kDa), chitinase GH=chit 5 (321.40 kDa) and tenebrin (370.49 kDa).The proteins identified only in pellet were: histone (12.37 kDa), ribosomal protein (16.61 kDa), transporter (19.34 kDa), cathepsin L-like cysteine proteinase (37.31 kDa), melanin-inhibiting protein (39.69 kDa), serpin 40 (43.56 kDa), sodium/potassium ATPase alpha subunit (45.75 kDa), early-staged encapsulation-inducing protein (62.45 kDa) and vitellogenin (206.07 kDa). Proteins found in both supernatant and pellet fractions were hemolymph proteins, desiccation stress protein, triosephosphate isomerase, putative trypsin-like proteinase, putative serine proteinase, masquerade-like serine proteinase homologue, alpha-amylase, beta-glucosidase, tyrosine hydroxylase, cockroach allergen-like proteins, prophenoloxidase, Hexamerin 2, early-staged encapsulation-inducing protein, and melanization-related protein. These proteins had Mw's ranging from 6.6 kDa to 168 kDa. The data show further that a number of proteins, like hemolymph, occur in different isoforms. Proteins identified in defatted & ground *T. molitor* consisted of all proteins found in supernatant and pellet fractions, plus larval cuticle protein (12~18 kDa), membrane alanyl amino peptidase (105.9 kDa), and prophenoloxidase activating factor (43.75 kDa) (results not shown). In neither of the fractions, analysed muscle proteins were identified using the *T. molitor* database that contains 240 proteins.

When using the database *Insecta (50557)* (Table 4.2A) to check for the presence of muscle proteins, 21 types of muscle proteins were identified. In the pellet fraction, 18 types of muscle proteins were identified, including actin and its fragment (30 - 40 kDa), α-actinin-4 (106.8 kDa), myosin heavy chain (262 kDa), myosin-2 essential light chain (16.8 kDa), tropomyosin 2 (32.5 kDa), troponin I (23.8 kDa), troponin T (47.3 kDa), putative troponin C (18.3 kDa) and putative actin indirect flight muscle (42 kDa). Seven types of muscle protein were identified in the supernatant fraction, including α-actinin (107.2 kDa), tropomyosin 2 (Lethocerus indicus) (32.5 kDa) / (Nilaparvata lugens) (32.3 kDa), muscular protein 20 (20.3 kDa), myosin heavy chain (262.3 kDa), and actin-4 (41.9 kDa).

The *insecta* database was also used to identify the most abundant proteins present in *T. molitor* based on iBAQ values (Table 4.2B). The supernatant contained protein identified for *T. molitor* as labelled TENMO, *e.g.* hemolymph protein (fragment) and putative trypsin-like

proteinase, but also protein identified from other insects (*e.g.* muscular protein 20, arginine kinase, and heat shock protein 70). In comparison to proteins identified in the supernatant fraction, muscle proteins like tropomyosin 2 and actin, were more abundant in the pellet. These muscle proteins were not identified from the *T. molitor* database. For the supernatant fraction most proteins that were identified were also characterized. However, in the pellet fraction seven putative uncharacterized proteins were found in a large quantity (iBAQ) among the identified proteins. According to family and domain databases from Uniprot, these putative uncharacterized proteins were very likely actin/actin-like and tropomyosin. This information indicates that muscle proteins were the most abundant proteins found in pellet. As expected, for defatted & ground *T. molitor*, the same types of proteins were found as in the combination of supernatant and pellet fractions. Unfortunately, to date, the *Insecta* database is not complete and therefore proteins not present in the database will have escaped from being identified. Ten percent of the recorded MSMS spectra were identified when the *Insecta* database was used. This rather low percentage also indicates that the database is not complete.

Table 4.2. A: Identified muscle proteins of defatted & ground whole *T. molitor*, supernatant and pellet fractions (UniProt: taxonomy 50557, *Insecta*); B: Most abundant proteins identified of defatted & ground the whole *T. molitor*, supernatant and pellet fractions (UniProt: taxonomy 50557, *Insecta*) as determined by LC-MS/MS. Highlight: putative uncharacterized proteins identified based on family and domain databases from Uniprot.

A

	Muscle proteins	**Accession**	**Mol. weight [kDa]**	**Log10 (iBAQ defatted Yellow *T. molitor*)**	**Log10 (iBAQ Pellet)**	**Log10 (iBAQ Supernatant)**	**Sequence coverage [%]**
1	Putative tropomyosin-2-like isoform 1 (Triatoma infestans) Putative tropomyosin 1 (Fragment) (Rhodnius prolixus)	A0A023F9N9_TRIIF; R4G8P9_RHOPR;	30.8	5.03	6.42		28.8
2	Actin (Fragment) (Phaedon cochleariae)	A4KXN5_PHACE;	14.9	4.70			38.5
3	Putative actin indirect flight muscle (Fragment) (Anopheles aquasalis); Actin (Culex quinquefasciatus)	T1DQP1_ANOAQ; B0WZI6_CULQU;	42.3		6.23		35.5
4	Alpha-actinin, sarcomeric (Drosophila melanogaster)	P18091_ACTN_DROME;	107.0	5.17		5.34	10.9

5	Tropomyosin 2 (Lethocerus indicus)	B7ZGK8_9HEMI;	32.5	6.18	7.33	4.85	27.1
6	Actin (Fragment) (Luciola cruciate)	C4TJD9_LUCCR	8.3	4.68			46.5
7	Beta actin (Polyrhachis vicina); Actin (Riptortus pedestris); Actin-4 (Bombyx mori)	D0ES27_9HYME; R4WHS2_9HEMI; S5M0Z6_BOMMO;	41.8	4.27	5.79		56.4
8	Muscular protein 20 (Fragment) (Elaphrus cupreus)	Q1XFP4_ELACU;	20.3	6.91	6.72	7.14	24.5
9	Alpha-actinin-4, (Pediculus humanus subsp. Corporis)	E0VM19_PEDHC;	106.8	5.73	5.83	5.88	22.9
10	Myosin heavy chain, muscle (Anoplophora glabripennis)	V5G100_ANOGL;	262.3	5.85	6.83	3.53	28.4
11	Tropomyosin 2 (Nilaparvata lugens)	V5JDH8_NILLU;	32.3	6.91	8.16	5.51	48.9
12	Actin (Fragment) (Timema monikensis)	F1C3U2_9NEOP;	30.9		7.24		55.6
13	Actin (Fragment) (Timema poppensis)	F1C3V8_TIMPO;	30.9		5.22		59.3
14	Putative tropomyosin 1 (Graphocephala atropunctata)	Q1W295_9HEMI;	32.6		7.42		27.5
15	Actin-4 (Bombyx mori)	S5M0Y7_BOMMO;	41.9	6.35	7.21	4.81	49.7
16	Tropomyosin-1, isoforms 9A/A/B	V5GNY3_ANOGL;	29.4		6.24		52

	(Anoplophora glabripennis)				
17	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (Anoplophora glabripennis)	V5GVT5_ANOGL;	72.9	4.84	9.1
18	Myosin-2 essential light chain (Simulium nigrimanum)	E2BYA7_HARSA;	16.8	5.26	17
19	Putative troponin C (Maconellicoccus hirsutus)	A2I491_MACHI;	18.4	7.05	16.2
20	Troponin I (Nilaparvata lugens)	C0M4Y2_NILLU;	23.8	6.73	8.4
21	Troponin (Glossina morsitans morsitans); Troponin T (Acromyrmex echinatior)	D3TS62_GLOMM	47.3	5.26	6.3

B

	Most abundant proteins in Defatted & ground *T. molitor*	**Accession**	**Mol. weight [kDa]**	**Log10 (iBAQ defatted *T. molitor*)**	**Sequence coverage [%]**	**Family and domain databases**
1.	TENMO 28 kDa	Q27013_TENMO	24.8	7.70	36.9	

	desiccation stress protein					
2.	TENMO 13 kDa hemolymph protein a (Fragment)	Q7YWD2_TENMO	13.2	7.68	78.2	
3.	TENMO 12 kDa hemolymph protein b	Q7YWD7_TENMO	14.1	7.50	57.9	
4.	TENMO Alpha-amylase	P56634_AMY_TENMO	51.2	7.50	18.3	
5.	RHOPR Uncharacterized protein (Fragment) OS=Rhodnius prolixus	T1HVE5_RHOPR	41.6	7.45	27.3	Actin/actin-like
6.	Arginine kinase (Fragment) OS=Doliopygus rhodesianus	F5BGW4_9CUCU	30.3	7.35	29.2	
7.	TENMO 12 kDa hemolymph protein c	Q7YWD6_TENMO	13.9	7.31	38.9	
8.	TENMO Hexamerin 2	Q95PI7_TENMO	84.5	7.20	16.5	
9.	TENMO 12 kDa hemolymph protein d	Q7YWD5_TENMO	13.9	7.06	43.7	
10.	Arginine kinase	D3Y4D1_HELAM	39.9	6.97	22.3	

	OS=Helicoverpa armigera					
11.	Tropomyosin 2 OS=Nilaparvata lugens	D6X4X3_TRICA	32.3	6.91	41.2	
12.	Muscular protein 20 (Fragment) OS=Elaphrus cupreus	D2A180_TRICA	20.3	6.91	19.6	
13.	Histone H4 OS=Apis mellifera	H9K697_APIME	21.2	6.90	22.9	
14.	TENMO Putative trypsin-like proteinase proteinase OS=Tenebrio molitor PE=2	A1XG57_TENMO	27.6	6.83	7.8	
15.	TENMO 86 kDa early-staged encapsulation inducing protein	Q9Y1W5_TENMO	90.6	6.78	13.8	
16.	Putative uncharacterized protein OS=Tribolium castaneum	D6W9T6_TRICA	20.4	6.74	7.3	EF-hand domain pair (including troponin C, and myosin essential chain)
17.	TENMO 12 kDa hemolymph	Q7YWD4_TENMO	13.8	6.64	52.8	

	protein e (Fragment)					
18.	TENMO 13 kDa hemolymph protein b (Fragment)	Q7YWD1_TENMO	14.7	6.64	57.3	
19.	Fructose-bisphosphate aldolase OS=Tribolium castaneum	D2A6E8_TRICA	39.8	6.61	9.3	
20.	Putative uncharacterized protein OS=Tribolium castaneum	D6X4X2_TRICA	75.2	6.50	21.4	Tropomyosin

	Most abundant proteins in supernatant	**Accession**	**Mol. weight [kDa]**	**Log 10 (iBAQ supernatant)**	**Sequence coverage [%]**	**Family and domain databases**
1.	TENMO 12 kDa hemolymph protein b	Q7YWD7_TENMO	14.1	8.02	57.9	
2.	TENMO 13 kDa hemolymph protein a (Fragment)	Q7YWD2_TENMO	13.2	7.99	78.2	
3.	TENMO Alpha-amylase	P56634_AMY_TENMO	51.2	7.86	13.4	
4.	TENMO 28 kDa desiccation stress protein	Q27013_TENMO	24.8	7.84	40.4	

5.	TENMO 12 kDa hemolymph protein c	Q7YWD6_TENMO	13.9	7.61	63.5
6.	Arginine kinase (Fragment) OS=Doliopygus rhodesianus	F5BGW4_9CUCU	30.3	7.56	35.6
7.	TENMO 12 kDa hemolymph protein d	Q7YWD5_TENMO	13.9	7.30	49.2
8.	TENMO Putative trypsin-like proteinase	A1XG57_TENMO	27.6	7.29	11.9
9.	Muscular protein 20 (Fragment) OS=Elaphrus cupreus	D2A180_TRICA	20.3	7.14	19.6
10	TENMO 12 kDa hemolymph protein e (Fragment)	Q7YWD4_TENMO	13.8	7.13	55.3
11	TENMO Putative serine proteinase	A1XG83_TENMO	28.2	7.11	14.8
12	Arginine kinase OS=Helicoverpa armigera	D3Y4D1_HELAM	39.9	7.09	22.3
13	Fructose-bisphosphate aldolase OS=Tribolium castaneum	D2A6E8_TRICA	39.8	6.91	9.3
14	TENMO 13 kDa hemolymph protein d (Fragment)	Q7YWC9_TENMO	14.7	6.85	54.2
15	Superoxide dismutase [Cu-Zn]	D2A2T2_TRICA	15.7	6.80	32

	OS=Tribolium castaneum					
16	TENMO 13 kDa hemolymph protein b (Fragment)	Q7YWD1_TENMO	14.7	6.65	57.3	
17	Arginine kinase OS=Locusta migratoria manilensis	A6M9J4_LOCMI	40.1	6.63	18.8	
18	TENMO Putative serine proteinase	A1XG73_TENMO	28.2	6.54	24.4	
19	Heat shock protein 70 OS=Mantichorula semenowi	D2Y0Z5_9CUCU	71.0	6.40	21.4	
20	Putative uncharacterized protein GLEAN_15595	D2A5U4_TRICA	16.0	6.36	19.9	single-domain von Willebrand factor type C proteins

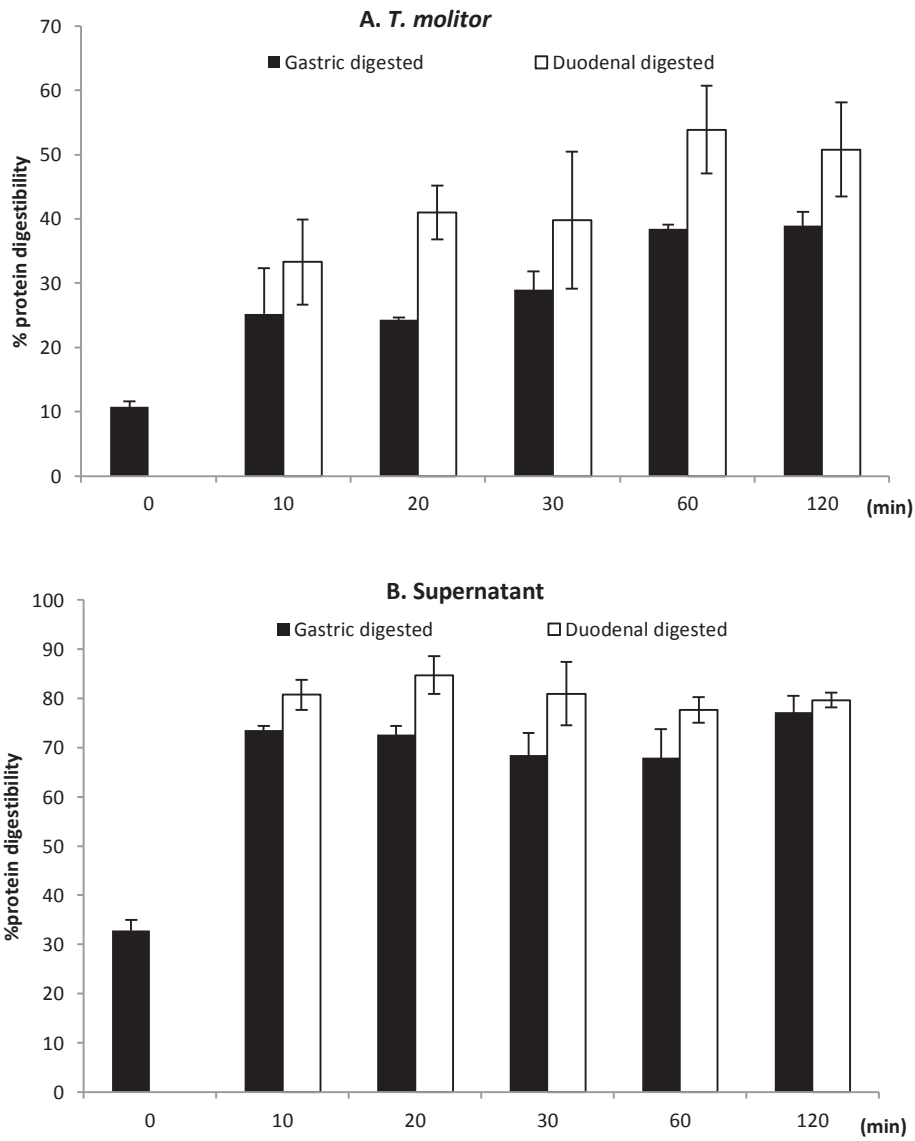
	Most abundant proteins in pellet	**Accession**	**Mol. weight [kDa]**	**Log 10 (iBAQ Pellet)**	**Sequence coverage [%]**	**Family and domain databases**
1.	RHOPR Uncharacterized protein (Fragment) OS=Rhodnius prolixus	T1HVE5_RHOPR	41.6	8.25	51.6	Actin/actin-like
2.	Tropomyosin 2 OS=Nilaparvata lugens	D6X4X3_TRICA	32.3	8.16	41.2	
3.	Histone H4 OS=Apis mellifera	H9K697_APIME	21.2	8.02	29.7	
4.	TENMO Hexamerin 2 OS=Tenebrio molitor	Q95PI7_TENMO	84.5	7.63	17.5	

5.	Putative uncharacterized protein OS=Tribolium castaneum Gent)	D6X095_TRICA	16.9	7.50	26.1	EF-hand domain pair (including troponin C, and myosin essential chain)
6.	Putative uncharacterized protein OS=Tribolium castaneum	D6W9T6_TRICA	20.4	7.50	7.3	EF-hand domain pair (including troponin C, and myosin essential chain)
7.	Putative tropomyosin 1 OS=Graphocephala atropunctata	Q1W295_9HEMI	32.6	7.42	26.4	
8.	TENMO 28 kDa desiccation stress protein	Q27013_TENMO	24.8	7.35	23.6	
9.	Tropomyosin 2 OS=Lethocerus indicus	B7ZGK8_9HEMI	32.5	7.33	26.1	
10.	TENMO 86 kDa early-staged encapsulation inducing protein	Q9Y1W5_TENMO	90.6	7.31	26.1	
11.	TENMO 56 kDa early-staged encapsulation-inducing protein	Q9Y1W6_TENMO	62.5	7.28	7.3	
12.	Actin (Fragment) OS=Timema monikensis	F1C3U2_9NEOP	30.9	7.24	53.8	
13.	Putative uncharacterized protein OS=Tribolium castaneum	D6WF19_TRICA	41.8	7.24	51.6	Actin/actin-like
14.	Actin-4 OS=Bombyx mori	S5M0Y7_BOMMO	41.9	7.21	44.9	
15.	Putative uncharacterized protein OS=Tribolium castaneum	D6X4X2_TRICA	75.2	7.20	21.4	Tropomyosin

16.	TENMO Putative trypsin-like proteinase	A1XG57_TENMO	27.6	7.19	11.9	
17.	TENMO 12 kDa hemolymph protein b	Q7YWD7_TENMO	14.1	7.14	41.3	
18.	Putative uncharacterized protein OS=Tribolium castaneum	D6WI56_TRICA	60.1	7.08	13.1	Myosin_tail
19.	Uncharacterized protein OS=Dendroctonus ponderosae	U4UBC2_DENPD	32.7	7.06	31.2	Tropomyosin
20.	ATP synthase subunit alpha	D6WSI9_TRICA	59.5	6.98	23	

3.3 Protein digestibility determination by OPA assay

Using equation 2, protein digestibility of the ground *T. molitor*, supernatant, pellet and residue fractions from gastric-duodenal digestion was calculated (Figure 4.1).



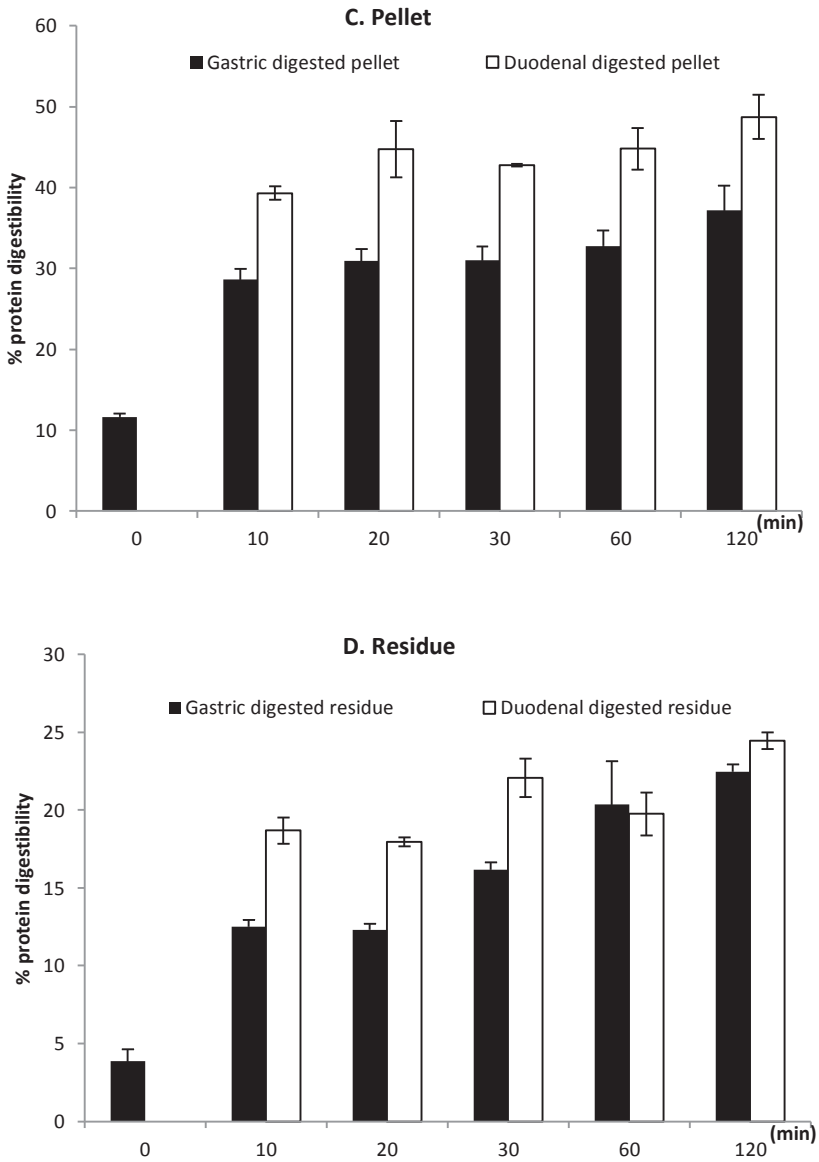


Figure 4.1: Protein digestibility of the ground *T. molitor* and its protein fractions (expressed according to equation 2) after gastric digestion (incubating from 10 minutes to 120 minutes) followed by duodenal digestion (incubating 120 minutes) (mean ± S.D., n=2).

Protein digestibility of defatted and ground *T. molitor*, increased from around 24% to 39% with increasing gastric digestion time (10 - 120 min) (Figure 4.1A). Subsequently, after two hours duodenal digestion, protein digestibility of all fractions obtained after gastric digestion increased to values ranging from 33% to 54%. The initial amount of free NH_2 group expressed as a percentage of total free NH_2 was around 11% in defatted & ground *T. molitor*.

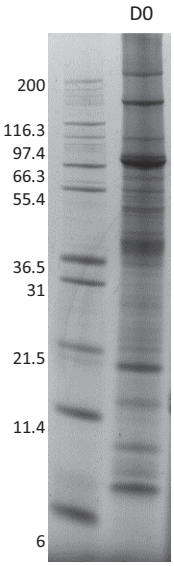
Protein digestibility of supernatant fractions was around 75% after gastric digestion and was nearly 85% after duodenal digestion (Figure 4.1B). Increasing gastric digestion time from 10 min to 120 min did not clearly increase protein digestibility of the supernatant fraction. The initial content of free NH_2 groups expressed as a percentage of total free NH_2 groups was found to be around 33%.

Protein digestibility of the pellet fraction increased from 29% to 37% with increasing gastric digestion time (Figure 4.1C). Subsequently, protein digestibility after duodenal digestion was around 45% for pellet. The initial content of the amount of free NH_2 group as a percentage of total free NH_2 groups was 12%.

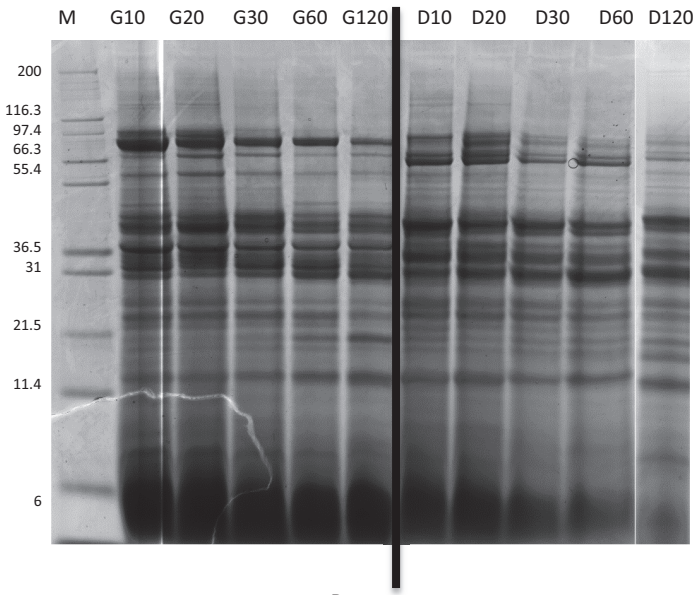
For the residue, protein digestibility increased from 13% to 23% with longer gastric digestion time (Figure 4.1D). Duodenal digestion compared to gastric digestion alone increased digestibility values, except for t = 60 min. The initial percentage of free NH_2 groups in residue was 4%. In comparison to water-soluble protein fractions (supernatant), proteins in pellet as well as in residue fractions showed relatively lower digestibility after gastric digestion and duodenal digestion.

3.4 SDS-PAGE

Reduced SDS-PAGE using 12% Bis/Tris gels (Figure 4.2) shows the protein band patterns of ground *T. molitor* and its protein fractions (supernatant, pellet and residue) after gastric digestion (incubating from 0 minutes to 120 minutes), and subsequently followed by duodenal digestion (incubating 120 minutes).

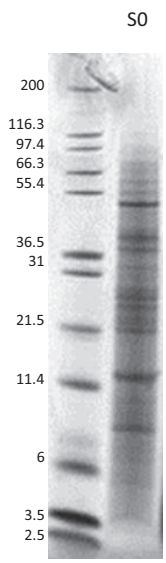


A

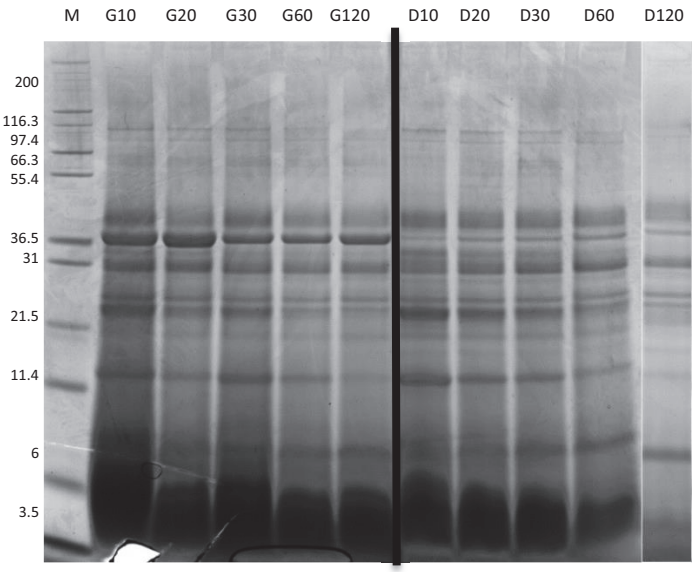


B

Defatted *T. molitor* (A and B)

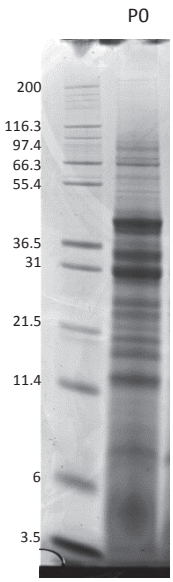


C

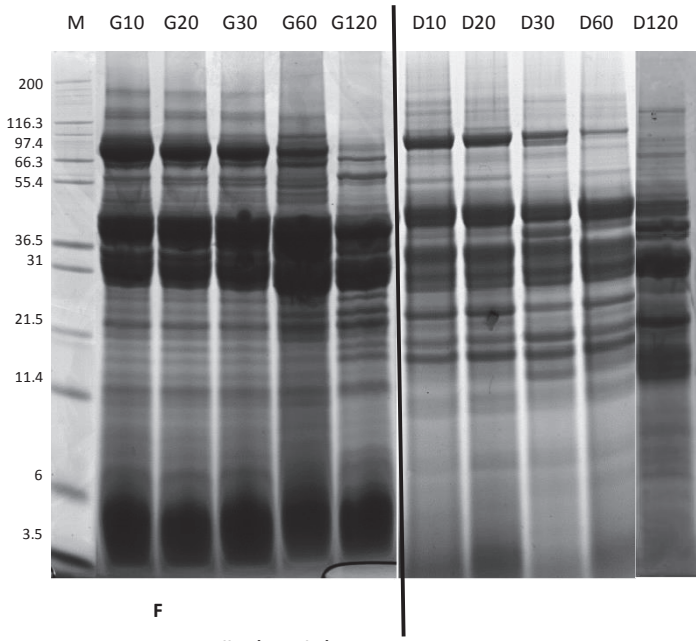


D

Supernatant (C and D)

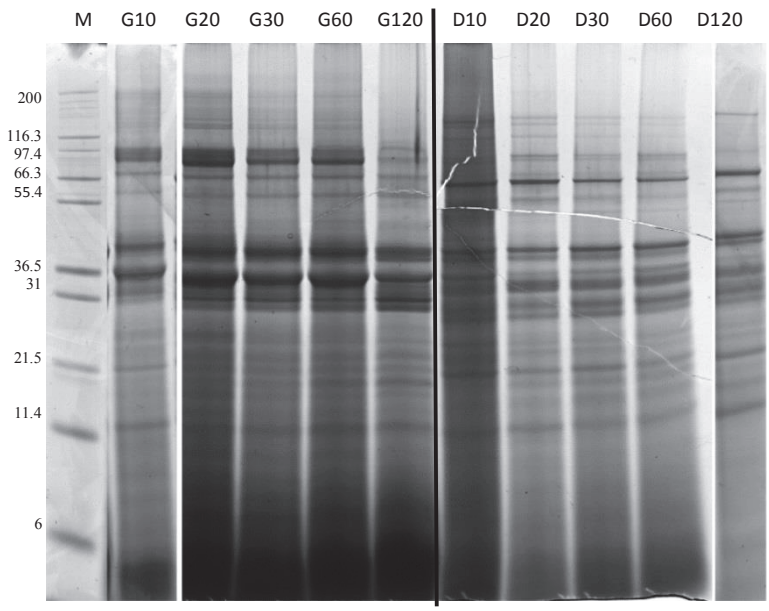


E



F

Pellet (E and F)



G.

Residue (G)

Figure 4.2: The band patterns of the ground *T. molitor* and its protein fractions after gastric digestion (incubating from 10 minutes to 120 minutes) and subsequent duodenal digestion (incubating 120 minutes) as determined by reduced SDS-PAGE.

For defatted & ground whole *T. molitor*, it is clear from the gels that the overall intensity as well as the band pattern changed upon digestion time (Figure 4.2A and 4.2B). The major bands of the initial defatted ground whole *T. molitor* had Mw of 151, 124, 80, 30 - 50, 17, 12, 10 kDa (Figure 4.2A). Protein bands with Mw of 124 and 151 kDa were not observed after gastric digestion (10 min to 120 min) (Figure 4.2B). Instead, bands appeared in the range of 30 - 50 kDa, as well as protein bands at size of < 6 kDa. Furthermore, the bands ranging from 30 to 50 kDa remained the same after duodenal digestion. Next to that, a band at around 80 kDa became less intense upon increasing gastric digestion time, and was completely absent after duodenal digestion. The protein band at around 12 kDa does not disappear after both gastric and duodenal digestion.

Band patterns of the supernatant fraction were in a range of < 97 kDa, and prominent bands distributed at around 58, 45, 40, 30, 19, 13 and 8 kDa (Figure 4.2C). Protein bands at around 13, 30, 40 and 45 kDa remained not only after gastric digestion, but also after duodenal digestion (Figure 4.2D). The intensity of two bands decreased. A band at 58 kDa was absent after gastric-duodenal digestion. A single protein band at 8 kDa disappeared, instead “smear” bands smaller than 6 kDa appeared, especially after the first 10 min of gastric digestion.

In the initial pellet fraction, the major proteins were visible at 75, 46, 36, 30, 24, 23, 19, 17, 13 kDa and bands of < 6 kDa (Figure 4.2E). The intensity of the initial pellet fraction was lower than that extracted after gastric-duodenal digestion, due to its poor solubility during sample preparation. The intensity of a band at 75 kDa was slowly decreasing with increasing gastric digestion time (Figure 4.2F). The pattern and intensity of bands ranging from 30 kDa to 50 kDa remained largely the same after gastric-duodenal digestion, which was similar to the trend in defatted ground whole *T. molitor*.

The water-insoluble residue protein fraction was also investigated in terms of molecular weight distribution. However, the initial residue was not visible when applying on the SDS-PAGE gels, due to its poor solubility in water. Therefore, the protein pattern of residue extracted after gastric-duodenal digestion was present without the initial protein bands in residue (Figure 4.2G). The protein bands were found at round 95 - 80, 70, 49, 39, 29, 19, 13 and < 6 kDa after the first 10 min gastric digestion. Most of the major bands were visible over the range of 70, 49, 39, 29, 13 and strong "smear" bands < 6 kDa upon increasing digestion. However, several bands at 95 - 80 kDa were less intense with increasing gastric digestion time, and subsequently disappeared after duodenal digestion.

4 DISCUSSION

4.1 Protein content determined by total nitrogen vs amino acid content

Protein content of *T. molitor* was determined by total nitrogen content (Dumas) multiplied by a protein factor of 6.25. However, Hall & Schönfeldt (2013) stated that the protein content as determined by total nitrogen is not accurate due to chemical and compositional differences between proteins, as well as the presence of non-protein nitrogen. Lysine, tryptophan, histidine and arginine contain additional nitrogen atoms in comparison to other amino acids. It means that if *T. molitor* contains large amounts of these high nitrogen-containing amino acids, the amount of nitrogen analysed would result in inaccurate protein content. In our previous study (Yi et al., 2013), the total amount of amino acids found in defatted & ground *T. molitor* was 910 mg/g protein, and such value did not end up to 1000 mg/g protein which could be explained by the presence of non-protein nitrogen. Besides, concentrating on the influence of side-chain differences between amino acids, the sum of total amount of amino group nitrogen could be calculated by using the amount of each amino acid divided by molecular weight of each amino acid, and then multiplied by molecular weight of nitrogen. Using the data of Yi et al. (2013), the rough sum of total amount of amino group nitrogen in defatted & ground whole *T. molitor* is calculated to be 128 mg/g crude protein extract. However, a precise value for a protein factor could not be given due to the presence of non-protein nitrogen, uncertainties in ash content, and

occurence of free amino acids as reported for other protein sources (Karman & Van Boekel, 1986; Lourenço et al., 2002).

4.2 Protein digestibility by OPA essay and SDS-PAGE

Protein fractions of *T. molitor* were digested more after the *in vitro* duodenal process than after the *in vitro* gastric process (Figure 4.1), which is qualitatively confirmed by the intensity of protein bands of SDS-PAGE, except for the supernatant fraction. Furthermore, strong intense protein bands < 6 kDa appeared in most fractions, which explains the increase of free NH_2 groups after gastric-duodenal digestion. Next to that, the initial content of free NH_2 in supernatant showed a very high value of 33% in comparison to the pellet and residue as determined by OPA essay.

The specific digestibility of soluble versus insoluble proteins by *in vitro* method has not been reported for *T. molitor* before. However, studies on *in vitro* digestion of other animal sources that are used as a whole for fish feed (including *i.e.* fish larvae, and cod filet) have been reported (Tonheim et al., 2007). These authors measured nitrogen content of the TCA-soluble nitrogen in order to determine protein digestibility. Alike our results, proteins of water-soluble fraction of the live feeds (*Artemia* and *Calanus*) were more digestible than those of water-insoluble fraction. Similar to our data, the initial TCA-nitrogen content of water-soluble fraction in *Artemia* was found to be around 38%, which can be explained by proteolysis. Tsybina et al. (2005); Goptar et al. (2013); Verhoeckx et al. (2014) mentioned that the major digestive peptidases of *T. molitor* are cysteine peptidases (mainly cathepsin L) and serine peptidases (including four trypsin-like and five chymotrypsin-like serine peptidases), as well as membrane-bound amino-peptidase. These present in the midgut. According to Bishop (1923), protein autolysis could indeed occur in insect body, as was shown for bee larvae. This autolysis occurred due to the degradation of muscle or skin protein by endogenous enzymes (Mukundan, Antony, & Nair, 1986). In addition Tonheim et al. (2007) mentioned that autolysis could occur, even though extraction took place at low temperature. This could explain the high initial content of free NH_2 groups found in supernatant of *T. molitor*.

4.3 Protein identification by LC-MS/MS, SDS-PAGE, and digestion

Muscle proteins

Myofibrils, the most abundant protein in muscular tissue, mainly consist of myosin heavy chain/light chain (~43%), actin (~20%) and other minor proteins such as, tropomyosin (~5%), troponins (~5%), and α-actinin (~2%) (Liu et al., 2011; Marion, 2008). Myosin heavy chain and light chains from sardines (*Sardinella longiceps*) showed molecular weights of 205, 31, 23, and 22 kDa (Mathew & Prakash, 2006); myosin from white mackerel muscle had three light chain subunits with Mw of 26.5, 20, and 17.5 kDa (Watabe & Hashimoto, 1980). Furthermore, Mw of light chains of carp ranged between 16 and 26 kDa (Okagaki et al., 2005). The exact molecular weights of myosin vary among species. Corresponding to myofibril proteins found in *T. molitor*, myosin heavy chain had a molecular weight of 262.3 kDa, and myosin light chain of 16.8 kDa, as is shown in Table 4.2A. Further, based on the data myosin heavy chain was not identified as a major protein in pellet fractions in Table 4.2B.

Identification and digestion of proteins in pellet fraction

Muscle proteins (especially myofibrillar protein) are classified as salt-soluble or insoluble fractions (Fox, Condon, & Foundation, 1982). From the LC-MS/MS results (Table 4.2), proteins were identified in the water-insoluble protein fraction (pellet), including actin and its fragment (30 - 40 kDa), α-actinin-4 (106.8 kDa), myosin heavy chain (262 kDa), myosin-2 essential light chain (16.8 kDa), tropomyosin 2 (32.5 kDa), troponin I (23.8 kDa), troponin T (47.3 kDa), putative troponin C (18.3 kDa) and putative actin indirect flight muscle (42 kDa). Using intensity based absolute quantification (iBAQ) data as quantitative data, those proteins were found in high amount in pellet fraction, in comparison to those in supernatant or defatted & ground whole *T. molitor*. Those larger amounts of muscle proteins in pellet fraction (especially actin and its fragment, tropomyosin and troponin T) were distributed from 30 to 50 kDa as determined by LC-MS/MS, corresponding to the strong intensity of bands between 30 - 50 kDa in pellet as determined by SDS-PAGE. In addition, those bands in pellet (30 - 50 kDa) showed less intensity after duodenal digestion than after gastric

digestion. Furthermore, these muscle proteins (30 - 50 kDa) could be gradually digested with increasing digestion time as observed by OPA results, but this was not clearly confirmed by SDS-PAGE. Similar results were found by Santé-Lhoutellier et al. (2008) for muscle protein (30 - 50 kDa) in lamb, actin (~50 kDa), troponin T (~44 kDa), tropomyosin (~40 kDa) was determined by SDS-PAGE.

In our study, among all muscle proteins identified, protein tropomyosin was found as one of the most abundant proteins in pellet (LC-MS/MS). Liu et al. (2011) and Verhoeckx et al. (2014) mentioned tropomyosin not only as part of myofibrillar protein, but also as the major allergen in fish, shrimp and crab. Furthermore, tropomyosin in pacific white shrimp, as well as in grass prawn was hardly degraded by pepsin (*in vitro* gastric digestion) (from t=0 to t=60 min), but gradually degraded by trypsin and α-chymotrypsin (*in vitro* duodenal digestion) (from t=0 to t=240 min) as determined by SDS-PAGE (Liu et al., 2011). These findings are in line with the band patterns found at around 35 kDa in pellet fraction of *T. molitor* based on SDS-PAGE (Figure 4.2F). It showed that proteins at around 35 kDa in pellet fractions were likely not degraded with time increasing in gastric digestion, but still visible after duodenal digestion.

Next to that, actin (~42 kDa) in pacific white shrimp was found to be gradually digested by pepsin, as well as by trypsin (t=120 min), and completely digested by α-chymotrypsin (t=120 min). However, according to our SDS-PAGE results, the intensity of bands (30 - 40 kDa) identified as actin with its fragment in pellet fraction (Figure 4.2F) was also reduced after duodenal digestion in comparison to that after gastric digestion, but not completed digested (t=120 min). The different protein band pattern could be explained by different positions at which amino acids are cleaved *in vitro* by trypsin and chymotrypsin. It is known that pepsin splits proteins to smaller parts which increases its accessibility, but does not digest proteins to amino acids (Akimov & Bezuglov, 2012); trypsin cleaves peptide bonds on the carboxyl side of arginine or lysine; and chymotrypsin usually cleaves peptide bonds on the carboxyl side of aromatic amino acids (phenylalanine, tryptophan and tyrosine) or leucine (Olsen, Ong, & Mann, 2004; Swanson et al., 2010).

In comparison to myofibrillar proteins from other meat sources, Storcksdieck, Bonsmann, & Hurrell (2007) reported that digestion of myofibrillar protein extracted from fresh beef, chicken, lamb, and pork or frozen cod fillets, resulted in high amounts of low molecular weight peptides < 10 kDa. That was based on centrifugation and ultrafiltration through 10 kDa molecular weight cut-off membranes after using pepsin, as well as after using pepsin/pancreatin. The amounts of peptides (> 10 kDa) were found in beef, chicken, cod, lamb and pork after pepsin only or pepsin/pancreatin digestion based on the change of nitrogen content (Storcksdieck, Bonsmann, & Hurrell, 2007). Furthermore, the nitrogen content of all meat extracts ranged from 55% to 65% of total nitrogen after using pepsin only, which was slightly lower than the range from 66% to 79% observed after using pepsin/pancreatin as determined by the Kjeldahl method (N × 6.25). In our study, the values for protein digestibility found in pellet ranged from 29% to 37% by using pepsin only, and were around 45% by using pepsin/pancreatin, which is relatively low in comparison to meat extracts as mentioned above. Alike our results, a strong "smear" group < 6 kDa was also observed in the pellet after pepsin digestion. However, this group of bands remained the same for pepsin and pepsin/pancreatin digestion, and showed less intense bands by using pepsin/pancreatin (Figure 4.2F). Regarding the myofibrillar proteins in pellet of *T. molitor*, the major bands remained often the same for *in vitro* gastric-duodenal digestion, although less intense bands were found after duodenal digestion.

Identification and digestion of proteins in supernatant fraction

In supernatant (water-soluble protein fraction) after gastric-duodenal digestion, major bands were found at 13, 19, 30, 40, and 45 kDa by using SDS-PAGE, likely corresponding to hemolymph protein (~13 kDa), muscular protein 20 (~20 kDa), putative serine proteinase (~28 kDa), arginine kinase (~40 kDa), and alpha-amylase (~50 kDa) identified by LC-MS/MS. The band at 13 kDa (hemolymph protein ~13 kDa) was digested with increasing digestion time (t=120 min) after *in vitro* gastric digestion. *In vitro* duodenal digestion did not seem to add substantially to digestion of this protein. Beside this band, the bands ranging from 20 to 50 kDa show a completely different pattern after duodenal digestion than after gastric digestion. Furthermore, in comparison to the initial protein pattern of supernatant, bands

with molecular weight > 50 kDa were hardly observed after gastric digestion or duodenal digestion. Proteins at molecular sizes > 50 kDa apparently could be digested easily by pepsin (t=10 min). Those water-soluble proteins consist of sarcoplasmic proteins as a major portion of muscle proteins which consist of glycolytic enzymes, myoglobin and other proteins present in intracellular fluid of muscle (George Jesslin et al., 2013). As mentioned by Verhoeckx et al. (2014), sarcoplasmic Ca binding proteins in water-soluble fraction of *T. molitor* were found at a molecular weight of 109.9 kDa. Storcksdieck, Bonsmann, & Hurrell (2007) mentioned that sarcoplasmic proteins are easily digested in comparison to myofibrillar protein, *e.g.* nitrogen content after digestion was 67% for chicken, 89% for beef, 88% for lamb and 87% for pork of total nitrogen after pepsin/pancreatin digestion. It likely contributed to high digestibility of water-soluble protein fraction.

In addition, Verhoeckx et al. (2014) reported that putative allergens found in water-soluble protein extract from *T. molitor* contained cationic trypsin (26.5 kDa), arginine kinase (40.1 kDa) and tubulin α-1 chain (50.6 kDa), alpha-amylase (51.7 kDa), ovalbumin-like (43.2 kDa) as determined by LC-MS/MS based on database homology with metazoan proteins. In our study, these putative allergic proteins could be found in both supernatant and pellet fraction as determined by LC-MS/MS based on the *T. molitor* database. However, these proteins were found to be more abundant in supernatant fraction based on database *Insecta* and the ranking of IBAQ. Direct comparisons between insect proteins and other proteins are difficult because many factors like composition of the digestive fluids used in each step, types of enzyme or enzyme concentrations have impact on determining absolute digestibility values as reviewed by Hur et al. (2011).

Proteins in residue fraction

Next to supernatant and pellet fractions, the band patterns of the residue were similar to the pellet, but the intensity of those bands was lower after duodenal digestion according to SDS-PAGE. The initial protein band in residue was not found in either LC-MS/MS or SDS-PAGE, due to its poor solubility during sample preparation.

5 CONCLUSIONS

This study produced data on protein digestibility of defatted & ground whole *T. molitor*, its water-soluble protein fraction (supernatant) and water-insoluble protein fractions (pellet and residue) after *in vitro* gastric-duodenal digestion.

With respect to protein identification and relative quantification as determined by LC-MS/MS, the most abundant proteins identified in supernatant were hemolymph protein and putative allergens (*e.g.* arginine kinase), which correlated to the band patterns (< 50 kDa) based on SDS-PAGE. For the pellet fraction, the most abundant proteins were muscle proteins, including actin and its fragment, tropomyosin and troponin T, mainly ranging from 30 to 50 kDa, corresponding to the strong intensity of bands (30 - 50 kDa) based on SDS-PAGE. These proteins could be degraded more after duodenal digestion than after gastric digestion.

The digestibility of the water-soluble protein fraction (supernatant, about 80%) was higher than that of water-insoluble protein fraction (pellet 50% and residue 24%) after *in vitro* gastro-duodenal digestion as determined by the OPA essay. High amounts of free NH_2 groups in supernatant (around 33%) were found before digestion, which is likely due to autolysis. Furthermore, increasing digestion time had no clear impact on protein digestibility of supernatant and impact on protein digestibility of pellet and residue. These findings suggest that the water-soluble protein fraction was more easily digested than water-insoluble protein fraction found for gastric and duodenal digestion. This study gives insight in the bulk protein composition of *T. molitor* and the *in vitro* digestibility, thereby contributing to knowledge needed for future food applications of this insect species. Overall, we conclude that a major gap in knowledge is filled concerning protein composition of an insect like *T. molitor* and its digestibility. The findings are helpful in addressing the question whether or not insect proteins are a promising new source of food proteins.

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Supplementary

Identified proteins of supernatant and pellet fractions from *T. molitor* (UniProt: taxonomy 7067, *T. molitor*).

Proteins found only in supernatant fractions		
No.	Accession	Protein Descriptions
1.	Q27011	Mw 14.02 kDa_TENMO 12 kDa hemolymph protein
2.	Q08596	Mw 16.05 kDa_TENMO C protein
3.	A1XG71	Mw 27.97 kDa_TENMO Putative serine proteinase
4.	A1XG83; A1XG84	Mw 28.15 kDa_TENMO Putative serine proteinase
5.	A1XG92	Mw 32.65 kDa_TENMO Putative cathepsin B-like like proteinase
6.	Q7YZB9	Mw 39.55 kDa_TENMO Chitinase
7.	D1MYQ7	Mw 41.04 kDa_TENMO Serpin1
8.	B1B5K0	Mw 41.97 kDa_TENMO 41 kDa zymogen
9.	B1B5K2	Mw 51.00 kDa_TENMO GNBP1
10.	G3XGC4	Mw 96.34 kDa_TENMO 93 kDa Serpin
11.	B4UWK6	Mw 179.04 kDa_TENMO E cadherin
12.	Q8MP05	Mw 321.40 kDa_TENMO Chitinase GN=chit5
13.	Q6R5A9	Mw 370.49 kDa_TENMO Tenebrin
Proteins found only in pellet fractions		
No.		Protein Descriptions
1.	A7X812	Mw 12.37 kDa TENMO Histone H3 (Fragment)
2.	Q27021	Mw 16.61 kDa_TENMO 60S ribosomal protein L27a
3.	Q0G8K8	Mw 19.34 kDa_TENMO Transporter
4.	Q86FS6;Q7YXL3; Q7YXL4	Mw 37.31 kDa_TENMO Cathepsin L-like cysteine proteinase
5.	Q4LE89	Mw 39.69 kDa_TENMO Melanin-inhibiting protein
6.	D1MYQ4	Mw 43.56 kDa_TENMO Serpin40
7.	Q6W970	Mw 45.75 kDa TENMO Sodium/potassium ATPase alpha subunit
8.	Q9Y1W6	Mw 62.45 kDa_TENMO 56 kDa early-staged encapsulation-inducing protein
9.	Q64EZ1	Mw 206.07 kDa TENMO Vitellogenin

Proteins found in both supernatant and pellet fractions		
No.		Protein Descriptions
1.	E7CIJ5	Mw 6.6 kDa TENMO 14-3-3 zeta
2.	Q7YWD2	Mw 13.17 kDa TENMO 13 kDa hemolymph protein a (Fragment)
3.	Q7YWD4	Mw 13.84 kDa TENMO 12 kDa hemolymph protein e (Fragment)
4.	Q7YWD4	Mw 13.94 kDa TENMO 12 kDa hemolymph protein d (Fragment)
5.	Q7YWD6	Mw 13.97 kDa TENMO 12 kDa hemolymph protein c (Fragment)
6.	Q7YWD7	Mw 14.14 kDa TENMO 12 kDa hemolymph protein b (Fragment)
7.	Q7YWD1;Q7YWD0	Mw 14.68 kDa TENMO 13 kDa hemolymph protein b (Fragment)/ Mw 14.45 kDa TENMO 13 kDa hemolymph protein c (Fragment)
8.	Q7YWC9	Mw 14.74 kDa TENMO 13 kDa hemolymph protein d (Fragment)
9.	Q27014;Q27013	Mw 24.83 kDa TENMO 28 kDa desiccation stress protein
10.	Q8MPF2	Mw 26.68 kDa TENMO Triosephosphate isomerase
11.	A1XG55;A1XG58; A1XG56;A1XG57	Mw 27.06 kDa TENMO Putative trypsin-like proteinase
12.	A1XG73;A1XG75; A1XG74	Mw 28.18 kDa TENMO Putative serine proteinase
13.	Q8I6J9	Mw 48.82 kDa TENMO Masquerade-like serine proteinase homologue
14.	P56634	Mw 51.24 kDa TENMO Alpha-amylase
15.	Q9GSE6	Mw 57.76 kDa TENMO Beta-glucosidase
16.	C8CE48	Mw 60.67 kDa TENMO Tyrosine hydroxylase
17.	Q7YZB8	Mw 65.48 kDa TENMO Cockroach allergen-like protein
18.	O97047	Mw 79.15 kDa TENMO Prophenoloxidase
19.	Q95PI7	Mw 84.54 kDa TENMO Hexamerin 2
20.	Q9Y1W5	Mw 90.62 kDa TENMO 86 kDa early-staged encapsulation inducing protein
21.	Q9NDN7	Mw 167.82 kDa TENMO Melanization-related protein

CHAPTER 5

INSECT LIPID PROFILE: AQUEOUS VS ORGANIC SOLVENT-BASED EXTRACTION METHODS

Tzompa-Sosa, D.*, Yi, L.*, van Valenberg, H., van Boekel, M. A. J. S, Lakemond, C. M. M. (2014). Insect lipid profile: aqueous vs organic solvent-based extraction methods. **Food Research International**, 62(0), 1087-1094. *Authors equally contributed to this work.

Insect lipid profile: aqueous vs organic solvent-based extraction methods

ABSTRACT

In view of future expected industrial bio-fractionation of insects, we investigated the influence of extraction methods on chemical characteristics of insect lipids. Lipids from *Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus* and *Blaptica dubia*, reared in the Netherlands, were extracted by two industrial extraction processes (aqueous & Soxhlet) and one laboratory method (Folch extraction). Chemical characterization in terms of fatty acid composition (GC-FID), triacylglycerol profile (GC) and lipid classes (TLC) was performed on all the extracted lipids. The major findings on lipid chemical characterization were: 1) *T. molitor* had the highest lipid content around 13%; 2) the highest yield was obtained using Folch extraction, and the lowest yield using the aqueous method (from 19% to 60% related to the lipid recovery of Folch extraction); 3) ω-3 fatty acids, which are related to health benefits, were most abundant in lipids from aqueous extraction, while ω-6 fatty acids were most abundant in Folch extractions, except for *B. dubia*; 4) lipids from Folch and Soxhlet extractions contained free fatty acids and partial glycerides, which were absent in aqueous extractions. 5) triacylglycerol distribution is similar among insect species, with high levels of ECN 50-54 and low amounts of ECN 36-38. In conclusion, aqueous extraction gave the lowest lipid yield, but provided a lipid extract low in ω-6/ω-3 ratio and with less polar lipids than Soxhlet and Folch extractions. These characteristics are desirable in edible lipids. This is the first time that the triacylglycerol profile of insect lipids is reported. It is also the first time that C18:1 and C18:2 are reported as separated isomers and that trans isomers of C16:1 and C18:1 are reported in insect lipids.

KEYWORDS

Insect lipid; Edible insects; Triacylglycerol profile; *Tenebrio molitor*; *Alphitobius diaperinus*; *Acheta domesticus*; *Blaptica dubia*; ω-3/ω-6 ratio

1 INTRODUCTION

Most of the attention on insects as a food source focuses on protein content. However, lipids are also a main component of insects and are produced during protein isolation (Yi et al., 2013). Lipids are a source of energy and of essential fatty acids (FA), therefore they could be used to combat malnutrition in developing countries (Smit, Muskiet, & Boersma, 2004). In populations with inadequate total energy intake, such as seen in many developing regions, dietary fats are important macronutrients that contribute to increase energy intake to more appropriate levels (FAO, 2010). Insect lipids can contribute to human nutrition by supplying energy and essential fatty acids (Ramos-Elorduy, 2008). Generally, the lipid content of insects ranges from less than 10% to more than 30% on a fresh weight basis and are relatively high in the unsaturated C18 FA, including oleic acid (18:1 cis 9), linoleic acid (18:2 cis 9,12) and linolenic acid (18:3cis 9,12,15) (DeFoliart, 1991).

Approximately 1900 insect species are consumed globally as human food in the world *e.g*. in Africa, Asia and Latin America (van Huis, 2013). As a food source, insects are potentially nutritious, rich in protein, minerals and vitamins. Insect production is a potential agricultural business because insects have a high nutritional value and their rearing has a low environmental impact (Oonincx et al., 2010; Ramos-Elorduy, 2008; van Huis, 2013). Despite this fact, in most developed countries people dislike eating insects due to their “dirty” and ”scary” image (Chen, Feng, & Chen, 2009). Extracting insect proteins and using these as a food ingredient may increase consumer acceptance. Recently, a study has been performed on extraction and characterization of proteins from five different insect species using an aqueous extraction method (Yi et al., 2013). Next to several protein-rich fractions, a lipid fraction was obtained as a by-product from the extraction. We studied the lipid composition of the extracts of four insect species obtained by this aqueous method and compared it with two other extraction methods: Soxhlet, a method with industrial application and with Folch an analytical method usually applied at a laboratory scale. Both Soxhlet and aqueous lipid extractions are of industrial relevance.

Insect lipid profile: aqueous vs organic solvent-based extraction methods

Lipid content and types of lipids in insects vary according to their species and life stage. Total lipid content for caterpillars (Lepidoptera) ranges from 8.6 to 15.2 g/100 g fresh In contrast, grasshoppers and related species (Orthoptera) have a relatively low lipid content, which range from 3.8 g to 5.3 g /100 g fresh insects (Bukkens, 1997). Insect crude lipids obtained by organic solvent-based extractions are constituted of several types of lipids *e.g.* triacylglycerols, phospholipids, sterols, glycolipids. Although several types of lipids are present in the extracts, about 80% of the lipid content is present in the form of triacylglycerols (Gilby, 1965), which serve as energy deposit for periods of high energy demand for example for prolonged flights (Beenakkers, Vanderhorst, & Vanmarrewijk, 1985). The second most important lipid class in crude insect lipid consist of phospholipids, which have an important role in membrane cell structure. The content of phospholipids in crude fat is usually below 20% but its content varies between life stage and insect species (Ekpo, Onigbinde, & Asia, 2009; Gilby, 1965). Cholesterol is the most important type of sterol present in insects. Cholesterol is also part of the structure of cell membranes. It serves as a precursor for vitamin D, bile salts and steroid hormones. Ekpo, et al. (2009) studied the cholesterol content in lipids of termites (*Macrotermes bellicosus*), a caterpillar (*Imbrasia belina*), and beetle larvae of *Oryctes rhinoceros* and *Rhynchophorus pheonicis*, which are four types of insects consumed in Nigeria. They found that the average cholesterol content in insect lipid fraction was 3.6%. The presence of several types of lipids is justified by the biological role of each one of them. On the other hand, the absolute lipid content of insects depends mainly on their life stage and on their physiological requirements (Beenakkers, Vanderhorst, & Vanmarrewijk, 1985).

Lipids from vegetable and animal sources are industrially extracted with non-polar solvent or with aqueous extractions (Dijkstra & Segers, 2007). Non-polar solvent extractions are based on the capacity of the non-polar solvents to dissolve lipids while aqueous extractions are based on the insolubility of lipids in water (Ricochon & Muniglia, 2010). The use of different lipid extraction methods results in different yields (Perez-Palacios et al., 2008) and in extraction of different lipid classes (Christie, 1993). Similar lipid extraction yields have been reported for meat and meat products by using Soxhlet and Folch extractions. However,

Folch is an analytical method applied mainly in the laboratory, while Soxhlet is an extraction method widely used in industry. The solvents used include carbon disulfide, petroleum naphtha, benzene, trichloroethylene, alcohol, pentane, supercritical carbon dioxide, and especially commercial hexane (Kemper, 2013). Lower yields have been consistently reported with aqueous based lipid extraction when compared with non-polar solvent extractions (Ricochon & Muniglia, 2010). Due to safety, quality, and environmental issues, aqueous extractions are industrially applied to extract animal fat and vegetable oils.

Several studies have been published on fatty acid composition of insects. While most of these studies focused on wild-caught edible insects (Bukkens, 1997; Rumpold & Schluter, 2013), some included reared insects to be used as animal feed (Finke, 2002) using mainly organic solvent-base extractions. It is known that the method of extraction affects the types of lipids extracted; hence, the FA composition of the lipids is expected to be affected. The degree in which the FA profile is affected will depend on the proportion of lipid classes originally present in the sample and this varies from species to species. So far no assessment on the effect of extraction method of insect lipid composition has been done. Such knowledge is of relevance in view of future expected industrial bio-fractionation of insects. For these reasons, it was decided to perform a complete chemical lipid analysis on reared insects commercially available in the Netherlands and to assess the effect of the lipid extraction method used. The four insect species under study are reared as food for human consumption and for insectivorous animals.

2 MATERIALS AND METHODS

Four insect species were selected based on their commercial availability in the Netherlands. The insect species studies were: Yellow mealworm (*Tenebrio molitor*) and Lesser mealworm (*Alphitobius diaperinus*) (Coleoptera); house cricket (*Acheta domesticus*) (Orthoptera); and the Argentinian cockroach (*Blaptica dubia*) (Blattodea). The first three insect species are considered for human consumption and the fourth as animal feed. All insect species were obtained from the commercial supplier Kreca V.O.F. (Ermelo, the Netherlands). The feed for *T. molitor* and *A. diaperinus* consisted mainly of wheat, wheat bran, oats, soy, rye, corn,

carrot and beer yeast, and the feed for *A. domesticus and B. dubia* consisted mainly of carrot and chicken mash.

2.1 Extraction methods

All live insects were fastened for about 24 h. After this period, the insects were stored for half an hour in the freezer at -50 ºC. Next, the frozen insects were put in liquid nitrogen and subsequently ground using a blender (Braun Multiquick 5, 600 W, Kronberg, Germany). The frozen, ground insects were freeze-dried until arriving at stable weight and its moisture content was determined (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands). Insects were stored at -20 °C for subsequent operations. All experiments were performed in triplicate.

2.1.1 Insect lipids extracted with Soxhlet extraction

Total lipid content was determined using representative samples of 10 g freeze-dried insect powder. The lipids were extracted using a Soxhlet apparatus for 6 hours (Biosolve, CAS nr. 110-54-3) using petroleum ether (CAS nr. 0101316465) as a solvent. Afterwards, petroleum ether was removed using a rotary evaporator (R420, Buchi, Switzerland) at 350 mbar in a water bath at 40 °C for about 30 min, and further increase up to 60 °C until no solvent was seen. The lipid extracts were stored under nitrogen atmosphere at - 20 ºC for further analysis.

2.1.2 Insect lipids extracted with Folch extraction

Total lipid content was determined using representative samples of 5 g freeze-dried insect powder. Ground insects were mixed with 200 mL dichloromethane/methanol (2:1) solution. The mixture was shaken for 20 seconds. Next, the mixture was sonicated (Sonicator Elma transsonic T700, Germany) for 10 minutes and then shaken for 2 hours on a rotary shaker (Edmund Bühler Gmbh SM-30, Hechingen, Germany). After adding 25 mL demineralised water, the mixture was centrifuged at 1006 g for 20 min at room temperature. The upper layer containing non-lipid compounds was removed from the bottle using a glass pipette.

Subsequently, the lower layer containing the lipids solubilized in organic solvents was filtered using a paper filter. To avoid losses, the bottles and the filters were flushed with dichloromethane two times. Next, the organic solvents were evaporated for 1.5 hour using a rotary evaporator at 800 mbar flushed with nitrogen and with a water bath at 40 °C. The amount of Folch lipid extracts (FLE) was calculated. The lipid extracts were stored under nitrogen atmosphere at - 20 ºC for further analysis.

2.1.3 Insect lipids extracted with an aqueous extraction

The extraction method was based on an aqueous method described by Yi et al. (2013) with some modifications. First, 200 g frozen insects were mixed with 600 mL demineralized water and blended for one minute, followed by 15 minutes sonication. The obtained insect suspension was sieved through a stainless steel filter sieve with a pore size of 350 µm. After that, the insect suspension was centrifuged at 15,000 g for 30 min at 4 ºC. Three fractions were obtained from the filtrate. From top to bottom: the lipid fraction, the supernatant, and the pellet. Subsequently, the lipid fraction was centrifuged at 15,000 g for 30 minutes at 40 ºC. After the second centrifugation, a transparent anhydrous lipid extract was obtained in the upper layer of the centrifuged tube. Two other fractions were obtained, these were a thin cream layer and a lower layer with water and water soluble compounds. The amount of aqueous lipid extracts (ALE) was calculated. The lipid extracts were stored under nitrogen atmosphere at - 20 ºC for further analysis.

2.1.4 Feed lipid extracted with Soxhlet extraction

Insect feed was freeze-dried until arriving at stable weight (GRI Vriesdroger, GR Instruments B.V., Wijk bij Duurstede, the Netherlands). Afterwards, lipids were extracted using a Soxhlet apparatus for 6 hours (Biosolve, CAS nr. 110-54-3) using petroleum ether (CAS nr. 0101316465) as a solvent. Afterwards, petroleum ether was removed using a rotary evaporator (R420, Buchi, Switzerland) at 350 mbar in a water bath at 40 °C, and further increased up to 60 °C until no solvent was seen. The lipid extracts were stored under

nitrogen atmosphere at - 20 ºC for further analysis. All experiments were performed in duplicate.

2.2 Chemical characterization

2.2.1 Determination of fatty acid composition

The FA composition of the lipid extract from four insect species and insect feeds were analysed as fatty acid methyl esters (FAME) prepared by transesterification in accordance with international standard ISO5509:2000(E). The determination of FA composition was performed by means of gas chromatography with flame ionization detector (GC-FID) (Thermo Scientific Trace GC Ultra) using a WCOT fused silica column (100m x 0,25 mm i.d., Coating Select Fame, Varian, the Netherlands) in accordance with international standard ISO15885:2002(E)IDF184:2002(E).

2.2.2 Qualitative determination of lipid classes

A qualitative determination of lipid classes of insect lipid extracts was achieved by silica gel thin layer chromatography (TLC). The extracted lipids and reference compounds were dissolved in dichloromethane (40 µg/µL). In short, 5 µL of lipid extracts and reference compounds were applied in spots to a silica gel plate of 20x20 cm, with a thickness of 500 µm (Analtech Inc., Newark, DE). The plates were developed in glass chambers using hexane/diethyl ether/acetic acid (70:30:1, v/v) as mobile phase (Kaluzny et al., 1985). The following references for lipid classes were used: L-α-phosphatidylcholine (CAS nr. 8002-43-5) for phospholipids, monopalmitin (CAS nr. 32899-41-5) for monoacylglycerols, dipalmitin (CAS nr. 26657-95-4) for diacylglycerols, free cholesterol (CAS nr. 57-88-5) for free sterols, palmitic acid (CAS nr. 57-10-3) for free fatty acids (FFA), tripalmitin (CAS nr. 555-44-2) for triacylglycerols, and cholesteryl butyrate (CAS nr. 521-13-1) for sterols. Iodine vapour (CAS nr. 7553-56-2) was used to reveal the lipid classes.

2.2.3 Determination of triacylglycerol (TAG) profile by equivalent carbon number (ECN)

Equivalent carbon number (ECN) was determined in insect lipid extracts. ECN is a quantitative determination of the size distribution of the TAG in lipids. This information complements that of the FA composition. The determination of TAG profile was performed by means of gas chromatography with flame ionization detector (GC-FID) (CP-380, Varian) using a WCOT SimDist fused silica column (5 m x 0,53 mm i.d., DF=0,17µm, Varian, The Netherlands) in accordance with the reference method of ComissionRegulation(EC)No.273/2008 Annex XX. A certified reference of TAG containing a wide range of TAG (from 24 to 54 carbons) was used as calibration standard (IRMM/BCR519, Fluka, the Netherlands). The ECN of a triacylglycerol is an indirect way to determine the molecular weight of TAG, because it is related to the number of carbons and to the number of double bonds in the TAG. ECN is determined by the formula ECN=N-2n in which N is the number of carbon atoms in the three FA that make up the TAG and 'n' is the total number of double bonds present in the TAG (Christie & Han, 2010).

2.3 Statistical Analysis

To test for significant differences between the extraction methods, two-way ANOVA was performed, followed by pos-hoc LSD test. IBM SPSS statistics software (version 21; IBM Corp., Armonk, NY) was used. A significance level of $p<0.05$ was used throughout the study.

3 RESULTS AND DISCUSSION

3.1 Total lipid content and extraction yield from different extraction methods

The proximate lipid content of four insect species was determined on live weight basis by using Folch, Soxhlet and an aqueous based method (Table 5.1). Extracted yield was calculated based on total lipid content of SLE/FLE as well as that of ALE/FLE (Table 5.1).

Table 5.1: Total lipid content of four insect species after aqueous, Soxhlet and Folch extraction expressed on live weight basis. Extraction yields of aqueous and Soxhlet extractions relative to Folch extraction (mean ± S.D., n=3).

Insect species	Extracted lipid (g/100 g fresh insects)			Yield (%)	Yield (%)
	Aqueous	Soxhlet	Folch	(ALE/FLE)	(SLE/FLE)
T. molitor	7.8±0.4^{A}	12.7±2.4^{B}	12.9±0.2^{B}	60.3±0.4	98.4±2.4
A. diaperinus	5.5±1.0^{A}	10.7±0.5^{B}	9.4±1.0^{B}	58.3±1.4	113.5±1.1
A. domesticus	1.6±0.1^{A}	6.0±0.3^{B}	8.0±1.1^{C}	19.2±1.1	74.8±1.1
B. dubia	3.1±0.3^{A}	7.6±0.2^{B}	7.5±0.3^{B}	40.9±0.4	100.5±0.4

Statistical analysis: One-way ANOVA-LSD. Figures with different letters between rows are significantly different ($p<0.05$)
ALE, aqueous lipid extracts; SLE- Soxhlet lipid extracts; FLE- Folch lipid extracts

The proximate total lipid content of four insect species ranged from 1.6% to 7.8% using aqueous extraction, from 6.0% to 12.7% using Soxhlet extraction and from 7.5% to 12.9% for Folch extraction. As expected, the highest quantities of lipids were obtained using Folch extraction as well as Soxhlet extraction, and the least amounts were extracted using the aqueous method. No significant difference on lipid content was seen between Soxhlet and Folch extractions, except for *A. domesticus* ($p < 0.001$). The lipid content obtained by aqueous method was significantly different from Soxhlet and Folch (Table 5.1). Aqueous extraction led to 40.9%, 58.3% and 60.3% of lipid yield (ALE/FLE) for *B. dubia, A. diaperinus T. molitor*, respectively. In contrast, the lipid yield of *A. dometicus* was only 19%. A similar trend was seen for Soxhlet extraction, with Soxhlet extraction the lipid yield obtained was very close to the yield obtained by Folch extraction, reaching almost 100% for *T. molitor*, *A.*

diaperinus and *A. domesticus*; however for *B. dubia* the lipid yield was of only 74.8%. The crude fat content of *T. molitor* was about 12.7 g/100 g fresh insects, which is comparable to previous findings (Finke, 2002; Ghaly & Alkoaik, 2009; Jones, Cooper, & Harding, 1972). The crude fat content for *A. domesticus* (adult) was comparable to the ranges described in literature, which is approximately 6-7 g/100 g insects (Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002). For *A. diaperinus* and *B. dubia*, no crude fat data are available in literature.

3.2 Chemical characterization: Influence of extraction method on lipid composition of insect lipid extracts

3.2.1 Fatty acid composition

The FA profiles found in the four insect species as analysed by GC-FID (Table 5.2) are in accordance with the profiles found in previous studies on lipids where high amounts of unsaturated FA (USFA) were found relative to saturated FA (SFA) (Finke, 2002; Paoletti, 2005; Rumpold & Schluter, 2013; Thompson, 1973). The most abundant USFA in each of the four insect lipid extracts was C18:1 cis9 and C18:2 cis9,12; the most abundant SFA was C16:0. These three FA accounted from 84.7 to 89.8 g/100 g of the FA in the lipid extracts (Table 5.2). In the four insect species studied, the average amount of USFA ranged from 64 g/100 g in *A. diaperinus* and *A. domesticus* to 75 g/100 g in *T. molitor* and *B. dubia*. Other minor FA present in insect lipid extracts included trans, ω-3 and CLA FA.

Table 5.2: Fatty acid composition (g/100g) of lipids extracted from four insect species (mean ± S.D.) extracted with an aqueous and two solvent lipid extraction methods (n=2).

	Feed		*Tenebrio molitor*			*Alphitobius diaperinus*			*Acheta domesticus*			*Blaptica dubia*		
Fatty Acid	A[1]	B[2]	Soxhlet[3]	Folch	Aqueous	Soxhlet[3]	Folch	Aqueous	Soxhlet[3]	Folch	Aqueous	Soxhlet[3]	Folch	Aqueous
C12:0	0.89	0	0.23	0.14	0.28	0	0	0	0.30	0.16	0.27	0.16	0.00	0.20
C14:0	0.96	0	3.11	3.18	3.60	0.65	0.63	0.73	1.80	1.55	1.65	1.05	1.12	1.22
C16:0	17.21	12.60	18.52	17.31	17.96	25.18	23.33	24.68	25.99	23.69	24.81	18.05	20.18	19.30
C16:1 trans 9	0	0	0.70	0.69	0.65	0.74	0.70	0.83	0.68	0.60	0.75	0.23	0.09	0.26
C16:1 cis 9	0.59	0	2.09	2.05	2.25	0.22	0.11	0.26	2.09	1.78	2.04	5.17	4.95	5.51
C17:0 anteiso	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05
C17:0	0	0	0	0	0	0.40	0.39	0.39	0.20	0.11	0	0.21	0.12	0.22
C17:1 cis 9	0	0	0	0	0.10	0	0	0.06	0	0	0	0.18	0.09	0.18
C18:0	1.40	2.80	2.43	2.82	2.53	8.55	8.65	7.78	6.09	6.76	4.61	3.71	4.27	3.67
C18:1 trans 11	0	0	0	0	0	0	0	0	0.21	0.12	0.23	0	0	0
C18:1 cis 9 / C-18:1 trans 12	15.43	25.17	49.50	49.15	49.15	38.49	37.35	39.20	29.14	26.63	30.23	51.38	51.51	49.48
C18:1 trans 15 / C-18:1 cis 11	1.07	1.04	0.22	0.12	0.23	0.34	0.17	0.37	0.82	0.81	0.77	0.61	0.58	0.61
C18:2 cis 9,12 (ω-6)	54.99	53.76	21.82	23.35	21.67	23.28	26.79	23.61	29.11	34.35	31.80	17.36	15.65	17.62
C18:2 cis 9, trans 11	0	0	0	0	0	0	0	0	0	0	0	0.15	0.08	0.18
C18:3 cis 6,9,12 (ω-6)	0	0	0	0	0	0	0	0	0	0	0	0	0.10	0
C18:3 cis 9,12,15 (ω-3)	5.61	4.50	0.84	0.85	0.89	1.14	1.14	1.24	1.56	1.59	1.74	1.24	1.05	1.28
C20:0	0	0.17	0	0	0	0.38	0.36	0.37	0	0.09	0	0.14	0.08	0.14
C20:1 cis 11	0.61	0	0	0	0.05	0	0	0	0	0.06	0	0	0	0.12
C20:4 cis 5,8,11,14 (ω-6)	0	0	0	0	0	0	0	0	0	0.09	0	0	0	0
C20:3 cis 11,14,17 (ω-3)	0	0	0	0	0	0	0.08	0	0	0	0	0	0	0
C20:5 cis 5,8,11,14,17 (ω-3, EPA)	0.77	0	0	0	0	0	0	0	0.64	0.46	0.75	0	0	0
C22:6 cis 4,7,10,13,16,19 (ω-3, DHA)	0.49	0	0	0	0	0	0	0	0	0	0	0	0	0
Total SFA	20.45	15.56	24.28	23.52	24.43	35.14	33.35	34.05	34.37	32.35	31.33	23.30	25.77	24.78
Total USFA	79.55	84.45	75.17	76.21	74.99	64.19	66.33	65.55	64.23	66.48	68.28	76.31	74.08	75.22
Total CLA FA	0	0	0	0	0	0	0	0	0	0	0	0.15	0.08	0.18
Total ω-3 FA	6.87	4.50	0.84	0.86	0.89	1.14	1.22	1.24	2.20	2.04	2.48	1.24	1.05	1.28
Total ω-6 FA	54.99	53.76	21.82	23.35	21.67	23.28	26.79	23.61	29.11	34.44	31.80	17.36	15.74	17.62
Total MUFA	17.69	26.20	52.51	52.01	52.43	39.77	38.31	40.70	32.92	29.99	34.01	57.56	57.21	56.14
Total PUFA	61.86	58.25	22.66	24.20	22.56	24.42	28.01	24.85	31.31	36.48	34.28	18.75	16.88	19.08
ratio ω-6/ ω-3	8.00	11.96	25.98	27.15	24.35	20.42	21.96	19.04	13.26	16.85	12.82	14.06	14.95	13.76
Total unknown	0	0	0.56	0.27	0.58	0.67	0.32	0.41	1.41	1.17	0.39	0.39	0.15	0

[1] Feed A- *Tenebrio molitor* and *Alphitobius diaperinus*
[2] Feed B- *Acheta domesticus* and *Blaptica dubia*
[3] Petroleum ether was used as solvent for extraction of lipids.
Values are means of duplicate analysis

In the current study, C18:1 cis9 and C18:1 trans12, and C18:1 cis 11 and C18:1 trans15 co-eluted in the GC column. Since the cis isomers are the most abundant in nature we expect that the detected peak in the chromatogram corresponds mainly to the cis isomers. Lately the chromatographic techniques in lipid analysis had become more powerful. Better isomer separations have been achieved and the detection limit has dramatically decreased. Therefore, it is now possible to detect very low amounts (from 0.01 g/100 g of lipid approximately) of a FA present in the lipid fraction. These advances in the techniques allow us to challenge previous knowledge regarding trans FA in unprocessed lipids. In the present study, we confirm the presence of C16:1 trans9 and C18:1 trans11 in the lipid extracts of the insects under study. C16:1 trans9 has been previously reported in ruminant milk fats (Destaillats et al., 2002) but not in insect lipids. C16:1 trans9 and C18:1 trans11 could be products of some bacteria biohydrogenation because trans isomers cannot be synthesized *de novo* by the insect. It is well known that insect gut possess a wide microbial biota with potentially complex interactions with the diet, insect developmental age or genotype (Douglas, 2013). Therefore, it is plausible that these microbes produce trans FA. Moreover, neither C16:1 trans9 nor C18:1 trans11 were present in the insect diet (Table 5.2). C18:1 trans11 was found only in *A. domesticus* lipid extracts which makes this FA specific for this insect species. The proportion of C18:1 cis9 in the lipid extracts was higher as compared with the proportion of this FA in the diet. Moreover, C18:2 cis9,12 and C18:3 cis9,12,15 in the lipid extracts strongly decreased as compared with the proportion of this FA in the diet. A possible synthesis pathway for C18:1 cis9 in insect lipids is that C18:2 cis9,12 and C18:3 cis9,12,15 are completely biohydrogenated to C18:0 by insect gut microbiota. Afterwards, C18:0 is desaturated by Δ9 desaturases producing C18:1 cis9. Previously, Thompson (1973) suggested that C18:1 cis9 is the product of Δ9 desaturase which adds a double bond to C18:0. However, a little is known about the biohydrogenation of unsaturatred C18 to C18:0 in insect gut. It is known that ruminal microbial biohydrogenation of unsaturated C18:0 produces C18 trans isomers. In addition, this trans isomers are found only in *A. domesticus* (C18:1 trans11) and in *B. dubia* (C18:2 cis9trans11). To confirm our suggestion, studies on biohydrogenation of FA in insect gut are required.

The most abundant ω-3 FA found in insect lipids was C18:3 cis9,12,15 (α-linolenic acid), which was present (0.84 - 1.74 g/100g) in all four insect species. Previous work on terrestrial and aquatic insects have reported only traces (0.09-0.28 g/100g) of EPA (C20:5 cis 5,8,11,14,17) (Fontaneto et al., 2011) and DHA (C22:6 cis 4,7,10,13,16,19) in insect lipids or even reported their absence. In this study we found EPA in lipids extracted from *A. domesticus* (mean=0.62 g/100g). EPA was not found in the other three insect species studied. The presence of EPA and DHA in insect lipids has been related to their diet and to the need of these FA for specific physiological purposes (Beenakkers, Vanderhorst, & Vanmarrewijk, 1985; Fontaneto et al., 2011; St-Hilaire et al., 2007). EPA was not present in the feed from *A. domesticus*, indicating that this insect was able to biosynthesize EPA probably from C18:3 cis9,12,15 (Christie, 2013). Previous studies showed that *Gryllus assimillis,* an insect species related to *A. domesticus*, is able increase its EPA content when C18:3 cis9, 12,15 is increased in the diet (Komprda et al., 2013). In our study, C18:3 cis9, 12, 15 was also present in *A. domesticus* diet and it is likely that this FA is a precursor of EPA.

The highest ω-6/ω-3 ratio in insect lipids was found in *T. molitor*, where the ratio was of about 27:1. *A. domesticus* was the insect with the lowest ω-6/ω-3 ratio (17:1). The ω-6/ω-3 ratio of the four insect species is higher than the ratio recommended by FAO, which is 10:1 (FAO, 2010). The high amounts of C18:2 cis9,12, which is the main ω-6 FA present in our lipid extracts, could be responsible for the high ω-6/ω-3 ratio. In our study, C18:2 cis9,12 was the most abundant FA in both diets accounting for more than 53% of the FA present in the feed (Table 5.2). Since the FA composition of the insect lipids is influenced mainly by insect feed (Bukkens, 1997), an insect diet with low amounts of C18:2 cis9,12 might reduce the presence of this FA in insect lipids with a consequent reduction in ω-6/ω-3 ratio.

Higher amounts of PUFA and ω-6 FA were extracted by Folch compared to Soxhlet or aqueous extraction, except for *B. dubia*. Omega-3 FA content was higher in lipids obtained by aqueous extraction compared to those from Folch or Soxhlet method. These differences in FA have a direct effect on the ω-6/ω-3 ratio. The ω-6/ω-3 ratios of the lipids from the aqueous extraction were lower than the ones from Folch extraction. *A. domesticus* showed the greatest difference between extraction methods.

3.2.2 Lipid classes

Lipid classes are separated in silica gel TLC plates according to their degree of polarity: Polar lipids such as phospholipids will stay close to the origin while non-polar lipids such as triacylglycerols will run to the upper part of the plate. As expected, triacylglycerols were the largest and strongest spot at the upper part of all the TLC plates indicating that this is the most important lipid in the extractions (Figure 5.1). The method of extraction affected the lipid composition of the lipid extracts. The methods that involve organic solvents are able to extract a wide range of lipid classes while the aqueous method extracted mainly non-polar lipids, namely triacylglycerols, carotenoids and cholesterol esters (Figure 5.1). In our samples, phospholipids were present in organic solvent extracts but not in the aqueous extraction. It is likely that during the aqueous extraction the phospholipids, which are the most polar lipids, remain in the aqueous layer because in the presence of water, phospholipids hydrate and become water soluble (Dijkstra, 2011). Hydration does not occur when organic solvents are used, therefore, all phospholipids will stay in the solvent layer.

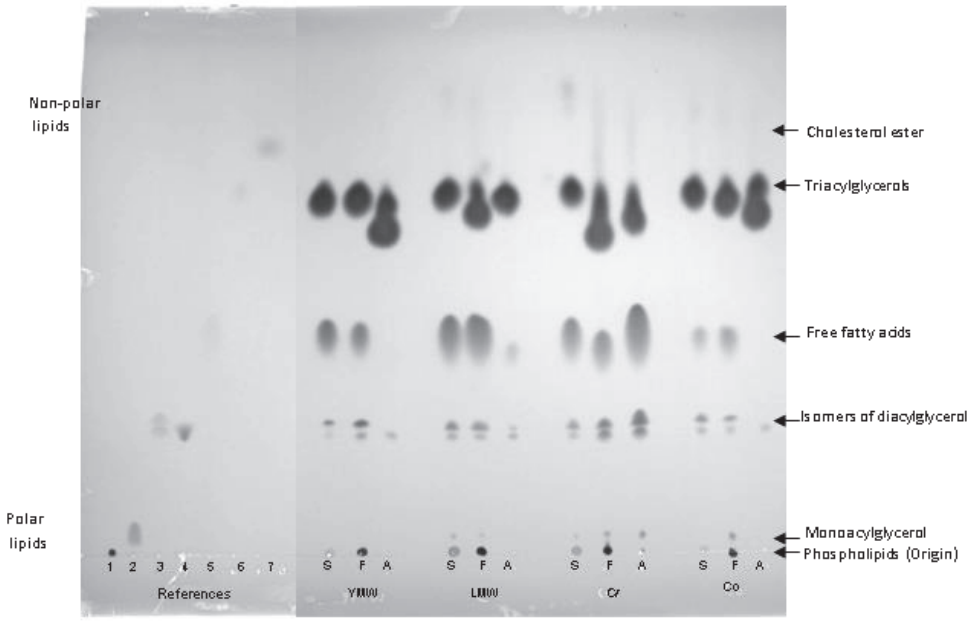


Figure 5.1. Lipid class separation of extracted lipids from four insect species in silica gel thin layer chromatography. Mobile phase used- hexane: diethyl ether: acetic acid (70 : 30 : 1). References: 1. L-α-phosphatidylcholine (phospholipids), 2. monopalmitin (monoacylglycerol), 3. dipalmitin (diacylglycerol), 4. Free cholesterol, 5. Palmitic acid (free fatty acid), 6. tripalmitin (triacylglycerol), 7. Cholesterylbutyrate. Samples: S- Soxhlet extraction with petroleum ether, F- Folch extraction, A- aqueous extraction, YMW- *Tenebrio molitor*, LMW- *Alphitobius diaperinus*, Cr- *Acheta domesticus*, Co- *Blaptica dubia*.

Monoacylglycerols, diacylglycerols and FFA were extracted with Soxhlet and Folch methods. These three types of glycerides are typical products of lipolysis, which can be formed during sample preparation (Kramer & Hulan, 1978). Kramer and Hulan (1978) showed that the temperature, at which the sample is homogenized, prior to enzymatic inactivation by the extracting solvents, affects the lipolitic activity and therefore the amounts of lipolized products. During grinding and drying of our samples for Folch and Soxhlet extractions, our samples were kept frozen at temperatures below -20°C to reduce degradation of the compounds. However, by TLC we show that FFA, monoacylglycerols and diacylglycerols were present in our lipid extracts. One plausible explanation for the presence of partial

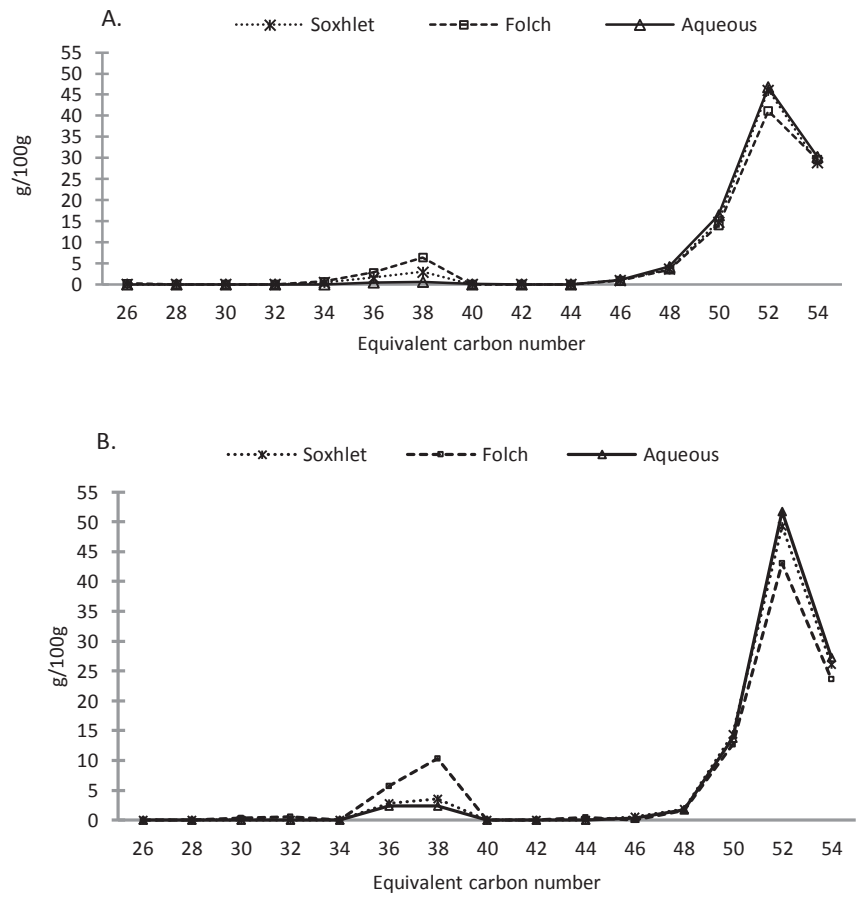
glycerides is that diacylglycerols are present in the insects itself. It is known that diacylglycerols are the main form in which lipids are transported in insects (Horne, Haritos, & Oakeshott, 2009), therefore, they will be present in the lipid extracts. However, the presence of FFA cannot be explained in this way and high amounts are not expected in tissues because they perturb the membrane structure (Christie, 2012). Another plausible explanation for the presence of partial glycerides is that these insects contain cold active lipases. These cold active lipases have a low optimum temperature, have high activity at very low temperatures and are active under low water conditions (Joseph, Ramteke, & Thomas, 2008). The conditions during freeze-drying of our samples could be appropriate for the activation of cold active lipases that will degrade the lipids in our insect samples. If Soxhlet extraction is used for industrial extraction of insect lipids, a refining process will be necessary to eliminate phospholipids, FFA and partial glycerides such as mono- and diacylglycerols .

The lipids extracted from aqueous extraction lacked mono-, diacylglycerols and contained less FFA than Soxhlet and Folch extractions. This could be explained by the fact that in the presence of sodium or calcium salts, FFA form soaps and increase their solubility in water (Rustan, 2005). Calcium and sodium are micronutrients commonly found in insects (Bukkens, 1997). These salts could have been solubilized in the supernatant of our aqueous extractions, which could have bind FFA forming soaps, which in turn will have increase the solubility of these lipids. Mon- and diacylglycerols are insoluble in water but can form stable emulsions (Dolezalkova et al., 2013; Miklos, Xu, & Lametsch, 2011). If mono- and diacylglycerols were produced during sample preparation, it is likely that they remain in the cream layer seen after the second centrifugation during the aqueous extraction.

In lipid from *A. domesticus* extracted from aqueous extraction polar and non-polar lipids were found; we suggest that *A. domesticus* contains phospholipids with a low hydration capacity that will allow them stay in the lipid phase. The reason for the presence of partial glycerides and FFA in these insect lipids is unclear. If *A. domesticus* lipids extracts free of phospholipids are desirable, a change in the pH during extraction can be applied (Dijkstra, 2011).

3.2.3 Determination of equivalent carbon number (ECN)

The distribution of FA within the glyceride molecule is genetically controlled; therefore, each oil and fat has a unique and typical pattern (Bonvehi & Coll, 2009). The information on ECN helps to identify the presence of foreign lipids and it is typically used in the industry to detect adulterations. The extracts from the four insect species in this study shared similar patterns (Figure5.2).



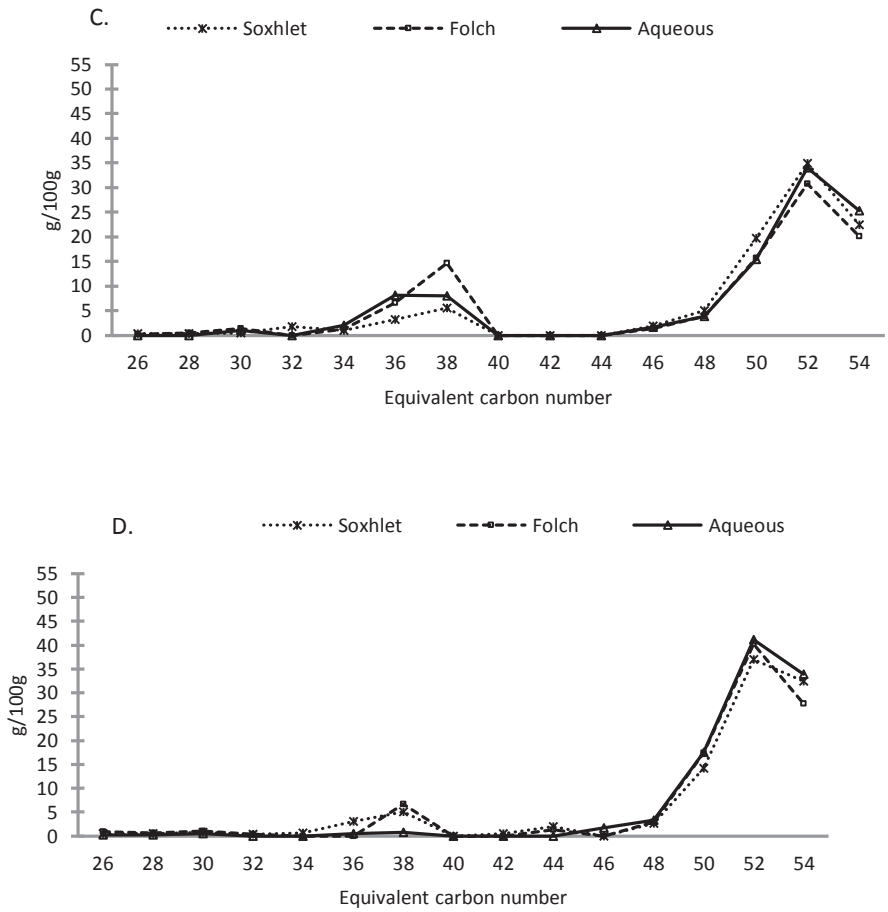


Figure 5.2: Triacylglycerol composition of insect fat expressed as equivalent carbon number (g/100g). Lipid extracted by Folch, Soxhlet and by aqueous method. A. *Tenebrio molitor*; B. *Alphitobius diaperinus*; C. *Acheta domesticus*; D. *Blaptica dubia* (n=2).

All four lipids had large concentrations of glycerides with an ECN of 50-54 and low concentrations of glycerides with ECN of 36-38. *A. domesticus* lipid extracts had the highest amount of glycerides with ECN of 36-38. The ECN pattern of our extracted lipids from insects differs from most vegetable oils and animal fats (Bergqvist & Kaufmann, 1993; Bonvehi & Coll, 2009). The most abundant glycerides in vegetable oils have an ECN from 44-48

(Bonvehi & Coll, 2009), while the most abundant glycerides in tallow have an ECN of 48-50 (Bergqvist & Kaufmann, 1993). Vegetable oils and animal fats had no glycerides below ECN 38, except for bovine milk fat (Haddad et al., 2012; Kuksis, Marai, & Myher, 1973), which has a large amount of short and medium chain FA. Regarding differences among extraction methods, we observed that the ECN pattern changed with the type of extraction. Glycerides with ECN of 36-38 were extracted with Folch and Soxhlet extractions but not in aqueous extraction. It is likely that phospholipids and diacylglycerols are the responsible glycerides for the peaks at ECN 36-38 because no short chain FA, which are typically in this range of ECN, were found in any of these lipids. Moreover, with lipid class determination, we show that phospholipids and diacylglycerols were present in Folch and Soxhlet but not in aqueous extractions (Figure 5.2). Phospholipids and diacylglycerols are glycerides with two FA which will give a lower carbon number than triacylglycerols which have three FA.

Further, the ECN analysis allows quantification of free cholesterol, which cannot be synthesize de novo and is synthesize from plant sterols. Cholesterol was present in amounts lower than 3.6% (Table 5.3). The highest proportion of free cholesterol in all four insect species was seen for the Folch extraction and the lowest proportions were found for aqueous extraction. Our results are in agreement with the data found by Ekpo et al. (2009) who studied the cholesterol content on four types of oil insects consumed in Nigeria.

Table 5.3: Free cholesterol (g/100 g) in lipids extracts from four insect species (mean ±S.D, N=2).

Insect species	Aq	S	F
T. molitor	N.D.	0.41±0.02	0.59±0.01
A. diaperinus	0.37± 0.01	1.51±0.01	1.76±0.05
A. domesticus	0.67± 0.01	3.32±0.03	3.58±0.03
B. dubia	0.24± 0.001	0.49±0.01	0.91±0.004

N.D. -not detected

4 CONCLUSIONS

We extracted lipid fractions from four insect species by using two industrially relevant methods (Soxhlet and aqueous extractions) and an analytical method (Folch extraction). *Tenebrio molitor* contained the highest amounts of lipids among four insect species. With respect to the extraction yield, the highest yield was obtained using Folch extraction. The lowest yields for lipid extractions were obtained using the aqueous method. Relative to the lipid recovery of Folch extraction, aqueous extraction could recover 40 - 60% for *T. molitor*, *A. diaperinus* and *B. dubia*. The lowest lipid yield, around 19%, was obtained for *A. domesticus*. The lipid yield of Soxhlet extraction was close to 100% for *T. molitor*, *A. diaperinus* and *A. domesticus*, and about 75% for *B. Dubia.*

The method of extraction affected the quantity and types of lipids and the FA profile of the insect lipid extracts. Higher ω-6/ω-3 FA ratios were extracted by Folch method while aqueous extraction had the lowest ratio, hence having the highest ω-3 FA content. Essential FA was present in the extracted lipids from *A. domesticus*. Lipid extracts obtained by Folch and Soxhlet contained polar and non-polar lipids while lipid extracts obtained by aqueous method contained mainly non-polar lipids. Therefore, if Soxhlet extraction is the chosen method for insect lipid extraction, a refining process should follow the extraction since FFA and monoacylglycerols are undesirable in lipid extracts. This process will not be necessary with an aqueous extraction because the lipids from these extractions lack phospholipids and partial glycerides. The ECN pattern in insect lipid extracts is similar between all four insect species. They are rich in CN 50-54 and have low amounts of CN 36-38. This pattern is unique and does not resemble any other vegeTable or animal lipid source. It the first time that a detail isomer separation of C18:1 and C18:2 is reported and that trans isomers of C16:1 and C18:1 are reported in insect lipids.

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CHAPTER 6

GENERAL DISCUSSION

1 INTRODUCTION

With an increasing world population, alternative protein sources are needed to replace conventional animal-derived proteins. Insects are among the novel protein sources that are suggested for food and feed in the European market (van der Spiegel, Noordam, & van der Fels-Klerx, 2013). Insects have a protein content comparable to common sources of conventional proteins (Barker, Fitzpatrick, & Dierenfeld, 1998; Finke, 2002; Jones, Cooper, & Harding, 1972; Ghaly & Alkoaik, 2009), as was confirmed in this thesis. In many countries of South America, Africa and Asia, people habitually use insects as an animal protein source. However, in the western world there is generally a bias against insects as food, especially when the insects are offered in a recognizable form (egg, larvae, pupae or adult). Therefore, extraction of ingredients from insects in order to use them as ingredients in food applications could be a promising route. The main objective of this thesis was therefore to investigate the functionality of protein and lipid fractions from insects aimed at obtaining ingredients for food use.

The work performed in this thesis was part of the project "Sustainable production of insect proteins for human consumption (SUPRO2)" funded by the Dutch Ministry of Economic Affairs. The SUPRO2 project consisted of a food science related part (this thesis) and an entomological related part in order to cover both rearing and processing aspects within an insect production chain. The rearing part included production of edible insects from side streams (*e.g.* the use of food waste as insect feed), investigating feed conversion efficiency, and an exploration into greenhouse gas and ammonia production by insect species in comparison to conventional livestock.

The objective of this thesis was first to evaluate if insect proteins have potential as a future ingredient in food (chapter 2). The second objective was to study the yield of protein extraction from *T. molitor* while preventing browning. Variations in pH and salt content were studied as relevant factors for protein yield of the water-soluble fraction (chapter 3). The third objective was to identify proteins and to study protein digestibility (*in vitro*) of the ground whole *T. molitor* and its fractions (supernatant, pellet and residue) obtained after

aqueous extraction (chapter 4). Besides studying the potential of insects as a novel protein source, insect lipid as a by-product of protein production was also investigated. We discussed the influence of extraction methods on chemical characteristics of insect lipids, as well as its quality as food ingredient (chapter 5). The present chapter starts with a summary of the main findings, followed by discussion of the results. Future prospects and main conclusions of the research are discussed next.

2 MAIN FINDINGS

Limited knowledge is available on fractionation of insects for human consumption, including extraction and characterization of insect protein. The main findings of this thesis are summarized in Figure 6.1.

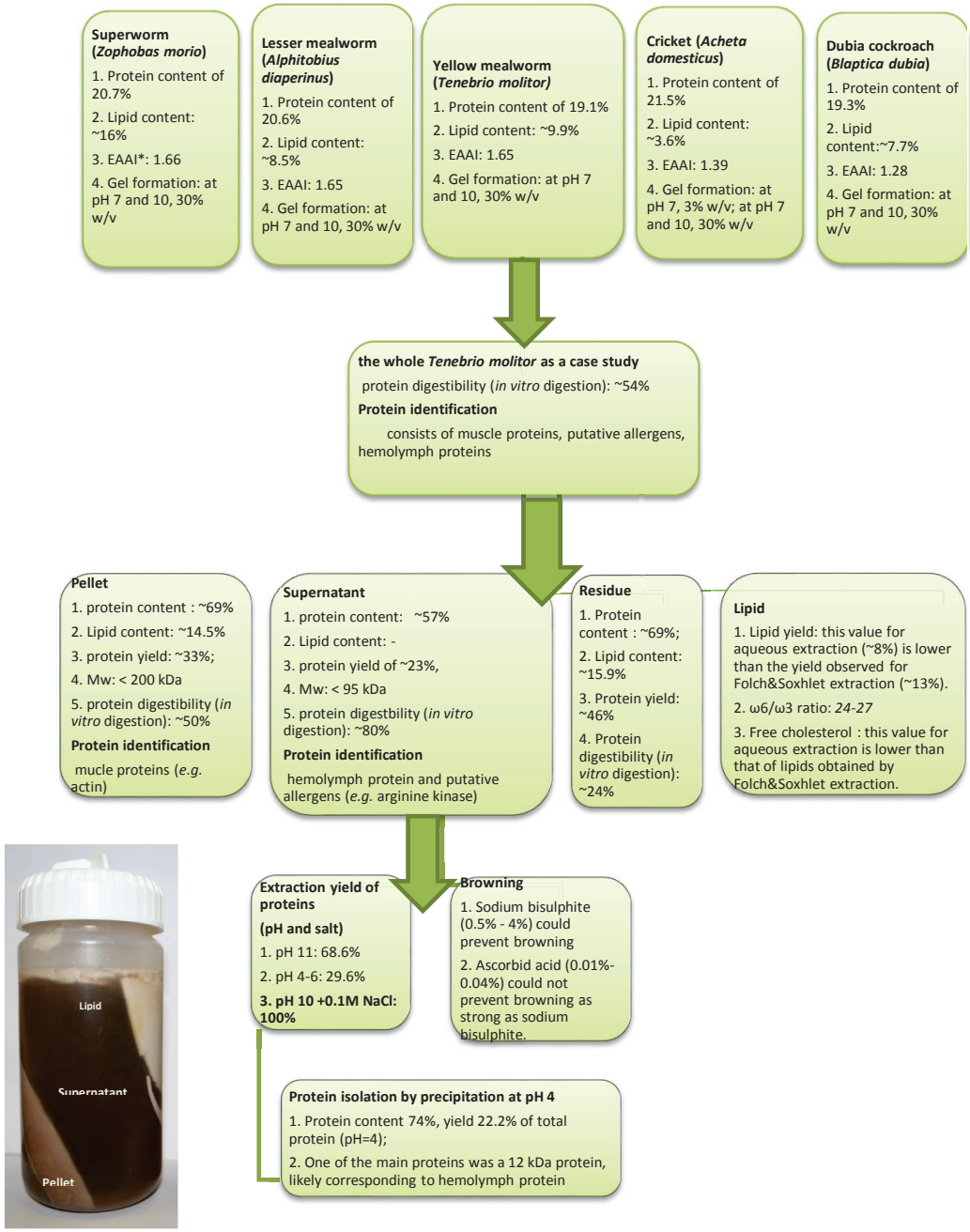


Figure 6.1: The main findings of this thesis (*Essential amino acid index: EAAI; for calculation FAO/WHO/UNU1985 essential amino acid requirements for humans are used as a reference)

In chapter 2, we focused on extracting and characterising proteins from several insect species. Protein characteristics and functionality were determined and evaluated for each of the five insect species (*T. molitor*, *Zophobas morio*, *Alphitobius diaperinus*, *Acheta domesticus, and Blaptica dubia*). In order to evaluate protein quality, crude protein content and amino acid composition were determined and discussed in comparison to well-known protein sources (soybean protein and casein). We found that essential amino acid levels in all insect species were comparable with soybean proteins, but were lower than for casein. In addition, next to a fat fraction, three protein fractions (supernatant, pellet and residue) were obtained by using aqueous extraction. In order to study functional properties of insect proteins, the ability of the protein in the supernatant to form a gel or foam was experimentally tested at different pH values and protein concentrations. The major findings were that supernatant fractions did not form stable foams at 3% (w/v), no gels at pH 3, 5, 7, and 10, except for gelation for *A. domesticus* at pH 7. At 30% (w/v), gels were formed at pH 7 and pH 10, but not at pH 3 and pH 5. After aqueous extraction, a supernatant, pellet, and residue were containing 17 - 23%, 33 - 39%, 31 - 47% of total protein, respectively. Also, severe and rapid browning was observed during extraction of protein, which appeared to be due to polyphenol oxidation.

In chapter 3, *T. molitor* was selected to further study the yield of the water-soluble fraction by purification while preventing brown colour formation during protein extraction. Protein yield of the water-soluble fraction was influenced by pH and salt concentration. The highest amount of protein extracted was 68.6% at pH 11, and the lowest one was 29.6% at pH 4 - 6. Salt addition gave the highest protein yield up to 100% at 0.1 M NaCl, at pH 10. The increased solubility corresponded partly to a protein band above 200 kDa, likely corresponding to myosin heavy chain protein. The problem of how to maintain protein quality by preventing browning during extraction was tackled by using sodium bisulphite (studied from 0.5 - 4%). Upon further purification by acid precipitation at pH 4, an isolate with a protein content of 74%, representing 22% of total protein present in *T. molitor* was obtained, thereby extracting a 12 kDa protein as one of the main proteins, likely corresponding to hemolymph protein.

The nutritional value of a food protein depends on amino acid composition, but also on protein digestibility. Chapter 4 focused on protein identification and digestibility (*in vitro*) of the whole *T. molitor* and its protein fractions (supernatant, pellet and residue) obtained by aqueous extraction. More proteins were digested with increasing digestion time (10, 20, 30, 60, and 120 minutes) for gastric digestion in pellet and residue fractions, the digestion of the supernatant fraction was time independent in the time range studied. Water-soluble protein fractions (supernatant: 80%) were digested more than water-insoluble protein fractions (pellet: 50% and residue: 24%) after *in vitro* gastro-duodenal digestion. The most abundant proteins were hemolymph protein (~ 12 kDa) & putative allergens (*e.g.* arginine kinase ~ 30 kDa) in supernatant, and muscle proteins (*e.g.* actin 30 - 50 kDa) in the pellet, as was concluded from LC-MS/MS & SDS-PAGE data (Chapter 4).

Next to several protein-rich fractions, a lipid fraction was obtained as a by-product using aqueous extraction. Chapter 5 dealt with the differences in extraction yield, fatty acid composition, presence of lipid classes, and equivalent carbon number patterns of lipids obtained by the aqueous method and compared with two other extraction methods: Soxhlet and Folch. Aqueous extraction gave the lowest lipid yield with a lower ω-6/ω-3 ratio and presence of less polar lipids. A lower ω-6/ω-3 ratio corresponds to a better lipid nutritional value (Simopoulos, 2002).

3 DISCUSSION AND INTERPRETATION OF RESULTS

3.1 Protein extraction and fractionation

Extraction and fractionation techniques for proteins are generally based on their physicochemical and structural characteristics such as solubility, hydrophobicity, molecular weight, isoelectric point (pI) (Toldrá et al., 2013). Extraction practices using an aqueous extraction are very common for extraction and purification of animal proteins (Boland, 2002). Protein concentrates (48 - 70% protein) or isolates (85 - 90% protein) obtained after an aqueous method can differ in techno-functional properties and protein quality due to the presence of other ingredients, *e.g.* lipids, carbohydrates, polyphenols, ash. Our findings in chapter 3 should that the protein concentrate and isolate of *T. molitor* had a protein

content of 52% and 74%, respectively. The protein content of the concentrate in *T. molitor* was in the range for protein concentrates in general, but that of the isolate in *T. molitor* was lower than commonly found for protein isolate (Toldrá et al., 2013). With regard to insect protein extraction, only one study exists, which used an aqueous method for Mexican fruit fly larvae (*Anastrepha ludens*). Protein concentrate and isolate obtained from these larvae contained 65% and 87% protein, respectively (Del Valle, Mena, & Bourges, 1982). These values were higher than what we found for concentrate and isolate of *T. molitor*. Further, according to Del Valle, Mena, & Bourges (1982), a maximum protein yield of 95% could be obtained by using aqueous alkaline extraction (pH 10) (Del Valle, Mena, & Bourges, 1982). Similarly, we could recover about 95% of total crude proteins for *T. molitor* by using aqueous alkaline extraction (pH 10) (Chapter 3).

In general, common protein extraction methods for plant, meat or fish use alkaline conditions for aqueous extraction (Hultin & Kelleher, 1999, 2000; Nolsøe & Undeland, 2009; Feng et al., 2004; Kristinsson & Ingadottir, 2006; Kristinsson et al., 2005; Undeland, Kelleher, & Hultin, 2002; Ghaly & Alkoaik, 2010). Next to alkaline extraction, sodium and calcium salts are also widely applied to extract proteins from plant or animal food (Horax et al., 2010; Karaca, Low, & Nickerson, 2011). Modifying pH and salt concentration affects protein yield and purity (Toldrá et al., 2013) and also protein functionality (Dissanayake et al., 2013). Muscle proteins extracted from meat or fish are generally produced at pH values ranging from pH 10.5 to 11.5. Myosin, a major muscle protein, is the main protein recovered in such processes (Kristinsson & Hultin, 2003). Its functionality is highly sensitive to pH and salt concentration. Quite likely, freeze-dried protein supernatant of all five insect species re-solubilized at pH 10 (described in chapter 2) may have contained myosin, because of the gel formation observed. Indeed, myosin was identified in protein supernatant extracted as determined by LC-MS/MS (chapter 4). Another possibility is that hemolymph protein as major proteins in supernatant (chapter 4) could possess gel forming ability, and this has not been reported in literature before.

In order to obtain higher protein yield, we used alkaline conditions in combination with additional salt as described in chapter 3. The yield of extracting *T. molitor* proteins (Chapter

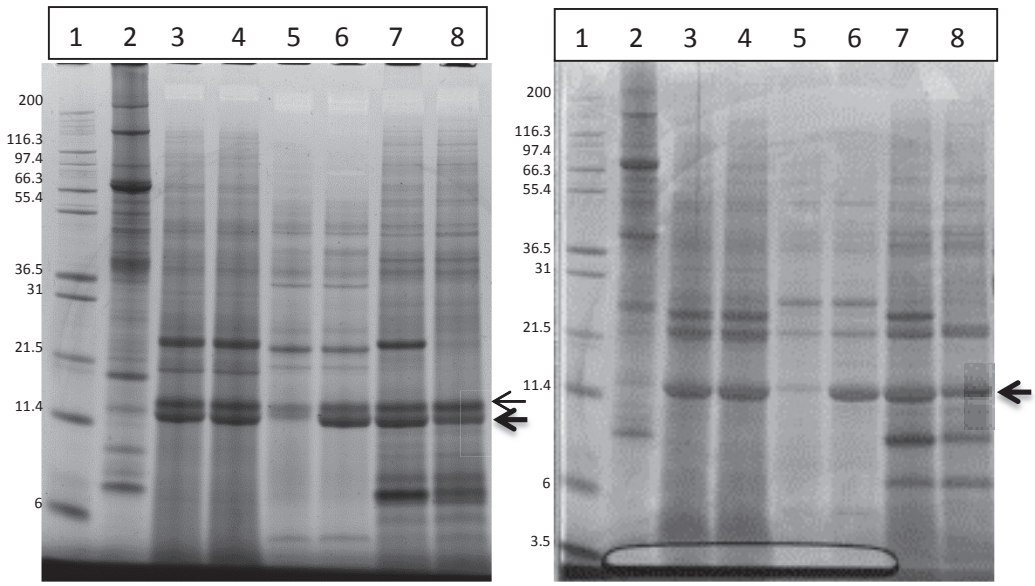
3) gives similar results as for extraction of conventional protein sources. The significance of this finding is that the food industry could apply aqueous extraction for insect proteins under alkaline conditions. By using such a procedure simultaneously, an oil fraction could be extracted as well, being an environmentally friendly and safe method in comparison to solvent-based extraction (O'Brien, 2008). This aqueous procedure for lipid extraction leads to a different quantity and a different fatty acid profile of the insect lipid extracts in comparison to solvent-based extraction. Namely, lipids with higher ω-6/ω-3 fatty acid ratios were extracted by the Folch method, while lipids obtained by aqueous extraction had the lowest ratio, hence having the highest ω-3 FA content. As mentioned by Simopoulos (2002), a lower ratio of ω-6/ω-3 fatty acids (ratio < 5) could reduce the risk of several chronic diseases, especially in western diets (current ratio is 15-16.7). The ratio recommended by FAO is 10:1 (FAO, 2010). The ω-6/ω-3 ratio for *Acheta domesticus* and *Blaptica dubia* (ratio 13-17) was below the current ratio in the Western diet, while for *Tenebrio molitor and Alphitobius diaperinus* (ratio 19-27) it was above (chapter 5). A reduction in ω-6/ω-3 ratio probably could be achieved by changing FA composition of insect feed (Oonincx, 2014, unpublished results).

Protein extraction at high pH condition may cause undesirable reactions, such as racemization of amino acids, formation of toxic compounds (lysinoalanine), reduction of digestibility, loss of essential amino acids, and therefore decrease in nutritive value (de Groot & Slump, 1969; Toldrá, et al., 2013). *Tenebrio molitor* protein yield was found to be maximum at pH 11, a pH value for which these reactions are expected to occur. Protein denaturation and undesirable chemical effects will also occur for extraction at lower pH values, but less (Betti & Fletcher, 2005). Therefore, as mentioned by Berk (1992) for soy protein production, the range between pH 7.5 and pH 9.0 is most preferred in practice. Besides pH, thermal treatment also influences protein denaturation (Dissanayake et al., 2013). Heat treatment is very commonly used for safety and quality reasons, i.e. reduction of microbiological load and inactivation of enzymes. It also induces protein denaturation, and Maillard reactions if reducing sugars are present, which can be undesirable. In preliminary experiments, a portion of 20 g *T. molitor* was blanched for one minute in boiling

water in order to inactivate polyphenoloxidases. The protein content of this supernatant fraction was about 18% on a dry basis (Dumas), which was a more than twice as low in comparison to the protein content of extracts without heating (~52%). It is an indication that heat treatment induces insect protein denaturation leading to formation of insoluble aggregates. Other methods for longer shelf life, next to heating, were mentioned by Veldkamp et al. (2012), such as drying (*e.g.* freeze-drying) or using high-pressure treatment. The drawback of these methods could be reduced protein functionality due to structural changes of protein (Veldkamp et al., 2012).

3.2 Protein purification of *Tenebrio molitor*

When water-soluble protein fractions have been extracted from insects using aqueous extraction, further protein purification can be applied. Separating proteins from non-protein materials is very general and almost a central procedure in biochemistry (Dennison, 2003). Various precipitation procedures are widely used in protein purification. The common methods applied in food applications are isoelectric precipitation (pI), ammonium sulphate precipitation, and solvent precipitation (Smith & Nielsen, 2010). Besides acid precipitation, which is performed at pH 4 and described in chapter 3, several preliminary experiments were done using ammonium sulphate precipitation at 80% & 90% saturation, and ethanol precipitation using concentrations of 80% & 100% and the results are described here. The disadvantage of solvent precipitation is that organic solvents (ethanol) decrease solubility of proteins in the native state after purification (Yoshikawa et al., 2012). By using ethanol and ammonium sulphate precipitation 7-14% of total protein present in Yellow mealworm could be extracted, which is low in comparison to the value of 22% obtained after acid precipitation (pH=4). The protein purity of the fractions obtained by ethanol and ammonium sulphate precipitation ranged from 47-60%, which is also low compared to the value of 74% for acid precipitation. Figure 6.2A and 6.2B illustrate the molecular weight distribution by using reduced and non-reduced SDS-PAGE of the protein fractions, obtained after acid precipitation (pH 4), ammonium sulphate and ethanol precipitation.



A non-reduced B reduced

Figure 6.2: Proteins extracted by acid precipitation, ethanol precipitation and ammonium sulphate precipitation: characterization by using 12% Bis/Tris NuPAGE gels by using MES running buffer under non-reducing and reducing conditions. Mark12™ Unstained Standard (2.5 - 200 kDa) was applied as a reference. The samples ranged from left to right: 1: standard, 2: control (crude Yellow mealworm), 3: Acid precipitation (F) (pH=4) with freeze-drying step before acid precipitation, 4: Acid precipitation (NF) (pH=4) without freeze-drying before acid precipitation, 5: AS80 (ammonium sulphate precipitation with saturation of 80%), 6: AS90 (ammonium sulphate precipitation with saturation of 90%), 7: E80 (ethanol precipitation at concentration of 80%, and 8: E100 (ethanol precipitation at concentration of 100%). The bold arrow is at molecular weight of ~13 kDa, and the black arrow is at molecular weight of ~12 kDa. F: Freeze-dried NF: non-Freeze-dried.

Major bands were found with molecular weight of 6 - 25 kDa for all protein extracts obtained from both reduced and non-reduced conditions. The band at around 6 kDa was only clearly present for ethanol precipitation. Bands with molecular weights (< 6 kDa), observed for all three precipitation methods, are likely linked to digestion by proteases as

was also suggested in Chapter 3. Englard, Seifter, & Murray (1990) suspected that the presence of ammonium sulphate may inhibit protease activities. That could be the reason why bands with molecular weights (< 6 kDa) were found at a low intensity after ammonium sulphate precipitation. The band at around 25 kDa was absent for extraction with 100% ethanol, but clearly present for extraction with 80% ethanol for both reduced and non-reduced SDS-PAGE. Besides this band, a protein band at around 13 kDa extracted (black arrow) for ethanol (80%) and ethanol (100%) was observed after non-reduced SDS-PAGE, but not found after reduced SDS-PAGE. The protein band of 22 kDa extracted by all purification methods at non-reducing condition SDS-PAGE could be likely identified as muscular proteins (20). Furthermore, a band at about 12 kDa (the bold arrow), likely hemolymph protein (identified by LC-MS/MS, chapter 4) was observed after all precipitation procedures. These preliminary findings suggest that the type of purification methods used determines the composition of protein fractions obtained. To obtain certainty on what type of proteins were exactly present in which fractions LC-MS/MS experiments need to be performed on these fractions. Proteins bands ranging from 12-14 kDa could correspond to hemolymph proteins (identified by LC-MS/MS, chapter 4). However, when identifying proteins by using LC-MS/MS, the limited amount of known proteins in the *T. molitor* database (240) hampers interpretation.

3.3 Nutritional properties of insect proteins

Protein quality can be evaluated by amino acid composition, and digestibility. For instance, the lysine content of rice proteins is more than that of wheat (50% higher). Therefore, rice protein is considered as a better quality protein than wheat. In literature no direct comparisons are made between insect protein composition and conventional protein sources (plant and animal protein) with respect to nutritional value. In Chapter 2, based on the analysis of essential amino acids of the five insect species, protein composition of these species was compared to casein and soybean. These are conventional protein sources, which are known for their high nutritional value. In addition to that, table 6.1 compares beef, wheat flour, and egg white with *T. molitor* proteins in terms of essential amino acids. It is clear that the level of EAA (essential amino acid) of *T. molitor* is comparable to soybean

and beef, much higher than wheat flour, but lower than casein and egg white. The same trend can be observed for the EAAI values. The amino acids of plant protein that are limiting, like lysine in wheat protein and methionine in soy protein, are found in higher amounts in *T. molitor* protein.

Table 6.1: Essential amino acids (EAA) of casein, beef and egg white, [A,B] and wheat flour and soybean, [A,C] and daily requirement for adults, [D] in comparison to *T.molitor* as an example of insect protein.

Essential amino acids (mg/g crude protein)	Casein	Beef	Egg white	Wheat flour	Soybean	1985 FAO/WHO/ UNU	*T. molitor (Defatted)*
Histidine	32	32	23	22	25	15	29
Isoleucine	54	42	53	33	47	30	43
leucine	95	78	88	69	85	59	73
lysine	85	79	70	27	63	45	54
Methionine + Cystine	35	33	66	39	24	22	26
Phenylalanine + Tyrosine	111	70	91	78	97	38	100
Threonine	42	42	47	29	38	23	39
Tryptophan	14	10	15	11	11	6	12
Valine	63	45	68	43	49	39	61
Sum of EAA	**531**	**431**	**519**	**350**	**439**	**277**	**437**
Essential Amino Acid Index (EAAI)	**1.93**	**1.60**	**1.95**	**1.30**	**1.56**	**1.00**	**1.60**

A: Friedman, 1996
B: Sarwar, Christensen, Finlayson, Friedman, Hackler, Mackenzie, et al., 1983
C: Young & Pellett, 1991
D: FAO/WHO/UNU, 1985

EAAI was used as a measure to compare insect protein to conventional proteins in the current study. However, the Protein Digestibility-Corrected Amino Acid score (PDCAAS) has been suggested by FAO/WHO as the preferred method for measuring protein quality (Schaafsma, 2000). The values of PDCAAS are based on the ratio between the amount of the first limiting essential amino acid in test protein and that in reference protein. This ratio

is corrected for the true digestibility of the test protein, as determined by an *in vivo* assay in rats (Smith & Nielsen, 2010). In comparison to EAAI, the advantages of PDCAAS are that information is provided on both amino acid composition and protein digestibility *in vivo*. The values obtained for *in vivo* protein digestion in rats are comparable to those of humans (Smith & Nielsen, 2010). The PDCAAS method focusses on the first limiting essential amino acid, not all essential amino acids are taken into account, which could be seen as a disadvantage (Smith & Nielsen, 2010). Further, an *in vivo* assay as a part of calculation to PDCAAS usually takes time and is costly. In contrast, EAAI provides an overall prediction of protein nutritional value by accounting for all essential amino acids. Direct comparisons on the relation between EAAI and PDCAAS scores are not reported in literature as far as we know.

Besides our analysis on amino acid composition (Chapter 2), data on the digestibility of *T. molitor* protein (Chapter 4) gave additional insight in protein quality. This is the first time that protein quality of *T. molitor* was investigated in terms of digestibility. The different fractions from *T. molitor* were analysed based on the amount of free amino groups released by digestive enzymes and detected by the *in vitro* OPA assay (chapter 4). Results show that insect proteins are indeed a promising new source of food proteins. However, protein digestibility by an *in vivo* assay could probably provide more accurate insights in relation to protein quality of insects for human consumption.

3.4 Sustainability of insect protein production

Sustainable food production is not only related to environmental friendly production methods, but also to food availability (Zhu, van Wesenbeeck, & van Ierland, 2006). New and sustainable sources of proteins require appropriate processing technologies to maintain protein quality. Examples of such sources are insects, duckweed and algae as mentioned by Science and Technology Options Assessment http://www.europarl.europa.eu/stoa/. These alternative protein sources are expected to enter the market as replacers for animal-derived proteins (van der Spiegel, Noordam, & van der Fels-Klerx, 2013), and are needed to help to solve the upcoming shortage of food proteins. Protein production should be designed using

low cost raw materials, and should result in high protein quality (van der Spiegel, Noordam, & van der Fels-Klerx, 2013).

Oonincx et al. (2010) concluded that insect rearing could be used as a sustainable, more environmentally friendly alternative for the production of animal protein with regard to greenhouse gas and NH_3 emissions. Nowadays, the global meat and dairy production chains are held responsible for approximately 18% of the greenhouse effect and 8% of all water consumption according to the FAO report 'Livestock's Long Shadow' http://www.fao.org/docrep/010/a0701e/a0701e00.HTM. Currently the majority of edible insects is collected in the wild. Schabel (2010) said that uncontrolled and unsustainable harvesting of insects in the wild will lead to overcollecting, forest destruction, and extinction of species. Therefore, rearing edible insects on an industrial scale will be required. However, this should be benign to the environment, be cost-effective, and food safety should be guaranteed (Rumpold & Schlüter, 2013).

Furthermore, insect production can be performed in a sustainable way. For instance, the use of House fly or Black soldier fly larvae could be applied to recycle organic waste to produce protein and also lipid as a useful by-product. In general, the benefits of insect rearing are that they are easily reared in a short period due to their short life cycle and high intrinsic growth rate, and also they require much less land than chickens, pigs and cattle (Oonincx & De Boer, 2012). These authors further mentioned that the land used for producing 1 kg protein from *T. molitor* only requires 43% of that for milk, and only 10% of that for beef. We can conclude that many indications exist that insect rearing is a sustainable practice. For future processing of insects in order to obtain food ingredients, sustainability is one of the dimensions that will likely get attention. In this thesis, conventional ways of extraction were used to be able to make comparisons with conventional protein sources. We used aqueous extraction for obtaining different protein fraction, and also a lipid fraction was obtained as a by-product. It seems that aqueous extraction is an optimal method in comparison to solvent extractions for sustainable protein production.

The purpose of this thesis was to extract and purify proteins from insects in order to evaluate the potential of the use of insects in human food and this was only done at

laboratory scale. The techniques for processing procedures namely freeze-drying, dialysis and centrifugation need to be reconsidered when upscaling for industrial production will be performed. Producing protein concentrate of insect could be seen as more sustainable than production of protein isolates, since less processing could be considered as more sustainable. Further, sodium bisulphite could effectively prevent browning of water-soluble protein fraction of *T. molitor* as described in chapter 3. However, sodium bisulphite is known to cause health effects to some people, for instance wine sensitive asthmatic (Lester, 1995; Vally & Thompson, 2001). In addition, Sereewatthanawut et al. (2008) mentioned that insect processing with regard to water use needs to consider eco-efficient methods. Washing out alkali used in a production process of protein fractions will cause large amounts of wastewater.

4 FUTURE PROSPECTS

In this thesis, fractionation of insects has been studied. The data on the characteristics of proteins and lipid fractions obtained show the potential of using insects as a food source. However, some challenges for food technologists remain, namely to:

- investigate how to obtain higher purity protein fractions and to study functional properties of these protein fractions
- prevent browning by a more sustainable method
- keep intact proteins by inhibiting proteases and polyphenol oxidase (PPO) during fractionation

In chapter 2, for five insect species, supernatant as water-soluble protein fraction was studied in terms of functionality of foaming and gel formation. However, protein purity is known to have a substantial impact on its functionality, e.g. foaming or gel formation. Therefore, crude protein extracts could be further purified to aim at even higher purity (> 85%), which is relevant for food applications, but possibly a less sustainable practice.

Another relevant extension is to investigate in more detail the browning reaction that occurred in *T. molitor*. Sodium bisulphite could prevent browning reaction at concentrations

ranging from 0.5% to 4% (chapter 3). In foods, sulphite is widely used to inhibit enzymatic browning (Kuijpers et al., 2013). However, the critical concentration of sodium bisulphite for inhibiting PPO reaction, and the mechanism of inhibition should be further investigated. In addition, other natural agents (*e.g.* N-acetyl-L-cysteine) could be tested for preventing browning reaction (Velisek, 2013). Enzymatic browning is known as a major quality problem in the processing of fruits, vegetables and seafood. Tyrosinase, a major polyphenol oxidase, is found in potato and mushroom. It is known to cause browning and thereby major losses during processing (Kuijpers et al., 2013).

In chapter 3 and 4, the supernatant fraction showed a strong wide "smear" at molecular weights < 6 kDa as determined by SDS-PAGE, likely corresponding to the presence of digested proteins as was also clear from the fact that nearly 30% free NH_2 groups were found in supernatant before gastric-duodenal digestion. A possible explanation is the presence of proteases in insect midgut lumen, which lead to lower protein yield and quality during extraction. Follow-up research should include the addition of protease inhibitors during the extraction to prevent protein breakdown. Commonly used protease inhibitor cocktails have a broad inhibitory specificity, i.e. towards serine proteases, cysteine proteases, metalloproteases, and calpains. Protease inhibitors are commonly used to protect the integrity of proteins during protein extraction and purification process as described by García-Carreño, An, & Haard (2000).

Concerns on consumer acceptance

Another aspect to take into account is consumer acceptance of insects as food, in order to better understand and improve the acceptability of foods containing whole insects, ground insects, insect protein fractions and insect chitin in the western food supply. In fact, a project entitled "Customisation of insect proteins: interplay between functional properties, sensory performance and consumer acceptance of insect proteins and insect protein containing food" is currently running and is an extension of our research with the objective to study consumer acceptance of insects as food.

Concerns on food safety aspects

Last but not least, future research in this field must take into account food safety in terms of microbiological aspects (Klunder et al., 2012), and in terms of allergenicity, the latter aspect already being initiated for *T. molitor* (Verhoeckx et al., 2014).

In our research performed at laboratory scale, we consistently used around 200 g fresh insects per batch as starting material. Such an amount is far less than the quantity required for industrial scale production. Therefore, for insect companies, insect rearing is indeed required in a larger amount before starting extraction. Next to them, for food companies/industries, some challenges remain next, to:

- design insect protein production process on an industrial scale using sustainable procedures, *e.g.* in relation to water/energy use and feasibility
- find specific food applications for insect protein fractions, with respect to protein functional and nutritional characteristics, *e.g.* use as a meat-replacer
- reduce the cost of production of insect proteins in practice with the ultimate objective to make the price more competitive in comparison to production of conventional meat in the future
- interact with governments with the goal of adjusting the present regulations and legislation that could be an obstacle for using insect protein as an alternative protein source.

These challenges are in line with the ones formulated at the first international conference "Insects to feed the world", FAO & Wageningen University, 2014, http://www.wageningenur.nl/en/show/Insects-to-feed-the-world.htm (access in October 2014).

5 CONCLUSIONS

The studies reported in this thesis provide insight in the possible use of insect protein for human food. The thesis contributes to fill the knowledge gap with regard to protein quality and functional properties of these proteins, i.e. their ability to form gels and their digestibility. Furthermore, it showed the potential of aqueous extraction of insect proteins,

a process which is of relevance for industrial bio-fractionation of insects. Next to that, substantial information was obtained on the effect of pH in combination with salt to protein yield, which is important for further upscaling. Because of the similar characteristics between *T. molitor* protein and conventional meat protein sources, *e.g.* fish muscle proteins and meat muscle proteins, insects have potential to be used as a novel meat source. Besides protein extraction, the lipid fraction that is obtained as a by-product may also be used as additional food ingredient.

Although much more research on insect proteins and insect fractionation is needed, the present study gives a first insight in its potential for food use.

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SUMMARY IN ENGLISH

Insects are now seriously considered as an alternative and additional source of protein in developed countries in view of an increasing world population and the environmental problems caused by conventional cattle. People in the western world have, on average, a strong bias against insects as food, which will hamper them to eat insects, especially when the insects are offered in a recognizable form (including egg, larvae, pupae or adult). However, it is also possible to extract proteins from insects for further use in food products. When protein extraction is performed, a lipid fraction will remain as a by-product from the extraction.

Very little information from a food science point of view is available on characteristics and functionality of extracted insect proteins and lipids. The aim of this thesis was to investigate protein and lipid extracts from insects with respect to chemical and physical properties, and to evaluate their quality and nutritional value for food consumption.

The study in **chapter 2** focused on protein extraction using an aqueous procedure, and protein characterization in terms of amino acid compostion, protein content and gel formation. Five insect species including *Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus, and Blaptica dubia* were studied Crude protein content of five insect species ranged from 19 - 22%, which is comparable to conventional animal protein sources. Next to that, essential amino acid levels of all insect species studied were comparable with soybean proteins, but lower than for casein. For each insect, three protein fractions were obtained after aqueous extraction, including one water-soluble fraction (supernatant), and two water-insoluble fractions (pellet and residue), containing 20%, 40%, 40% of total protein, respectively. Supernatant fractions formed stable gels at pH 7 and pH 10 when used in a concentration of 30% (w/v), while the supernatant from *A. domesticus* also gelled at pH 7 at a concentration of 3% (w/v). We observed that *T. molitor* supernatant had the darkest colour (dark brown) among all insect supernatant fractions. The brown colour was assumed to be the result of enzymatic browning, i.e., oxidation of polyphenols.

In **Chapter 3,** we investigated how pH and NaCl affected protein yield of water-soluble protein fractions of *T. molitor* as target protein source, while preventing brown colour formation during protein extraction in order to obtain high protein quality. Minimum

solubility was found at pH 4 - 6 with a recovery of 29.6% and maximum solubility was found at pH 11 with a recovery of 68.6%. Furthermore, extracting protein at 0.1 M NaCl, pH 10 gave the highest recovery up to 100% (Dumas analysis). We also found that the amount of total monomeric phenolic content of supernatants at pH 2 - 7 was higher (around 36 mg) than that at pH 8 - 11 (ranging from about 18 to 27 mg). Furthermore, sodium bisulphite (studied from 0.5 - 4%) could prevent brown colour formation, but ascorbic acid (studied in the range 0.01 - 0.04%) was much less effective. After acid precipitation at pH 4, an isolate with a protein content of 74% was obtained. This isolate contained 22% of total protein present in *T. molitor.*

The aim of **chapter 4** was to identify proteins using LC-MS/MS and to investigate protein digestibility (*in vitro*) of the whole *Tenebrio molitor* and its fractions (supernatant, pellet and residue) obtained using aqueous extraction as described in chapter 2. Proteins were more digested after pepsin/pancreatin digestion than after only pepsin digestion. The digestibility (estimated using the OPA method) of the supernatant fraction (~80%) was much higher than that of pellet fraction (~50%) and residue fraction (~24%) after *in vitro* gastro-duodenal digestion. Furthermore, the protein content of defatted pellet and residue (~80%) was higher than that of supernatant (~57%). The most abundant proteins were hemolymph protein (~ 12 kDa) & putative allergens (*e.g.* arginine kinase ~30 kDa) in supernatant fraction, and mainly muscle proteins (*e.g.* actin 30 - 50 kDa) in the pellet fraction.

A by-product from protein extraction was a lipid fraction, of which we analyzed the properties in **chapter 5** for four insect species (*Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus and Blaptica dubia*). We studied the lipid composition of the extracts obtained by using aqueous extraction and compared it with two other extraction methods: Soxhlet, a method with industrial application and with Folch an analytical method usually applied at a laboratory scale. Both Soxhlet and aqueous lipid extractions are of industrial relevance. Extracted lipids were characterized in terms of fatty acid composition, extraction yield, presence of lipid classes, and equivalent carbon number patterns of lipids. *Tenebrio molitor* had the highest lipid content around 13% among all insects by using Folch extraction. The highest yield was obtained with Folch extraction, and the lowest yield using aqueous

method (from 19% to 60% related to the lipid recovery of Folch extraction). This study concluded that ω-3 fatty acids were most abundant in lipids from aqueous extraction, while ω-6 fatty acids were most abundant in Folch extractions, except for *B. dubia*. Lipids from Folch and Soxhlet extractions contained free fatty acids and partial glycerides, which were absent in aqueous extractions. Triacylglycerol distribution is similar among insect species, with high levels of ECN 50-54 and low amounts of ECN 36-38. Overall, aqueous extraction gave the lowest lipid yield, but provided a lipid extract low in ω-6/ω-3 ratio and with less polar lipids than Soxhlet and Folch extractions. These characteristics are desirable in edible lipids.

Chapter 6 discussed the main findings of this thesis and gave implications regarding to future insect production. In conclusion, the studies reported in this thesis provide insight in the possible use of insect protein for human food. The work presented in the thesis contribute to fill the knowledge gap with regard to protein quality and functional properties of these proteins, i.e. their ability to form gels, their nutritional value and their digestibility. Furthermore, it showed the potential of aqueous extraction of insect proteins, a process which is of relevance for industrial bio-fractionation of insects. Next to that, substantial information was obtained on the effect of pH in combination with salt to protein yield, which is important for further upscaling. Because of the similar characteristics between *T. molitor* protein and conventional meat protein sources, *e.g.* fish muscle proteins and meat muscle proteins, insects have potential to be used as a novel meat source. Besides protein extraction, the lipid fraction that is obtained as a by-product may also be used as additional food ingredient.

Although much more research on insect proteins and insect fractionation is needed, the present study gives a first insight in its potential for food use.

SAMENVATTING

In de westerse wereld is er groeiende belangstelling voor insecten als alternatieve eiwitbron. Eén van de redenen is de toenemende milieuproblematiek als gevolg van traditionele veehouderij, maar ook de groeiende wereldbevolking is een belangrijke factor in deze. In het algemeen zijn westerse volkeren niet gewend aan het eten van insecten, en dit gaat vaak samen met een sterke weerstand hiertegen, vooral wanneer insecten herkenbaar zijn in het voedsel. Echter, wanneer eiwitten worden geëxtraheerd uit insecten kunnen deze in onherkenbare vorm in levensmiddelen worden gebruikt, wat kan bijdragen aan consumentenacceptatie. Het extraheren van eiwitten levert een vetfractie op als bijproduct. Er is weinig bekend over de karakteristieken en de functionaliteiten van geëxtraheerde insecten-eiwitten en -vetten. Functionaliteit kan bijvoorbeeld betrekking hebben op verteerbaarheid, gelerend vermogen, oplosbaarheid, waarbij elke toepassing in levensmiddelen weer een andere functionaliteit kan vereisen. .

Het doel van dit onderzoek betrof het extraheren van eiwitten en vetten uit insecten en het karakteriseren van de verkregen fracties met betrekking tot chemische en fysische eigenschappen om zo inzicht te krijgen in kwaliteit en nutritionele waarde met betrekking tot gebruik in voedsel.

Het onderzoek in **Hoofdstuk 2** richtte zich op eiwitextractie met water gebruik makend van vijf verschillende insectensoorten, namelijk *Tenebrio molitor, Zophobas morio, Alphitobius diaperinus, Acheta domesticus,* en *Blaptica dubia*. De aanwezige eiwitten werden gekarakteriseerd qua aminozuursamenstelling, eiwitgehalte, en gelvormende eigenschappen. Het ruwe eiwitgehalte van deze vijf insectensoorten varieerde van 19-22%. Deze waarden zijn vergelijkbaar met die van conventionele dierlijke eiwitbronnen. De gehaltes aan essentiële aminozuren waren vergelijkbaar met die van soja-eiwitten, maar lager dan die in caseïne. Voor elk insect werden drie eiwitfracties verkregen na extractie met water; een water-oplosbare fractie (supernatant), en twee water-onoplosbare fracties (pellet en residu), die respectievelijk 20%, 40% en 40% van het totale eiwit bevatten. De supernatant fracties van alle insectensoorten vormden stabiele gelen bij pH 7 en pH 10 in een concentratie van 30% w/v. Het supernatant van *A. domesticus* vormde ook een gel bij pH 7 bij een concentratie van 3% (w/v). Het *T. molitor* supernatant had de donkerste kleur

(donker bruin) in vergelijking met de vier andere supernatanten. De bruine kleur is waarschijnlijk het gevolg van enzymatische bruinkleuring (oxidatie van polyfenolen). Een donkere kleur kan toepassingsmogelijkheden in voedsel limiteren.

In **Hoofdstuk 3** is voor *T. molitor* onderzocht hoe het modificeren van pH en NaCl concentratie de extractie van water-oplosbaar eiwit beïnvloedde, terwijl ook onderzocht werd hoe de bruinkleuring kon worden tegengegaan om zo eiwit van hoge kwaliteit te verkrijgen. Een minimale oplosbaarheid werd gevonden bij pH 4-6, waarbij 29,6% van het totaal aanwezige eiwit werd geëxtraheerd. Maximale oplosbaarheid werd gevonden bij pH 11 met een opbrengst van 68,6%. Extractie bij pH 10 in aanwezigheid van 0.1 M NaCl gaf de hoogste opbrengst van 100%, zoals bepaald met de Dumas analyse. De totale hoeveelheid fenolen bij pH 2-7 was hoger (ongeveer 36 mg galluszuur equivalenten/g gevriesdroogd SUP A) dan het gehalte bij pH 8-11 (variërend van ongeveer 18 tot 27 mg galluszuur eq./g) hetgeen een aanwijzing was dat bruinkleuring meer optreedt bij hogere pH. Toevoegen van natriumbisulfiet (0,5-4%) kon bruinkleuring voorkomen, maar ascorbinezuur (0,01-0,04%) was veel minder effectief. Na precipitatie bij pH 4 werd een eiwit isolaat verkregen met een eiwitgehalte van 74%. Dit isolaat bevatte 22% van het totale aanwezige eiwit in *T. molitor*.

Het doel van **hoofdstuk 4** was om eiwitten te identificeren met behulp van LC-MS/MS en het onderzoeken van *in vitro* eiwitverteerbaarheid van 1. intacte *T. molitor* en 2. de na waterige extractie verkregen fracties (supernatant, pellet en residu). Een hogere verteerbaarheid werd gevonden na toepassen van een combinatie pepsine/pancreatine in vergelijking met pepsine alleen. De *in vitro* gastro-duodenale verteerbaarheid (geschat met de OPA-methode die vrije aminogroepen meet) van de supernatant fractie (~ 80%) was veel hoger dan die van de pellet fractie (~ 50%) en de residufractie (~ 24%). Bovendien was het eiwitgehalte van het ontvette pellet en residu (~ 80%) hoger dan dat van het supernatant (~ 57%). Het supernatant bevatte hemolymph eiwit (~ 12 kDa) en enkele (mogelijke) allergenen, bijvoorbeeld arginine kinase ~ 30 kDa. Het pellet bevatte vooral spiereiwitten zoals actine 30 - 50 kDa.

Als bijproduct van eiwitextractie werd een vetfractie verkregen, waarvan voor vier insectensoorten (*Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus* en *Blaptica dubia*) de eigenschappen zijn bestudeerd (**hoofdstuk 5)**. De samenstelling van de door waterige extractie verkregen vetfracties werd vergeleken met twee andere winningsmethoden: Soxhlet, een methode met industriële toepasbaarheid en Folch extractie, een analysemethode gewoonlijk gebruikt op laboratoriumschaal. Zowel Soxhlet als waterige vet extractie hebben industriële relevantie. De geëxtraheerde vetten werden gekarakteriseerd in termen van vetzuursamenstelling, TAG-samenstelling, en de extractie opbrengst. *T. molitor* had het hoogste vetgehalte (13% bepaald met Folch extractie) in vergelijking met de drie andere insectensoorten. De hoogste vet opbrengsten werden verkregen met Folch extractie en de laagste met de waterige methode. Met behulp van de waterige methode werd 19% tot 60% van het vet geëxtraheerd, uitgedrukt als percentage van de opbrengst verkregen met behulp van de Folch extractie. Het gehalte aan ω-3 vetzuren was hoger na waterige extractie, terwijl het gehalte aan ω-6 vetzuren hoog was na Folch extractie, behalve voor *B. dubia*. Vetfracties verkregen na Folch en Soxhlet extractie bevatten vrije vetzuren en mono- en di-glyceriden. Na waterige extractie waren deze afwezig. De samenstelling van de triacylglycerol (TAG) moleculen was vergelijkbaar voor de vier bestudeerde insectensoorten. TAG moleculen met ECN 50-54 kwam veel voor in tegenstelling tot ECN 36-38. Samenvattend, waterige extractie gaf de laagste vet opbrengst, maar de verkregen fracties bevatten vetzuren met een relatief lage ω-6 / ω-3 ratio, en een lager gehalte aan polaire vetten in vergelijking met fracties verkregen na Soxhlet- en Folch extracties. Deze karakteristieken zijn gewenst wanneer het gaat om eetbare vetten.

Hoofdstuk 6 bespreekt de belangrijkste bevindingen van dit proefschrift en beschrijft implicaties van deze bevindingen met betrekking tot toepassingen in levensmiddelen en de toekomstige insecten productie. De resultaten dragen bij aan kennis over eiwitkwaliteit en functionele eigenschappen ervan, waaronder het vermogen om gelen te vormen, de voedingswaarde en de verteerbaarheid. Verder is in dit proefschrift waterige extractie van insecten eiwitten bestudeerd, een proces dat van belang kan zijn voor industriële bio-

fractionering van insecten. Hierbij is kennis vergaard over het effect van extractie pH en het toevoegen van NaCl op de hoeveelheid oplosbaar eiwit, wat belangrijk is in verband met opschaling. Uit dit proefschrift blijkt dat de samenstelling van *T. molitor* eiwit vergelijkbaar is met conventionele eiwitbronnen, zoals vis en vlees. Dit ondersteunt de gedachte dat insectenproducten potentieel hebben als een alternatief vlees/visproduct, of daarin als ingrediënt verwerkt kunnen worden. De vet fractie die wordt verkregen als bijproduct na eiwitextractie kan eventueel ook worden gebruikt als voedselingrediënt.

Hoewel meer onderzoek naar insecteneiwitten en fractionering van insecten nodig is, geeft de huidige studie een eerste inzicht in de mogelijkheden voor gebruik van insecten in levensmiddelen.

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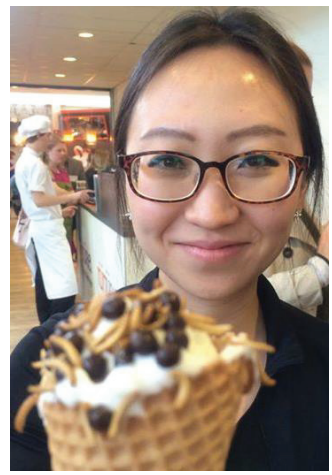
Liya Yi 依丽娅

ABOUT THE AUTHOR

Curriculum vitae



Liya Yi was born on April 26th 1985 in Hohhot, Inner Mongolia, China. She took an opportunity to enter a Bachelor study programme between China Agricultural University (2003-2005) and Wageningen University (2005-2007). After obtaining her Bachelor degree in 2007, she continued her study in Master programme of Food Technology at the Wageningen University. During her study, she did her Master thesis on a topic of "Microcapsules based on protein-carbohydrate complexes" on Food Physics Group. In addition, she worked the internship on "Protein-Carbohydrate interactions occurring in tube feeds during processing" in Product Development Department, Danone Liquid Advanced Medical Nutrition, Zoetermeer, the Netherlands (March - August, 2009). In 2009, she obtained her Master degree in Food Technology at the Wageningen University. Afterwards, she worked as an assistant researcher of food physics group to continue her thesis work at the Wageningen University from November 2009 to March 2010.

After short traineeship at Research & Development of Alpuro breeding company, she started her PhD at the Food Quality and Design Group, Wageningen University in 2010. This PhD project was to extract proteins from insects, in order to characterize the obtained protein fractions and to establish their functional properties, which was in part of SUPRO2 project (Sustainable production of insect proteins for human consumption). During her PhD years (2010-2014), she awarded as "Best Poster Overall" in FOOD Denmark PhD Congress in 2010. Furthermore, she coached many BSc & MSc students for their thesis, as well as assisted Master course. She gave poster/oral presentations in many international conferences, and participated to PhD trip in UK/Thailand/Singapore with her colleagues. Currently, she is working as a postdoctoral researcher at Zetadec B.V., Wageningen, the Netherlands.

List of publications

Peer-reviewed journals

Yi, L., Lakemond, C. M. M., Sagis, L. M. C., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2013). Extraction and characterisation of protein fractions from five insect species. *Food Chemistry, 141*, 3341-3348.

Yi, L., van Boekel, M. A. J. S., Lakemond, C. M. M. (2014). Extracting *Tenebrio molitor* protein while preventing browning: pH and NaCl affect protein yield. *PLOS ONE (Submitted)*

Yi, L., van Boekel, M. A. J. S., Lakemond, C. M. M. (2014). Protein identification and *in vitro* digestion of fractions from *Tenebrio molitor*. *(To be submitted)*

Tzompa-Sosa, D.*, **Yi, L.*,** van Valenberg, H., van Boekel, M. A. J. S, M. A., Lakemond, C. M. M. (2014). Insect lipid profile: aqueous versus organic solvent-based extraction methods. *Food Research International, 62(0), 1087-1094.* *Authors equally contributed to this work.

Humblet-Hua, N. P.; Sagis, L. M. C.; Scheltens, G.; **Yi, L.;** van der Linden, E (2009). In *Encapsulation systems based on proteins, polysaccharides, and protein-polysaccharide complexes*, 5th International Symposium on Food Rheology and Structure, Zurich, Switzerland, pp 180-183.

Conference abstracts

Yi, L., Lakemond, C. M. M., van Huis, A., & van Boekel, M. A. J. S. (2010) Characterization and functional properties of protein fractions from insects. FOOD Denmark Congress 2010, Copenhagen, Denmark. (Poster presentation)

Yi, L., Lakemond, C. M. M., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2012) Extraction and characterization of proteins from five different insects. 9th International Conference on Protein Stabilisation. Lisbon, Portugal. (Poster presentation)

Yi, L., Lakemond, C. M. M., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2013) Extraction and characterization of proteins from five different insects. 59th International Congress of Meat Science and Technology. Izmir, Turkey. (Poster presentation)

Yi, L., Lakemond, C. M. M., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2013) Extraction and characterization of proteins from five different insects. 1st International Conference on Global Food Security, Noordwijkerhout, the Netherlands. (Poster presentation)

Yi, L., Lakemond, C. M. M., van Boekel, M. A. J. S. (2013) *In vitro* digestibility of water-soluble and water-insoluble protein fractions of the Yellow mealworm (*Tenebrio molitor*). Food Structures, Digestion & Health Conference. Melbourne, Australia. (Poster presentation)

Yi, L., Lakemond, C. M. M., van Boekel, M. A. J. S. (2013) *In vitro* digestibility of water-soluble and water-insoluble protein fractions of the Yellow mealworm (*Tenebrio molitor*). 3rd International conference on Food Digestion. Wageningen, the Netherlands. (Poster presentation)

Yi, L., Lakemond, C. M. M., Eisner-Schadler, V., van Huis, A., & van Boekel, M. A. J. S. (2014) Extraction and characterization of proteins from five different insects. 1st International Conference of Insects to Feed the World. Ede, the Netherlands. (Oral presentation)

Overview of completed training activities

Discipline specific activities

Courses

- Advance food analysis (2010), VLAG Wageningen
- Reaction Kinetics in Food Science (2012), VLAG Wageningen
- Industrial proteins (2013), VLAG Wageningen
- Compositional analysis of lipids (2013), Ghent University

Conferences

- FOOD Denmark PhD congress: Functional Foods and Sustainable Food Production (2010), Copenhagen University, Denmark. (Poster presentation)
- 9th international Protein Stabilization (2012), Lisbon, Portugal. (Poster presentation)
- COST Protein Workshop (2012), Amsterdam, the Netherlands. (Poster presentation)
- 59th International Congress of Meat Technology and Science (2013), Izmir, Turkey. (Poster presentation)
- 1st International Conference on Global Food Security, Noordwijkerhout, the Netherlands. (Poster presentation)
- Food structure, Digestion and Health (2013), Melbourne, Australia. (Poster presentation)
- 3rd International Conference on Food Digestion (2014), Wageningen, the Netherlands. (Poster presentation)
- 1st International Conference of Insects to Feed the World (2014), Ede, the Netherlands. (Oral presentation)

General courses

- VLAG PhD Introduction Week (2010), VLAG, Maastricht
- Techniques for Writing and Presenting Scientific Papers (TWP) (2010), WBS, Wageningen
- EndNote X4 (2010), WUR, Wageningen
- Academic Writing II (2011), WUR, Wageningen
- Scientific Writing (2012), WGS, Wageningen
- Scientific Publishing (2012), WGS, Wageningen
- Applied Statistic (2012), VLAG, Wageningen
- Statistic course for analysis data (2012), VLAG, Wageningen

Optional courses and activities

- Preparation of PhD research proposal (2010-2011)
- PhD trip of Food Quality and Design Department to the United Kingdom (2012)
- PhD trip of Food Quality and Design Department to Thailand and Singapore (2014)
- Meetings and Seminars in Food Quality and Design (2010-2014), Wageningen, the Netherlands
- Meetings in SURPO2 project (2010-2014), Wageningen, the Netherlands

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