UNIVERSITÀ DI PARMA

PhD in Food Science

Cycle XXXII

INSECT BIOREFINERY FOR GENERATING HIGH-ADDED-VALUE PRODUCTS SUCH AS PROTEIN AND CHITIN

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Abbreviations list

AIPP	Acid isoelectric point precipitation
AA	amino acid
BG	Brewery grains
BSF	Black soldier fly
DDA	degree of deacetylation
DDGS	Distiller`s Dried Grains with Solubles
DM	Dry matter
EAA	Essential amino acids
EFA	Essential fatty acids
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion rate
FTIR	Fourier-transform infrared spectroscopy
LM	Lesser mealworm
MGC	Minimal gelling concentration
MUFA	Mono unsaturated fatty acids
NDY	Non-dialyzed yield
OBC	Oil binding capacity
P	Pellet
PUFA	Poly unsaturated fatty acids
SDS-Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SN	Supernatant
TGA	Thermogravimetric analysis
TLC	Thin layer chromatography
UP	Upper layer
WBC	Water binding capacity
XRD	X-Ray Diffraction
YM	Yellow mealworm

Abstract

Introduction: Over the last decade, academic research on the insect value chain is growing exponentially, especially on larvae of the black soldier fly (for feed applications) and mealworms (for food applications), and an increasing amount of commercial insect rearing facilities are being established in Europa. Insects are being approached as a new mini-livestock and since they are rich in protein and lipids, they can potentially decrease future food and feed shortages. In addition, they can be reared on a large range of organic material, including underspent waste streams. This way, they can be employed to recycle nutrients from side-streams back into the market as insect biomass.

However, even though the insects' nutritional value is ideal for feed/food applications, the composition of the insect biomass is not necessarily so and thus extractions may be needed to (1) tailor the composition of a diet/food product and (2) increase the acceptability for human consumption. The latter was suggested after establishing a higher acceptancy when insects are not recognizable and incorporated into a familiar food product. The three main compounds are protein, lipids, and chitin. These compounds could have, next to nutritional value, other purposes. Proteins, for example, can have techno-functional properties that could be an added value to a food product. This aspect is still an unstudied domain for insect proteins. Moreover, chitin is a valuable biopolymer that can be applied in many applications and the market is expected to grow rapidly in the near future.

For these reasons, the fractionation, characterization, and valorization of insect fractions are gaining more attention. However, published data on insect-based fractionation processes are not covering all aspects, for instance (1) the focus is on extracting one compound, neglecting the impact of the extraction method on other

compounds (proteins, lipids or chitin), (2) data is often limited to analytical fractionation and (3) it is scattered over different insect species

The ***general aim*** of the thesis is to increase (academic) knowledge on different aspects of the insect-based value chain with a special focus on scalability for an easy transition of lab-scale findings to the industry. Two insect species were studied i.e the larvae of the black soldier fly and the lesser mealworm. The main focus was put on engineering a biorefinery approach with a focus on the recovery of multiple compounds (chitin, proteins, and lipids) that could be used as raw materials for feed, food, and technical applications. The study started with investigated if the insect biomass was impacted by the composition of the feed to clarify if the insect biomass was homogenous year-round. Second, the fractionation approach was investigated which started with mechanical treatment to obtain a dechitinized fraction and a chitin-rich fraction. Next, the two subfractions were further fractionated in protein, lipid, and chitin enriched samples. The characteristics and possible functionalities of these fractions were evaluated in the study.

Does a varied insect feed composition impact the composition of the insect biomass? Reported data on larvae of the black soldier fly reared on side-streams have indicated that the composition of the larvae can change depending on the diet composition. These findings were cross-checked for the lesser mealworm. The mealworms were reared on 18 different diets composed of side-streams to (1) determine the nutritional composition of the larvae and (2) study the effect of dietary changes on the larval nutrient composition. The lesser mealworm proved to be of good nutritional value with essential amino acid profiles comparable with that of beef. The side-stream based diets varied on dry matter basis in protein (16 % to 34 %) and lipid content (2 % to 19 %). The nutrient content of the larvae reared on diets that supported good growth ranged between 35 % and 45 % of protein, 22 %

and 26 % of lipid and 4 % to 6 % of chitin on a dry matter basis. No significant correlations were identified between the larval protein, amino acids, lipid content, nor fatty acid profile and that of the diet. However, other compounds such as carbohydrate and minerals were not studied but or needed for determining the optimal nutritional requirements of the lesser mealworm.

Would the use of organic acids improve fractionation in an acid-based biorefinery approach to obtain lipid and protein-enriched fractions? A fractionation approach was studied for dechitinized insect biomass of the larvae of the black soldier fly. The study aimed at the development of a robust fractionation process for wet insect biomass, targeting enriched protein fractions as well as enriched lipid fractions. The added value of different organic acids for an acid isoelectric point precipitation (AIPP) was evaluated and compared with an inorganic acid (HCl). A beneficial effect of organic acids on the lipid extraction yield (shift from 35 % to 45 %) was observed, which did not negatively influence the protein extraction efficiency. One organic acid, in particular, lactic acid, increased lipid purity from 75 % to 85 %. Protein fractions with a purity of 60 % proteins were achieved. In conclusion, the use of some organic acids at low pH (pH 2) resulted in (1) a higher lipid purity in the lipid-enriched fraction and (2) had similar results in protein fractionation as the inorganic acid. The process can be further optimized, especially towards the recycling of additive and an economic study toward the benefits of organic acids is adviced.

Is the newly developed biorefinery approach comparable to traditional defatting methods? This new fractionation approach was compared to solvent-based extraction, supercritical CO_2 extraction (SFE) and heat treatment. Dechitinized biomass of the lesser mealworm biomass was used and the methods were compared based on extraction yield, purity, and impact on protein solubility. AIPP was most successful in generating a protein-enriched fraction (71 % protein),

followed by SFE (67 %), heat extraction (62 %) and solvent extraction (60 %). However, a lower yield and protein recovery were observed for AIPP and heat treatment. The most soluble protein-enriched fraction was SFE defatted meal (100 % soluble), then the supernatant of the heat treatment (89 %), followed by a hexane-defatted meal (73 %) and the AIPP generated fraction (30 %). SFE and AIPP treatments were able to recover most of the lipid (85 %), followed by the hexane extraction (61 %) and heat treatment (59 %). In conclusion, SFE is suggested as the best method for obtaining a high degree of defatting as well as good preservation of the protein solubility, whereas the AIPP treatment is more promising considering high protein content fractions and easy upscaling but does not preserve techno-functional properties.

Could a modified biorefinery approach to obtain chitin and chitosan from insects be more sufficient than the traditional crustacea-based purification method? A new fractionation approach was also developed for the chitin-rich black soldier fly biomass. The aim was (1) to tailor the traditional methods (based on crustacea) towards insect chitin, (2) decrease the amount of process waste which would be beneficial towards upscaling, (3) to recuperate proteins and (4) to produce chitosan with different degrees of deacetylation (DDA). The result proved that a process with less chemical and/or milder process parameters was feasible for larvae of the black soldier fly and that pure chitin and chitosan fractions could be achieved with a DDA ranging between 30 % to 90 %. In addition, the alternative method allowed the recovery of proteins.

What are the techno-functional properties of insect proteins and what is the impact of the extraction method? Techno-functional properties (TFP) of the native proteins of the dechitinized lesser mealworm biomass (before fractionation) were measured and compared to the properties of the protein-enriched fraction

generated by the AIPP approach, an SFE, and heat treatment. The study confirmed that proteins derived from the lesser mealworm contained techno-functional properties and the native proteins had a good solubility and gelling ability which illustrates the potential of lesser mealworm proteins as a functional ingredient in food/feed applications. In addition, the oil binding capacity was higher compared to reference proteins caseinate, egg white and albumin. Depending on the fractionation approach, the TFP were either increased or decreased. The gelling ability and oil binding capacity were increased by SFE and the defatted meal from the SFE was able to form a foam. The AIPP treatment decreased most TFP expect for the WBC and OBC which were increased whereas the heat treatment resulted in a protein fraction that had lost all its techno-functional properties. These findings are a first step towards tailoring the techno-functional properties of proteins to specific product-based needs.

What is the effect of different life stages on the chitin characteristics? Lastly, chitin samples were collected along different stages the lifecycle of the black soldier fly (larvae, prepupae, pupae, flies, shedding & cocoons) and examined for differences. The chitin content in the collected biomass ranged between 8 % and 24 %, with sheddings and cocoons being most rich in chitin. Purified chitin was subjected to a physicochemical evaluation based on FTIR, XRD, and TGA as well as a deacetylation step. The data indicated that BSF chitin was α-chitin with FTIR profiles matching closely to shrimp chitin and showing some differences compared to squid pen chitin (ß-chitin). Small physicochemical differences were observed among the different BSF samples. Prepupae and cocoon chitin was more crystalline while chitin from larvae and sheddings had a lower thermal degradation temperature. In addition, sheddings were more difficult to purify. Further processing to chitosan showed that a deacetylation degree of 89 % could be obtained for all samples after 3 hours, although prepupae and pupae chitin was found to be more reactive in the

deacetylation process. Overall, the small differences in physicochemical properties that were detected between the BSF chitin samples did not prevent further processing of chitin to chitosan with the same degree of deacetylation via the same treatment.

In general, within this thesis, data were generated that contribute to close the knowledge gap related to the insect-based value chain. Data indicated that the lesser mealworm could be reared on side-streams without changing the larval composition and thus alterations the side-stream based diet throughout the year (for example due to seasonal availability) are possible. Furthermore, the thesis illustrates a new, two-step insect fractionation approach where 76 % of all proteins, 73 % all lipids and, 75 % of the BSF chitin could be recovered. Lastly, the thesis describes the techno-functional properties of the lesser mealworm and points out that different chitin containing waste-streams originating from the black soldier fly rearing industry could be processed to a homogenous chitosan product and valorized. Further research is advised on upscaling and process optimization, especially towards using green solvent, decreasing and recycling of additives/ wastewater and increasing the purity of the fractions. Next, a good understanding of the nutritional value, functionalities and characteristics is required for product developments and needs to be further expanded. Lastly, a techno-economic study is advised to establish the economic viability of the process and the insect value chain.

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Chapter 1.

INTRODUCTION

1.1 Insects in a new perspective

1.1.1 2050 faces some global problems

The world's population is expected to grow rapidly and will reach a staggering amount of 10 billion people by 2050. In the same time period, nowadays underdeveloped countries will grow and change from a cereal-based diet to a livestock-based diet [1]. Predictions indicate an increase of 70 % in food production between 2007 and 2050 [1], resulting in an even higher pressure on natural resources (degrading ecosystem, soil depletion, erosion), land and water use (loss of tropical forest and depletion of freshwater reserves) and the need for more sustainable innovations in agricultural industries [2],[3]. Future land and water requirement are available on a global scale, but the bottleneck is on a national level. For example, most of the available land is located in Latin America and sub-Saharan Africa where other factors such as the infrastructure limit its use [3]. With an eye on the future, a special focus is given to animal-derived proteins, since predictions point to a doubling in consumption by 2050 [1],[4]. However, animal-based food products have a higher ecological footprint and land use is already a problem in the European Union (EU). 75% of its protein-rich-animal feed is nowadays imported from south America since most agricultural land is used for livestock rearing itself [2],[5]. Henchion et al. (2017) reviewed these problems and suggest some new and emerging sources of proteins, including insects [2].

Insect rearing is more sustainable than traditional livestock since less land, water, and feed is required to produce 1 kg of insect-based protein compared to 1 kg of beef, pig or poultry-based proteins (see Figure 1-1). Insects are cold-blooded, so more energy can be stored in the body causing the feed-to edible biomass conversion (feed conversion ration, FCR) to be lower than traditional livestock [6]. For example, commercially bred yellow mealworms have an FCR of 2.2 on the standard diet and for black soldier fly larvae bred on chicken feed an FCR of 1.8 has

been reported, whereas commercially bred poultry and cereal beef have an FCR of 2.3 and 8.8, respectively [7]. In addition, a higher percentage of insects is edible and most edible species have a high protein content (> 35 % on dry matter basis [8]). Entomophagy, or better said the eating of insects, has been performed by humans for millennia. More than 2000 insect species are documented as edible and entomophagy is nowadays already practiced in different parts of the world such as Australia, Mexico, Thailand, Japan and throughout the African continent. Insects are either seen as a vital nutritional ingredient in the diet or as a snack or delicacy [6]. Due to these reasons, organizations such as the Food and Agriculture Organization of the United Nations (FAO) and the European commission are supportive to include insects in Western diets [2].

Figure 1-1 Sustainable insect rearing: resources needed to produce 1 kg of each protein source [9]

1.1.2 From livestock farms to mini-livestock farms

In general, most edible insects outside the EU are collected by 'wild picking'. This term refers to the collection of 'free, uncultivated' insects and the practice is

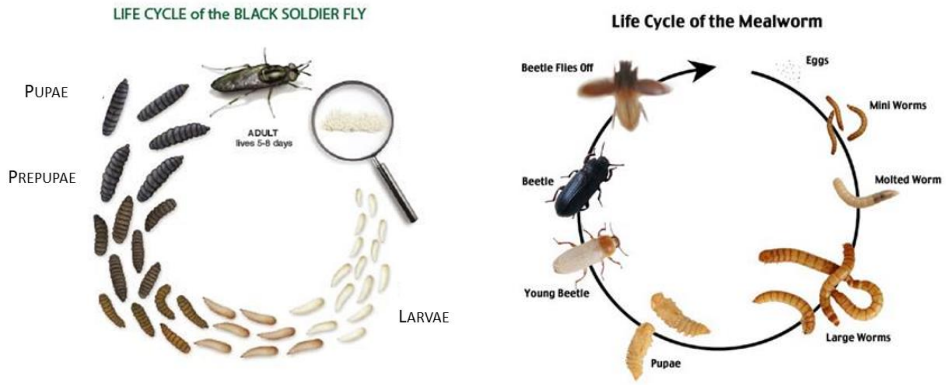
dependable on seasonal availability, the population size and the ease of collecting [6]. However, in the last decades, a small number of insect species have been domesticated and are now reared in a human-controlled environment. For example, Thailand is rearing the house cricket, the palm weevil and the giant water bug for human consumption [10],[6]. Western countries also had a history of insect rearing but for different purposes. Insect species such as crickets, mealworms, locusts, maggots of the housefly and cockroaches were being reared for fish bait or as feed for reptiles, amphibians, birds and fish. Later, dried insects were also included in cat and dog feed and other insect species were reared for biological pest management [6]. In conclusion, western countries already had a basic knowledge of insect rearing, implying the switch to rear for feed and food applications to be relatively easy. Most frequently reared insects for human consumption are cricket, locusts and mealworm species [11] whereas feed applications generally involve larvae of the black soldier fly (BSF), mealworms and larvae of the housefly [11]. The larvae of the BSF and the lesser mealworm (LM) are further discussed in detail down here.

1.1.2.1 Larvae of the black soldier fly

The BSF, *Hermetia illucens*, is native to America but is now found worldwide in tropical and sub-tropical areas [12]. It usually inhabits an environment rich in decomposing material such as manure, spoiled fruit & vegetables and dead animals [13],[14]. In the past, they were found abundantly in open-sided caged layer houses, were the larvae thrived on poultry manure [15]. Due to its unique ability to be reared on a broad range of possible organic streams, this insect is particularly interesting. Every year, the number of published articles about the BSF increases and more companies are starting to rear this insect for commercial purposes [11]. Due to their powerful mouthparts and digestive enzymes, the larvae can consume feed more quickly and efficiently than any other known species of fly [16] and they can be reared on a wide range of waste streams going from kitchen waste to manure. Rearing conditions for the larvae involve a temperature between 27 °C and 32 °C

[17], [18], a shaded environment [19],[20], and a moisture content of the substrate between 60 % and 90 % [19],[21]. The latter was found an important factor since a non-ideal moisture content could result in larval death or larval escape by crawling out of the substrate [22]. This preference for high moisturized feed is most beneficial since some organic waste streams are of wet origin and thus a pre-drying step could be avoided. However, it also leads to the disadvantage of unwanted microbiological fermentation in the substrate over time [23]. Depending on these abiotic factors, and the presence of proteins and carbohydrates for growth [24],[22], the life cycle can range between 20 days and 3 months.

Larvae of the BSF tends to grow from instar to prepupae in 14 days to 2 months [25]. In this period, they go through five larvae stadia were the first four stages are difficult to differentiate, except for body size and molding between every stage (see Figure 1-2a) [26],[27]. The fifth instar results in a fully-grown larva (of about 2.5 cm in length and 0.5 cm in width) and after molding one last time, a prepupae emergence. The prepupae change color from light brown to dark brown and eventually black [19],[14],[28]. In this phase, the digestion track is emptied and removed and the prepupae migrates to a more dry location to pupate [29],[25]. Without molding, the prepupae becomes a pupa by altering the soft tissue from larvae to fly. This phase is indicated by a completely rigid state with no elasticity [14]. Generally, after 10 days, the fly crawls out and leaves an empty casing behind, called a cocoon [27],[30].



(a) *(b)*

Figure 1-2 Life cycle of (a) the black soldier fly [31] and (b) the lesser mealworm [32]

The larvae or prepupae are considered for commercial purposes and have a dry matter between 33 % and 41 % [33]. Depending on the diet formula, the life stage and the rearing conditions, the protein content varies between 36 % and 46 % on a dry matter basis and the lipid content between 18 % and 37 % [33],[34],[7],[35].

1.1.2.2 The lesser mealworm

The LM, *Alphitobius diaperinus*, is believed to originate from sub-Saharan Africa but is now found worldwide. The mealworm usually inhabits poorly maintained grain processing plants, but can also be found in poultry houses [36]. The adults (beetle) and the larvae are commonly seen as a pest insect since they can be vectors of pathogens and can cause structural damage to the poultry housing infrastructure. However, lately, they are also reared for PET food and are considered for future human consumption [6], [37].

The larvae hatch from eggs and have a development time of about 35-40 days to grow into the last instar larvae (see Figure 1-2b) [38],[39]. There are approximate 6 to 11 instars and with every stage, they mold. The larvae have a segmented body and grow to be 7 to 11 mm in length. In each instar phase, the color changes from

white to light brown as they age. The pupae are about 6 to 8 mm in length and underwent some morphological changes. The adult beetle is dark of color and is about 6 mm [37],[36]. The LM has a dry matter between 30 % and 35 % [31],[40], a protein content of about 48% and [41] a lipid content between 13 % and 25 % on dry matter basis [37],[38],[40],[42].

1.2 Boundaries and opportunities

1.2.1 An opportunity for a circular economy

Insects fit in well with the circular economy strategy of the EU. Two of the key elements in the action plan are to (1) have better waste management and (2) start investigating waste as new resources and secondary raw materials [43].

Firstly, the EU generates approximately 100 million tonnes per year of food waste and this number is growing [44]. Food waste is generated throughout the food supply chain starting from harvesting, to processing, storing, packaging, purchasing and eventually consuming. Bruising, early spoilage, process losses, overplus in the supermarket & the consumers and poor food preparation all lead to food waste [45]. This food waste is disposed of through landfilling, incineration, partially recuperated for livestock feed, or recycled for biogas production via composting. The latter is recommended by the EU over incineration but not often employed [46] because of the high capital costs, the sensitivity and long duration of the process, and its performance is largely dependent on the feedstock characteristics [47],[48], [49],[50]. An overplus on manure, other organic waste streams, can also pose a problem depending on the country. Generally, it is used as a fertilizer, but negative side effect limits its use. For example, when more manure is produced than can be spread over the land or if the ground is already rich in nitrogen, eutrophication occurs [51]. For most organic waste, including manure, insects can be employed to obtain better waste management. Studies on the BSF report a waste reduction of 60 % on restaurant waste [52],[52], 66-79 % on municipal waste [53] and 56 % on

manure [15],[54]. In addition, the larvae provide a decrease in smell, nitrogen, and phosphor of the residue after rearing [15]. These results represent the potential of the BSFL as a management tool. However, up till now, insects are seen as mini livestock where the legislation of livestock applies (Regulation (EC) No 1069/2009). This means that manure is not permitted as feed nor animal byproduct. Nevertheless, changes are being pursued regarding insect rearing and perhaps, when rearing on manure, tolerance is possible with the condition that the insect does not end up in the food supply chain. In the present, other organic waste streams that fall within the permitted livestock feed, that cannot be directly fed to traditional livestock (because of the low nutrient content, or morphological characteristics such as the moisture content for example) are being tested for insect rearing. For example, Nguyen et al. (2015) reported successful rearing of the BSFL on a mixture of fruit and vegetables and Oonincx et al. (2015) on mixtures of spent grain, beer yeast, cookie remains, potato steam peelings, beet molasses and bread [7],[55]. Depending on the waste stream characteristics, other insects than the BSFL such as the mealworm are also tested [7],[38],[56].

Secondly, by rearing insects on waste streams, insect biomass is being generated. These organic waste streams often still contain useful nutrients that are lost by applying traditional disposal techniques. Yet, since the biomass is often only available in small amounts, are seasonally dependent or heterogeneous, a biorefinery approach is difficult to design. However, by rearing insects on this waste, the nutrients are recycled and converted to insect biomass. This way, the second pillar for circular economy (waste as a new resource) is being adapted. By transforming waste streams into insect biomass, a new protein source can be generated with reduced use of raw feedstocks [11]. Because of the lower feed-to-edible biomass conversion ratio of insects compared to other livestock animals (1,7 for crickets versus 10 for beef), insects are a promising source for future food and feed applications [6].

1.2.2 Insects for food: the consumer's opinion

Human consumption of insects is limited by legislation because of the novel food law (EU Regulation No 2015/2283). Nevertheless, some insect-based products are already on the EU market, but human consumption remains part of a niche food sector [6]. The general population in the EU still has a certain resistance toward eating insects. Since they are not included in their common diet and because of their physical appearance, insects are presumed as dirty and scary [57]. However, studies have pointed out that the willingness to eat insects depends on the presentation of the food product. When the insect is not recognizable, for example in the form of a powder and incorporated in a familiar food product, the product is more acceptable for human consumption [58],[59],[10],[60]. It seems that for introducing insects in the Western market, insect processing and protein extraction would facilitate the procurement of insect-based food products. Feed applications are also limited by legislation and up till now, live insects are allowed for all livestock except ruminants and processed insect meal is allowed in fish feed (Regulation No 2017/893). However, predictions suggest that this will be extended for poultry and pigs.

Fractionation, although it results in a longer post-treatment, includes some additional benefits. Tailoring of the nutritional composition of the food or feed product is easier with relatively pure fractions. In fact, although insects are nutritional, the protein/lipid ratio is not ideal for feed applications. In addition, insects mainly contain proteins and lipids, but also a small but significant amount of chitin. Chitin is beneficial for feed applications in small amounts [61], but it is suggested that it negatively affects the digestibility and utilization of other macros- and micro-nutrient at higher concentrations [62]–[64]. By fractionation, chitin could be removed and fractions of purified insect proteins, lipids, and chitin, could be considered for other applications besides food and feed if they have a functional value.

1.3 Insects biorefinery

1.3.1 State of the art on insect biorefinery

A biorefinery approach refers to an upgrading process where a biomass feedstock is converted to different classes of biofuels and biochemicals through jointly applied conversion technologies. This means that a biorefinery should separate all biomass components resulting in high concentrated fractions with well-identified functions [65]. Up till now, most published studies focus only on one compound and the extraction procedure is often at the expense of the other compounds. Based on literature, either chitin is extracted, or protein & lipids are extracted, and the procedures are completely different. Nongonierma and FitzGerald (2017) concluded that the current extraction procedures for insect proteins (& lipids) generally includes the following steps: (1) drying of the insect biomass, (2) homogenization, (3) defatting since lipids interfere with protein extractions [66], (4) protein solubilization, (5) isoelectric precipitation of the proteins and (6) protein solubilization followed by a possible drying step [67] (see Figure 1-3). However, literature indicates that not all steps are always included in extraction procedures and details of individual steps can vary.

In respect to **drying & homogenization (step 1 & step 2)**, insects are generally freeze-dried [60],[68]–[70] and ground into smaller particles for better solvent contact. Indeed, dried biomass facilitates grinding and is often required for the defatting step. Yi et al. (2013), however, did not perform a pre-drying step but used frozen insects and water to homogenize the biomass [40].

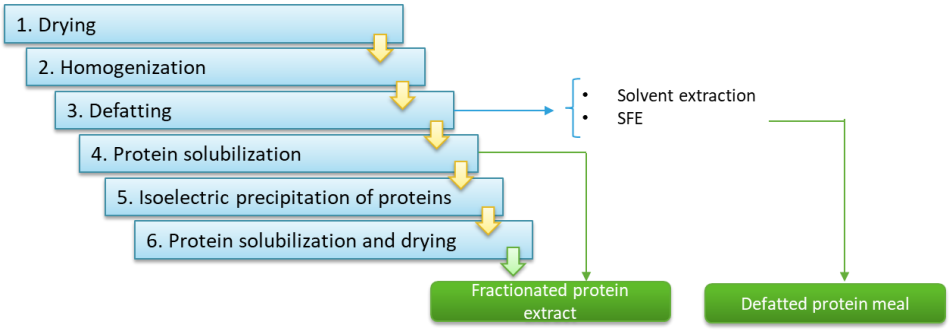


Figure 1-3 Schematic presentation of the general procedure for extracting proteins from insects

Defatting (step 3) is commonly performed by solvent extraction, mostly with hexane. Purschke et al. (2017), Choi et al. (2017), Ndiritu et al. (2017), Mishyna et al. (2019), Bußlet et al. (2016) and Purscke et al. (2018) all performed hexane extractions on different insect species at room temperature for 1- 36 h resulting in a defatted meal with a lipid content below 5 % [66], [69]–[72]. However, ethanol has also been reported [60] as well as supercritical CO_2 fluid extraction [73],[74]. Some studies only perform steps 1 to 4 and obtain a defatted protein meal [70]. Other studies combine the defatting step (step 3) with the **protein solubilization (step 4)**. Step 4 usually involves a centrifugal force to separate the solubilized protein from the solids (such as chitin) and this generates a floating lipid layer [40]. Mishyna et al. (2019) also skipped a separate defatting step and immediately perform an alkaline solubilization step. The same as Yi et al. occurred were a floating lipid layer was formed after centrifugation [71]. Generally, the pH of insects biomass is increased to perform an alkaline extraction in aqueous conditions [67]. NaOH is added to reach a pH between 9 and 10 and the reaction could be performed at elevated temperature (25 till 45 °C), usually for 1 hour [60],[69],[71],[75]. After a centrifugation step, the floating upper layer (UP) and pellet (P) are removed and the supernatant (SN) is freeze-dried or the fifth step is performed. The fifth step involves the **precipitation of protein (step 5)** by lowering the pH to the isoelectric point of the proteins, which is generally around pH 5 for insects. In this way, the purity of the

resulting protein fraction can be increased. A few publications report on alkaline isoelectric point precipitation that resulted in a protein extract with a lipid content below 14 % and an extraction yield of at least 24% [40],[70],[76]. Steps 4 and 5 perform a protein extraction and simultaneously fractionate proteins based on their solubility. The last step (step 6) includes **resolubilization** by bringing the pH to neutral and **drying (step 6)** of the obtained protein fraction [67].

The fractionation approach for chitin is totally different and usually only focused on chitin (will be further discussed later in '3.2 Isolation of chitin'. One article was found where all three (lipid, protein, and chitin) were separated. Yi et al. (2013) performed a filtration step after homogenization to separate chitin.

1.3.2 Applications for proteins and lipids

Due to legislation issues and insects being a relatively new source, insect-derived fractions on the market are scares. However, they have, besides a nutritional value, also techno-function properties that can have an added value to the food/feed product or can be employed in technical applications. For example, Yi et al. (2013) found excellent gelling properties for a protein extract from the house cricket and only a minimal concentration of 3 % was needed to form a gel [40]. Gould and Wolf (2018) measured good emulsification abilities of a yellow mealworm protein extract that was able to stabilize an emulation up to two months [77] and Zielinska et al. (2018) reported good foaming capacities of a protein extract from the cricket *Gryllodes sigillatus*. The authors also showed that depending on the insect species, the techno-functional properties can change significantly as this species exhibited mush better foaming capacities than the yellow mealworm for example [68]. Ndiritu et al. (2017) showed that the fractionation process also influences techno-functional properties since the aqueous extraction resulted in a protein fraction with much better emulsification and foaming abilities than the hexane based solvent extraction

[70]. Some potential applications for proteins with techno-functional properties are summarized in Table 1-1.

Table 1-1 Applications of protein and lipid fractions

	Food/feed applications	Non- food applications
	Proteins	
Nutritional value	Food/feed ingredient	
Proteins with a gelling capacity	An important feature for products such as cheese, yogurt and tofu [57] and adds to the texture of meat [78].	Film-forming properties in skin care products [79].
Proteins with a foaming capacity	Foams are often the base of a food product texture, especially in desserts (whip cream, ice cream, cakes), bakery and dairy products [57], [78]. In addition, bread is also a foam where gluten causes foam formation and an alternative for gluten is still highly sought after [80].	Surfactant in cosmetics and cleaning supplies [81] [79].
Proteins with an emulsifying capacity	Examples of food products that involve an emulsion are drinkable dairy products, salad dressing, sausages, soups, mayonnaise, cake batter,… [57], [78].	
Proteins with a water binding capacity	The capacity to bind water prevents drip by entrapping water and is useful in meat and bakery products [57]	Proteins are used in cosmetics since they have the ability to bind water to the horny layer skin [79]. Water absorbent material is also used in agriculture and horticulture applications [82].
Proteins with an oil binding capacity	Protein in food products that can trap oil plays an important role in ground meal formation and meat substitute products. Oils are often a flavor retainer and it increases the palatability of the food product [83], [68].	
Other characteristics		Vaz et al. (2003) pointed out the most common technical applications are adhesives, coatings, bioplastics, and surfactants [84]. Other include wood glue [85], bottle-labelling [81], inks and paint [85] and bioplastics [82]
	Lipids	
Nutritional value	Food/feed ingredient	
Other characteristics	Lauric acid has a biological activity (antimicrobial and prebiotic properties)[86]	Tao (2007) summarizes some applications for lipids [87]. They include coatings and polymers (paint, varnishes), lubricants, cosmetics /pharmaceuticals, moisturizing agent, odor and flavor, skin nourishment (essential fatty acids are absorbed through the skin) [88], drug delivery system (dermatological delivery), decrease blood pressure [89], surfactant and biofuels [90]

1.4 Chitin extraction and the conversion to chitosan

1.4.1 Chitin characteristics

Chitin is abundantly present in nature with an estimated annual production of 10^{11} to 10^{14} tons [91],[92] and it is present in at least 19 animal phyla, as well as in bacteria, fungi and algae [93]. It is suggested that a large portion of chitin is located in the oceans [94][95], as species such as corals [22],[23], sponges [93],[98], squids [8],[24], cuttlefish [101] and crustacea (like krill, crab, shrimp and lobsters) [99,][9],[19],[38]) all contain chitin. However, all Arthropoda, which includes insects, scorpions and spiders, contain chitin in their exoskeletons [33],[104]–[106],[107] [108]. Currently, the main sources for commercial chitin and chitosan production are crustaceans [109]. Crustacea are generally used for the simple reason that they are conveniently available as a waste stream from the seafood industry [110].

Chitin is a natural polysaccharide containing 2 monosaccharides, *N*-acetyl-D-glucosamine, and D-glucosamine connected by β-1,4-glycoside bonds. Depending on the frequency of the latter monosaccharide, the molecule is defined as chitin or as its derivate chitosan. Chitin contains mainly *N*-acetyl-D-glucosamine and can be transformed into chitosan by partial deacetylation of the monomer *N*-acetyl-D-glucosamine to D-glucosamine [111]. Generally, the molecule is defined as chitosan when a degree of deacetylation (DDA) of 60 % or 75 % is reached [112],[113],[114]. Chitin found in crustacea is mainly a heteropolymer with a DDA ranging from 5 % to 20 % [115].

Chitin has a structural function in exoskeletons where it is combined with proteins and minerals to form a stiff, strong material that is one of the most resistant organic material found [39],[53]. This way, the exoskeleton is tough and can give support and protection to the body [115]. The exoskeletons are composed of highly crystalline fibers containing chitin- sheets that are strongly connected through interaction with proteins and other fibers. Within a fiber, sheets of multiple polysaccharide chains are placed next to each other and one sheet consists of multiple parallel-positioned chitin chains [113]. Depending on the orientation of the sheets, three crystalline forms have been reported (α, β, and γ).

- The most abundant form is **α-chitin** that is present in almost all crustaceans, insects, fungi and yeast cell walls [111]. In this formation, the chitin sheets (3 sheets as an example in Figure 1-4a) consisting of parallel chitin chains (for each sheet, two chains are presented in Figure 1-4a) are positioned in an anti-parallel way, allowing a maximum formation of hydrogen bonding. More specifically, two intramolecular and two intermolecular bonding are formed: a first intermolecular bonding with a vertical neighbor chain (in the same sheet), and another with a horizontal neighbor chain form a different sheet [118],[119]. These hydrogen bounds create a remarkably high crystallinity resulting in stiff and stable material. Therefore, α-chitin is characterized as a non-reactive and insoluble product [110]. Since this form is the most common polymorphic, α-chitin has extensively been studied [113].

- On the other hand, in **β-chitin,** the chitin sheets are ordered in parallel (Figure 1-4b) with weaker intermolecular forces. This results in a softer material with a higher affinity for solvents and higher reactivity. It is proven to be soluble in formic acid and can be swollen in water [118]. This chitin form is present in squid pen, in the tubes of pogonophoran and vestimetiferan worms and in monocrystalline spines excreted by diatoms like *Thalassiosira fluviatilis* [111] [119].

- The third formation, **γ-chitin**, is less common. It is considered to be a mixture or an intermediate form of α- and β-chitin with both parallel and antiparallel arrangements [120]. More specifically, every third chitin chain has the opposite direction to the two preceding chitin sheets [114],[118] [119].

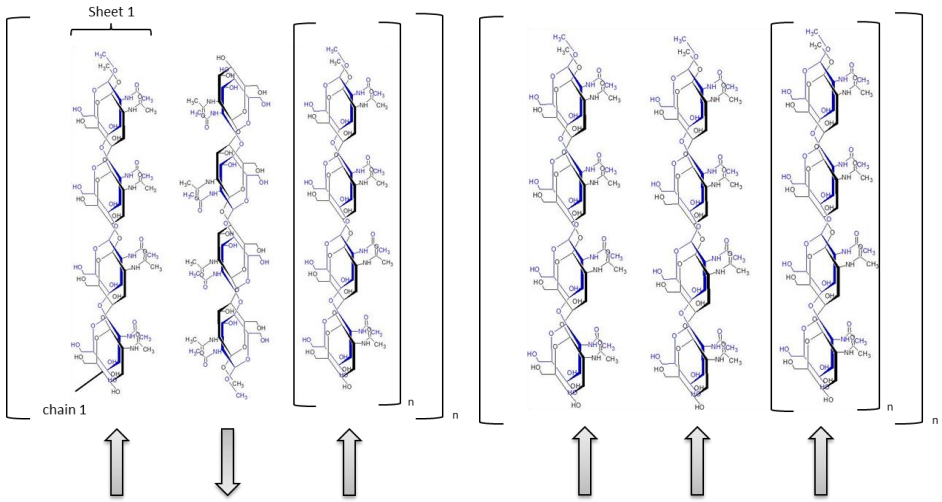


Figure 1-4 Schematic representation of (a) α-form and (b) ß-form of chitin [119]

1.4.2 Isolation of chitin

The isolation of chitin is a relatively laborious process. It is strongly bounded to protein and other compounds such as minerals and catecholamines. These impurities need to be removed to generate a high purity product for application development [121]. On a commercial scale, this purification process (for crustacean) is chemically-based and the extraction involves high consumption of hazardous chemicals and energy [8],[117],[63],[117].

Traditionally, a sequence of 5 processing steps is employed on crustacea shells comprising pre-treatment, deproteination, demineralization, decoloration and post-treatment processes (see Figure 1-5). The order of the steps can change according to the biomass type.

- **The pre-treatment** involves the removal of soft, unbounded compounds for example by pressing, boiling or scraping [123]. Afterward, the material is dried and reduced in size by grinding [105],[124],[125]. Published research on crustacea shells revealed that a large range in particle sizes in employed such as 60-120 µm [123], less than 250 µm [101], 0.5-1 mm [126], or 0.5-10 mm [127]. The impact of the particle size on further processing has not been studied extensively and opposite findings were found. Younes et al (2006) and Mahdy Samar et al. (2013) reported a better efficiency of purification when a smaller particle size was used [128],[129] whereas, Abdel-Rahman et al. (2015) reported a better demineralization and deproteinization with a particle size of 0.8-1 mm instead of a smaller sizes [130].

- For **demineralization**, two approaches have been reported in literature, being chemical or biological-based. For the chemical approach, HCl is the most commonly used reagent for removing minerals [117]. Depending on the mineral concentration present the biomass, the reaction time varies among species [100]. Biological demineralization is based on acid-producing biological processes using bacteria [92],[117],[103] or enzymes such as alcalase [117]. Lactic acid-producing bacteria are usually selected, for example, *Lactobacillus spp. B2* or *L. plantorum A3*. Since the acid also lowers the pH which activated proteases [102], demineralization and deproteinization may occur partially simultaneously. Khanafari et al. proved that demineralization of shrimp waste using fermentation could be evenly effective if not better than chemical demineralization [124].
- **Deproteinization** is usually performed by NaOH. Depending on the biomass, the process temperature, alkaline concentration and the alkali/biomass ratio changes [101]. Bastiaens et al. (2019) reported a variation in temperature (25-130°C), time (1 -24 hours) and concentration of NaOH (0.3- 12.5 M) that has been described in published research [119]. These harsh conditions result in

the degradations of proteins to amino acids and the formation of anti-nutritional compounds (such as lysinoalanine) [131] and thus the proteins cannot be valorized as a by-product. Biological deproteinization using (1) proteases (such as pepsin, papain, and trypsin) secreted by proteolytic bacteria in the fermentation medium or (2) isolated proteases (crude or purified) have been reported [117][103]. In comparison with the chemical deproteinization, the efficiency of the enzymatic method is lower, leaving approximately 5-11 % residual protein [102] [132].

- **Decoloration & other post-treatment processes** are not always employed. When desired, a decoloration step can be introduced to remove or recover pigments (for example, astaxanthin, a marketed fish food additive present in crustaceans [133]). Mild oxidizing treatments with hydrogen peroxide [134] or potassium permanganate [135] have been described in research articles (lab-scale) as well as solvent extractions with acetone [123], ethanol and chloroform [125]. Gómez-Ríos et al. (2017) employed plant process simulated data and used ethanol-water solutions 80 % for depigmentation [136].

- Finally, some **post-treatment steps** such as neutralization, drying, and milling may be required to finalize the chitin production process [132],[137].

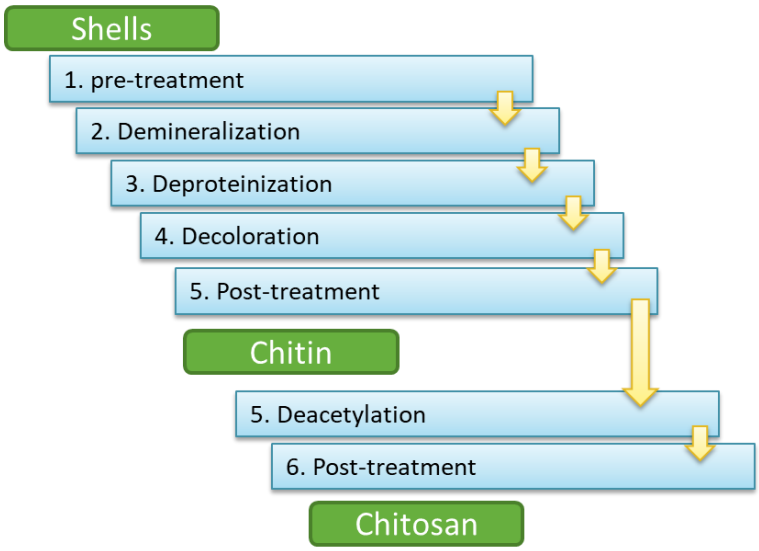


Figure 1-5 Schematic presentation of the isolation and conversion of chitin to chitosan

Some drawbacks of a chemically based chitin extraction are related to:

- Partial hydrolysis of the polymer during demineralization, depending on the process parameters employed [117],[121]
- Partial deacetylation and depolymerization of chitin during the harsh, alkaline deproteinization [102].
- No standardized procedure is available. Depending on the species, the crystallinity and the pre-treatment, the intensity of the different steps changes [134],[138]. Especially if the chitin source is seasonally depending and heterogeneous (different species together) as is often the case with crustacea.

Table 1-2 Chitin extraction from insect sources [119]

Insect	Insect stage	Pretreatment	Demineralization	Deproteinization	Posttreatment	ref
Colorado potato beetle	Larvae/adult beetle	Drained in 70 % (v/v) alcohol – washed – dried at room temperature – grounded – dried at 65 °C for 3 hours	20 g + 100 mL 2M HCl at 65-75 °C for 2 hours	50 mL 2 M NaOH at 80-90 °C for 16 hours	Filtered and washed– drained in chloroform (1), methanol(2) and water (4) for 1 hour - filtered and washed - dried at 60 °C	[139]
Crickets*: Gryllus bimacelatus*		Dried crickets - grounded - washed and dried at 60 °C	(3) oxalic acid - 3 hours at room temperature	(1) 40 g + 400 mL 1 M NaOH 95 °C for 6hours	(2) drained in ammonium persulfate solution (50 % (w/v)) at 50 °C for 6 hours (4) Dried at 60 °C overnight	[140]
Calliptamus. barbarus and *Otopharynx. decorus*		Grounded – dried at 70 °C for 30 min	2 g + 100 mL 1 M HCl at 100 °C for 30 min	1 M NaOH at 80-90 °C for 21 hours	Washed and dried at 55 °C - drained in chloroform (1), methanol (2) and water(4) for 1 hour	[141]
House fly : *Musca domestica*	Four-instar larvae	Washed with 15 % (w/v) aqueous NaCl - freeze dried - grounded	(3) 10 mg/mL oxalic acid aqueous for 3 hours	(1) 10 g + 100 mL 1 M NaOH at 95 °C for 6 hours	(2) 10 mg/mL potassium permanganate aqueous solution for 4 hours (4) Neutralized and freeze dried	[142]
Silkworm	Chrysalides	dried by lyophilization for 12 hours	1 M HCl at 100 °C for 20min (10 mL HCl/g)	1 M NaOH at 80 °C for 24 hours (10 mL NaOH/g)	Washed with Na_2CO_3 0.4 % (w/v) - dried at 80 °C	[143]
Blowfly: *Chrysomya megacephala*	Larvae	Washed with NaCl- dried at 50 °C- grounded	(3) oxalic acid (10 mg/mL) for 3 hours	(1) 10 g + 100 mL NaOH 1 M at 95 °C for 6 hours	(2) decolored with NaCl (0.5 %, w/v) for 3 hours (4) washed to neutral with distilled water and then freeze-dried in vacuum	[144]
Black soldier fly: *Hermetia illucens*	Pupal exuviae and dead imago	Cleaned – dried – grounded	1 M HCl for 1hour	1 M NaOH at 80 °C for 24 hours	1 % $KMnO_4$ and excess of $KMnO_4$ was removed by 4% (w/v) oxalic acid	[145]
House cricket: Brachytrupes *portentosus*	8 weeks old	Starved for 48 hours - frozen- washed with water - dried at 60 °C for 48 hours	(2) 200 mL 10 M oxalic acid at room temperature for 3 hours	(1) 20 g + 200 mL 1M NaOH at 95 °C for 6 hours	200 mL 1 % NaCl (1 %,w/v), at room temperature for 3 hours – filtered- washed to neutral pH- dried at 60 °C overnight	[146]
Beetle: *Holotrichia parallela*	Adult beetles	Starved for 48 hours- washed – frozen – defrosted - dried at 50 °C for 48 hours- grounded	5 g + 250 mL 1 M HCl at 100 °C for 30 min	1 M NaOH at 80 °C for 24 hours	Washed till neutral pH – 1 % (w/v) potassium permanganate for 1 hour - washed and dried at 5 °C	[137]
7 orthoptera species	Grasshoppers	Washed - dried at room temperature - grounded	2 g + 100 mL 4 M HCl at 75 °C for 1 hour	50 mL 2 M NaOH at 175 °C for 18 hours	Filtered – washed - drained in chloroform (1), methanol (2) and water (4) – washed with distilled water- dried at 60 °C for 24 hours	[125]
Desert locus, beetles, honey bee	Exoskeletons	Scraped free from loose tissue – washed – dried - grounded	1 M HCl at room temperature (15 mL/g)	1 M NaOH at 100 °C for 8 hours – repeated several times	Filtered - washed till neutral pH- washed with hot ethanol and boiled in acetone – dried in a vacuum oven at 50 °C	[105]
Melolontha melolontha	Adults	Killed using a killing agent – washed – dried at 60 °C for 24 hours - grounded	2 g + 50 mL 4 M HCl at 75 °C for 2 hours	4 M NaOH at 150 °C for 18 hours	Filtered - washed till neutral pH - drained in chloroform (1), methanol (2) and water (4) for 20 min – washed – filtered - dried at 60 °C for 24 hours	[106]

For insects, the isolation procedure usually mimics the procedure performed on crustacea shells. Only a few articles could be found on chitin extraction from insects and are summarized in Table 1-2, indicating that insect chitin is a relatively new field. For this reason, no standard or engineered procedure for insects was found [119].

1.4.3 Conversion to chitosan

Depending on the application, either chitin or chitosan is desired. Due to its crystalline nature, chitin is insoluble in many common solvents, including water [147]. Among the derivatizations, N-deacetylation is the most simple modification [148]. Chitosan is generally insoluble in organic solvents and water, but soluble in diluted acid solutions with a pH below 6. A pH lower than the pKa of the amino group (6.3) results in the protonation of these amines and electrostatic repulsion between the positively charges groups increase the solubility. Depending on the DDA, the exact pKa and in turn, also the solubility of chitosan will change [111],[149]. Besides the increased solubility, chitosan is an important derivative of chitin since the amino group enables easy chemical modification and generate new or improved properties [111],[112].

Deacetylation is usually performed in three steps, starting with purified chitin that is processed by (1) a pre-step, (2) alkaline deacetylation and (3) a post-treatment (see Figure 1-5).

- A pre-step could mean a step that lowers the crystallinity of chitin and thus improve the access of the solvents for deacetylation [150], or a pre-swelling of chitin to increase the amorphous zones [114]. However, this is not always employed.
- Secondly, the alkaline deacetylation is performed. Deacetylation is a two-step nucleophilic substitution reaction. Firstly, nucleophilic addition of hydroxide on the carboxy groups [134] occurs while in the second step, an amine and acetate are formed (see Figure 1-6). The reaction is generally performed based on

Kurita (1993) with a high aqueous NaOH solution (30-60 m%), long reaction times (1-80 hours) and at high temperatures (80-160°C) [117],[99] [114],[151],[152],[153].

- The post-treatments generally involves a washing step to neutral pH and drying [134], [139].

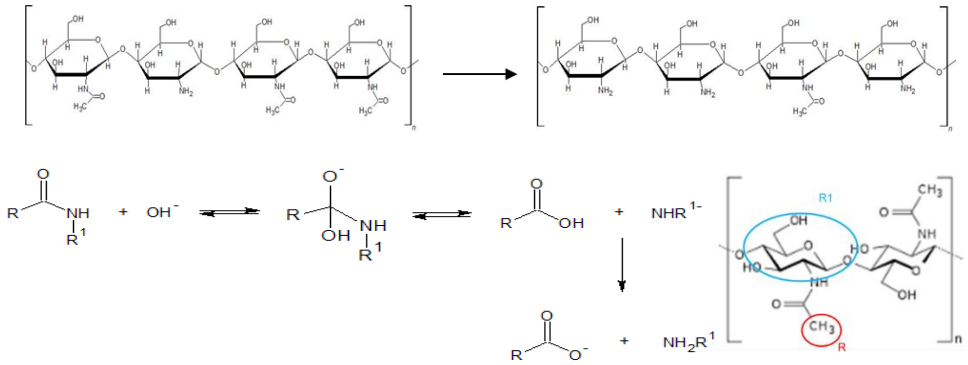


Figure 1-6 Reaction mechanism of the deacetylation step [119].

Some examples of deacetylation procedures of crustacea and insects are given in Table 1-3.

Table 1-3 Examples of different deacetylation procedures [119].

Origin	Biomass type	Deacetylation approach	Ref.
Crustaceans & mollusca	Crabs, lobsters, crayfish, shrimp, cuttlefish, squid	50 w/w% NaOH, 80°C, 60 mL/g, under nitrogen atmosphere (Kurita) - 0.5 g scale	[134]
Crustaceans	Prawn shells	25-50 % NaOH; 80-100 °C; 2-5-10 hours; solid/Liquid 1:5	[123]
	Lithodes antarcticus, Paralomis granulosa Palinurus vulgaris	50 w% NaOH; 110 °C; 4 hours; 10 mL/g – under N_2 atmosphere	[126]
	Shrimp waste	50 w% NaOH; 105 °C; 1-4 hours; 20 mL/g	[154]
	Shrimp waste	421 g/L NaOH; 130 °C; 16.5 g/L	[155]
	Shrimp shells	40-50 w% NaOH; 90 °C; 1.5-2 hours	[151]
Insects	Larvae and adult beetles of the potato beetle	50 % NaOH (w/v 1:20) at 100 °C for 3 hours	[139]
	Grashoppers (Calliptamus. barbarus and Otopharynx. Decorus)	50 % NaOH (w/v 1:15) at 130 °C for 2 hours	[141]
	Larvae of the blowfly	670 g/L NaOH at 90 °C for 9 hours (with the renewal of alkali every 3 h)	[144]
	Cricket (*Gryllus bimaculatus*)	50 -67 % NaOH at 95 °C for 9 h	[140]
	House fly	400 mg/L NaOH at 70 °C for 8h	[142]

Drawbacks of the deacetylation procedure are associated with the following aspects:

- In respect to chain degradation, Galed et al. (2005) reported degradation for three different crustacea samples (two crabs species (*Paralomis granulosa, Lithodes antarcticus)* and a lobster (*Palinurus vulgaris*)) during deacetylation under 110 °C for 4 hours with 50 m% NaOH [126].
- Since chitin usually consists of crystalline zones and amorphous zones, deacetylation mainly impacts the amorphous zones leading to a heterogeneous more block-like deacetylation pattern [114],[120].
- Deacetylation requires high NaOH concentrations and thus generates high amounts of alkaline wastewater [122]. Alternative methods such as enzymes proved less effective [102],[152].

- Depending on the nature of the chitin source and the physical structure (often impacted by the pre-treatment and isolation process), the efficiency of the deacetylation differs [102],[134],[156]. For example, different crustacea species underwent the same pre-treatment, isolation and deacetylation process but it took squid chitin 1 hour, grey shrimp 2 hours, pink shrimp and prawns 3 to 4 hours and lobster or red crab 6 to 7 hours to reach the same DDA [134].

1.4.4 Applications

Chitin and chitosan have a broad range of properties, depending on the DDA, the molecular weight and the solubility. They are already used in applications for cosmetics, (waste) water treatment and biomedical applications such as wound dressings [102],[157]. Based on the extensive literature related to other applications [111] and the market growth expectancy of the two main sectors (healthcare and wastewater), the chitin market is expected to grow 3.3 times by 2027 [158].

The main sectors that have an increased interest in these molecules for (future) applications are (1) biomedical, (2) cosmetics, (3) food and feed, (4) (waste) water treatment and (5) agriculture. Many reviews on potential applications exists [133], [114],[157],[159] some examples are listed below.

Biomedical applications:

- Chitin and chitosan have some antimicrobial properties and have been reported to inhibit fibroplasia in would healing. In addition, the molecules promote would healing and boost the immune system. For this reason, they are incorporated into wound-dressing materials as fibers [133].
- More research is being done on controlled drug delivery systems. Chitin/chitosan can absorb or encapsulate drugs and by slow diffusion through the polymer material, drugs can be controllably released. Chitin/chitosan is of

particle interest because of their gel-forming ability and the swelling in an acid environment [133].

- Chitin/chitosan also have anti-coagulant and anti-thrombogenic properties that could be employed for pharmaceuticals [114].

Applications in cosmetics [160]:

- Chitosan finds many applications in the field of dentistry. For example, chitosan can be added to toothpaste for plaque formation prevention and avoidance of biofilm formation of bacteria.
- Hair products containing chitin/chitosan enhance the adhesion of other ingredients to the hair.
- The filming properties and emulsifier activity of chitin/chitosan can be used for cosmetic products.
- Since chitosan has a high moistening effect, it is often added to skin products. In addition, chitosan films block UV light and can be used as a sunscreen.

Food and feed:

- Chitosan oligosaccharides have been proven to be prebiotic and thus can help prevent over-antibiotic use in feed.
- Chitin and chitosan absorb lipids, including cholesterol. By adding these polymers to the diet, fewer lipids can be absorbed by the digestion system [133].
- Chitosan is also a good emulsifying agent and can be used in food products [114].
- These polymers can be added to food products to prevent spoilage since they have antimicrobial and anti-oxidant properties [114]. Studies have also investigated the use of chitin and chitosan for edible films and coatings [161].

Applications in (waste) water treatment:

- Chitin and chitosan are excellent chelators and can entrap metals, dyes, phenols, and anions [133] [162].
- They can also be used as a flocculant to clarify water and reduce odors [114].

Agricultural [159]:

- Chitosan stimulates cell production in plants and can be used to treat wounded bark tissue. In addition, chitosan is known to enhance defense responses to microbial infections.
- Since they have good film properties, chitosan can be used for seed-coating to enhance crop production.
- The same principle as a controlled drug release can be employed to release fertilizes and nutrients into the soil.

1.5 Scope of the thesis and specific aims

Since insects are a new resource on the market, the main aim of the thesis is to further expand the scientific knowledge to facilitate the introduction of insects to the market. The different aspects that are associated with the establishment of an insect-based value chain are presented in Figure 1-7 and comprise the 1) compiling of the insect feed with the necessary dietary nutrients and possible integration of underspent side-streams, 2) breading to rearing of insects, 3) first stage processing of insect biomass for direct utilization which may include separation from the substrate and possible killing, hygienisation & drying or 4) a secondary fractionation step to obtain crude extract that could be further tailored to high concentrated fractions of proteins, lipids and chitin that can be utilized for different application fields with step 5) for characterization of the fractions and 6) for product development. This thesis was mainly focused on fractionation and characterization of insect biomass. A biorefinery approach was engineered with a focus on the recovery of multiple compounds that could be used as raw materials for feed, food and technical applications. Attention was given to scalability and industrial applicability with a special focus on the minimalization of waste streams.

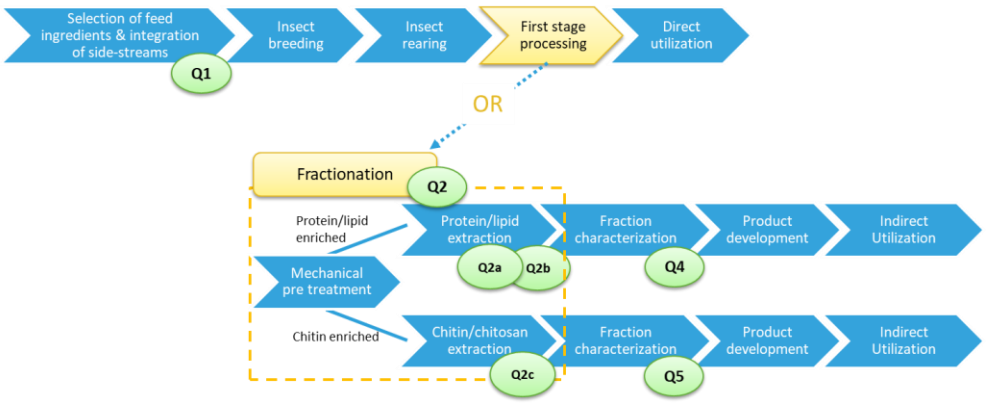


Figure 1-7 Insect value chain with an indication of the different research questions.

The specific aims of this PhD work are linked to the following research questions that were the basis of the work performed:

- Research question 1 (Q1): **Does a varied insect feed composition impact the composition of the insect biomass?** Insects have been proposed to be reared on side-streams but because of reasons described in '1.3.1 An opportunity for a circular economy', the composition of the side-streams may vary in time. In general, insects can be reared on side streams but the efficiency of rearing when the diet changes and especially the impact on the insect biomass composition is a point of attention. Indeed, some studies indicated that the insect's composition can change according to the diet and thus may influence the characteristics of the end fractions. This hypothesis was verified, and the results are described in chapter 2. More specifically, different diet mixtures (based on selected side-streams that are nowadays allowed for insect rearing) were fed to the LM and the composition of the LM and the diet were measured in terms of protein & lipid content and the amino acid & fatty acid profile.

- Research question 2 (Q2): **Can a biorefinery, in the true sense of the word, separate <u>all</u> (major) components with minimal generation of waste?** Up till now, biorefinery of insects usually focusses only on one compound, often at the expense of others. The scope of the current work was to fractionate the insect biomass in different fractions (protein-enriched fractions, lipids enriched fraction & chitin enriched fractions) and maximize the valorization of these fractions. The biorefinery involved different steps starting with a mechanical separation of the solids and liquids. The solids contained the chitin containing exoskeletons, and the liquid concentrated all free lipids and proteins. Secondly, the two fractions were treated separately where a new approach for the separation of lipids and proteins in the liquid fraction was proposed in chapter 3 and an alternative procedure for extracting chitin from the solid fraction

where a maximal protein recuperation was envisioned is discussed in chapter 7. For this part specific sub-questions were given focus:

- **Q2a: Would the use of organic acids improve fractionation in an acid-based biorefinery approach to obtain lipid and protein-enriched fractions?** Processes found in literature usually involve alkaline isoelectric precipitation, but this can negatively impact the protein quality. Since organic acids are allowed in food and feed industry, an acid-based isoelectric precipitation was investigated in chapter 3. The distribution of the proteins and lipids was studied as well as the yield & purity of the end products in function of meeting the demands set by the feed industry and minimizing non-usable fractions.

- **Q2b: Is the newly developed biorefinery approach comparable to traditional defatting methods?** Many insect rearing facilities market defatted insect meal. The process efficiency of the new biorefinery process was compared to the traditional defatting techniques employed for obtaining this defatted meal. The distribution of lipid and protein and the yield & purity of the end fractions were compared in chapter 4.

- **Q2c: Could a modified biorefinery approach to obtain chitin and chitosan from insects be more sufficient than the traditional crustacea-based purification method?** Chapter 7 investigates a new biorefinery approach for the solid chitin-rich fraction where a maximal recuperation of by-products such as proteins was envisioned during the chitin extraction and a minimal generation of chemical waste streams. This process was compared to the traditional purification in terms of chitin purity, the impact on the degree of deacetylation and residual protein concentrations.

- Research question 4 (Q4): **What are the techno-functional properties of insect proteins and what is the impact of the extraction method?** The techno-functional properties of LM were investigated in chapter 5. Results of the native protein properties (after the mechanical pre-step) were compared to extracted protein properties that were extracted by either the new biorefinery approach or a traditional defatting method.

- Research question 5 (Q5): **What is the effect of different life stages on the chitin characteristics?** Different life stages of the BSF were collected. After a traditional chitin extraction, the physicochemical characteristics were studied by employing FTIR, XRD, and TGA and the impact on further processing was evaluated by deacetylating chitin to chitosan. This study will be discussed in chapter 6.

The thesis was partially embedded in the H2020-BBI project InDIRECT where a two-step valorization approach was studied to convert underspent side streams to a new feedstock. The process involved (1) the rearing of two insect species (larvae of the BSF and LM) on these side-streams for converting side-stream biomass in insect biomass and (2) a biorefinery of the insect biomass to extract proteins, lipids, and chitin. Within the thesis, both insect species were used but each chapter reports the data of one insect species. Crucial findings were also confirmed with the other species. Figure 1-8 illustrates the different chapters and points out which insect was studied.

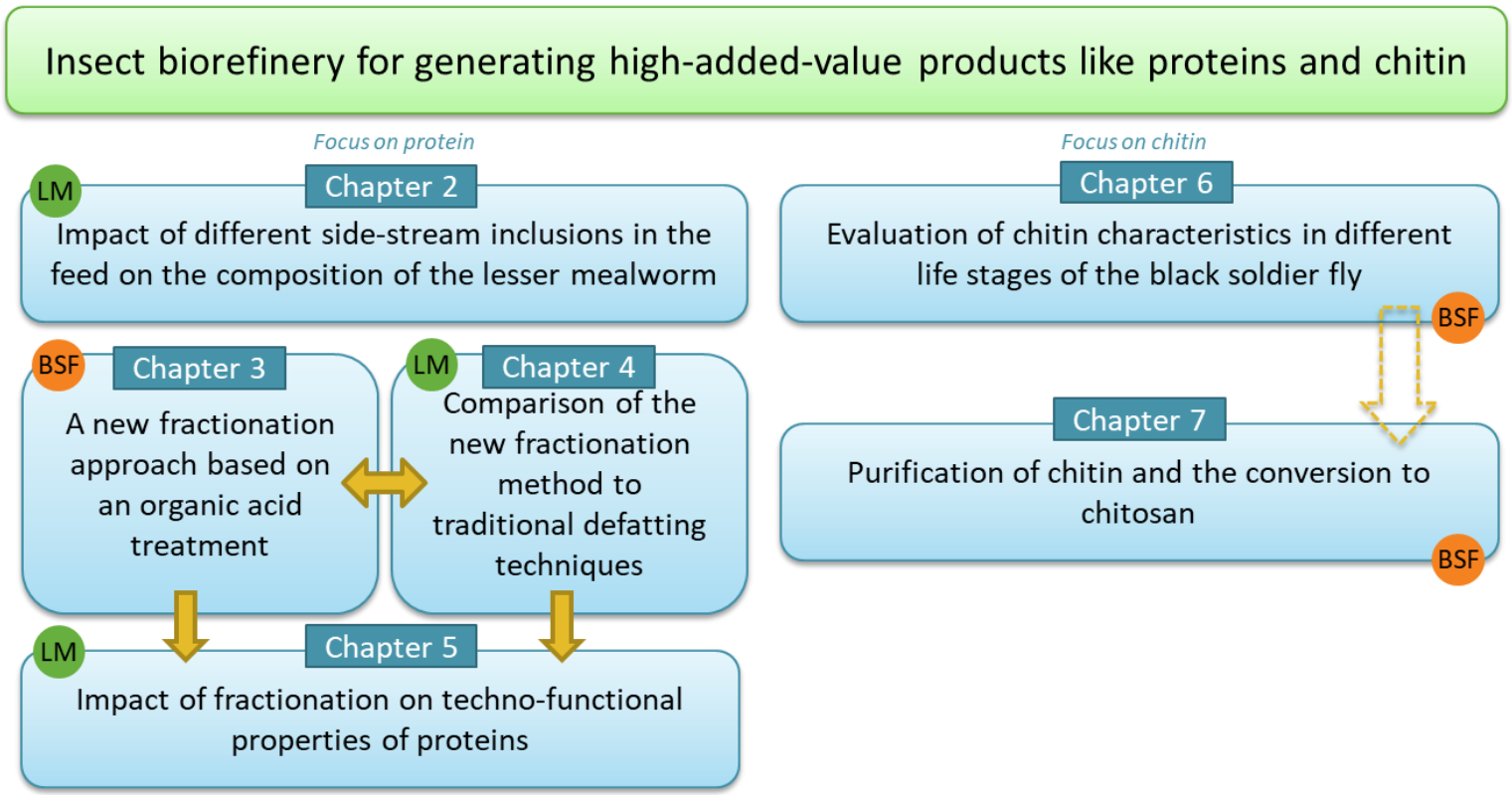


Figure 1-8 General overview of the thesis

Chapter 2.

IMPACT OF DIFFERENT SIDE-STREAM INCLUSIONS IN THE FEED ON THE COMPOSITION OF THE LESSER MEALWORM

Parts of this chapter has been sent for publication (L. Soetemans, N. Gianotten, L. Bastiaens. Agri-food side-stream inclusion in the diet of Alphitobius diaperinus: Impact on larvae composition, submitted)

2.1 Introduction

Growing concerns about future protein shortages are inducing a search for alternative protein sources. Insects offer an opportunity since they are rich in proteins (ranging from 13 % to 77 % on dry matter basis) and could provide a sustainable and local protein source. The larvae of the BSF, common housefly larvae, silkworms and yellow mealworms (YM) have been described as promising species for industrial food or feed applications and have a protein content of 56 %, 62 %, 54 % and 52 % on dry matter basis, respectively [6], [42], [163]. Recently, a considerable amount of literature was published on the BSF and YM, but information on the LM is still rather limited. Nevertheless, the latter species is reported to be rich in proteins, is suitable for human consumption and has in comparison to the YM a shorter development time (66 days versus 117 days) [38], [42]. Van Broekhoven et al. (2015) even found a higher protein content of the LM compared to the YM when reared on the same diet [38]. The rearing of insects is commonly performed with grain-based diets. However, food by-products also are being introduced in the diet to avoid future competition for grains and to obtain a sustainable, low-cost diet. In addition, by using (underspent) side-streams, side-stream nutrients can be recycled back in the market as insect proteins. However, the impact of the feed on the composition of the insect biomass is not yet clear and reports on a possible effect have been published for some species. Research on the larvae of the BSF, for example, proved that the lipid content and fatty acid profile were influenced by the diet [7], [33], [164]. This could generate opportunities to tune the nutritional value of the insects for example in essential amino acid (EAA) composition or fatty acid profile [165]. Published data on the compositional changes in mealworms are however contradictory. Some studies observed no changes in larval protein and lipid content when providing different diets [7], [166] whereas Van Broekhoven et al. (2015) did measure a variable larval lipid content with different diets. However, a direct connection between the dietary lipid content was not found as two diets with

similar lipid content provided larvae with a significantly different amount of lipids [38]. In addition, Dreassi et al. (2017) and Van Broekhoven et al. (2015), measured differences in the fatty acid profile when the YM was reared on different diets. These findings were partially confirmed by Oonincx et al. (2015) who found only a slight variation in fatty acids C16:0 and C18:2 and stated that the larval fatty acid did change over different rearing diets but the changes did not follow the fatty acid profile changes of the diet [7].

In a prior research article, Gianotten et al. (submitted) described the impact of these diets on larval growth. 6 agri-food side-streams (corn Distiller`s Dried Grains with Solubles (DDGS), rice bran, wheat middlings, corn gluten feed, brewery grains (BG) and rapeseed meal) that are allowed according to the feed legislation were tested as a singular feed or as mixtures and the larval growth was evaluated by monitoring the larval yield, efficiency of conversion of ingested feed and larval weight. The larvae were able to grow on all diets, but differences in growth were observed. Two side-streams, wheat middlings and rapeseed meal, proved to support good larval performance when used as a single ingredient and a combination of the two with BG as moisture & nutrient source provided the best larval growth. In addition, low percentages of the wheat middlings could be replaced by rice bran without affecting larval growth and resulted in a reduction in production costs. Even though rapeseed meal is a significantly more expensive side-stream compared to wheat middlings, partial replacement (5 % to 10 %) was still cost-beneficial since rapeseed meal was able to increase the larval yield. In conclusion, the results show that agri-food side-streams have the potential to be included in insect diets and this offers the opportunity to search for cheaper and underspent side-streams to avoid competition with other feed applications and improve the economics and sustainability of insect rearing.

While Gianotten et al. (submitted) described the impact of these diets on larval growth, the current study investigates the effect of the diets on the nutrient composition of the larvae. In addition, the LM is among the seven insect species on a list that allows the use of their proteins in feed for aquaculture animals (Regulation No 2017/893). Yet, published data on the nutrient composition is scarce. The current study will also report the composition of larvae that were reared on the different diets.

2.2 Materials and methods

2.2.1 Insects and diets

The LM was cultivated on different side-stream compositions as described by Gianotten et al. (submitted). Briefly, wheat middlings, rice bran, rapeseed meal, corn DDGS, and corn gluten feed were selected as side-streams and carrot or brewery grains as a source of moisture. Carrots were assumed not to contain considerable amounts of nutrients supporting the growth of the LM which was based on the results from Oonincx et al. (2015) who stated that the carrots did not influence the crude protein, crude lipid and fatty acid profile [7]), while brewery grains are known to support the growth. After 28 days, the LM was harvested, separated from the rearing substrate, frozen and later freeze-dried. A selection of the diets evaluated by Gianotten et al. (submitted) was considered in the current study. Details on the different side-stream mixtures that were fed to LM and the relative larval yield (compared to diet 2) obtained are summarized in Table 2-1.

Table 2-1 Overview of all tested diets with the inclusion percentage of side-streams and a summary of the rearing evaluation.

% FW	Wheat middlings	Rice bran	Rapeseed meal	DDGS	Corn gluten feed	Moisture delivery agent	% Yield*
Diet1	100					carrots	74[4]
Diet2 (ref)	100					BG	100[2]
Diet3		100				Carrots	4[4]
Diet4		100				BG	40[4]
Diet5			100			carrots	40[4]
Diet6				100		carrots	20[4]
Diet7				100		BG	79[3]
Diet8					100	BG	84[3]
Diet9	95	5				BG	101[2]
Diet10	90	10				BG	101[2]
Diet11	85	15				BG	98[2]
Diet12	80	20				BG	100[2]
Diet13	95		5			BG	105[1]
Diet14	90		10			BG	106[1]
Diet15	85		15			BG	106[1]
Diet16	80		20			BG	95[3]
Diet17	50			50		BG	95[3]
Diet18	50				50	BG	91[3]

1 excellent yield, 2 normal yield, 3 tolerable yield, 4 bad yield

* calculated and evaluated according to Gianotten et al. (submitted)

2.2.2 Composition analysis

Freeze-dried LM and the side-streams used were subjected to a set of analyses to determine their composition. Dry matter (DM) was determined after drying the samples at 105 °C for 48 hours and ash content after mineralization at 550 °C for 6 hours. All composition data for the larvae and feed are reported on a dry matter basis. Soxhlet extractions with diethyl ether for 6h were performed to determine the lipid content gravimetrically. The fatty acids profile was measured on Soxhlet extract by GC-FID after methylation with NaOH/MeOH and H2SO4/MeOH (pre-

treatment according to ISO 12966-2:2011 and AOCS Ce 1b-89). Analyzation with GC-FID was performed with a FAMEWAX column (30mx0.32mm, 0.25 µm df) at a constant flow rate of 1 ml/min helium. The split/splitless injector was set at a temperature of 245 °C and the split flow at 75 ml/min. The FID detector was set at 250°C. The internal standard was methyl heptadecanoate and the standards lauric acid and oleic acid (50 µg/g -20000 µg/g) underwent the same methylation pre-treatment as the samples. Amino acids were determined on defatted samples after acid hydrolysis with phenol-HCl (6N) for 23 hours at 110°C under a nitrogen environment (pre-treatment according to ISO 13903:2005). Amino acids were subsequently analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection in Chromeleon software. The columns Dionex AminoPac PA-10 (2x250 nm) and Dionex AminoPac PA-10 Guard (2x50 mm) were used at 30 °C. The mobile phases (0.250 ml/min) consisted of (A) Milli-Q water, (B) 250 mM NaOH, (C) 1M NaOAc, (D) 0.1 M Acetic acid; gradient : 76 % Eluent A and 24 % B (0-2 min), 64 % eluent A and 36 % eluent B (2-11 min), 40 % eluent A, 40 % eluent C and 20 % eluent A (11-47 min), 100 % eluent D (47.1 -49.1 min), 20 % eluent A and 80 % eluent B (49.2 – 51.2 min), 76 % eluent A and 24 % eluent B (51.3 -76 min). Tryptophan was not measured given its notorious tendency to be degraded during acid hydrolysis. During the acid hydrolysis, asparagine and glutamine were converted to aspartic acid and glutamic acid, respectively. The acid hydrolysis was performed in a nitrogen environment, resulting in minimal degradation of methionine, which could then be determined. The total protein content was calculated as the sum of mmols of the individuals anhydrous amino acid residues (subtracting one molecule of water from the molecular weight of each amino acid) per g sample. In this study, the term ‘essential amino acids’ refers to amino acids that are essential for humans or insects and will be specified. Quantification of the chitin content was performed as described by D’Hondt et al. (D’Hondt et al., submitted). Briefly, chitin was hydrolyzed with 6 M HCl for 6h at 110 °C. The released glucosamine was subsequently quantified by LC-MS (Waters UPLC BEH HILIC 2.1 ×

100 mm, 1.7 µm column at 40 °C, isothermal gradient elution using water with (A) 20 mM ammoniumformiate and 0.1% formic acid and (B) acetonitrile with 0.1 % formic acid with gradient settings: 5-25 % A (0-3 min), 25 % A (3-4 min), 25-5 % A (4-4.1 min), 5 % A (4.1-7 min)). Quantification was performed against a set of standard solutions.

2.2.3 Statistical analysis

All measurements were performed in triplicates unless stated otherwise. The Pearson correlation coefficient was calculated on replicates separately to evaluated correlations.

2.3 Results

2.3.1 Composition of the side-streams and insects diets

The composition of all feed ingredients (wheat middlings, rice bran, corn gluten feed, rapeseed meal, and BG) were characterized in terms of protein & lipid content on DM basis, amino acid profile and fatty acid profile (Table 2-1). DDGS and rapeseed meal were the most protein-rich (30 % protein) ingredients, while rice bran contained the highest lipid content (19 %). Wheat middlings, rapeseed meal, and corn gluten feed contained very low lipid content (≤5 %) while DDGS proved to be a feed ingredient rich in both lipids and protein (14 % and 30 %, respectively). The amino acid profile of all ingredients revealed that arginine and glutamate were most dominant. YM requires the same 9 EAA as humans, plus arginine, and the same was presumed for the LM [167],[168]. Rapeseed meal and DDGS were most nutritional in terms of the presence of EAA for insects (188 g/kg and 202 g/kg, respectively). The fatty acid profiles indicated that C16:0; C18:1 and C18:2 were mainly present for all side-streams. Since no literature was found on the essential fatty acids (EFA) for insects, the fatty acids essential for human consumption were also considered essential for insects in the current study. Rice bran contained more lipids than DDGS

but a better nutritional value in terms of the sum of all EFA (61 g/kg versus 45 g/kg, respectively) was found for DDGS.

The compositions of the mixed diets were theoretically calculated based on the proportion of each feed ingredient given (see Table 0-1, Table 0-2, Table 0-3). The protein content (on DM basis) in the diets ranged between 14 % and 29 % (see Figure 2-1) and the lipid content (on DM basis) between 2 % and 19 % (see Figure 2-2). Considering the presence of EAA (on DM basis) for insects, diet 5 (100 % rapeseed), 6 and 7 (100 % DDGS) provided the most nutritional diet (> 170 g/kg EAA) whereas diet 1,2,9-11 and 18 were the poorest (< 100 g/kg EAA). In terms of EFA, C18:2 and C18:3 were present in high amounts in all diets except for a low amount of C18:2 for diet 5 (100 % rapeseed meal). The sum of all EFA (on DM basis) was between 2.3 g/kg and 61.0 g/kg where diets 6, 11 and 15 were rich in EFA and diets 1, 5, 8 were poor. Brewery grains, providing moisture but also nutrients, had the highest C18:3 concentration and was presumed to be the main provider of this fatty acid in all diets. In conclusion, the 18 diets that were tested differed in origin (different side-streams) and also in nutritional composition. Diet 2, containing wheat middlings mixed with brewery grains, was pointed out by Gianotten et al. (submitted) as a reference diet that provided a good yield and healthy larvae. This diet will also be addressed as a reference diet in the current study.

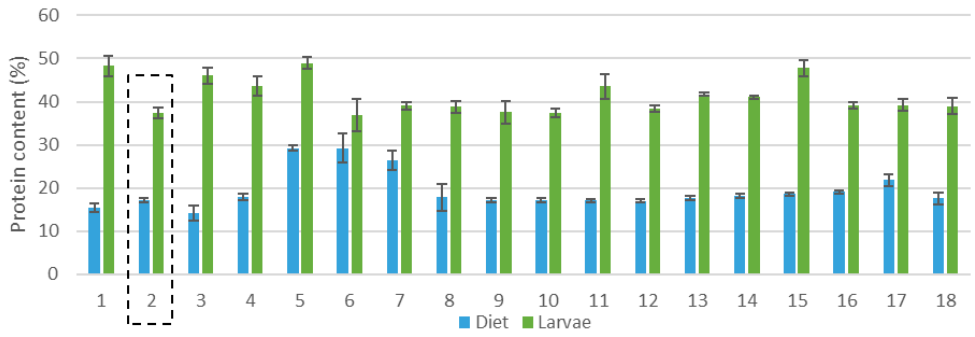


Figure 2-1 Protein content (% on dry matter basis) of the larvae and their diet. Diet 2 (reference diet) is framed.

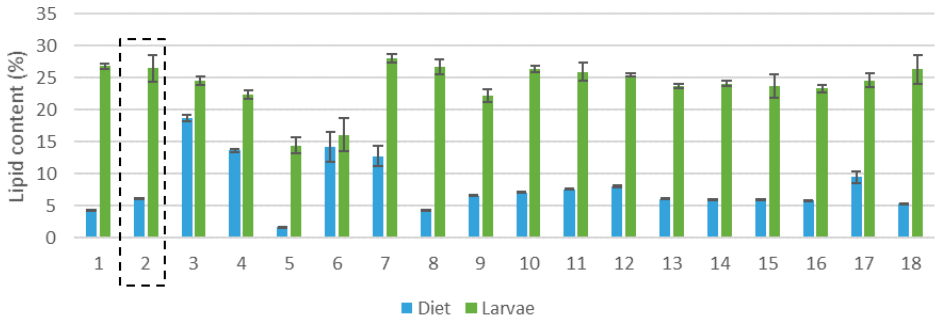


Figure 2-2 Lipid content (% on dry matter basis) of the larvae and their diet. Diet 2 (reference diet) is framed.

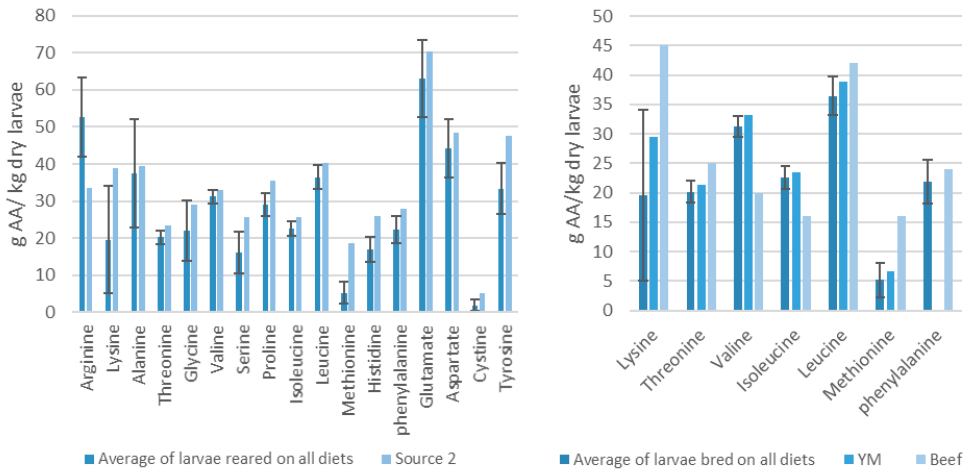
2.3.2 Composition of the larvae

The LM reared on 18 different diets, had a dry matter content between 23 % and 33 %. The protein content (on DM basis) of the larvae ranged between 35 % and 49 % (see Figure 2-1 and Table 0-4), the lipid content (on DM basis) between 14 % and 28 % (see Figure 2-2 and Table 0-5) and the ash and chitin (both on DM basis) content between 4 % and 7 % and between 4.2 % and 6.2 %, respectively. This composition is comparable to data found in literature. Yi et al. (2013) determined a dry matter of 35.2 % while Van Broekhoven et al. (2015) reported a value between 30 % and 33 % for different breeding conditions [38], [40]. The protein content (on DM basis) in literature for the LM ranges from 48 % [41] to 65 % [38], [42], [76], [169]. This broad range can be explained by a different larval age [170] or a different method of data processing or analytical method. The protein content is often determined based on nitrogen measurements where nitrogen content is converted to protein content by multiplying with 6.25. Due to the presence of chitin, the 6.25 factor results in an overestimation for insect biomass. Janssen et al. (2017) calculated a more accurate conversion factor of 4.86 and when employing this conversion factor, the authors found a protein content (on DW basis) of 48.8 % in comparison to a protein content of 63.8 % when the conversion factor of 6.25 was used [41]. The results of Janssen et al. (2017) were more in line with the protein content measured in the current study (where the protein content was calculated based on the total amino acid

analysis). However, the amino acid profile does not contain tryptophan and thus may lead to a slight underestimation. In view of the lipid content, published data indicate a lipid content (on DM basis) between 13 % and 25 % [37], [38], [40], [42] which is matching well with the findings of the current study. All rearing trials were ended after 28 days and for some conditions, a low larval yield was observed which means that the larval weight (mg per larvae) was low. This could imply that the larvae of these trials were not yet mature and thus in another larval stage. As the composition, and especially lipid content, of the larvae, can differ between development stages [22,23], a lower larval yield corresponded in some cases (diets 5 and 6) with a lower lipid content, explaining the large variance in lipid content. Yet, for diets 3 and 4 also a low larval yield was measured while an average lipid content was measured. Janssen et al. (2017) found a similar chitin content between 4.4 % and 9.1 % for the LM [41],[41] and an ash content of 4.1 % for the LM was reported by Bosh et al. (2014) [42]. Carbohydrates, besides chitin, are present in insects, for instance about 10 % was reported by Janssen et al. (2017) for the LM [41]. Non-chitin carbohydrates as well as tryptophan were not quantified in the current study and may explain the incomplete mass balance.

The most dominant amino acids in the larvae (higher than 32 g/kg on DM basis) were glutamate, arginine, aspartate, alanine, leucine, and tyrosine (Figure 2-3a). The diets also contained a high concentration of arginine, leucine, glutamate, and aspartate but not for tyrosine and alanine This observation suggests that the latter compounds are of importance for the larvae and were concentrated or synthesized by the larvae. In fact, tyrosine is known to be involved in the production of melanin that is employed for cuticular hardening, wound healing and innate immune responses with insects [171],[172]. The average amino acid profile of the larvae reared on the different diets was similar to the data reported by Despins and Axtell (1995) [169] except for arginine and methionine. These differences could originate from the analytical method since analytical limitation occurs for arginine, methionine and

cysteine. Even though the hydrolyzes were performed under nitrogen, minimizing degradation, it could be that degradation did occur and thus an underestimation of the methionine in the current study was measured. Janssen et al. (2017) also found the same amino acids (except for arginine) to be dominant in the LM just as Bosh et al. (2016) for the YM [41],[173]. The sum of the human EAA indicates that diet 1 (100 % wheat middlings), 5 (100 % DDGS), and 15 (15 % rapeseed meal inclusion) resulted in larvae with a high nutritional value (>200 g EAA/kg DM) whereas diets 9 and 10 resulted in larvae with the lowest nutritional value (<150 g EAA/kg DM). A small underestimation of the total sum of the EAA is expected since tryptophan was not measured. Figure 2-3b shows the EAA profile of the YM and beef for human consumption. A similar profile between the LM (data of the current study) and the YM was found. Van huis et al. (2013) did report a much higher leucine content for the YM but Bosch et al. (2016) and Heidari-parsa et al. (2018) reported similar leucine contents as the LM in the current study [6], [173], [174]. When comparing the profile of the LM with beef, lower values of lysine and methionine were observed for the LM, but higher for valine and isoleucine.



(a) (b)

Figure 2-3 (a) Average amino acid profile of LM larvae reared on the diets in the current study compared to data found in literature of the LM (Source 2: [169]*). (b)*

Comparison of the essential amino acids in LM (measured in the current study) with YM and beef [6].

In respect to fatty acid profiles, the most dominant fatty acids of the larvae were C18:2, C18:1, and C16:0 (> 10000 mg/kg on DM basis) and to a lesser extent C18:0, C18:3 and C16:1 (> 1000 mg/kg on DM basis) (see Table A5). Van Broekhoven et al. (2015) and Tzompa-Sosa et al. (2014) found the same dominant fatty acids being, palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) [38], [76]. When comparing to the YM, similar results were found [174],[175],[7]. The total saturated fatty acids (SFA) ranged between 7 g/kg and 74 g/kg, the total monounsaturated fatty acids (MUFA) between 41g/kg and 109 g/kg and the total polyunsaturated fatty acids (PUFA) between 23 g/kg and 91 g/kg. Essential fatty acids, C18:2 and C18:3 were present in high amounts for all diets, except for diets 5, 6, 9, 12 and 16 that reared larvae with a small decrease in C18:2. Diets 6 and 7 (both containing 100 % DDGS) resulted in larvae with an elevated amount of C18:3.

By comparing the diets nutritional profile (with respect to the protein & lipid content and the EAA & EFA profile) to the reference diet (diet 2), it seems that some diets were to increase the nutritional value of the larvae. The larvae reared on the reference diet had a protein content (on DM basis) of 37 % and a lipid content (on DM basis) of 27 %. Diets 1, 3 to 5, 11 and 13 to 15 resulted in larvae with a significantly increased protein content up to 50 %. The nutritional value in terms of the presence of human EAA was increased for larvae reared on diets 1, 3 to 5, 7, 8, 11 and 15. No diet was able to increase the larval lipid content. The sum of the EFA for the larvae reared on the reference diets was 60 g/kg and diets 7 (100 % DDGS) and 18 (50 % corn gluten feed incorporation) were able to grow larvae with a significantly higher amount.

2.3.3 Correlations between the diet composition and the larvae composition

When comparing the protein content of the larvae with the protein content of the diets (Figure 2-1), it is clear that the larvae have a higher protein concentration than the feed on which they were grown. This is reflected in the high larval protein content in comparison to the content of the diets. This was also reported by Stull et al. (2019) who measured a high protein content in YM larvae bred on the low-nutrient and low-protein feed of stover [176]. In addition, it seems that variations in dietary protein amount were not translated into the larval biomass. For instance, diets 6, 7 and 8 had a decreasing protein content while the larvae exhibited a similar protein content. The Pearson correlation coefficient (R) indicated that no linear correlation was found between the protein content of the diet and that of the larvae (R = -0.0004). Van Broekhoven et al. (2015) also found no clear impact of the dietary protein content that ranged from 12 % to 39 % and the larval content that remained rather constant (32.2 ± 1.6 %). Behmer (2009) stated that insects regulate their nutrient intake when the opportunity is given [177]. To the author's vision, this statement is applicable in optimal conditions, were all required nutrients are abundantly available. However, some restrictions would impact nutrient intake. For example, when an insufficient concentration of an important dietary compound is available, the insect switches to a 'survival mode' where the intake of other nutrients will be increased [38],[178],[179]. For example, during a lipid shortage, carbohydrates are converted to lipids by insects [180],[181]. In addition, the nutritional value of the diets is of importance. Insects, like humans, require EAA that are needed for protein build up [167],[168].

In the current study, no clear impact of the EAA concentration in the insect diets was detected on the corresponding larval protein content. For example, diets 6 to 8 had a decreased EAA concentration but no effect of this decrease was translated in

the larval protein content which stayed constant. This can indicate that the necessary amounts of the EAA were already present in diet 8 (lowest EAA containing diet). On the other hand, diet 15 had a lower total EAA concentration compared to diet 17 but the larvae generated by diet 15 also had a significantly higher protein content. In this case, it could be that the digestibility of the dietary EAA was different since the diet ingredients were different and the digestibility was better for proteins in diet 15. Tryptophan, an EAA for insects was not measured and could also explain some differences.

Amino acids from the diet are digested, absorbed and metabolized to rebuild insect proteins. This may explain the varying concentrations of individual amino acids in larvae grown on different diets. For example, the larval concentration (on DM basis) of non-essential amino acids (for insects) alanine varied between 32 g/kg and 61 g/kg, the larval concentration of glutamate between 51 g/kg and 89 g/kg and the tyrosine concentration between 20 g/kg and 43 g/kg. Ramos-Elorduy et al. (2002) also found variating amino acid concentrations when YM were reared on different diets [56]. However, no clear correlation was observed with the dietary concentrations ($R<0.4$). Variations in the insect EAA concentrations were also observed and slight correlations were found with the dietary concentrations for EAA threonine ($R= 0.58$) and isoleucine ($R= 0.49$) (Figure A1). No correlation was observed for arginine ($R = -0.03$) even though the larval concentration varied significantly (36- 74 g/kg on DM for arginine). EAA valine, histidine, phenylalanine, lysine, and leucine also did not show a correlation between the dietary and larval content ($R= 0.13$, 0.11, 0.10, 0.28 and -0.36, respectively). Nevertheless, the digestibility of the dietary amino acids was not examined which could impact possible correlation and non-correlations. For methionine, on the other hand, the concentration in the larvae varied significantly (0-10 g/kg) even though the dietary concentration was constant.

In terms of lipid content, the data in Figure 2-2 indicate that the larvae also concentrated or metabolized lipids. This conclusion is based on a significantly increased lipid content of the larval biomass compared to the diets they were reared on. Fluctuations in the dietary lipid content were not translated into fluctuations of lipids in the larval biomass, which was confirmed by the very low Pearson correlation coefficient (R -= 0.01). However, an excess of carbohydrates in the diets can be converted to lipids. Carbohydrates were not measured in this study, but wheat middlings, the basal ingredient in the mixed diets, is known to be rich in carbohydrates (sum of starch and sugar 28.1 %, table A1). When presented diets were short in lipids, carbohydrates made it possible for the insect to metabolize their necessary lipid concentration. For instance, diets 5 (100 % rapeseed meal) and diet 6 (100 % DDGS) generated larvae with a significantly lower lipid content, which corresponded with a low carbohydrate and lipid content in both substrates. Diet 7, however, consisted also of 100% DDGS but the moisture source BG provided extra carbohydrates (6 %) and lipids (10 %), resulting in larvae with an average lipid content. Corn gluten feed also contained a low amount of lipids, yet, larvae reared on diet 8 (corn gluten + BG) had an average lipid content. The carbohydrates in corn gluten (17.7 %) and BG (6.1 %) probably compensated for the low dietary lipid content. On the other hand, rice bran is rich in lipids as well as in carbohydrates, yet, the surplus of the two nutrients did not result in higher lipid-containing larvae. In addition, diets containing 5 % to 20 % rice bran, had a slightly increased dietary lipid content but the larvae, however, only increased in lipid content up to a 15 % inclusion. This suggests a self-regulating system of the larvae and that the lipid/ carbohydrate concentration of diet 11 is sufficient. In conclusion, the larval lipid content was found to be influenced by the lipid as well as the carbohydrate content of the diet, as well as an upper limit. Van Broekhoven et al. (2015) reported that the lowest lipid-containing diet resulted in a significant decrease in larval lipid content (single analysis only). The other diets had a slightly different lipid content, but an increase led even to a small decrease in larval lipid content (5.0 – 5.8 % dietary lipid

with resulting 24.3 – 18.1 % larval lipid). The authors did state the possibility that the low nutritional quality of the feed could be responsible for the low larval lipid content. The YM diets containing different lipid content (0.5 -9.3 %) were reported not to cause significant changes in larval lipid content [166]. Our study suggests that carbohydrates in the diets may have played a role in stabilizing the larval lipid content.

The dominant fatty acids measured in the diets (C16:0; C18:1 and C18:2) were also present in the larvae at high amounts. However, for most fatty acids, no significant correlations ($R < 0.5$) were observed. This can be explained by the fact that most fatty acids are metabolized by the organism. The fatty acid C20:4, for instance, was not detected in the diet but was found in the larvae. For insects, and the LM, in particular, no literature was found on the designation of insect EFA. According to the current study, three fatty acids were found that showed a slight linear correlation. Fatty acids C14:0, C18:3 and C20:2 all had a Pearson correlation factor of 0.5 indicating a positive correlation of the fatty acid concentration in the diet to the concentration in the larvae. However, as previously described, the carbohydrates in the diet were probably also used to generate fatty acids. An influence of these carbohydrates could not be excluded, and is probably also responsible for a large range in other larval fatty acid concentration such as C14:1 (ranging from 0-52.6 g/ kg on DM), C16:0 (0.6-60.9 g/kg on DM), C16:1 (0.4-3.7 g/kg on DM), C18:0 (3.5-18.6 g/kg on DM), C18:1 (32.3-89.7 g/kg on DM) and C18:2 (1.1 -88.5 g/kg on DM). Van Broekhoven et al. (2015) found similar results and concluded that the fatty acid composition in the diet influenced the larval fatty acid composition but not in the same trend and that physiological regulation of the larval fatty acid composition takes place [38]. The same statement was reported by Dreassi et al. (2017) [166]. In conclusion, no or only slight linear correlations were found between the fatty acid composition of the diet and the larvae.

2.4 Discussion

One of the goals of the current study was to evaluate the impact of changes in the feed composition on the composition of the larval biomass. The protein and lipid content, as well as the amino acid and fatty acid concentrations in different diets and corresponding insect biomass, were measured and compared. Generally, the same amino acid and fatty acid profile were detected in all insect samples, indicating that the general composition was rather stable. However, based on concentrations of the different compounds, some variations were detected that could mostly not be correlated with the dietary concentrations. These observations indicate that insects, like all living organisms, possess a self-regulating system for controlling their biomass composition to a certain extent. Nevertheless, for some specific compounds (like threonine, isoleucine, and C18:3), slight trends were observed.

However, even though the larval composition (in terms of components types) was found stable, nutrients are also required for the growth of insects. Gianotten et al. (submitted) described that higher larval yields were obtained for certain diets. Figure 2-4 summarizes the relationship between the % larval yield versus proteins, lipids, the sum of insect EAA, the sum of human EFA, the theoretically calculated carbohydrates and the sum of the lipid and carbohydrates (energy sources) in the diet. The SFA, MUFA and PUFA profile was very similar to that of the lipid graphic (data not shown). The curves for the protein content, lipid content and the sum of EAA suggest that, for the diets evaluated in the current study, optimum larval yield was achieved with 17 % to 22 % dietary protein, 6 % to 8 % dietary lipid, 18 and 20 % dietary carbohydrates and 55 to 60 g/kg EAA. It must be realized that the diets were not optimized for maximal growth, but the diets were designed to study the use of specific side-streams, hence other nutrients (other than protein, lipid and carbohydrates) that were not measured in the current study can also influence larval growth. Even though the larval composition remained similar, higher doses of these nutrients in the diet will negatively impact the larval yield. A possible explanation for this observation could be the wellbeing of the larvae in the growth medium. For

example, a more lipid-rich environment may change the texture of the substrate. Alternatively, the larval yield may also have been negatively influenced by inhibiting components that were dosed along. Figure 6c & f suggest that diets 1, 4 and 3 contained sufficient calories (carbohydrates & lipids) to support good larval growth, implying that associated the larval yield remain was due to a non-energy related factor. Based on the results illustrated in Figure 2-4, diet 15 had the optimal composition.

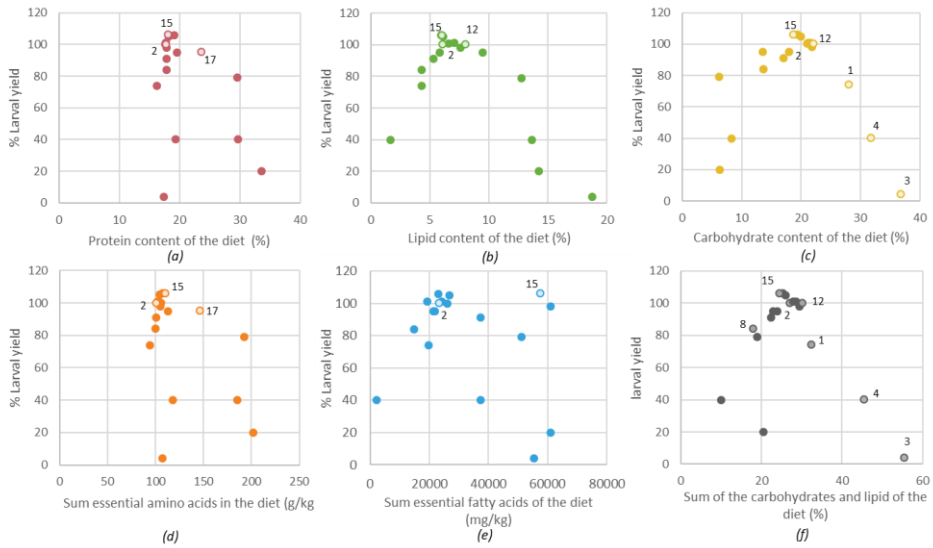


Figure 2-4 The relation between the dietary nutrient component and the larval yield Hollow bullets were indicated by their diet number. Diet 2 = reference diet.

When the larvae are to be considered as a protein source for food or feed purposes, the optimal nutritional value of the larva biomass (protein, lipid, EAA, and EFA for human consumption) is important, but also the larval yield. Figure 2-5 illustrates the relationship between the nutritional value and % larval yield. Based on larval protein content, diet 15 (15 % rapeseed inclusion) resulted in the most promising diet, referring to the best ratio between larval yield (106 %) and protein content (45.1 %). For diets 1, 3, 4 and 5 a poor yield (< 80% of reference diet) was observed but still a high protein content, potentially referring to enhanced uptake of proteins as a

surviving mechanism to compensate for other lacking nutrients. Figure 2-5 also depictures the relation between the larval lipid content and the % larval yield. The larvae exhibited a lipid content between 14 % and 28 %. By excluding the diets that resulted in poor yield, the larval lipid content ranged between 22 % and 28 %. Diet 14 and 15 (10 % and 15 % rapeseed inclusion) seem to be the most promising based on lipid content. Diet 15 also had an increased amount of EAA and a similar amount of EFA compared to the reference diet. Considering the relatively larval yield, protein content and EAA content for humans, diet 15 is presumed a good possibility for rearing.

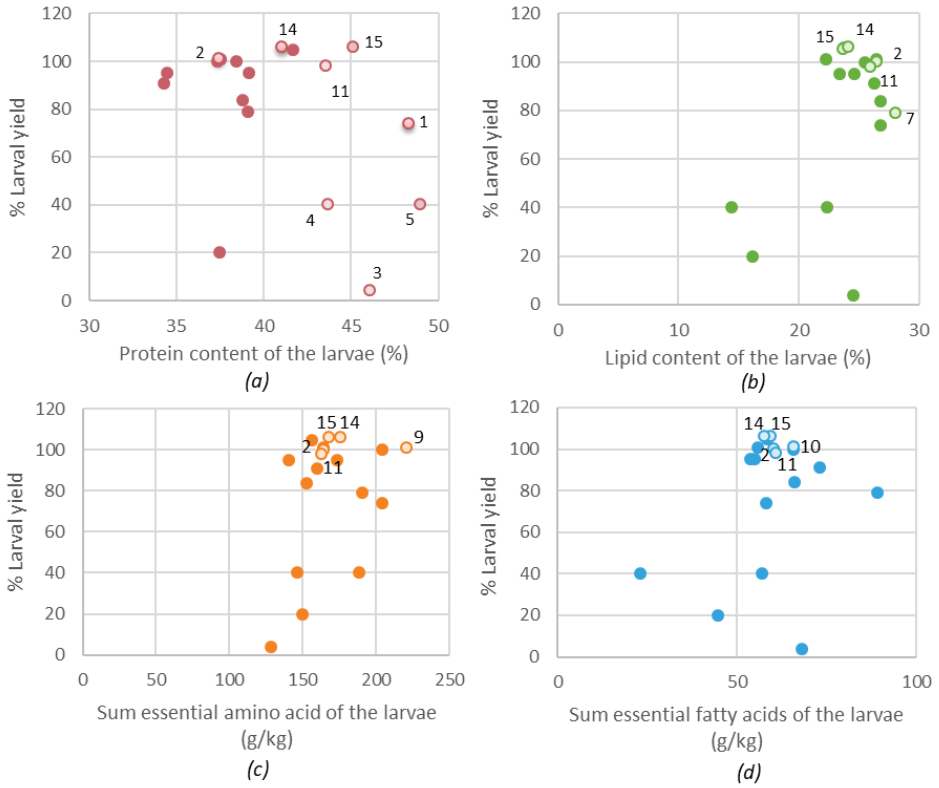


Figure 2-5 The relation between the larval nutrient component and the larval yield. Hollow bullets were indicated by their diet number. Diet 2 = reference diet.

Gianotten et al (submitted) revealed that the cost of producing LM biomass could be beneficially influenced by including rice bran (up to 20 %) or rapeseed meal (up

to 10%). In respect to rice bran, inclusions (up to 20 %) did not significantly change larval yield but the cost price of rice bran is lower compared to the reference diet. For this reason, high inclusions of the side-stream lowers production costs. Rapeseed meal, on the other hand, is much more expensive than the reference diet but inclusions up to 15 % increased the larval yield and an economical benefit was calculated for inclusion up to 10 % whereas inclusion of 15 % resulted in a similar cost price as the reference diet. Figure 2-6 illustrates the results when these calculations were applied to protein level. All inclusions were economically beneficial, except for a 20 % replacement of the reference diet by rapeseed meal. Considering rice bran, only diet 11 (15 % inclusion of rice bran) resulted in larvae with an elevated protein content and were economically most beneficial. The elevated protein content of larvae reared on diet 11 could not be explained by increased dietary compounds concentration (protein, lipid or carbohydrates) and thus explanations should be found elsewhere. Since the yield was the same for all diets, it could be assumed that 80 % of the reference diet was sufficient in delivering all necessary nutrients and that 20 % of the reference diet was inessential. However, this 20 % may have provided another function (non-nutritional) for the larvae, for example, it may influence the density of the growth medium, the light permeability, … and that 15 % of rice bran inclusion had optimal settings.

Diet 15 (15 % inclusion of rapeseed) was economically even more beneficial than the reference diet (79 % of the cost price of diet 2). An elevated larval yield compared to the reference diet was observed (except for 20 % inclusion, which explains the increased cost price) and elevated larval protein content in diet 15 (15 % inclusion of rapeseed) was measured. In this case, the presence of anti-nutritional factor glucosinolates may explain the observation. Pracros et al. (1992) reported negative effects of glucosinolates, on the growth performance of the yellow mealworm. Different rapeseed meals with variating concentrations of the compound were tested and only for one diet (concentration of glucosinolate of 26

µmol/ 100g dry diet) a decrease in larval performance was noticed due to metabolic problems. The larvae were able to survive but had a delay in weight gain. As the concentration of glucosinolates is different depending on the rapeseed species, no direct comparison to the current study is possible [22]. However, the data acquired in the current study suggest that this threshold is reached with a 20% inclusion of the rapeseed meal used in the current study.

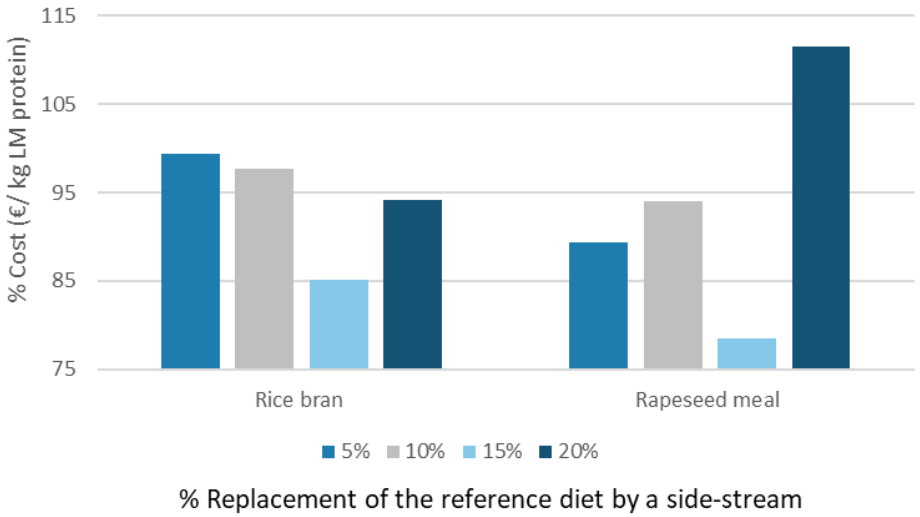


Figure 2-6 Changes in cost price producing LM proteins with diets 9 to 16 compared to the reference diet.

2.5 Conclusions

The study confirmed and complemented published data on the composition of the LM. Depending on the diet, the LM had a protein content between 35 % and 49 % (between 34 and 45 for well-performing diets), a lipid content between 14 % and 28 % (between 22 % and 26 % for well-performing diets) and a chitin content between 4 % and 6 %. The most dominant amino acids in the larvae were arginine, alanine, leucine, glutamate, aspartate, and tyrosine. The most dominant fatty acids were C16:0, C18:1, and C18:2. The rearing of larvae on underspent side-streams proved to be a good approach for introducing recycled nutrients to the market. The larvae have a higher density in proteins and lipids and as a result, also in EAA and EFA. In

this way, the nutritional value of the side-streams was indirectly raised. With respect to the impact of varying feed ingredients (side-streams) and associated varying concentrations of nutrients, a slight effect on the larval nutrient concentrations was observed but no direct link to the dietary concentrations could be made. However, the different feed ingredients did influence the larval yield. Within the limitations of the study (the type of side-stream, inclusion rates…) a maximum larval yield was achieved with a diet containing 17 % to 22 % of protein and 6 % to 8 % of lipid. This finding gives rise to the opportunity to change the diet ingredients or inclusion rates, for example, according to the cost price of the side-streams or the availability, without influencing the larval yield and composition and thus guarantee constant larval biomass in composition. Within the study, diet 15 (wheat middling with 15% inclusion of rapeseed meal + BG) was considered the most interesting diet in terms of larval yield, larval protein content and cost price and rapeseed meal and rice bran were selected as interesting side-streams for future incorporation in insect feed formulations and further investigation on inclusion rates between 15 and 20 % of rapeseed meal or rice bran could be interesting from an economic perspective. As mentioned before, this study was designed for the purpose of investigating side-streams, not for standard, optimal insect rearing diets. For this, further investigation is needed on the effect of other compounds such as carbohydrates, vitamins and minerals, the energy balance and optimal C:N ratio to decrease possible (nitrous) emissions. With this information, a balanced diet can be composed and other side-streams can be incorporated.

2.6 Acknowledgments

The authors would like to acknowledge VITO colleagues Sam Vloemans, Miranda Maesen for their contributions related to the analytical work and this research was funded by BBI Joint Undertaking under the European Union's Horizon 2020, grant number No 720715 (InDirect project).

Chapter 3.

A NEW FRACTIONATION APPROACH

BASED ON AN ORGANIC ACID

TREATMENT

Part of the chapter has been accepted for publication (L. Soetemans, M. Uyttebroek, E. D'Hondt, L. Bastiaens. Use of organic acids to improve fractionation of the black soldier fly larvae juice into lipid and protein-enriched fractions accepted Eur. Food Res. Technol., 2019). The authors would like to acknowledge the journal for giving copyright permission and the final publication is available at https://link.springer.com/article/10.1007/s00217-019-03328-7.

In addition, parts of this chapter were used for a patent applications (E. D'Hondt, M. Uyttebroek, L. Soetemans, L. Bastiaens, A methode for the fractionation of a lipid fraction and a protein fraction from a lipid and protein containing biomass, PCT/EP2017/084694,2018.https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2018122294.)

3.1 Introduction

Chapter '1.3.1 stage of the art on insect biorefinery' gives an overview of the different processes that have been employed on insects to separate lipids from proteins. However, these extractions are generally performed on a lab-scale level and upscaling will cause some limitations. For example, drying (during step 1, Figure 1-3) is often an energy-consuming step and difficult to scale up when no high temperatures are allowed [182]. In addition, solvent fat extraction (step 3, Figure 1-3) requires pre-dried insect biomass and are often not ecofriendly or food-friendly [67],[183]. Regarding step 4, it was already mentioned that alkaline conditions can impact protein quality. Despite the high yield of soluble proteins, anti-nutritional compounds can be formed such as lysinoalanine that negatively impacts protein digestibility and amino acid availability in food [131].

In order to make the fractionation procedure more applicable for feed/food industries on a large scale, this study focusses on avoiding a drying step and possible losses in nutritional value. Since organic acids are allowed in the feed and food industry, this chapter investigates the performance of fractionation at low pH with the use of organic acids where lipids are removed without the use of solvents. Earlier, Liang and Hultin (2005) studied the impact of citric, lactic and malic acid on the separation of phospholipids lipids from solubilized proteins in fish muscle [184]. A positive impact of the organic acids was reported. Malic acid gave the best results for lipid removal, followed by citric acid and lactic acid. For protein extraction, citric and malic acid resulted in the same yield, but a lower yield was noted with lactic acid [184]. The focus of that paper was on separating phospholipids from membrane proteins while in the current study, all lipid classes are present and expected to separate. In addition, HCl is traditionally used for solubilization of proteins in the acid region while this paper investigates the potential of organic acids.

The current study aimed to develop a method to separate proteins from lipids in an aqueous environment while preserving the techno-functionalities of the proteins. For all obtained fractions the distribution of lipids and proteins was evaluated not only to investigate the purity but also the maximum yield. The preservation of the techno-functionalities was evaluated based on the solubility of the proteins.

3.2 Materials and method

3.2.1 Materials

Living larvae of the BSF, bred on broiler feed, were kindly provided by Millibeter (Belgium). BSF juice was obtained after a mechanical separation (based on WO 2019081067 [185]), where the exoskeletons were removed. The insect juice was frozen immediately and stored at -20 °C in vacuum bags till use. The bags were defrosted in room temperature water for 10 min before use. Lactic acid (Alfa Aesar 85.0-90.0 %), acetic acid (Merck Millipore, 30 %), citric acid (Merck, citric acid monohydrate), succinic acid (Amresco), valeric acid (Aldrich,>99 %), oxalic acid (JT Baker, oxalic acid dihydrate) and HCl (Merck, 37 %) were tested. During Soxhlet, diethyl ether (Merck) was used as extraction media.

3.2.2 Fractionation procedure

The extraction media that were evaluated as triplicates are summarized in Figure 3-1. Defrosted insect juice was mixed with the solvent at a liquid(w): solid(w) ratio of 17.6 ± 0.5 (200 ml assay). A concentration of 0.5 M organic acid was used, except for valeric acid which was used at 0.33 M (because of the saturation point). For some test conditions, the pH was kept at pH 2 by measuring the pH every 10 minutes and adjusting it with 2.5 M HCl. These conditions are indicated by '+HCl' in Table 3-1. The mixture was stirred for 30 min in total at 100 rpm on a shaking plate (Gerhardt laboshake) at room temperature. After centrifugation (Eppendorf 5810- swing-out A-4-81 rotor) at room temperature (3220 g, 30 min) 3 layers were obtained (Figure 3-1). The upper layer (UP) was scooped off with a spatula. The SN1 was decanted

and the pellet was collected and resuspended at pH 7. The SN1 fraction was adjusted to pH 5 with NaOH (40 v% or 50 w%) and was subsequently centrifuged at room temperature (3220 g, 15 min) to obtain two fractions, being a supernatant (SN2) and a pellet (P2). Both fractions were resuspended at pH 7. Selected tests were repeated at a fixed temperature of 25 °C using angled head centrifugation (Thermo Scientific, Sorvall lynx 6000 – F14-14x50 cy rotor).

Table 3-1 Extraction media and characteristics used in this study.

Condition	Solvent	pH of acids	pKa of acid	pH of acid-juice mixture before HCl correction	± Amount of HCl (2.5 M) needed
1*	Water			6.9	
2	Water + HCl			2.1	16 g
3	Lactic acid	1.9	3.8	3.3	
4	Lactic acid + HCl	1.9	3.8	3.3	10 g
5	Acetic acid + HCl	2.5	4.8	4.0	15 g
6	Citric acid + HCl	1.9	3.13	2.4	9.5 g
7	Succinic acid + HCl	2.3	4.21	3.6	16 g
8*	Valeric acid + HCl	2.6	4.82	4.6	17 g
9*	Oxalic acid + HCl	1.3	1.25	1.6	

** conditions not studied in detail*

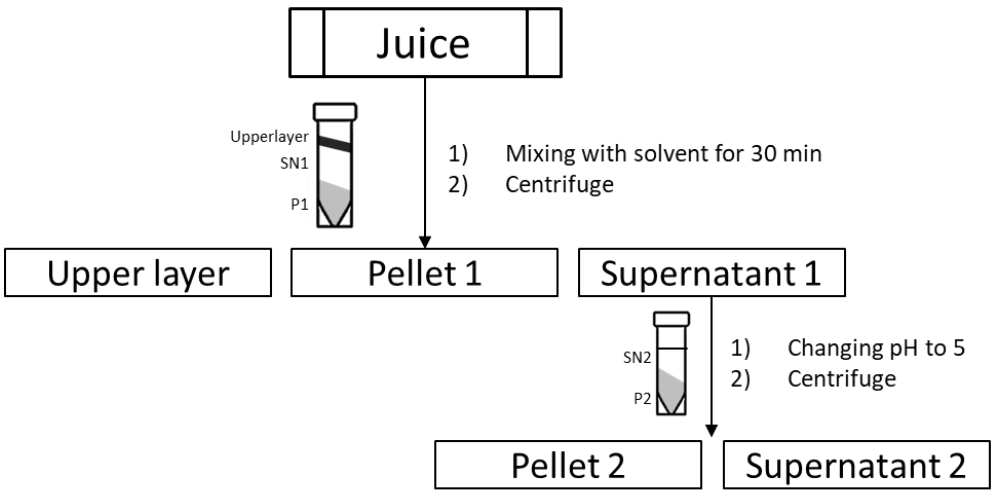


Figure 3-1 Scheme of the extraction procedure.

The distribution of the obtained fractions (on DM basis) was evaluated by calculating non-dialyzed yield (NDY) for the different fraction using the following formula (1):

$$NDY\ \% = \frac{fraction\ (g\ DM)}{juice\ (g\ DM)} * 100 \quad (1)$$

During the screening test, the UP (rich in oil) and P2 (rich in proteins) received the most attention. For a selection of well-performing conditions, a more detailed mass balance was established after a desalting step by dialyzing all 4 fractions (Spectrum™ Labs Spectra/Por™ 6 1000 D MWCO Standard RC Pre-wetted Dialysis Kits). Dialyzed yield, recovery and lipid/protein distribution were calculated using the following formulas (2), (3) and (4):

$$Dialyzed\ Yield\ \% = \frac{fraction\ (>1kDa)(g\ DM)}{juice\ (>1kDa)(g\ DM)} * 100 \quad (2)$$

$$Recovery\ (\%) = Yield\ upper\ layer\ (\%) + Yield\ pellet\ 1\ (\%) + Yield\ pellet\ 2\ (\%) + Yield\ supernatant\ 2\ (\%) \quad (3)$$

$$Lipid\ or\ protein\ distribution\ \% = \frac{amount\ of\ lipid\ or\ protein\ in\ fraction\ (g)}{amount\ of\ lipid\ or\ protein\ in\ juice\ (g)} * 100 \quad (4)$$

3.2.3 Sample characterization

Dry matter was measured either after oven drying at 105 °C (juice and UP) for 48 h or after freeze-drying (P1, P2, and SN2)(Christ, gamma 1-16 LSC) for 36 hours. Ash content was determined gravimetrically after incineration (550 °C) in a muffle furnace. The crude lipid content was determined by Soxhlet extraction with diethyl ether for 6 hours and expressed on dry matter. Quantification of the chitin content was performed as described by D'Hondt et al. (D'Hondt et al., submitted) and was further elaborated in Chapter 2. Crude protein content of the fractions was calculated based on nitrogen measurements (Dumas method, Elementar, vario EL cube). The correction factor of 6.25 was applied to convert the nitrogen content to protein content, yet, it is known that this is not the most correct factor. However, since the thesis will calculate the protein content of different, fractionated proteins and not all were analyzed from amino acid composition, the same conversion factor of 6.25, which is also still applied in recent publications for insect proteins, is applied throughout the thesis. pH solubility curves were measured at pH-values ranging

between pH 2 and pH 10. Dispersions in demineralized water were stirred at different pH for 30 min, then the pH was checked and adjusted if needed with 0.1 M NaOH or 0.1 M HCl, followed by another 30 min of stirring. Afterwards, samples were centrifuged at 4 °C for 20 min at 5910 g. The total nitrogen was determined by a chemiluminescence detector (Multi N/C 3100 Analytik Jena). The sample was oxidized by catalytic combustion in an oxygen atmosphere at 800°C, to produce nitrogen oxides as is described by EN 12260:2003 (Water Quality-Determination of nitrogen- determination of bound nitrogen (TNb), following oxidation to nitrogen oxides). The formed measuring gas was dried and entered the reaction chamber of the chemiluminescence detector. There the nitrogen monoxide present in the measuring gas was oxidized with ozone into activated nitrogen dioxide. By emitting light photons (luminescence) the molecules of the nitrogen dioxide returned to their original state. The luminescence was detected using a photomultiplier and is proportional to the nitrogen monoxide concentration. Sample vials were filled with about 25 ml sample and placed into the sample carrousel. About 1000 µl of sample was injected in the system. The catalytic combustion of the sample in an oxygen atmosphere (160 mbar) was carried out at 800°C. A linear calibration curve between 1 and 10 mg/l of nitrogen was set up using a mixture of ammonium sulfate (Merck) and potassium nitrate (Merck) at 5 concentration levels (1 - 2.5 - 5 - 7.5 and 10 mg N/l). Solubility was expressed as percent of protein in the supernatant relative to the protein content of the sample. The total protein solubility at a specific pH (of all proteins in the juice) after fractionation was determined by adding the solubilities of the different fractions according to formula (5). The fraction solubility for a certain pH was calculated based on formula (6) where 'F' stands for the fraction (P1, P2, SN2).

$$Total\ solubility\ (\%) = \frac{(fraction\ solubility\ of\ P1 + fraction\ solubility\ of\ P2 + fraction\ solubility\ of\ SN2)}{100\ g\ juice * crude\ protein\ of\ juice\ (\frac{g\ protein}{100\ g\ juice})} * 100 \qquad (5)$$

$$Fraction\ solubility\ (g\ soluble\ protein) =$$

$$100g\ juice * yield\ of\ F\left(\frac{\text{g fraction}}{\text{100 g juice}}\right) * crude\ protein\ content\ of\ F(\frac{g\ protein}{100\ g\ fraction}\%) * solubility\ (\frac{g\ protein\ soluble}{100\ g\ protein}) \quad (6)$$

3.3 Results and Discussion

3.3.1 Characterization of the BSF juice

The BSF juice used for this study had a dry matter of 26 %. The crude protein and lipid content based on dry matter was 38 % and 35 %, respectively, as indicated in Table 3-2 along with other composition data. Chitin content of the juice was 0.52 % which is much lower compared to BSF that has a chitin content between 4.4 % and 9.1 % [41], [186]. The solubility curve of the proteins in the insect juice was U-form shaped (see Figure 3-4), indicating that BSF proteins have a high solubility at low and high pH (90 % soluble at pH 2 and 80 % at pH 9) and an isoelectric point around pH 5. Earlier, Bußler et al. (2016) reported a solubility curve for BSF with a different pattern. The protein solubility of the larvae meal was only 40 % at pH 2 and pH 9 and a minimum was reached at pH 4. However, the exoskeleton (containing chitin) was included in this experiment [187]. Chitin is a non-soluble polymer containing nitrogen that may lead to underestimation of the protein solubility if not taken into account when converting nitrogen measurements into protein content. To the best of our knowledge, no solubility curves for BSF juice (without the exoskeletons) have been described before in literature. The findings of Zielińska et al. (2018) on solubility for the mealworm proteins (extracted by alkaline isoelectric point precipitation) *Tenebrio molitor*, the cricket *Gryllodes sigillatus* and grasshopper *Schistocerca gregaria* were more similar to the results obtained in the current study. The isoelectric point for these insect proteins (without exoskeleton) was situated around pH 5 and a solubility up to 90 % was reached at pH 2 and 8 [68].

Table 3-2 Proximate composition of black soldier fly larvae juice (without the exoskeletons) on dry matter basis (mean ± S.D., n =3).

BSF juice	%
Dry matter	26.45 ± 0.42
Protein content	38.16 ± 0.53
Lipid content	35.98 ± 0.77
Ash content	10.07 ± 0.52
Chitin content	0.52 ± 0.06

3.3.2 Distribution of lipids & proteins among the fractions obtained

Even though protein extractions at alkaline pH results in a high amount of soluble proteins, protein digestibility, and nutritional value can decrease [188]. Hence, protein fractionation at acid pH may be a better approach. Based on the solubility curve, it can be suggested that at pH 2 proteins can be separated from other non-soluble particles with only 10 % of protein loss. To separate the proteins from other soluble molecules in a subsequent step, the pH can be adjusted to 5 to precipitate about 60 % of the proteins. This fractionation approach was verified using different organic acids. Since the pH of the mixtures after adding the organic acids raised and was different overall treatment (see Table 3-1), HCl was used to ensure all treatments had the same pH.

Nine test conditions were set up to evaluate the performance of six different organic acids, being lactic acid, acetic acid, citric acid, succinic acid, valeric acid, and oxalic acid. The aim was to fractionate the juice into a lipid-enriched fraction and a protein-enriched fraction. The distribution of the lipids and proteins among the 4 fractions is visualized in **Figure 3-2.** The lipids were predominantly present in the upper layer except for conditions 1, 8 and 9. For all other conditions, on average 64 % of all lipids were concentrated in this fraction with 75 % for condition 6 (citric acid) and 73 % for condition 4 (lactic acid). Figure 3-2B illustrates that the proteins were distributed over all fractions but were concentrated predominantly in P2 (up to 48 %, in particular for conditions 2, 5 and 6) and SN2 (up to 48 %). Some proteins were lost

in the UP layer or in P1. It is possible that these proteins were to heavily bounded to other compounds (for example hydrophobic proteins that are trapped in lipid-micelles or structural proteins that were located in cell membranes that had undergo glycosylation) and were not set free under the extraction conditions. The impact of acid versus neutral pH during extraction could be evaluated by comparing the results obtained under conditions 1 and 2. When working in an acidic environment, an upper layer was formed separating lipids that were first located in the pellet, and more proteins could be collected in pellet 2 (13 % versus 45%). This result was expected based on the solubility curve of the juice (**Figure 3-4**). Comparison between conditions 3 and 4 also indicated that lactic acid at pH 2 was more beneficial than at pH 3.3. At pH 2 (cond. 4), more lipids were collected in the upper layer and the protein distribution altered towards proteins in P2 and less in the UP. The influence of organic acids was studied by comparing them to an inorganic acid (HCl) at the same pH (cond. 2). When only inorganic acid was used (cond. 2), the distribution of lipids was less in the upper layer compared to 4 out of 6 organic acids, indicating an added value of these specific acids in collecting lipids in the UP. The difference was less pronounced in the protein distribution. In conclusion, the results of the screening test indicate that conditions 2, 4, 5, 6 and 7 may be of interest for the fragmentation of insect juice. When pure lipid fractions are desired, condition 4 (lactic acid) would be superior over condition 6 or 7 because of a lower protein distribution in the UP. Condition 5 (acetic acid) resulted in a lower distribution of proteins in the UP, same as condition 2, but still, a high distribution of protein in P2 was noted (48%).

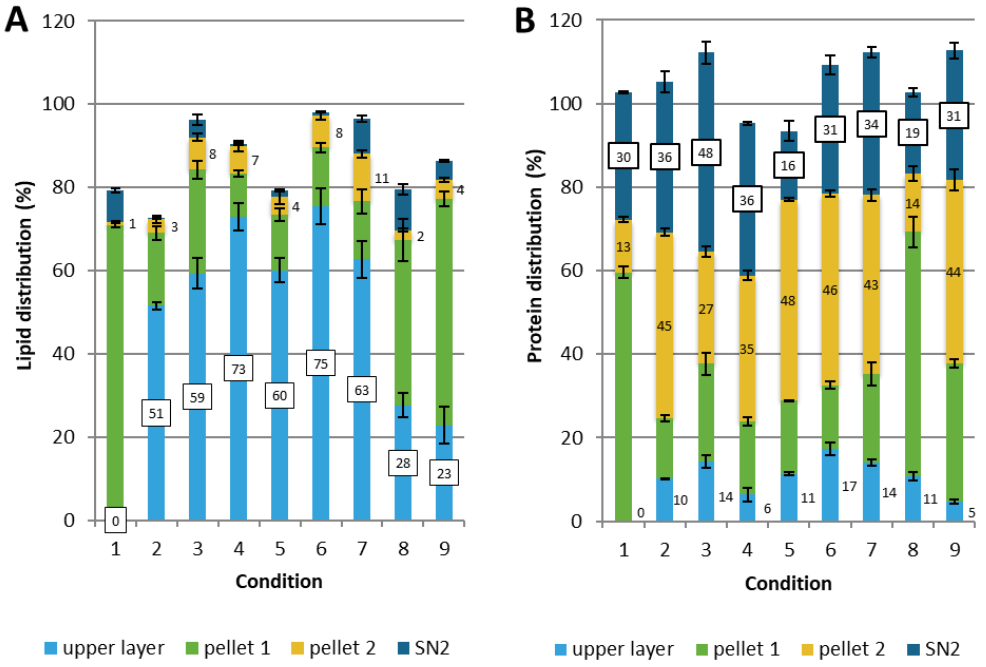


Figure 3-2 Lipid (A) and protein (B) distribution of the 4 generated fraction (mean ± S.D., n =3). cond. 1: water, cond.2: water +HCl, cond. 3: lactic acid, cond.4: lactic acid + HCl, cond. 5: acetic acid + HCl, cond. 6: citric acid +HCl, cond. 7: succinic acid +HCl, cond.8: Valeric acid +HCl, cond.9: oxalic acid +HCl

3.3.3 Yield and purity

In the previous section, the impact of organic acids on the distribution of lipids and proteins among the four generated fractions was evaluated. When envisioning the use of the fractions towards certain applications, also the size of the fraction (yield) and the concentration of lipids and/or proteins in the fraction (purity) are important criteria to evaluate the added value of organic acid assisted extractions. The compilation of accurate mass balances, however, was proven to be challenging due to the acids and alkaline that were added during the extraction. These chemicals were distributed over all fractions and were partially lost via evaporation during the drying of the fractions. Further, as proteins and lipids only represented 74% of DW juice, mass balances prepared based exclusively on these compounds are not accurate. Two types of mass balances were calculated to make the best estimates

for the yield of the different fractions. Firstly, NDYs were calculated based on the dry matter of the fractions after drying relative to the dry matter of juice used, which provides a realistic view of the obtained fractions. Next, for a selection of six most promising conditions (cond. 2,3, 4, 5, 6 and 7), yields based on the weight of dialyzed fractions were calculated relative to the weight of the dialyzed juice (named dialyzed yield) to verify the fate of the juice among the four fractions (see Table 3-3). Dialysis of the juice fraction resulted in a 79.0 ± 3.3 % recovery. This indicates the presence of small molecules (<1 kDa) such as minerals (ash content was 10 %), small peptides, free amino acids, small sugars, …Condition 1, 8 and 9 were excluded because of the poor lipid distribution. The added yields of the four fractions ranged between 107 % and 270 % and between 70 % and 98 % for non-dialyzed and dialyzes fractions, respectively. The total recovery based on dialyzed yield suggested some losses during the extraction procedure. Gas development, possibly caused by for instance enzymatic or microbial activity, was noticed during the extraction procedure. This may have contributed to the losses of mass. For the upper layer, which was observed for all test conditions except for conditions 1, the non-dialyzed yield varied between 12 % and 39 % DW of the initial juice and the dialyzed yield was slightly higher than one-third of the juice. The lipid content of the upper layer was between 66 % and 85 %. The highest percentage was obtained using lactic acid at pH 2 (cond. 4), followed by acetic acid (cond. 5), and lactic acid with no additional HCl (cond. 3). In addition, condition 4 realized an upper layer with a very small protein impurity (8%). The highest yield for the lipid-rich upper layer was obtained with succinic acid (46 %, cond. 7) followed by citric acid (41 %, cond. 6). Yet, these were not the purest fractions based on lipid content and thus not seen as an improvement of the fractionation procedure. Condition 4, on the other hand, contained a significantly higher % of lipids (73 % distribution) in the upper layer compared to condition 7 (63 % distribution) with a slightly lower dialyzed yield (37 % versus 46%).

In respect to protein purity, an enrichment of the protein content was obtained for all conditions in P2. Conditions 9, 5, 1 and 2 resulted in a protein content higher than 50 %. Despite its higher protein content in P2 for condition 1, water alone as extraction media is not advisable since the non-dialyzed yield was only 8 %. Protein fractions with low lipid content (< 5 %) were observed for cond. 2 and 5. The dialyzed yield of the protein-rich P2 was on average 25 % and was maximized with conditions 2, 6 and 7 to 27 %. Comparing non-dialyzed and dialyzed yields for conditions 6 and 7 revealed that significant amounts of compounds smaller than 1 kDa were present. This makes these fractions, despite their high non-dialyzed yield (48 % and 39 %, respectively) less interesting than condition 2 for the fractionation procedure. For both pellet 1 and 2, the sum of lipid and protein content was on average ± 55 %, indicating that other compounds, were concentrated in these fractions. Fraction P1 contained lower amounts of lipid and protein compared to the juice. These concentrations were even lower for fraction SN2, especially for lipids. The non-dialyzed yields of SN2 were significantly higher compared to the dialyzed yield. This indicates that SN2 consisted mostly of compounds smaller than 1 kDa and that only a maximum of 9% of the dialyzed juice was distributed in this fraction. In addition, this effect is also notable, but less pronounced with P1 (e.g. cond. 5, the yield decreased from 23.5 to 14.2 %) and P2 (the yield decreased from 32.7 to 23.2 % for cond. 5). These results indicate that a filtration step of these fractions will further purify the sample and thus also increase the protein/ lipid content. Filtration would result in the removal of organic and inorganic salt which can have a positive effect on feed applications. However, also other compounds such as small peptides, amino acids on other micronutrients would be lost.

Table 3-3 Extraction NDY, yield and crude protein and lipid content on dry basis of the four extracted fractions (mean ± S.D., n =3).

Fraction	Condition	acid	Average non-dialyzed yield (%)	Average dialyzed yield (%)	Average crude protein content (%)	Average crude lipid content (%)
UP	1	water	*	*	*	*
	2	HCl pH 2	18.62 ± 0.40	35.72 ± 1.19	16.02 ± 0.34	**75.05 ± 1.28**
	3	Lactic acid	28.22 ± 1.35	29.35 ± 1.40	19.31 ± 1.15	**75.57 ± 1.14**
	4	Lactic acid pH 2	30.77 ± 1.08	37.30 ± 1.26	8.03 ± 2.27	**85.27 ± 1.22**
	5	Acetic acid pH 2	27.83 ± 0.73	30.08 ± 1.96	15.54 ± 0.95	**77.64 ± 1.72**
	6	Citric acid pH 2	39.58 ± 0.57	41.05 ± 0.85	16.77 ± 1.34	**68.61 ± 4.47**
	7	Succinic acid pH 2	32.93 ± 1.99	45.89 ± 0.52	16.38 ± 0.35	**68.48 ± 0.88**
	8	Valeric acid pH 2	17.19 ± 1.58	*	24.00 ± 0.60	**66.24 ± 0.94**
	9	Oxalic acid pH 2	12.87 ± 1.90	*	13.81 ± 0.70	**72.55 ± 4.25**
P1	1	water	70.00 ± 1.35	*	32.69 ± 0.19	22.14 ± 0.52
	2	HCl pH 2	18.62 ± 0.42	10.47 ± 0.46	29.60 ± 0.83	33.82 ± 2.49
	3	Lactic acid	29.37 ± 3.32	22.44 ± 2.53	30.73 ± 0.04	30.68 ± 1.62
	4	Lactic acid pH 2	24.07 ± 4.32	14.16 ± 2.42	28.52 ± 5.13	16.01 ± 3.38
	5	Acetic acid pH 2	23.52 ± 0.84	14.19 ± 1.53	28.48 ± 0.92	20.64 ± 1.82
	6	Citric acid pH 2	24.23 ± 0.94	13.65 ± 0.83	24.17 ± 0.41	20.93 ± 0.90
	7	Succinic acid pH 2	27.77 ± 3.80	16.91 ± 3.74	29.38 ± 0.17	18.02 ± 1.67
	8	Valeric acid pH 2	69.75 ± 2.46	*	32.21 ± 0.89	23.40 ± 2.24
	9	Oxalic acid pH 2	56.60 ± 1.72	*	22.52 ± 0.31	**39.69 ± 0.21**
P2	1	water	8.20 ± 0.46		**59.26 ± 0.94**	17.69 ± 0.76
	2	HCl pH 2	30.11 ± 0.34	27.43 ± 3.01	**56.90 ± 1.53**	3.93 ± 1.21
	3	Lactic acid	22.92 ± 0.95	18.21 ± 0.75	**44.88 ± 0.65**	12.20 ± 0.94
	4	Lactic acid pH 2	30.80 ± 0.85	23.50 ± 1.22	**43.65 ± 0.83**	7.62 ± 1.31
	5	Acetic acid pH 2	32.68 ± 0.33	23.21 ± 1.25	**56.23 ± 0.46**	4.71 ± 1.87
	6	Citric acid pH 2	47.60 ± 0.48	27.40 ± 0.97	37.06 ± 0.50	5.76 ± 0.75
	7	Succinic acid pH 2	38.46 ± 1.58	27.33 ± 1.96	**42.94 ± 3.01**	10.66 ± 1.06
	8	Valeric acid pH 2	11.23 ± 0.79	*	**47.94 ± 2.56**	9.13 ± 1.93
	9	Oxalic acid pH 2	29.86 ± 1.72	*	**56.58 ± 1.45**	6.18 ± 0.40
SN 2	1	water	28.37 ± 0.47	*	**41.30 ± 0.65**	6.26 ± 0.67
	2	HCl pH 2	39.88 ± 2.52	3.05 ± 0.88	34.71 ± 0.19	0.25 ± 0.09
	3	Lactic acid	98.61 ± 5.41	6.27 ± 0.34	18.63 ± 0.23	1.55 ± 0.42
	4	Lactic acid pH 2	87.70 ± 4.67	8.54 ± 0.79	16.00 ± 0.98	0.25 ± 0.09
	5	Acetic acid pH 2	34.65 ± 6.06	2.80 ± 1.01	18.15 ± 0.57	1.48 ± 0.33
	6	Citric acid pH 2	158.91 ± 13.27	5.89 ± 1.57	7.44 ± 0.06	0.20 ± 0.04
	7	Succinic acid pH 2	122.91 ± 3.82	7.43 ± 0.72	10.67 ± 0.16	2.49 ± 0.18
	8	Valeric acid pH 2	49.99 ± 2.52	*	15.00 ± 0.77	8.03 ± 0.75
	9	Oxalic acid pH 2	76.23 ± 4.70	*	15.60 ± 0.10	2.54 ± 0.13

Comparison between condition 3 and 4 confirms the conclusions made based on the lipid and protein distribution. A more acidic pH resulted in a higher dialyzed yield of

P2 (24 % versus 18 %) with a lower lipid content (8 % versus 12 %), whereas the protein content was similar. In addition, the results for these conditions also indicate that a low pH favored lipid fractionation since the lipid content was higher in the upper layer at pH 2 (85 % versus 78 %). Literature on the BSFL reports the presence of a large amount of free fatty acids, depending on the killing method [86] [189]. Ushakova et al. (2016) reported 87 % of lipids to be free fatty acids when the larvae were killed by freezing. Caligiani et al. (2019) found a free fatty acid content of 78 % in larvae that were frozen. However, they stated that the storage length influenced the free fatty acids content (35 % immediately after freezing, 90 % after two months in frozen storage). Larvae that were first killed by blanching only showed traces of free fatty acids. The larvae in the current study where not frozen but mechanically killed. During this treatment, it cannot be excluded that lipases were activated and free fatty acids were formed. A possible explanation for a better separation in a more acidic environment is that these free fatty acids may become protonated and thus become less soluble in water. In addition, the acid environment will change the structure of proteins and perhaps decrease emulsifying properties making separation more easily. Focusing on the added value of organic acids assisted extraction of lipids, the dialyzed yields demonstrate that all organic acids (at pH 2) except for acetic acid (cond. 5) increased the upper layer yield compared to HCl (cond. 2). The inorganic acid (HCl) treatment at pH 2 resulted in a lipid content of 75 %. For most organic acids a similar or slightly lower lipid content was obtained, except for lactic acid (cond. 4). Here, an increased lipid content (85 %) and as such an increased purity (lower protein content) was observed. A similar observation was stated by Liang and Hultin (2005) where organic acid had a beneficial effect on separating phospholipids from soluble fish proteins. A better phospholipid removal was noticeable compared to a water treatment (both adjusted to the same pH with HCl). Malic acid gave the best results for phospholipid removal, followed by citric acid and lactic acid. In search of an explanation of the added value of the organic acids, Liang and Hulting hypothesized that the carboxylic groups of the acid were

involved in the separation. Phospholipids may interact under the applied conditions (pH3) with proteins via the basic amino acid residues and the polyanionic groups of the phospholipids. The presence of the carboxylic group of the organic acids may compete with the polyanionic groups of phospholipids [190]. However, in the present study, multiple lipid classes were present. Tzompa-Sosa et al. (2014) showed by TLC analysis that insect lipids consist mainly of triglycerides [76]. These insects were killed rapidly (30 min at -20 °C followed by nitrogen immersion). The same results were obtained after blanching (98 % triglycerides) [189]. TLC analysis in the current study (data not shown) also indicated a major presence of triglycerides (no polyanionic group) thus implying that the interaction between proteins and lipids in this study is different. Although the specific context is different, both studies observe an added value of organic acids for separating the lipids present in biomass. Since the current study indicates lactic acid to be better than citric acid in removing the lipids, it could be stated that lactic acid has a higher preference toward neutral lipids. In addition, under the applied conditions (pH 2), it is more likely that the acids were protonated (pKa of all acids was higher than 2, except for oxalic acid) and thus not negatively charged. The acidic pH may have stimulated the release of lipids from remaining tissue (located in P1 when fractionating at neutral pH) by, for example, changing the structure of bounded proteins. Nevertheless, pH cannot be the only explanation, as, lactic acid was proven to be beneficial compared to the use of inorganic acids or other organic acids. This indicates that lactic acid has another beneficial effect in separating neutral lipids from other insect biomass components.

For the protein-enriched fraction (P2), the use of organic acid did not increase the protein or lipid content in the fractions compared to water (cond. 1) or HCl (cond. 2). In comparison with condition 2, there was no added value of the organic acids. The purity of the P factions was maximal about 60 % proteins, which is comparable to other protein extractions of the BSFL. For example, Bußler et al. (2016) performed a hexane defatting resulting in a protein meal (still containing chitin) with a protein

content of 44.9 % and a crude fat content of 8.8 %. Osborne's fractionation after solvent lipid extraction created multiple fractions with a protein content range from 3-13 % [186]. Low-pressure defatting without the use of solvents resulted in a protein fraction containing 55 % proteins and 18 % fat and high-pressure defatting resulted in a fraction with 67 % crude proteins and 5 % crude fat [191]. For other insects such as the mealworm, for example, an aqueous extraction at pH 10 after a hexane extraction also delivered a protein fraction with 68 % protein and 0.4 % fat. Enzymatic extraction of proteins on crickets (*Gryllodes sigillatus*) did also not succeeded in obtaining a fraction higher than 70 % of protein and 8 % of fat [80]. Aqueous extraction of crickets (*Acheta domesticus*) resulted in multiple fractions comprising a fraction with 67 % crude proteins and 14 % crude fat [70].

3.3.4 Reproducibility

Extractions with HCl (cond. 2), lactic acid (cond. 4) and acetic acid (cond. 5) were repeated in triplicate under slightly different conditions to evaluate the reproducibility. For condition 4, the first repetition was performed with freshly prepared juice while for the second repetition, defrosted juice was used. Similar results were obtained (Figure 3-3) indicating that the freezing and defrosting process does not affect the fractionation procedure. Conditions 1 and 5 were performed with a different type of centrifuge and showed the same fractionation profile for all fractions except for condition 5, fraction SN2. However, this difference can be explained by the differences in additions acid/base for pH changes and the volatility of acetic acid. Overall, all three conditions show good reproducibility of the process. In addition, the protocol was also successfully executed for another insect, viz., the LM (data not shown).

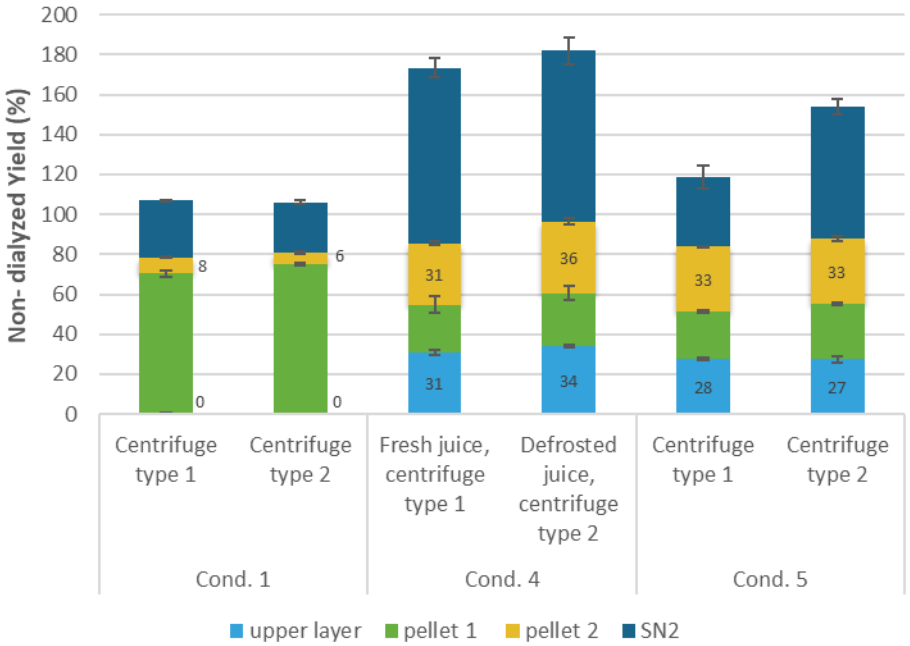


Figure 3-3 Reproducibility: NDY distribution after fractionation for 3 conditions performed in duplicate with slightly different conditions (mean ± S.D., n =3). cond. 1: water, cond.4: lactic acid + HCl, cond. 5: acetic acid + HCl, centrifuge type 1: Swing out centrifuge at room temperature, centrifuge type 2: angle head centrifuge at 25 °C.

3.3.5 Solubility curve assessment

Since most functional properties of proteins require the protein to be soluble [192], [193], the solubility was used as a criterion to evaluate the potential use of the fractionated proteins as functional proteins. The total protein solubility of P1, P and SN2 are given in Figure 3-4. The proteins in the UP were not investigated for functional properties and were therefore not included in the calculations. These proteins were extracted in a highly hydrophobic environment (caused by the lipids) and were assumed to be insoluble in water or trapped in micelles in the lipid-rich fraction. Based on Figure 3-4, it can be concluded that the acidic water treatment and acetic acid treatment resulted in soluble proteins, although partial loss in solubility was observed. At pH 2 and 9, ± 35 % of proteins were probably denatured or lost in the upper layer resulting in a lower solubility. At pH 5, no losses were

measured for the inorganic acid treatment, and at pH 6.5-7 a loss of approximately 13-15 % was recorded. No similar protein extractions of the BSFL were found in literature, however, data on extracted protein from the YM was found. Protein extraction at pH 10 after a defatting with ethanol resulted in 40 % solubility at pH 7 [60], similar to the results discussed here (Figure 3-4). Bußler et al. (2016) obtained a higher solubility, up to 60 % at pH 7, after alkaline extraction at 60 °C and hexane defatting. The solubility of the separate fractions indicated that SN2 was most soluble (around 80 % for pH 2-5 and 7). The protein-enriched fraction (P2) had a high solubility at pH 2 (60 %) but extremely low at pH 5 and 7 (around 10 %). Yet, the solubility experiment of SN1 indicated a solubility of 53 % at pH7. This leads to the hypothesis that the acid environment did cause structural changes in the proteins but did not cause denaturation. Only when the pH was adjusted to pH 5 the protein was denatured to a point were at neutral pH, no renaturation could occur. Further investigation of other techno-functional properties beside the solubility is needed to evaluate the effect of the treatment more in detail.

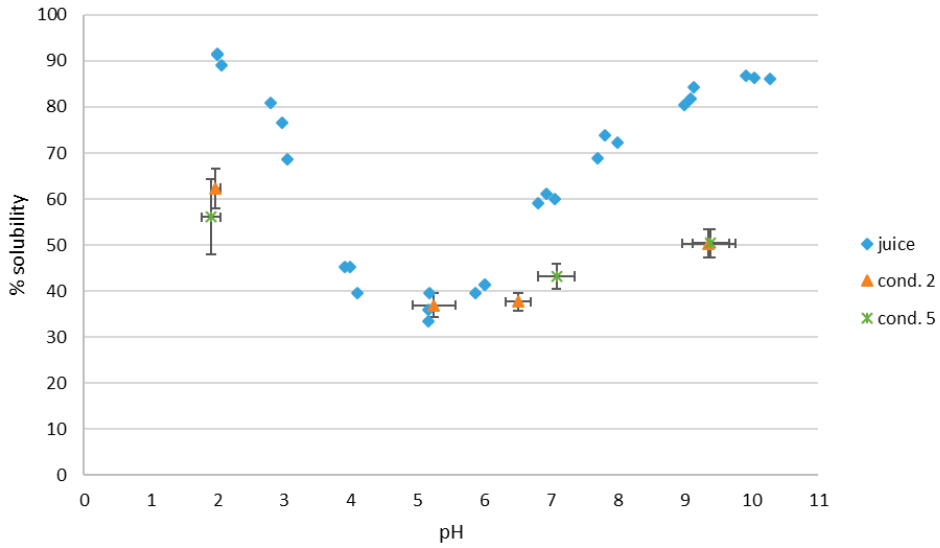


Figure 3-4 Protein solubility as function of pH of defrosted juice, and the summarized solubility after fractionation of P1, P2, and SN2. cond.2: water +HCl, cond. 5: acetic acid + HCl

3.4 Conclusion

The aim of this paper was to evaluate an alternative approach for the fractionation of wet biomass. Organic acids were evaluated to improve the acid isoelectric point precipitation (AIPP) to separate lipids from proteins. All acids (organic and inorganic) enabled to obtain a lipid-rich fraction, however, the use of lactic acid combined with HCl proved to be the best condition to obtain a lipid fraction with a purity of 80 % lipid. Multiple acids were capable to enrich the protein level in 1 fraction, however, when taking into account the yield and purity, acetic acid combined with HCl or HCl by itself gave the best results. The purity of the enriched protein fraction reached ± 60 %. The data also implied that pH has a significant effect on lipid separation whereas protein separation was less affected. The process proved to be robust and proteins were still partially soluble. The total protein solubility after fractionation was in the same order of magnitude compared to other protein extraction procedures.

3.5 Acknowledgments

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Chapter 4.

COMPARISON OF THE NEW FRACTIONATION METHOD TO TRADITIONAL DEFATTING TECHNIQUES

4.1 Introduction

In the previous chapter, an AIPP was studied on dechitinized juice from the BSF larvae [194]. This treatment was able to concentrate up to 73 % of the lipids in one fraction and thus could also be defined as a defatting method. Other defatting methods applied to insect biomass involve solvent extraction, supercritical fluid extraction (SFE) or alkaline isoelectric point precipitation (see chapter 1.3) but no published data on AIPP treatment applied on insect biomass was found. In addition, a heat treatment, although being a traditional defatting method that is often used for extracting lipids from slaughter waste or fish offal [195],[196], is also being applied to insects but no published data was found. Furthermore, the different reported defatting methods were performed on different insects (mealworms, crickets, grasshoppers, silkworms, stink bugs) which makes a comparison between defatting techniques difficult.

In the current study, these four different defatting approaches were applied to the same insect biomass, being dechitinised LM juice. The approaches that were evaluated were solvent-based extraction, SFE, heat treatment, and AIPP. The research approach that was followed consisted of two phases. Firstly, for each defatting approach different operational parameters were evaluated, while in a second phase the different approaches were compared based on the most optimal settings. The aim of the study was two-fold. The first aim was to elaborate and identify a method to generate defatted protein-enriched fractions to study in the next phase the techno-functional properties of LM insect proteins. For this rather analytical oriented aim, a high degree of defatting is required as well as good preservation of the techno-functional properties of the proteins. On the other hand, the second aim of the study was to compare the defatting approaches for fractionation of insect biomass via a cascading biorefinery into protein and lipid enriched fractions. For the latter aim, the scalability of the process and quality of

the lipid enriched fraction become important, besides the quality and extraction efficiency of the protein-enriched fraction. To meet with these two aims, the defatting approaches were evaluated by determining mass extraction yield, protein and lipid recovery in the different fractions, purity of the fractions and impact of the fractionation process on protein solubility. This multi-criteria evaluation is another aspect that differentiates the current study from previously reported data.

4.2 Material and methods

4.2.1 Materials

The LM larvae, bred on a standard grain-based diet, were kindly provided by a breeding company in the Netherlands (Protifarm). The larvae were starved for one day and killed during the mechanical treatment. The exoskeletons were removed based on the procedure described in WO 2019081067 [185] and the insect juice was immediately frozen and stored at -20 °C in vacuum bags till use. Before use, the biomass was defrosted in room temperature water for 10 minutes. Some defatting methods acquired water-free samples. For this reason, part of the juice was freeze-dried (Christ, gamma 1-16 LSC) for 36 hours. The following solvents were used: hexane (VWR, CAS 1.04374; 110-54-3), ethanol 90% (alfa aesar, cas 22930), acetone (VWR, cas 20067), diethyl ether (Merck, cas 60-29-7), lactic acid (Alfa Aesar, 85.0 - 90.0%) and HCl (Merck, 37 %).

4.2.2 Defatting methods

Four defatting methods were applied to the LM juice. In respect to solvent extractions, defrosted (8g) or freeze-dried (2.7 g) juice was mixed with the solvent in solid: liquid (w:w) ratios of 1:4 and incubated at room temperature for 2 hours on a shaker (Gerhardt laboshake) at 150 rpm. Centrifugation (Eppendorf centrifuge 5804.R) at 4500 g for 10 min at 4°C followed by decantation was employed to separate the solids from the liquid. The solvents were evaporated from the liquid phase by a nitrogen air stream until visually dry, followed by 24 hours in a vacuum

oven (20°C). These lipid fractions were presumed pure. The solids were placed in a vacuum oven (20°C) for drying. Three solvents were tested, being hexane, ethanol and acetone.

Defatting by SFE was performed on freeze-dried samples (4.5 g, packed without co-packing material in 10 ml extraction vessels) with either 100 m% CO_2, 15 m% acetone & 85 m% CO_2 or 15 m% hexane & 85 m% CO_2. SFE (Jasco) was performed at 325 bar, at 37 °C and at a solvent flow rate of 2 mL/min for 2 hours. Residual solvent was evaporated by a nitrogen airflow for 48 hours and the collected lipid fraction was presumed pure. The defatted sample was collected and dried in the vacuum oven.

Heat treatment was performed as a third defatting method. Defrosted juice (moisture content of 69 %, 20 g), with or without additional water (total moisture content of 77 %), was subjected to 120°C for 30 or 15 minutes at 2 bar in an autoclave (200V, Boxer, BRS). After cooling, the samples were centrifuged (thermos Scientific, Sorvall Lynx 600) at 4000 g for 30 min at 20°C. The upper layer was scooped off by a spatula and dried at 105 °C for 48 hours. The decanted supernatant and pellet were freeze-dried.

The fourth defatting method was an AIPP and is described in Chapter 3, page 80. The same procedure was executed on 50 g defrosted LM juice and two solvents were evaluated, being lactic acid (0.5M) and demineralized water (referred to as inorganic acid treatment). Briefly, both solutions (solvent + insect biomass) were pH adjusted by 6M HCl to reach pH 2. Next, the samples were centrifugated (Thermo Scientific, Sorvall lynx 6000 – F14-14x50 cy rotor) resulting in a floating lipid-rich layer (UP), a supernatant (SN1), and a pellet (P1). The supernatant was collected, and the pH was adjusted to pH 5 to precipitate proteins. After a second centrifugation, a supernatant 2 (SN2) and a pellet (P2) were obtained. The UP layers and P2 fractions were collected for further analysis. The extraction yield (based on dry matter (DM)) of all subsamples was calculated based on formula 7.

$$Yield(\%) = \frac{weight\ of\ the\ sub\ fraction\ (g\ DM)}{weight\ of\ the\ initial\ (juice)\ sample\ (\ g\ DM)}\ x\ 100 \quad (7)$$

4.2.3 Analysis

The lipid content was determined gravimetrically after a Soxhlet extraction (diethyl ether, 6h) and expressed as percentage lipid on dry matter basis of the juice sample. The protein content was measured via nitrogen element analysis (Elementar, vario EL cube) and converted as discussed in Chapter 2. All nitrogen in the sample was presumed to originate from proteins, peptides or amino acids since chitin, the most abundant source of nitrogen in insects besides proteins, was removed by the mechanical pretreatment. The protein content was expressed as percentage protein on dry matter basis of the juice sample.

4.2.4 Lipid and protein recovery

Lipid recovery for the solvent and SFE extraction was calculated based on formula 8 (the purity of the lipid subtraction was presumed 100 %) while for the heat treatment and isoelectric precipitation it was calculated based on formula 9. The protein recovery was calculated accordingly.

$$Lipid\ recovery\ (\%) = \frac{weight\ of\ the\ lipid\ sub\ fraction\ (g\ DM)}{weight\ of\ the\ initial\ juice\ (g\ DM)\ x\ lipid\ content\ juice(\%)} * 100 \quad (8)$$

$$Lipid\ recovery\ (\%) = \frac{yield\ subfraction\ (\%) x\ lipid\ content\ sub\ fraction(\%)}{100\ x\ lipid\ content\ initial\ (juice)\ sample\ (\%)} * 100 \quad (9)$$

4.2.5 Solubility curve

The protein solubility was determined as described in Chapter 3. When more than one defatted fraction was obtained (for the heat and isoelectric precipitation), the solubility of the separate fractions was reported (g soluble protein per 100 g protein in the initial sample) and a global solubility was calculated (of all subfractions). The effect of the extraction method was evaluated on the protein solubility increase or decrease compared to the original juice solubility (solubility of the subfractions – solubility of the juice at a certain pH).

4.2.6 SDS-Page

The sample was diluted to proximally 3 mg soluble protein/ml in demineralized water and centrifuged (3000 g, 5 min, 25 °C). Next, 70 µl of the sample was added *to* 25 µL XT sample reagent (Bio-Rad, CAS 2044-56-6) and 5 µl reducing agent (Bio-Rad, CAS 51805-45-9). After heating for 5 min at 95 °C, 25 µl was loaded tot the polyacrylamide gel (Bio-Rad, CAS 3450124). Coomassie blue (Bio-Rad, CAS 64-19-7) was used for coloring agent to visualize the protein fragments and a broad-range ladder (Bio-Rad, cas: 1610363) was used as a reference.

4.2.7 Statistical analysis

All measurements were performed in triplicates unless stated otherwise. The data were expressed as the averages with the standard deviation. The data were statistically processed by one-way analysis of variance (ANOVA) in Excel ($p< 0.05$) and the Tukey post hoc test was performed by using IBM SPSS software.

4.3 Results and discussion

4.3.1 Characterization of lesser mealworm juice

The composition of the LM juice was determined in terms of the lipid and protein content (on dry matter basis) of the juice (Table 4-1). Two batches of LM juice were produced in an identical manner but in a different time period (with a new batch of LM larvae). Small differences were observed that may be attributed to different instar larvae quality or naturally occurring variations in the feed ingredients. Both batches had a similar lipid content of 38 % and a slightly different protein content (47 % for batch A, 45 % for batch B).

Table 4-1 Lipid and protein content (on dry matter basis) of all samples and subfractions.

Procedure	Sample	% lipid content	% protein content
	Juice A	36,71 ± 1,15	47,10 ± 0,40
	Juice B	38,69 ± 2,29	44,56 ± 0,88
Solvent extraction	**Defatted meal by hexane ****	**20,33 ± 1,37**	**60,19 ± 0,76**
	Defatted meal by acetone (Dry)	**23,90 ± 0,18**	**59,94 ± 1,11**
	Defatted meal by acetone (wet)	**27,90 ± 1,03**	**56,79 ± 0,72**
	Defatted meal by ethanol (dry)	**24,78 ± 1,33**	**64,38 ± 0,63**
	Defatted meal by ethanol (wet)	**31,64 ± 0,40**	**55,48 ± 0,25**
SFE	**Defatted meal by hexane**	**2,46 ± 0,84**	**68,35 ± 1,25**
	Defatted meal by acetone	**5,14 ± 0,81**	**67,11 ± 0,53**
	Defatted meal by CO2 **	**5,01 ± 1,65**	**67,06 ± 1,92**
Heat treatment	30 min – 69 % Moisture - Lipid layer	67,12 ± 4,22	24,20 ± 3,09
	30 min – 69 % Moisture – P	36,80 ± 3,63	45,17 ± 1,16
	30 min – 69 % Moisture - SN	**12,54 ± 12,61**	**55,22 ± 6,75**
	30 min – 77 % Moisture - Lipid layer	70,47 ± 1,48	25,68 ± 1,83
	30 min – 77 % Moisture - P	33,27 ± 2,70	46,83 ±2,11
	30 min – 77 % Moisture – SN **	**3,18 ± 1,02**	**62,36 ± 0,26**
	15 min – 69 % Moisture - Lipid layer	65,61 ± 2,03	24,53 ± 1,01 *
	15 min – 69 % Moisture - P	32,88 ± 3,28	46,07 ± 2,95
	15 min – 69 % Moisture - SN	**4,48 ± 5,65**	**56, 56 ± 4,69**
AIPP	Lactic acid – Lipid layer	71,17*	19,19 ± 0,47
	Lactic acid – P1	22,42 ± 3,46	34,46 ± 0,42
	Lactic acid – P2	**11,19 ± 2,65**	**45,56 ± 1,89**
	Lactic acid – SN2	2,35 ±1,09	25,67 ± 0,25
	Inorganic acid – Lipid layer	85,50 ± 2,05	12,18 ± 0,88
	Inorganic acid – P1	24,67 ± 1,40	46,43 ± 1,34
	Inorganic acid – P2 **	**7,59 ± 1,60**	**70,54 ± 0,63**
	Inorganic acid– SN2	1,63 ± 0,52	55,32 ± 0,82

* duplicate; Protein-enriched fractions are marked in bold; **Best performing condition for each technique, abbreviations are explained in material and methods

The solubility of the proteins in the two batches of LM juice was determined before further fractionation and differed slightly (Figure 4-1). A high solubility was measured at acid and alkaline pH and the isoelectric point was found to be at pH 5. To the author's knowledge, no protein solubility curve for LM juice was reported before. However, information was found for YM which displayed compared to LM a lower protein solubility at acid and neutral pH [60],[72]. However, the chitin present in the YM meal reported in the literature may have led to an underestimation of the protein solubility by impacting the N-measurement, while the chitin and cuticle proteins were not included in LM juice (removed before by mechanical treatment)

in the current study. Chapter 3 reports a similar protein solubility for the BSF larvae juice.

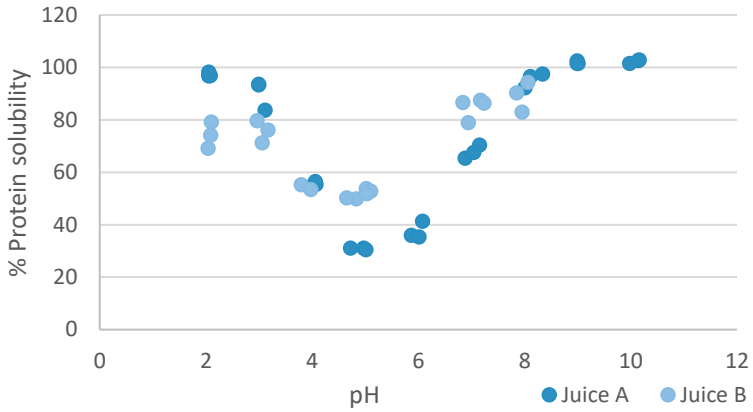

Figure 4-1 Solubility curve for two batches of LM juice.

In the current study, the solvent and AIPP extraction were performed on juice A and the heat and SFE treatment on juice B.

4.3.2 Solvent extraction

Within the solvent extraction approach, three different solvents were examined. Hexane, acetone, and ethanol were tested on freeze-dried juice and the latter two were also tested on wet, defrosted juice that had a moisture content of 69 %. The mass distribution of the juice over the obtained fractions on dry matter basis is illustrated in Figure 4-2. The solvent extractions resulted in two fractions were 78 % of the juice was concentrated in the defatted meal (protein-enriched fraction).

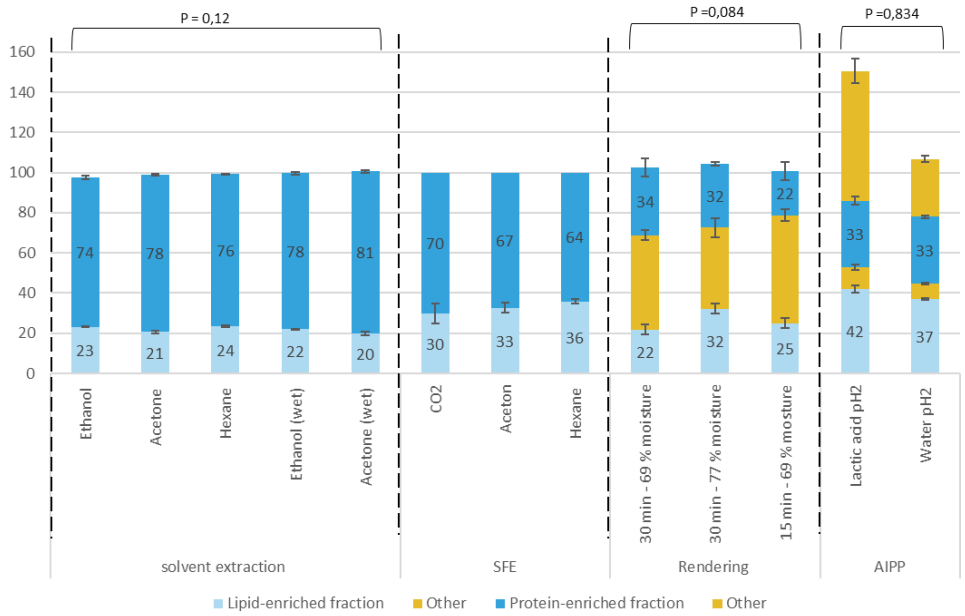

Figure 4-2 Mass yields of the subfractions generated after implementing different defatting approaches on LM juice. Anova analysis performed on the protein-enriched fraction.

The lipid recovery (Figure 4-3) indicates that small but significant differences were observed between the different solvents used. Hexane and ethanol (dry extraction) had a higher lipid recovery whereas acetone had a lower lipid recovery. This was not expected considering acetone is less polar than ethanol. The experiment was repeated but similar results were observed. TLC indicated that the same lipid classes were extracted, in similar proportions. The fat extracted by ethanol and acetone had a difference in appearance (ethanol had a much deeper yellow color) and both had a cloudy substance. A possible explanation could be that other solvent-soluble compounds were extracted, more by ethanol than acetone. The remaining lipids were located in the defatted meal that resulted in a lipid content between 18 % and 32 %, and the protein content between 55 % and 64 % (see Table 4-1).

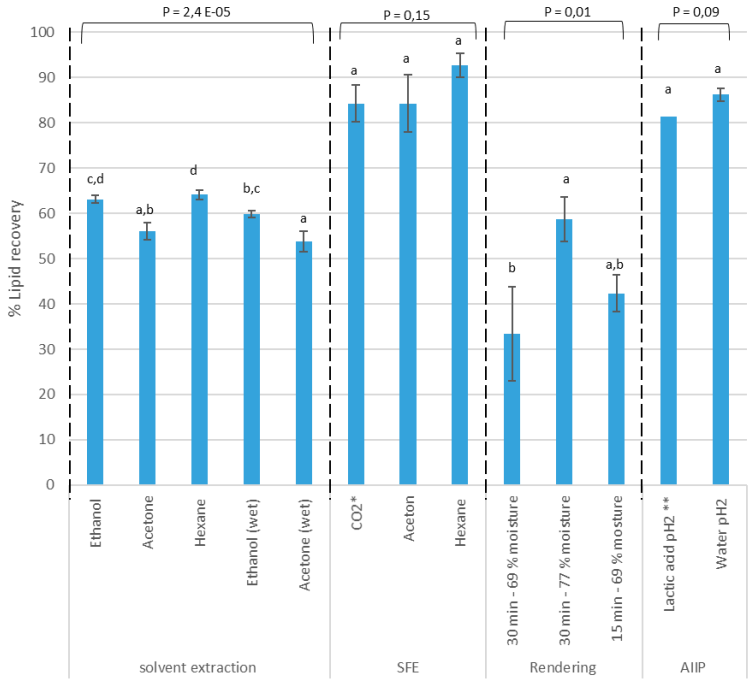

Figure 4-3 Lipid recovery from LM juice realized by different defatting approaches. duplo, ** single analysis*

Hexane is the most common solvent used for defatting but recently, due to the safety and environmental aspects, a search for alternative solvents is ongoing. Based on the current results, ethanol would be a good candidate. The use of ethanol increased the protein content in the defatted meal (64 % versus 60 %) but resulted in a slightly higher lipid residue (25 % versus 20 %). These findings are in line with L'Hocine et al. (2006) who reported ethanol as a good alternative for hexane to defat soybeans.

Hexane has a high preference for neutral (triglycerides) lipids while acetone and ethanol are more polar and can also extract polar lipid classes such as phospholipids. Previous research has indicated that the lipid classes of insects are mostly dominated by triglycerides and to a lesser extent (depending on the insect type) by phospholipids [197]. Cholesterol, free fatty acids, and wax esters are present in

minor concentrations [197],[198]. Tzompa-Sosa et al. (2014) reported a high abundance of triglycerides in LM but also the presence of free fatty acids and a small number of diglycerides and phospholipids [76]. Diglycerides were expected since they are the main form in which lipids are transported in insect tissue [198]. Free fatty acids were presumed to be a product of degradation by cold-active lipases. Caligiani et al. (2019) proved that the killing method can cause degradation of the lipids of the larvae of the BSF by activation of lipase during the freezing process, commonly used for killing. In the current study, the larvae were killed quickly by mechanical grinding and stored in vacuum bags at -20 °C. However, TLC showed that a large quantity of free fatty acids was present. Nevertheless, no major differences were observed between the three solvents, which suggests that the polarity of the solvent did not have a high impact under the test conditions applied. The results indicate that about 40 % of the lipids were not extracted, independent of the solvent choice. Literature data reported higher defatting efficiencies than acquired in the current study. Choi et al. (2017) for example, achieved a defatted meal with only 2 % lipids by a hexane extraction on the YM [66]. They did prolong the reaction time compared to the current study (3 times 12 h) as did Bußler et al. (2016) who generated a defatted meal with a lipid content of 3 % after a hexane extraction of 1 hour that was repeated twice [60]. Besides the difference in reaction time, the difference could also be originated from the difference in insect species or the killing method/pretreatment (YM in their studies was killed by freezing, dried and ground). Ethanol and acetone are two water-soluble solvents and were also tested on wet material which offers the potential to avoid an energy-consuming drying step. Nor for acetone, not for ethanol a significantly different lipid and protein recovery or extraction yield were measured for wet versus dried extraction (Figure 4 & Figure 5). However, wet extractions generated defatted meal with lower protein content and higher lipids content compared to the dry extractions with the same solvent (Table 1), pointing towards a lower defatting efficiency. The lipid extract exhibited a light brown color after the wet extraction which could be caused by residual proteins

in the lipid extract. An economic assessment may assist in choosing the most suitable extraction process (wet or dry) according to the application and extraction costs.

4.3.3 SFE extraction

SFE with supercritical CO_2 is known to only extract triglycerides. Therefore, co-solvents are often used to extract more polar compounds as phospholipids as well. In the current study, SFE was applied to dried LM juice using three different solvents, being 100% CO_2 and two conditions with 85 m% CO_2 and 15 m% of a co-solvent (hexane or acetone). Hexane was added to possibly increase extraction yield whereas acetone was added to improve the extraction of more polar lipid classes. Ethanol, also often used as a co-solvent was not tested since the solvent extraction already indicated a possible negative impact on protein solubility (discussed later). The data revealed that about 66 % of the juice was distributed in the defatted meal (Figure 4-2) and that extraction yields of the lipid fraction were statistically indifferent (P=0.09, data not shown) ranging between 30 % and 36%. The lipid recoveries varied from 83 % to 93 % and were statistically not different (P=0.15, Figure 4-3), regardless of the co-solvent that was employed.

About 10 % of the lipids were not extracted by the SFE compared to a Soxhlet extraction (6 h, diethyl ether). The lipid residue in the defatted meal after SFE was below 5 % (Table 4-1, based on Soxhlet) which is in line with the results reported by Sipponen et al. (2017) who found a 3.5 % lipid content in the defatted meal after SFE [199] and Purschke et al. (2017) who mentioned a 95 % lipid recovery [73]. The defatted meal had a protein content around 68 %. A possible increase could be employed when a more polar co-solvent is used.

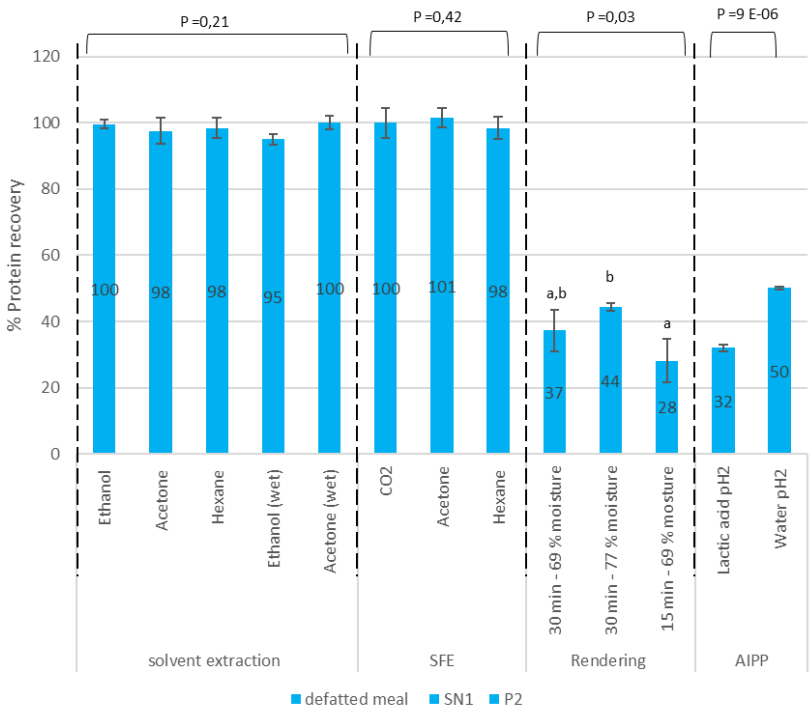

Figure 4-4 Protein recovery from LM juice realized by different defatting approaches.

4.3.4 Fractionation via heat treatment

For the heat treatment approach, a combination of heat and a small pressure was applied on LM juice to separate lipids. Evaporation of the moisture in the closed reaction system caused a pressure build-up. After treatment, the samples were centrifuged, and three subfractions were obtained viz., a floating UP, a pellet, and a supernatant. Three different setups were tested with (1) one condition involving a heating period of 30 min, (2) the second was performed at an elevated moisture content (77 % instead of 69 %), and (3) the third during a shorter time of 15 min at its original moisture content. Depending on the process condition, the mass distribution of the juice over the fractions varied. In general, the upper layer mass yield contained 22-32 % of the juice, the pellet 40-53 % and the supernatant 22-34 % (Figure 4-2). The floating upper layer was presumed to concentrate the free lipids and resulted in a lipid-enriched faction (at least 65 % lipid) while the pellet was

presumed to contain denatured protein and remaining tissue where the composition was similar to the juice (at least 32 % lipid). The upper layer and pellet had a lipid recovery of 33-59 % and 35-50 %, respectively and thus this fractionation approach resulted in a total 83- 94 % lipid recovery/ removal. But considering that the main aim of the study was to generate a defatted protein fraction, the most important subfraction was the supernatant. The protein recovery of the supernatant fraction was between, 28 % and 44 % (see Figure 4-4). Between the three different set-ups, it was clear that the second set-up performed significantly better. The processing time had no effect on the performance, but an improvement was achieved when the moisture content and as such the pressure was raised. The latter resulted in a better mass yield (32 %), a higher protein content (62 %) and lower lipid content (3 %) of the protein-enriched supernatant fraction.

4.3.5 Fractionation using AIPP

An AIPP was performed at pH 2 with or without the presence of lactic acid. The AIPP methods generated four fractions where the UP layer was considered a lipid-rich fraction and P2 the defatted, protein-enriched fraction. When comparing the extraction yields obtained in the current study, a > 100% cumulative mass yield was calculated (Figure 4-2) which can be explained by the addition of lactic acid and formation of salts by HCl and NaOH which could not be deduced in the calculations (as explained in Chapter 3). The extraction yield for the protein-enriched fraction (P2) was similar for the two set-ups but the yield for the UP fraction was higher when lactic acid was used. However, based on the composition of the upper layer and the similar lipid recovery, the data suggest that lactic acid was partially located in the lipid-enriched fraction, increasing the yield but decreasing the purity (71 % lipids versus 86%, Table 4-1). The upper layer was able to recover 84 % lipids and no significant difference was observed in the lipid recovery (P=0.09) between the two setups in contrast to the findings of Soetemans et al. (2019). They reported an improved lipid extraction when lactic acid was used for extracting lipids from larvae

of the BSF (73 % versus 51 % lipid recovery). The lipid recovery of the two other sub-fractions (P1 and SN2) was 10.8 % when lactic acid was added and 6.4 % for the inorganic treatment. Overall, the lipid recovery/removal was about 92 % for the AIPP treatment. In respect to the protein-enriched fraction P2, comparing the two treatments, the inorganic acid treatment seemed much more efficient in collecting the proteins in the second pellet than the lactic acid treatment (32 % protein recovery versus 50 %) resulting in a protein fraction with 70 % proteins compared to 45 % protein (Table 1).

4.3.6 Impact of the defatting technique on the protein solubility and protein distribution

The protein solubility was determined to obtain a first indication of the impact of the defatting approaches on the TFP of the protein fractions from LM juice. More specifically, the protein solubility was measured for selected samples at 3 pH-values, being pH2, pH5, and pH7 (Figure 4-6). Figure 4-5 illustrates the size distribution of the soluble proteins between 250 and 10 kDa. Based on the protein content and the protein solubility, a concentration of 3 mg soluble proteins/ ml was loaded on the gel. The proteins in the juice had a large distribution with dominant molecular weights of about 75 kDa, 50 kDa, 37 kDa, between 25 and 37 kDa and 10 kDa

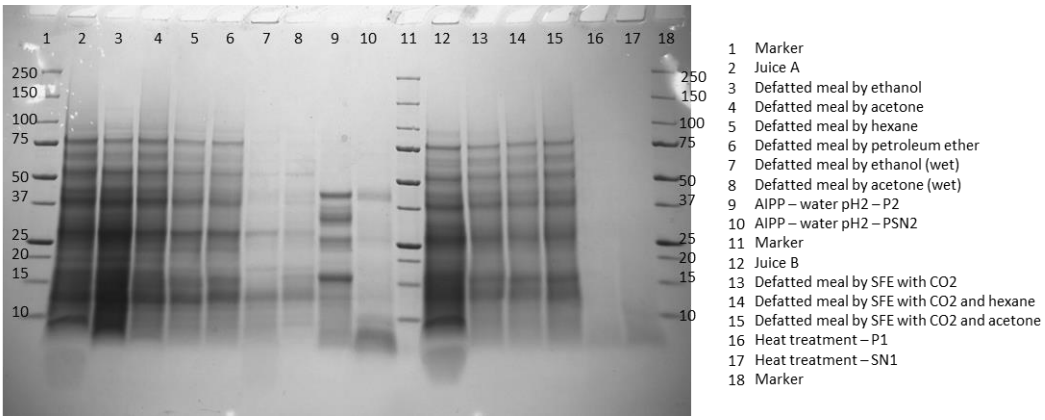

Figure 4-5 SDS-Page of all obtained fractions

Solvent-based extractions negatively impacted the protein solubility, but the impact was much less for highly non-polar solvents (like hexane). Non-polar solvents do not solubilize proteins and thus the native structure remains unchanged. The results for hexane were in agreement with Mishyne et al. (2019) who reported no differences in the protein solubility curve of the honey bee or grasshopper compared to the raw materials after a hexane defatting [200]. Ethanol, on the other hand, is an alcohol that is known to partially denature proteins by changing hydrogen bonding and penetration of the protein core causing noncovalent interaction and aggregation of proteins [201],[202]. This explains the reduced protein solubility that was observed in the current study after the dry and wet ethanol extraction. A dry extraction with acetone did not lead to major changes but the protein solubility decreased significantly in a wet extraction. Fukushima et al. (1968) stated that water-soluble solvents, in the presence of water, can cause denaturation. It was hypothesized that a hydrophilic shell originating from the hydrophilic amino acids is formed at the surface, with an outside hydration layer that prevents hydrophobic solvents to enter. However, water-soluble solvents make use of water to disturb this shell and thus can penetrate the hydrophobic core [203]. The solvent extractions caused little change to the SDS-PAGE compositional profile of the proteins extracted from freeze-dried juice (Figure 4-5). This is the first indication that no protein hydrolysis took place. The loading concentration was for all samples the same (theoretically calculated based on the protein content and solubility to 3mg/ml), yet the intensity of the band presented by the wet solvent extraction suggests a lower concentration of these protein sizes. This could indicate partial hydrolysis took place. Since no additional, smaller proteins were observed on the gel, the hydrolysates were probably smaller than 10 kDa. Further investigation is suggested and the presence of small oligo peptides could be confirmed by for example Gel Permeation Chromatography.

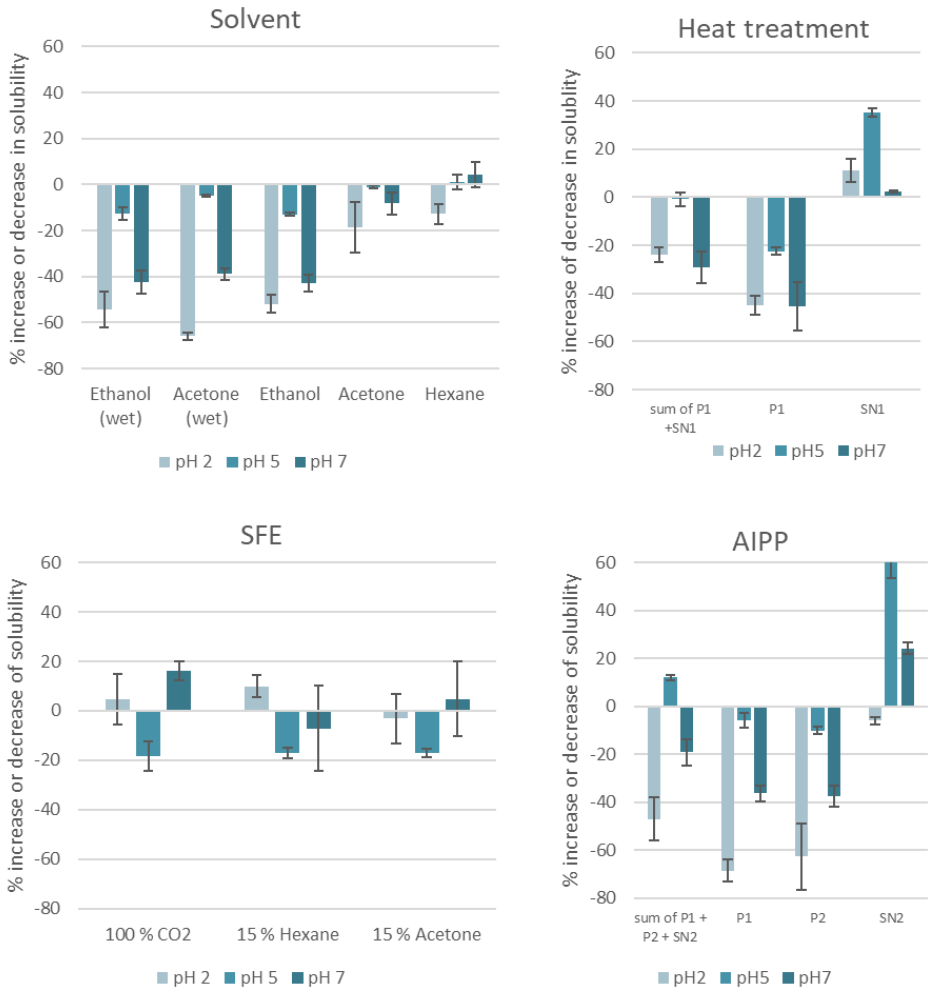

Figure 4-6 Impact of the defatting treatments on protein solubility.

SFE was shown to have a very limited effect on the protein solubility (less 20 % increase or decrease). Under pressure, hydrophobic interactions weaken, causing reconfirmation or denaturation and redistribution of non-polar compounds [204] [205]. SFE also did not alter the size distribution patterns, except for the proteins around 10 kDa that were no more present. Heat treatment and AIPP generated multiple protein fractions. As described before, the proteins were simultaneously fractionated based on their solubility. To evaluate the impact of the separation

technique, solubility profiles of all protein fractions were summed. The proteins in the lipid layer were considered insoluble since they were concentrated in a highly hydrophobic environment and were not included in the combined profile. Both treatments caused a decrease in protein solubility, especially for AIPP treatment. Proteins are known to denature during heat treatment since the in heat-induced flexibility can cause unfolding, increased hydrophobic bonding, and formation of aggregates with other proteins [206],[207],[208]. No bands were detected by SDS-Page for the soluble proteins in the fractions generated by the heat treatment which can point to protein hydrolysis [209] to smaller (than 10 kDa) peptides. The AIPP treatments probably caused irreversible denaturation by first forcing the reformation of the proteins in the acid environment where swelling and unfolding occurred due to strong intramolecular electrostatic repulsion, followed by aggregation and denaturation at the isoelectric point. Bußler et al. (2016) showed that proteins of the YM at acid pH were unfolded, exposing the hydrophobic residues, but soluble [72]. Assuming the same happened in the current study, the decrease in protein solubility probably occurred in the second stage when the isoelectric point was reached. At the isoelectric point, proteins would further denature, and the formation of aggregates driven by a decreased electrostatic repulsion would decrease the solubility. The size distribution of the two main protein fractions together generated by AIPP (P2 and SN2) showed the absence of larger proteins. AIPP-P2 had a different profile which was expected since 1) protein distribution over different fractions took place and 2) partial hydrolysis was expected due to the acid environment. The larger proteins (>50 kDa) that were present in the juice were not present and a new protein size was observed between 50 and 37 kDa, around 15 kDa and between 37 and 25 kDa. This suggests that either hydrolysis took place or that previously non-soluble proteins of that size were made soluble by the procedure. The second protein rich fraction was AIPP-SN2. Here, only two clear proteins sizes were observed at 50-37 kDa and below 10 kDa. The latter suggest protein hydrolysates.

4.3.7 Comparison of the four techniques

The best set-ups for all four techniques (hexane extraction, SFE without a co-solvent, heat treatment with elevated moisture content and an inorganic AIPP) are marked in Table 1 and were compared. The yield of the protein-enriched fraction (Figure 4-2) was significantly higher for the solvent-based extractions than the aqueous extractions. In addition, the latter relied on centrifugation to separate the lipids which simultaneously fractionated the proteins in soluble and non-soluble fractions, thus lowering the protein recovery and the extraction yield.

A hexane based solvent extraction was among the least efficient approaches for lipid removal (about 40 % was not removed) and the resulting protein extract still contained a high lipid content (20%). Yet, this technique is frequently used for upscaling purposes and further optimization of the process parameters (especially the processing time) may increase the recovery values. When only a nutritional purpose of the protein fraction is envisioned, hexane can be replaced by ethanol and even a wet extraction is possible. However, ethanol did negatively impact the protein solubility, making the protein fraction less suitable for some techno-functional applications. This treatment is advised for fractionating the inset biomass via a cascading biorefinery but not for analytical purposes. **SFE (100 % CO_2)** was proven most efficient to generate defatted protein fractions because of a high protein and lipid recovery, a high extraction yield and low lipid content in the protein fraction (5 %) while minimizing the impact on the protein solubility and distribution. A solvent hexane extraction and supercritical CO_2 are both highly non-polar solvents but an SFE extraction (without a modifier) resulted in a much higher lipid recovery compared to hexane solvent extraction. Since the hexane solvent extraction was performed at room temperature while SFE at 37 °C and 325 bar, the differences could be related to a higher solubility of the lipids at an elevated temperature and pressure or by the improved transport properties (due to the low viscosity and high diffusivity) of supercritical fluids [74],[73]. However, SFE is a rather expensive

treatment [210],[182] that is usually only applied for analytical purposes and high-end products [211],[212]. The necessary pre-treatment of drying makes this a less suitable technique for upscaling applications towards feed and food applications. However, for analytical purposes, this treatment is suitable. The heat treatment resulted in a protein fraction with enriched protein content and an increased protein solubility and generated a defatted fraction with the lowest amount of lipids (3 %). This technique is already performed on a large scale which makes the transition more easily. However, a presumption of protein hydrolysis to small peptides was raised which may impact some TFPs but on the other hand, could enhance protein digestibility and therefore the nutritional value of the fraction. The inorganic AIPP extraction was able to generate a protein fraction with the highest protein content (71 %) and a low lipid content (8 %). This procedure resulted in a similar extraction protein yield as the heat treatment and a similar extraction lipid yield and lipid recovery as the SFE, making this lipid-protein extraction ratio a good replacement for SFE. The envisioned protein fraction was mostly non-soluble and smaller proteins were measured compared to the juice. The separation of aqueous extractions (heat treatment & AIPP) is based on the disruption of cells and the disturbance of protein-lipid interactions causing the lipids to float to the surface. The lipid recovery data in Figure 4-3 suggest that the acid environment was much more efficient in releasing lipids than the heat treatment. Nevertheless, heat treatment results in the best defatted protein enriched fraction.

4.4 Conclusions

In conclusion, all defatting techniques were performed successfully on the juice of the LM. The first screening revealed that a solvent extraction with hexane performed best, a co-solvent for the CO2-SFE did not have an added value, a higher moisture content during the heat treatment had an added value and that an inorganic AIPP was must sufficient. The generated protein-enriched fractions from the selected treatment conditions contained 60-71 % of proteins and 2-20 % lipids

The aim was to (1) determine the best technique for analytical purposes and (2) study these techniques for implementing a fractionation approach via a cascading biorefinery into protein and lipid enriched fraction that could be upscaled. SFE proved to be most efficient for defatting of LM juice (66 % extraction yield, 100 % protein recovery, 67 % protein and 5 % lipid) and production fractions with a minimal impact on the protein solubility. However, an economic study is advised to evaluate the feasibility on an industrial scale since SFE is known to be an expensive treatment. Whereas the acid-based isoelectric point precipitation was identified as a suitable approach for large scale generation of crude protein (33 % extraction yield, 50 % protein recovery, 71 % protein and 8 % lipid) and lipid fractions (37 % extraction yield, 84 % lipid recovery) which uses less complex and expensive equipment. Further optimization of the latter approach to minimize the impact on the protein solubility is a point of attention.

4.5 Acknowledgments

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Chapter 5.

IMPACT OF FRACTIONATION ON TECHNO-FUNCTIONAL PROPERTIES OF PROTEIN

5.1 Introduction

Future predictions have indicated that a food shortage and especially protein shortages are inevitable and that insects are considered a good additional source. Nevertheless, as described in the previous chapter, entomophagy (human consumption of insects) is a taboo in Western countries. However, consumers have indicated they could overcome the cultural barrier when insects are processed in common food products. This led to a different approach were instead of employing intact insects as an ingredient or as a snack, insect meal (after grinding) or fractions have been proposed to be incorporated in food products. However, knowledge of their characteristics and possibly other functions is scares and incomplete.

For food applications, the nutritional value is the first important parameter. This involves the presence of essential amino acids and protein digestibility. Besides the nutritional value, proteins can also serve a non-nutritional purpose to the food product providing structure and stability. These non-nutritional functions occur on different levels. Firstly, the color of the food product, the sweetness, and the ligand-binding site (for flavor compounds for example) are happening on a **molecular scale**. Secondly, the arrangement of the protein into a structure is located on the **mesoscale**. In this phase, the non-nutritional function depends on the ability of the proteins to form and stabilize specific structures such as emulsions, foams, and gels. These properties will influence the physical and sensory propert²ies of the food product. And thirdly, on **the macroscale**, the interactions of the proteins with other ingredients will influence the overall properties of the food product. The final non-nutritional functions of the protein will be revealed at the macroscale and can differ from that at the mesoscale. For example, egg white proteins and whey proteins have the same foamability and have a similar structural transition during heating but by replacing egg white proteins with whey protein to produce an angel cake, the whey-based cake collapses upon heating. The difference lies in the interaction between

proteins and sugars. Sugar destabilizes the whey protein-based foam but not that of egg white [213]. Studies of the mesoscale are considered valuable for comparison among proteins and as a first indication of how they function [214],[213]. The first selection of ingredients for the formation of a food product is based on the mesoscale functionalities. However, as they say, “the proof of the pudding is in the eating”, the evaluation of the protein's functionality performance will be on the macroscale [213].

The current study focuses on the functional properties of insect proteins on the mesoscale. Firstly, **protein solubility** is seen as a critical functional property since it has a major influence on other functional properties such as foaming, gelling and emulsification. This functionality is usually studied first upon evaluating a new protein ingredient [68], [80]. The protein solubility is determined by the distribution of hydrophilic (on the outside) and hydrophobic (in the core) amino acid residues in the protein causing surface-active properties and electrostatic interactions between proteins [83]. Environmental factors such as pH and salts have a high influence on these characteristics and thus will also influence other functionalities. The pH changes the charge of the protein and in extreme cases, can cause partial unfolding, denaturation and the formation of precipitating aggregates. Mineral ions can either negatively influence functionalities for example by reducing the water availability, but can also positively influence functionalities, for example by the formation of an ionic double layer that increases electronic interactions [69]. **Protein that foam** can either be employed for the formation of a gas-in solid food product (for example bread) or a gas-in-liquid formation (for example whip cream). In literature, usually, the gas-in-liquid formation is studied. Foam formation on protein level involves three steps. Firstly, soluble proteins migrate to the air/water interface. Secondly, the proteins are absorbed form the solution to the air/liquid interfaced by simultaneously unfolding and partial denaturation in order to dehydrate the hydrophobic amino acid residues as they are the driving force of migration. This

lowers the interfacial surface tension of water and alters the viscosity. After that, the proteins are involved in preventing foam destabilization by forming an elastic film around the gas bubbles, preventing shrinkage of the gas bubbles and exhibit a low gas permeability [215],[214]. The presence of carbohydrates are known to either increase or decrease the foam stability [68], [214] as well as the presence of lipids [40]. Since proteins are amphiphilic and surface-active molecules, they can be used as **emulsifiers** in food products. A similar process happens during emulsification where the protein diffuse to the oil/water interface, unfold and non-polar amino acid residues are distributed to the oil phase and the oil droplet is coated with the proteins. The protein layer reduces the interfacial tension and forms a protective layer around the droplet. As with the foaming properties, a rapid migration to the interface is crucial together with a high flexibility to facilitate unfolding. The balance of the polar and non-polar forces in the protein maintain the emulsion stability [216],[217]. Electrostatic repulsions of the charged amino acid residues prevent coalescences and flocculation. Some sugars can increase emulsion stability by increasing the viscosity of the system [68]. Proteins can also act as a **gelling agent**. A gel in food systems is composed of liquid that exhibits solid-like properties were elastic properties arise from a protein network that acts as a skeleton entrapping the liquid phase [218]. During the gel formation, soluble proteins partially unfold and interact to form a three-dimensional cross-linked network. The unfolding is stimulated by either heat or by an acid/ alkaline environment. The network involves protein-to-protein interactions (mainly disulfide bridges and hydrophobic interactions) and protein-to-liquid interactions where the liquid presents the protein matrix to collapse and form rigid aggregates and the protein matrix prevents the liquid from flowing away. A gel can have different characteristics in strength and stability, all depending on the interactions, the strength of the junctions and the flexibility of the protein network which translates in different rheological textural properties in food [219], for example, yogurt is seen as a semi-solid gel whereas cheddar cheese is seen as a soft-solid gel [214]. The

water and oil binding capacity of proteins is tested on protein powder and evaluates the ability of the protein to hold water or oil against gravity. In the case for water, proteins capture waters by hydrogen bound, Van der Waals interactions and electrostatic attractions between the charged groups of water and amino acids [220]. The ability to entrap water is thus dependable on the amino acid profile, the conformation, and hydrophobicity [221]. Oil binding is a result of the hydrophobic amino acid residues or physical entrapment of oil where the capillarity interaction, density and the size of the powder particles play a major role [222],[60]. The ability of proteins to retain water or oil plays an important role in the textural properties[217]. In addition, oil can act as a flavor retainer and thus a high oil binding increases the sensory characteristic [83].

The previous chapter described different defatting methods to generate high-protein content fractions. The solubility curve already indicated an impact on protein characteristics. Since insect proteins are a new protein ingredient, further research on other functional properties on the mesoscale is needed for introducing and choosing these fractions as a new ingredient in a food product. This chapter will evaluate the different functional properties of different protein fractions obtained from the LM. In addition, the functionality test will be performed on both the native insect meal (before fractionation) and on the extracts so that the impact of the fractionation can be studied.

5.2 Material and method

5.2.1 Materials

The LM was kindly provided by Protifarm (The Netherlands) and reared on the standard Kreca diet. Living larvae were killed and fractionated by mechanical treatment (WO 2019081067 [185]). The liquid fraction (juice) was collected in a vacuum bag and immediately frozen by a quick freezer (Eco cell, MX85-40 A EC,

Claes Koeltechniek) to -30 °C. Afterwards, the liquid fraction was freeze-dried (Christ, gamma 1-16 LSC, 36 hours). All further fractionation techniques were performed on freeze-dried material. Reference proteins were purchased at sigma Aldrich, Egg whites from chicken (E0500), Albumin from chicken egg white (A5253) and Casein sodium salt from bovine milk (C8654).

5.2.2 Protein extraction methods

Based on the result of chapter 4, different methods were selected and performed on LM juice (see Figure 5-1). The first one is an SFE extraction by CO_2 (Separex) for 2 hours at 1000C and a flow of 6 kg/h. The freeze-dried sample was sieved to obtain 200 g sample between 0.5-1mm. A second extraction was performed by AIPP. Two protein fractions were obtained by 1) a one-step fractionation and 2) a two-step fractionation, respectively. Briefly, the freeze-dried sample was mixed with demineralized water at a 17.6 L:S ratio. The pH was lowered to pH 2 with 6M acid solution and homogenized at 350 rpm (IKA Eurostar 60) for 30 min. Afterwards, a centrifugation step (3220 g, 30 min, 4°C) created three layers, a floating lipid layer, a pellet, and a supernatant. The latter is the first protein fractions of the one-step approach. The second fraction was obtained by repeating the one-step approach and then adjusting the supernatant to pH 5 with 50 m% NaOH followed by a second centrifugation step (3220 g, 15 min, 4°C). The P2 was collected and the supernatant was discarded. Fraction SN1 and P2 were collected and freeze-dried. Lastly, heat treatment was performed. The freeze-dried liquid sample was mixed with water at a 1:3.2 ratio for 20 min. Afterward, the sample was heated to 120 °C for 30 min at 120 °C (autoclave, 200V Boxer, BRS) and the pressure in the container raised to 2 bar. After cooling, the sample was centrifuged (3220 g, 30min, 4°C) resulting in three fractions. The supernatant was collected and freeze-dried.

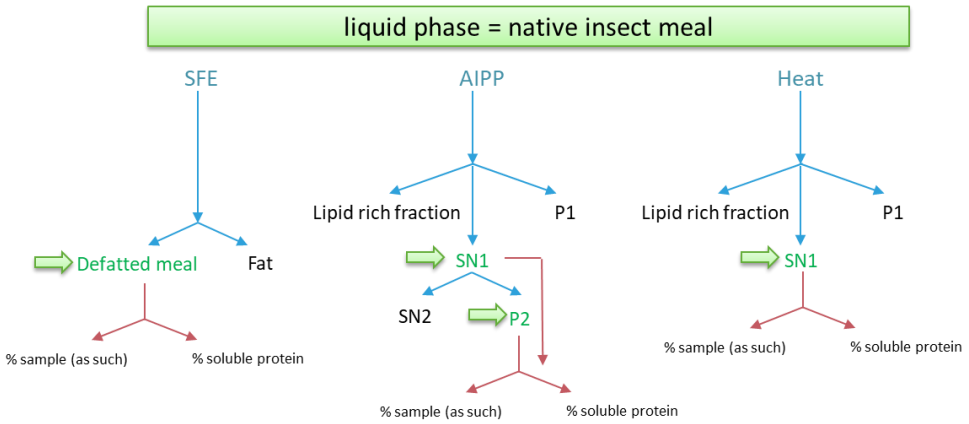

Figure 5-1 Schematic overview of the protein fractions that were collected and the tested samples

5.2.3 Proximate composition

Dry matter and ash content were measured gravimetrically by drying at 105 °C for 48h or at 550 °C for 6 h, respectively. The nitrogen content was determined based on DUMAS (Elementar, vario EL cube) and lipid content by Soxhlet (6h, diethyl ether). The amino acid composition was determined according to the method described in Chapter 2.

5.2.4 Sample characteristics

The percentage of insoluble material was determined gravimetrically after separating the pellet from the supernatant by centrifugation (5910 g, 20 min, 4 °C) and drying at 105 °C for 48 hours. The size of the proteins was determined via SDS-page that was performed as described in chapter 4 and around 3 mg soluble proteins/ml was loaded. SDS-page of the peptide fraction was performed with CriterionTM Tris-Tricine/peptide gels (Bio-Rad, cas 3450065). The sample was diluted to proximally 6 mg soluble protein/ml in demineralized water and centrifuged (3000 g, 5 min, 25 °C). Next, 5 µl of the sample was added to 4.75 µL Tricine sample buffer (Bio-Rad, CAS 161-0739) and 0.25 µl B-mercaptoethanol (Bio-rad, cas 161-0710). After heating for 5 minutes at 95 °C, 25 µl was loaded tot the gel and run in TTs buffer. A fixation solution of 40 % methanol and 10 % acetic acid was

used followed by a stain solution of 0.025 (w/v) Coomassie Blue G250 and 10 % acetic acid. Destaining was performed with 10 % acetic acid. A polypeptide ladder (Biorad, cas 161-0326, 26 kDa -1.5) was used as a reference.

5.2.5 Techno-functional properties:

After the removal of the exoskeletons, the proteins in the juice were presumed to be in their native state. The samples were not 100 % protein samples (neither the native meal nor the extracts) and will contain other compounds such as lipids, carbohydrates, and salts which will influence the properties. Therefore, the functionality of the sample, not the protein, was tested (see Figure 5-1, indicated in red). Firstly, the sample as such, containing insoluble compounds and a different protein concentration (according to the protein content and protein solubility) were tested. Secondly, to eliminate the effect of the insoluble compounds and to compare the properties of the proteins with the exclusion of protein concentration effects, samples were acquired after centrifugation with a constant theoretical concentration of the soluble proteins throughout all samples. However, the influence of carbohydrates, salts, and lipids could not be excluded.

(1) The **solubility** was determined according to Soetemans et al. (2019) [194].

(2) **Water and oil binding capacity** were determined based on Zhao et al. (2016) and Ogunwolu et al. (2009). Briefly, 1 g of sample was added to 10 ml water or oil in an overhead shaker for 5 min at 55 rpm (IKA Trayster digital). After a stabilization period of 30 min, the tubes were centrifuged at 3000 g, 30 min at 20°C. The supernatant was discarded, and the tubes were placed under an angle of 45 °C for drainage. After 10 min, the tubes were weighed. The water-binding capacity (WBC) or oil-binding capacity (OBC) was expressed as the amount of water or oil 1 g of sample (or protein) could retain [60][223].

(3) The **gelling capacity** was determined by solubilizing different concentrations (steps of 0.5 %) of the sample in milli-Q water at 30 °C for 30 min. Next, the samples were heated for 30 min at 95 °C and subsequently cooled in an ice bath. After 2 hours at 4°C, the sample was reversed and the minimal gelling concentration (MGC) was chosen as the smallest concentration where the samples did not loosen from the recipient. The procedure was performed on samples that were dissolved as such (with the presence of insoluble material) and with the supernatants after centrifugation (13000 g, 5 min, 25°C).

(4) **Foaming properties** were measured based on the method of Yi et al. (2015) and Schwenzfeier et al. (2013) [224][40]. A glass tube (diameter 2.1 cm) was designed in-house with a ruler printed on the side. A glass grit (diameter 1.5 cm) was placed at the bottom and air was blown in. 40 ml of protein solution was added and the volume was noted ($_{\text{Vliquid start}}$). The foam was created by bubbling air at a flow rate of 2L per hour for 1 minute. The volume of the liquid and foam phase ($V_{0\ \text{foam}}$) was noted 10 seconds after the airflow was stopped as well as after 30 min ($V_{30\ \text{foam}}$). The solution was either a 3 % sample solution as such or a 3 % soluble protein solution (centrifugation 3000 g, 15 min, 25 °C). The foam capacity and stability was calculated by formulas 10 and 11.

Foam capacity: $FC = \frac{V0\ foam}{V\ liquid\ start} * 100$ (10)

Foam stability: $FS = \frac{V\ 30\ foam}{V0\ foam} * 100$ (11)

5.3 Results and discussion

5.3.1 Proximate composition and characteristics of the obtained samples

The composition of the samples, before and after fractionation, is reported in Table 5-1. Freeze-dried juice had a protein content of 45 % and a lipid content of 43 % on

a dry matter basis. All protein fractions obtained after treatment displayed a decreased lipid content (a maximum of 19 % was detected) and increased or similar protein contents. The SFE resulted in the fraction with the highest protein content (57 %), followed by the heat extraction (55 %), the AIPP-P2 fraction (52 %) and the AIPP-SN1 fraction (45 %). The latter had a high protein recovery (73 %) but due to the presence of the added acids (ash content of 42 %), the protein content was low. The two-step AIPP was able to remove most of these added salts, resulting in an increased protein content, but also caused some loss of proteins and thus the protein recovery was much lower (32 %). The protein recovery of the SFE treatment was 85 % and 35 % for the heat treatment. The low recovery of the latter can be explained by the fractionation technique which was based on centrifugation force to remove the lipids and simultaneously fractionated the proteins based on solubility.

Table 5-1 Proximate composition of the samples, n=3.

	% DW	**% ash***	**% nitrogen***	**% lipid***	**% protein***
Juice	96,6 ± 0,7	4,2 ± 0,03	7,1 ± 0,0	43,2 ± 0,7	44,8 ± 4,9
SFE	90,7 ± 1,9	7,1 ± 0,7	**10,7 ± 0,0**	12,6 ± 0,2	**57,2 ± 1,2**
AIPP-P2	96,0 ± 2,2	13,9 ± 2,4	9,7 ± 0,0	10,4 ± 0,4	51,5 ± 2,7
AIPP-SN1	90,7 ± 2,5	**41,9 ± 2,4**	7,6 ± 0,0	8,3 ± 1,5	45,0 ± 1,1
Heat	84,9 ± 1,2	8,9 ± 0,6	9,3 ± 0,2	**18,7 ± 1,0**	55,1 ± 0,8

*Dry matter based.

Compared to the results obtained in chapter 4, the composition of the protein-enriched fractions was different. In the current chapter, the procedures were scaled to a higher volume and thus, small differences were expected.

- A pilot-scale SFE was used (Separex, 0.5 L extraction vessel) instead of the small-scale SFE (Jasco-SFE-semi-prep, 10 ml extraction vessel) and the flow rate was different (6 kg/h (=129 ml/min) instead of 2 ml/min). These changes resulted in a lower lipid removal (the lipid content of the defatted meal was 13 % instead of 5 % on a small scale).

- The heat treatment on a larger scale resulted in a fraction with a lower protein content (58 % based on nitrogen multiplied by 6.25, as was calculated in chapter 4 versus 62 %) and higher lipid content (19 % versus 3 %). Nevertheless, the process circumstances were the same. In this case, the higher volume of the sample probably resulted in a longer heat and cooling down period which could have influenced the fractionation.
- The AIPP-P2 fraction in chapter 4 had a higher protein content of 71 % versus 61 % but a similar lipid content. The method was also scaled up and the pH adjustment procedure was slightly altered.

Table 5-2 reports the amino acid profiles of the obtained samples. The amino acid profile of the juice, the SFE fraction and the AIPP-SN1 fraction were similar. This was expected since the protein recovery of the SFE faction and AIPP-SN1 was quite high. These treatments did not fractionate the proteins, but mostly removed lipids and thus the same proteins were present in the samples. The AIPP-P2 fraction exhibited a different profile and was relatively more rich in polar (41 % versus ± 34 %) and negatively charged amino acids (24 % versus ± 20 %). The fraction obtained after the heat treatment also differed and was richer in positively charged amino acids (39 % versus ± 30 %).

Table 5-2 Amino acid (AA) profile of the samples, n=2.

(mg/g sample)	Juice	SFE	AIPP-P2	AIPP-SN1	Heat
Arginine*	139,1 ±6,6	150,9 ±1,0	88,6 ± 1,3	124,0 ±3,8	195,6 ±4,5
Lysine* [E]	25,3 ± 4,6	35,2 ± 1,0	32,8 ± 0,0	29,9 ± 1,4	32,6 ± 0,2
Alanine	17,7 ± 1,6	24,7 ± 1,0	24,5 ± 1,9	21,2 ± 0,0	25,3 ± 0,0
Threonine [P E]	20,9 ± 4,5	26,3 ± 0,0	24,1 ± 1,0	19,0 ± 0,1	21,5 ± 0,1
Glycine	26,0 ±13,9	23,4 ± 3,1	23,6 ± 1,0	18,8 ± 1,0	24,3 ± 1,5
Valine [E]	26,5 ±11,4	28,0 ± 0,1	28,1 ± 1,3	22,8 ± 0,3	24,5 ± 0,3
Serine [P]	15,8 ± 0,9	21,7 ± 1,7	20,7 ± 1,1	17,2 ± 0,2	18,4 ± 0,2
Proline	22,6 ± 0,9	33,1 ± 0,5	23,6 ± 3,4	24,0 ± 1,4	31,3 ± 0,0
Isoleucine [E]	16,8 ± 1,2	25,6 ± 2,8	26,1 ± 3,6	20,6 ± 0,7	19,7 ± 1,1
Leucine [E]	24,2 ± 0,9	37,4 ± 3,4	37,1 ± 3,9	29,5 ± 0,7	29,1 ± 1,4
Methionine [E]	5,4 ± 0,4	7,8 ± 0,4	7,6 ± 1,8	6,3 ± 0,1	7,8 ± 0,5
Histidine* [E]	14,8 ± 0,8	21,0 ± 0,6	18,8 ± 0,7	16,3 ± 0,3	15,8 ± 0,6
Phenylalanine [E]	23,8 ± 1,4	32,8 ± 0,8	37,4 ± 1,4	24,6 ± 0,4	22,4 ± 0,4
Glutamate** [P]	54,5 ± 3,1	77,7 ± 1,8	78,0 ± 4,5	61,3 ± 5,2	74,9 ± 7,3
Aspartate** [P]	43,5 ± 2,6	61,4 ± 1,2	63,5 ± 2,7	46,7 ± 0,8	54,7 ± 4,8
Cystine [P]	3,6 ± 0,6	4,5 ± 0,3	3,7 ± 0,5	2,5 ± 1,1	2,3 ± 0,3
Tyrosine [P]	35,0 ± 2,7	46,8 ± 0,5	55,7 ± 3,2	33,3 ± 0,4	33,1 ± 0,1
% polar AA [P]	33,7 ± 1,0	36,2 ± 0,0	41,4 ± 0,3	34,8 ± 0,2	32,3 ± 1,3
% positively charge AA *	34,9 ± 1,6	31,4 ± 0,2	23,6 ± 0,9	32,9 ± 0,3	38,5 ± 1,1
% negatively charge AA **	19,1 ± 1,0	21,1 ± 0,0	23,8 ± 0,0	20,9 ± 0,7	20,4 ± 1,6
% EAA [E]	30,5 ± 1,4	32,5 ± 0,7	35,7 ± 0,4	32,6 ± 0,2	27,4 ± 0,2

The sum of the EAA (without tryptophan) pointed out that the SFE fraction and AIPP-P2 were more nutritional (considering the presence of the EAA) than the other samples (± 213 mg/ g sample versus ± 169 mg/g sample). However, these samples also contained more protein and thus an increase of EAA was expected. In respect to the relative amount of EAA, AIPP-P2 also had an increased value (36 %) and was more nutritional compared to juice (31%), SFE fraction (33%), the AIPP-SN1 fraction (33%) and especially the heat fraction (27 %) which was the least nutritional protein fractions obtained.

Besides the composition, also other characteristics of the samples differed. The native pH of the sample was for the juice, SFE and the heat fraction about pH 6.1-6.4 whereas pH 5.7 was noted for P2 and pH 2.5 for AIPP-SN1. These differences can be explained by the fractionation and drying conditions. The AIPP fractions were freeze-dried either at acid pH (SN1) or at the iso-electric point at pH 5 (P2).

Table 5-3 also reports soluble fraction at native pH and protein solubility. The sample and proteins of AIPP-SN1 and heat were highly soluble. Juice and SFE exhibited a relative good sample solubility and 50 % of the proteins were soluble at native pH. Whereas AIPP-P2 had a poor sample and protein solubility. These results were taken into account for calculating a specific soluble protein concentration.

Table 5-3 Characteristics of the samples, n=3.

	% soluble fraction at native pH?	**Native pH**	**% soluble protein at native pH**
Juice	57,2 ± 1,9	6,4	51,5 ± 0,7
SFE	61,4 ± 0,8	6,4	53,3 ± 0,3
AIPP-P2	35,2 ± 0,9	5,7	20,0 ± 0,6
AIPP-SN1	97,1 ± 0,1	2,5	86,7 ± 0,1
Heat	90,3 ± 0,4	6,1	73,3 ± 4,6

Figure 5-2 illustrates the protein sizes of the soluble proteins present in the fractions. The juice and SFE samples showed the same patterns as was reported in chapter 4, and little differences were observed between the two. The main protein molecule sizes were 75 kDa, 50 kDa, 37 kDa, and around 25 kDa, and to a lesser extent 15 kDa and 17 kDa. Only the proteins of 10 kDa were not detected in comparison to the data in chapter 4. No explanation was found for the absence of these proteins. However, the juice of the present study was derived from a different LM batch and the storage time in the freezer was much shorter. It could be that during the mechanical pre-treatment and storage, some protein hydrolysis took place with the juice used in chapter 4. In respect to the AIPP-SN1 fraction, a higher concentration was loaded (6 mg/ml) and a similar pattern to the juice was observed, indicating that the acid treatment did not cause hydrolysis. Regarding the size distribution of the soluble proteins of AIPP-P2 and the heat fraction, a higher concentration was also loaded in order to make the proteins visible (6 mg/ml instead of 3 mg/ml). This indicates a low concentration of these proteins. The AIPP-P2 soluble proteins had a protein size between 37 and 50 kDa, around 17 kDa and at

6.5 kDa whereas the heated sample only had soluble proteins between 37 kDa and 50 kDa. These two samples were expected to have a different pattern since the proteins were fractionated. However, if a 3 mg/ml concentration was loaded, no bands were visible indicating that the soluble proteins (nitrogen) were not in the form of proteins (higher than 3.5 kDa). It could be that a high concentration of free amino acids are present and thus cause an overestimation of the protein solubility.

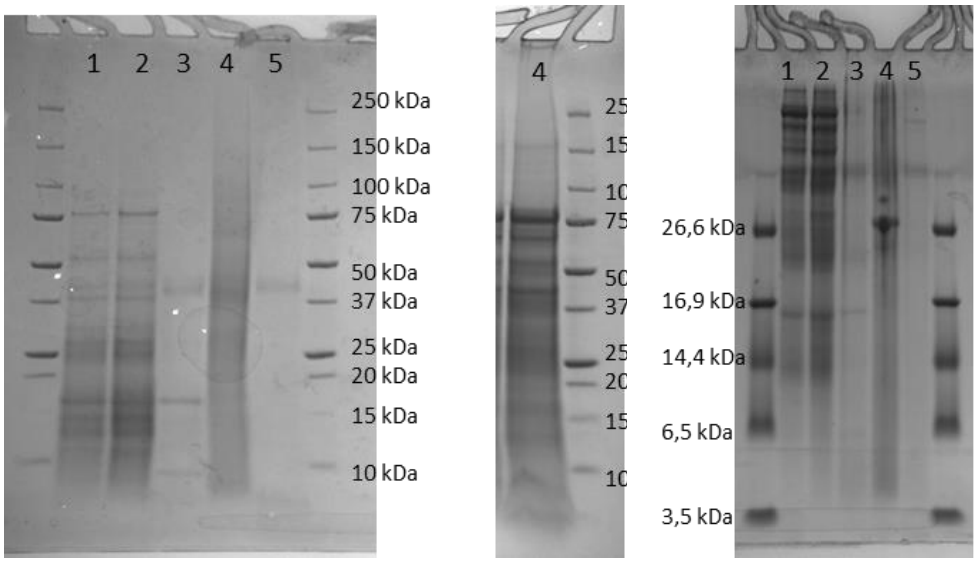

Figure 5-2 Protein size distribution of the soluble fraction of LM samples 1) juice, 2) SFE, 3) AIPP-P2, 4) AIPP-SN1, 5) Heat.

5.3.2 Techno-functional properties

5.3.2.1 Solubility

Figure 5-3a illustrates the three different protein solubility of reference proteins caseinate, egg white and albumin. Caseinate showed a typical U-curve with a clear drop in protein solubility around the isoelectric point (pI) (pH 4.5) and high protein solubility at acid and alkaline pH. Albumin showed a similar curve but less pronounced and a pI was observed at pH 5 where the solubility decreased but

throughout the whole pH range, a minimal protein solubility of 55 % was obtained. The protein solubility curve of egg white does not show a minimum at the pI and obtained a high solubility over the whole pH range.

Figure 5-3b reports the protein solubility profile of the LM samples. LM juice had a similar solubility profile as albumin with a minimum solubility of 30 % and a pI at pH 5. After freeze-drying, the solubility at acid and neutral pH decreased by at least 10%. For the proteins of the SFE fraction, an increased solubility was observed at acid and neutral pH whereas the heat treatment generated a protein fraction that was highly soluble throughout the whole pH range (much like egg white). The latter was expected since the fractionation procedure only extracts soluble proteins. Kang et al. (2016) described the effect of SFE on bovine liver proteins and also found an increased protein solubility at neutral pH [225]. The AIPP-SN1 fraction exhibited a higher solubility than the juice proteins whereas the AIPP-P2 fraction exhibited a much lower solubility. These results were in line with the results obtained in chapter 4 and with the result of Rawdkuen et al. (2009) who also reported a major decrease in protein solubility of tilapia surimi after a two-step AIPP extraction [226].

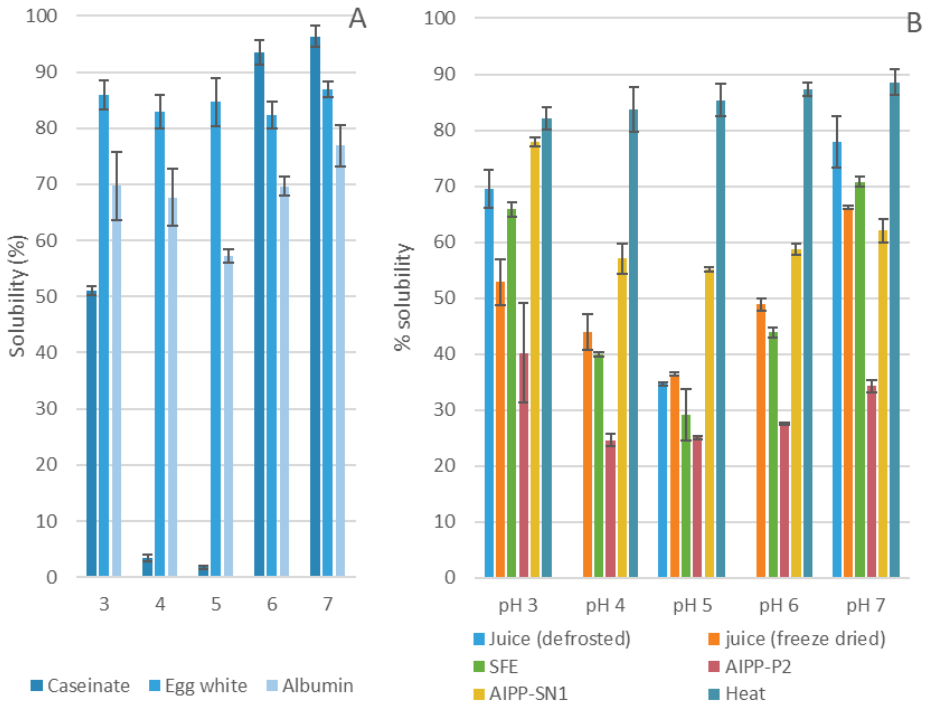

Figure 5-3 Protein solubility of (a) the reference proteins and (b) the LM samples, n=3.

5.3.2.2 Water and oil binding capacity

Figure 5-4 illustrates the water and oil binding capacity (WBC) of the reference proteins and the LM samples. Of the reference proteins, only caseinate was capable to bind water. Egg white, albumin and also the heat fraction and the SN1 fraction exhibited a high solubility and thus could not retain water. The proteins of P2 fraction exhibited the best WBC and the SFE fraction also had an increased WBC compared to the juice. Kang et al. (2016) also reported an increase in WBC of the liver proteins after the SFE extraction [225].

No published data was found on the WBC of LM proteins. However, some reports were found on the YM. Zielińska et al. (2018) reported a WBC of 3.95 g/g for YM proteins obtained after an alkaline IPP and 1.29 g/g for the YM meal (grounded

insects) [68]. The juice (0.9 g/g) and P2 (3.4 g/g) in the current study had a comparable WBC.

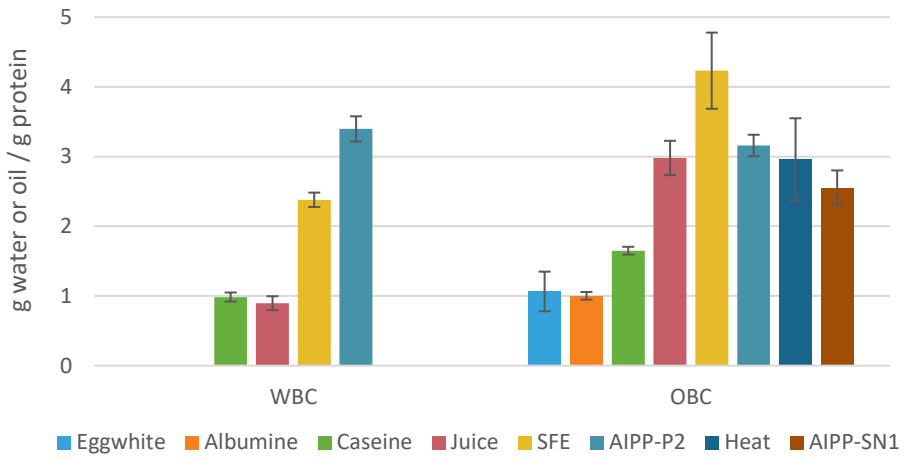

*Figure 5-4 Water or oil binding capacity expressed in g/g proteins. The protein content of the LM samples was calculated based on the analysis of the amino acid and for the reference proteins on the nitrogen measurement (*6.25), n=3.*

Figure 5-4 also reports the OBC. All insect-based samples performed better than the reference proteins for which an OBC below 2 g oil/g protein was found. The LM samples juice, P2, heat, and SN1 all had a similar OBC whereas the SFE fraction, in contrast to the findings of Kang et al. who found no significant differences before and after SFE, showed the highest OBC. Zielińska et al. reported a lower OBC for YM meal (grounded insect) of 1.29 g/g while in the current study for LM juice (not including the chitin, and the cuticle proteins) an OBC of 3 g/g was obtained. The differences could be related to the differences in species since Zielińska et al. also proved that the OBC could differ significantly between species or the different pre-treatment.

5.3.2.3 Gelling capacity

Egg white and albumin are known for good gelling properties and exhibit a low MGC. Only 1 % of the albumin sample was needed to form a gel. Some LM samples also showed good gelling properties based on the results reported in Table 5-4. Especially the SFE fraction showed a low MGC of 3 %. The MGC of LM juice was double but still lower than the MGC of caseinate. It seems that a higher lipid content did not interfere with the gelling properties as LM juice had a lipid content of 42 % but still exhibited a low MGC. Yi et al. (2013) also showed that the LM had gelling abilities with a 30 % soluble protein solution of the supernatant after an aqueous extraction at neutral pH could form a gel [40] and Zhong et al. (2008) also found an improved gelling ability of whey proteins after SFE [205]. Fraction AIPP-SN1 also showed a lower MGC than caseinate whereas the fractions AIPP-P2 and heat had poor gelling abilities and a higher concentration than 50 % of the sample was needed.

The SFE fraction had a lower MGC than juice but this was expected since the (soluble) protein content is much higher. In order to compare the gelling properties of the proteins, an experiment was performed with the supernatant of the samples. However, the same trends were observed. Soluble proteins of P2 were tested to a 5 % concentration and no gel was formed. This was in contrast to the findings of Cortés-Ruiz et al. (2008) and Hrynets et al. (2010) who reported improved gelling properties of proteins extracted by AIPP from giant squid and turkey [227],[228]. However, during the AIPP, the pH was lowered to 3 whereas in the current study the pH was adjusted to 2. In addition, the pH of the samples was adjusted to neutral before gelling. The more acid environment could have resulted in more conformation changes and the pH has a high influence on the gelling property.

Table 5-4 Minimal gelling concentration of the reference and LM samples, n=3, nd= not determined.

MGC	As such (% sample)	Soluble protein (% soluble protein)
Egg white	1.5	nd
Albumin	1.0	nd
Caseinate	12.1 ± 0,2	nd
Juice	6,7 ± 0,2	1.70 ± 0,07
SFE	3,0 ± 0,01	1,078 ± 0,004
AIPP-P2	>50	> 5,1
AIPP-SN1	7,3 ± 0,5	3.2
Heat	>50	> 5,1

5.3.2.4 Foaming

All LM samples were examined for foaming properties but only the SFE sample was able to form a foam with a foam capacity of 108 ± 15 % (3% soluble protein). Kang et al. (2016) also reported a significantly improved foamability of the liver proteins after SFE [225]. However, the analysis needs to be repeated since a large variation was observed in foam stability and formation. Figure 5-5 illustrates a very compact foam with small bubbles in replica A whereas replica B has a more loose foam with large air bubbles. Yi et al. (2013) also found no foaming ability for the LM with a 1.7 % soluble protein solution of proteins extracted by an aqueous extraction [40].

Figure 5-5 Picture of foam created by 3 % soluble protein SFE

5.3.3 Impact of defatting approach on TFP

The LM samples were compared in their native state, meaning that for instance, the pH was different among samples. However, the pH and also the ionic strength can influence the TFP greatly and thus de influence of the defatted approach on the TFP of the samples (not proteins) was evaluated in this stage of the study. Table 5-5 summarizes the results for the techno-functional properties (TFP) of the LM samples and the TFP of each technique was compared to the native (juice) samples TFP.

Table 5-5 Summary of the TFP of the LM samples

	Solubility	WBC	OBC	Gelling	Foaming
Juice	+	+	+	++	-
SFE	+	++	++	+++	+
AIPP-P2	--	+++	+	--	-
AIPP-SN1	++	--	+	+	-
Heat	++	--	+	--	-

In respect to the LM fraction defatted by SFE, structural changes in the conformation of the proteins were expected since the TFP changed while the amino acid composition and protein size pattern were similar. These conformation changes occurred when the proteins came into contact with the non-polar supercritical CO_2 solvent under a pressurized environment probably causing the non-polar amino acid side-chains that were located in the core of the proteins to become more exposed. This formation would increase the availability of more hydrophobic amino acids which explains the increase OBC. However, the increased OBC could also be explained by the differences in the physical characteristics. The freeze-dried juice was sieved to gain a particle size of 0.5-1mm which was a restriction of the SFE treatment. In addition, the defatted meal was subjected to a higher pressure and the defatted powder obtained from SFE was harder and less voluminous than the other freeze-dried fractions. The OBC is known to be strongly influenced by the capillarity and density of the powder and thus this could have also caused the increased OBC. The conformation changes of the proteins may have stimulated

protein-to-protein interactions for gel formation. The proteins exhibited high gelling properties and potentially, the reconformation has exposed more methionine and cysteine and thus enhanced the sulfide bridges resulting in better gelling behavior. The WBC was also increased compared to the juice fraction, explicable by the removal of lipids [229].

The AIPP-P2 fraction showed a higher WBC which indicated that more water-binding sites were made available by the treatment. The solubility curve already indicated that the proteins in P2 were denatured and precipitated which increased the binding sites for water [230],[225]. Nazareth et al. (2009) stated that the WBC is influenced by the extent of denaturation and the type of protein aggregation. As such, the reconformation of the proteins in P2 caused by the extraction conditions were in favor of the WBC. In addition, P2 contained more polar amino acids that probably increased the interactions between water and proteins. The sample was not able to form a gel due to 1) a limited solubility of the proteins which resulted in a low protein concentration for gel formation and 2) the low-sized protein molecules, as was visualized via SDS-page and 3) the disturbance of the abundant non-soluble particles to form a protein network. The sample was also tested after centrifugation (removal of the non-soluble particles) and at a soluble protein concentration of 5 % but the proteins were still not able to form a gel which could indicate that dense aggregates had formed with poor gelling behavior which often occurs at pI (this samples native pH is 5.7) [40].

The TFP of the AIPP-SN1 fraction also differed from that of the juice. The AIPP probably caused partial denaturation due to the acid environment which decreased the gelling property. The TFP could also have been affected by the high salt concentration and the native pH (pH 2.5). The AIPP-SN1 fraction exhibited a higher solubility than the juice proteins whereas the AIPP-P2 fraction exhibited a much lower solubility. In Chapter 4 a hypothesis was formulated to explain the decreased

solubility of the AIPP-P2 proteins based on the results of Bußler et al. (2016) who measured soluble but unfolded protein at acid pH [72]. More specifically, it was hypothesized that the irreversible denaturation and precipitation occurred at the pI. The data in Figure 5-3 confirmed this since the AIPP-SN1 proteins are highly soluble. In addition, the SDS-Page also illustrated that no hydrolyzes occurred at the acid stage. However, by performing the two-step AIPP, the proteins have a net-zero charge at pI and the electrostatic repulsion is decreased. At this point, the partially denatured proteins can form aggregates that precipitate and are irreversibly (for the most part) denaturized. No SDS-Page was performed on the denatured proteins in P2 but a higher concentration of large-sized proteins would be expected.

The proteins in the heat defatted fraction were highly soluble but no other TFP were determined. The SDS-Page results indicated that the soluble proteins were mostly free amino acids (or peptides with a molecular weight lower than 3.5 kDa). This explains the absence of TFPs since, for most TFPs, the length of the protein impacts, for example, the ease of forming a protein network during gelling or the covering of the interfacial layer at oil in water or air in water formations [80].

5.4 Conclusions

The study confirmed that the type of fractionation approach can significantly impact these techno-functional properties. Juice of the LM, before fractionation, had no foaming ability but good gelling and oil binding properties which makes these proteins interesting for incorporation in meat (substitute) products since these two properties improve meat texture and palatability. An SFE treatment resulted in a protein fraction with an increased foaming and gelling ability. These properties are partially interesting for solid-foam food products such as cakes, desserts, …. The AIPP treatment generally resulted in protein fractions with decreasing TFP while the heat treatment completely lost all its techno-functional properties.

5.5 Acknowledgments

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Chapter 6.

EVALUATION OF CHITIN CHARACTERISTICS IN DIFFERENT LIFE STAGES OF THE BLACK SOLDIER FLY

6.1 Introduction

The chitin market is expected to grow and more chitin/ chitosan-based applications will found their way to the market. To keep up with this demand and the exploration of new applications, there is a search for more and new chitin sources [231] and insect-based chitin is investigated as a potential source [232]. The number of industrial insect farms is growing worldwide and with this new livestock chain, new byproducts are formed such as cocoons, sheddings from molting and dead insects, all containing chitin. In addition, indications of chitin being a non-digestible fiber that negatively influences the digestibility of proteins and lipids, have led to a tendency of removing chitin for food and feed applications. Industrial isolation/production of chitin employs crustacea waste that is associated with a discontinuous supply, due to seasonal variations and high heterogeneity of the biomass (different concentrations of impurities and chitin crystallinity) since different species are combined. These disadvantages would be tackled considering insect-based products, since rearing companies rear year-round and keep insect species separately. A best-case scenario would be to combine all chitin-rich byproducts to employ one purification process to obtain a robust, homogeneous product with well-defined characteristics. In this case, the characteristics of chitin in all life stages should be similar.

In the current study, chitin from different life stages of BSF biomass (larvae, prepupae, pupae, sheddings of the larvae, cocoons, and flies) was extracted for examining their physicochemical properties. More specifically, purified chitin samples of BSF biomass, as well as shrimp and squid chitin, were subjected to FTIR, XRD, TGA and a deacetylation step to evaluate among other their chitin formation and crystallinity. To our knowledge, no research has been done on the chitin extracted from all life cycle stages of the BSF except for the study of Wάsko et al. (2016) who examined the flies and the pupae of the BSF [135]. In addition, the chitin

content of the raw samples was measured and reported. To our knowledge, this is the first study that reports on the physicochemical properties of the prepupa, pupae, sheddings, and cocoons of the BSF.

6.2 Material and methods

6.2.1 Starting material

The BSF larvae were purchased from a local breeder (Millibeter, BE). The larvae were harvest in the last larval stage, removed from the substrate and delivered alive. After a few hours, the bins contained sheddings that were collected by gently blowing air on top. Some larvae were further reared in small containers on moisturized broiler feed to collect all life stages. The prepupae were collected after they crawled out of the substrate and were distinguished by the dark color, a flexible body and they stayed relatively still unless exposed to bright light. A considerable amount of prepupa was allowed to search a pupation spot in cardboard pieces. The pupal stage was indicated after an examination where the pupae had to meet four conditions, (1) being stiff, (2) exhibiting an s-shape (see Fig.1) (3) white eyes (see Figure 6-1) and (4) absence of a dark digestion track. Some pupae were left to pupate and after flies emerged, the cocoons were identified (small hole in the top) and collected. Flies were collected after death. Commercial chitin was purchased from Sigma-Aldrich (from shrimp shells-C9213), while squid pen was kindly provided by a local restaurant (1.3 kg of fresh squid provided 1.05 g wet squid pen).

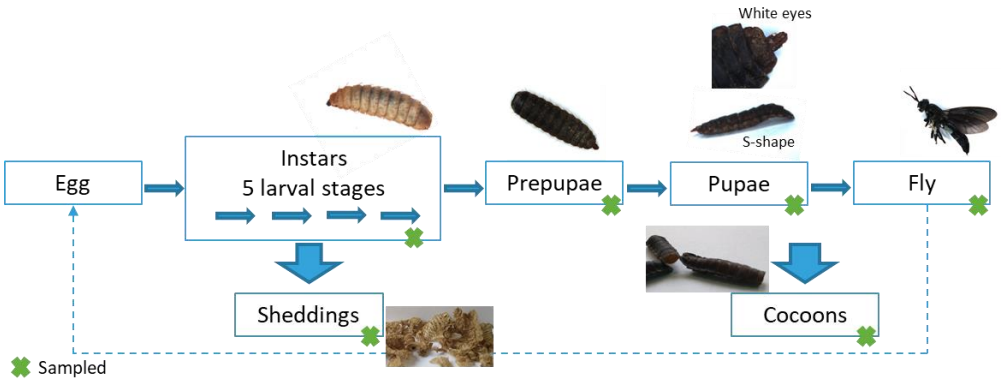


Figure 6-1 Different life stages of BFS and their characteristics for identification

6.2.2 Extraction of chitin

The collected insect-based samples were dried (105 °C – 48 h) and grounded in a mortar to break the exoskeleton. Next, chitin was extracted in triplicate for every life stage by a multi-step process. First, demineralization was performed at a solid: liquid ratio of 1:10 (m/v) with HCl 1M at room temperature for 1 hour. Deproteinization included a 1M NaOH treatment (solid:liquid ratio of 1:25 (m/v);1h at 80°C) that was repeated until the absence of color (12 times). Solids comprising the chitin were separated from the liquid by filtration (pore size 25 µm (49 PA (25/14), Solana). Finally, the chitin was washed with demi water till neutral pH and dried at 105°C for 48h.

6.2.3 Characterization of extracted chitin

Fourier-transform infrared spectroscopy (FT-IR) spectra of the purified chitin samples were measured in an ALPHA spectrometer under Attenuated Total Reflectance (ATR) mode (Bruker, Belgium) with a resolution of 4 cm-1 and 32 accumulations. The data was processed in OPUS software. All samples were measured in triplicates to guaranty reproducibility.

X-Ray Diffraction (XRD) measurements were performed in triplicates on the purified chitin samples. Prior to the measurement, each sample was further grounded in a mortar. XRD patterns were recorded with an Empyrean system equipped with a

cobalt tube. Data were collected at 40 mA, 45 kV and a scan speed of 0.067335 °/s with a PIXcel detector and a scan angle between 0 and 45 °. Highscore Plus (PANalytical) software was used to convert the cobalt measurement to copper. The crystallinity index (Icr) was calculated from the intensity (counts) at 15,0° (Iam, amorohous phase) and 19, 3° (I110) °2θ as indicated in formula (12).

$$Icr = \frac{I110 - Iam}{I110} * 100 \quad (12)$$

Thermogravimetric analysis (TGA) was obtained by Netzsch-STA-449C. The conditions were the following: about 15 mg of sample, temperature ramp from 25°C to 625°C at 10°C/min, nitrogen atmosphere with a flow of 70 ml/min. The chitin samples after purification were measured in triplicates. All samples were measured in triplicates

6.2.4 Deacetylation

Deacetylation was performed by applying a heat treatment at alkaline pH (1:30 sample:50 m% NaOH (m:v) ratio, 90°C, 1 or 3hours, under stirring) on dried, purified chitin samples in pressure-resistant glass vials. Next, samples were filtered over a Whatman 589/2 (4-12 µm pore) with water till a neutral pH was reached, washed three times with ethanol and dried for 48h at 105°C. Deacetylation and analyses were performed in triplicates unless stated otherwise.

6.2.5 Chemical analysis

The chitin-containing biomass samples were dried at 105°C for 48h and dry matter (DM) was determined gravimetrically. The chitin content (on dry matter basis) in the biomass was determined by two different methods. The first method involves a gravimetric analysis. The biomass obtained after the purification (demineralization and deproteinization) was presumed pure chitin and the proximate chitin content was calculated based on dry matter as detailed in formula 13.

$$Crude\ or\ approximate\ chitin\ \% = \frac{m\ after\ demineralization\ and\ deproteinisation\ (dm)}{m\ biomass\ (dm)} * 100 \quad (13)$$

The second method was based on the quantification method described by D'Hondt et al. [233], were the (non-purified, dried) biomass was first hydrolyzed to release glucosamine and acetate followed by an LC-MS analysis to determine the glucosamine concentration and HPLC-RID for acetate measurements. The degree of deacetylation (DDA) was calculated based on this quantification as described by D'Hondt et al. (2019).

6.2.6 Statistical analysis

All measurements were performed in triplicates unless stated otherwise. The data were expressed as the averages with the standard deviation. Significant differences were evaluated by Anova in Excel ($P<0.05$) and a Tukey post hoc test was performed by using IBM SPSS software. All measurements of FTIR, XRD, and TGA, were done in triplicates and a singular measurement of every sample is shown after evaluating the similarity of the triplicate.

6.3 Results and discussion

6.3.1 Chitin content of non-purified samples

The dry content and chitin content for all BSF samples as well as the squid pen are summarized in Table 1. The dry matter of the BSF samples varied between 30 % and 94 % (Table 6-1), with the lowest value for the BSF larvae and the highest for the BSF cocoons. The chitin content of BSF samples was determined via the two methods (gravimetrically after purification or via a glucosamine determination, described in material and methods). In general, during the first phases of the insect life cycle, the chitin content increased slightly (from 8 to 11 %), to decrease again in flies (<8 %). Significantly higher values were measured in the sheddings, cocoons (> 23 %) and the squid pen (36 %). Method 2 was not applied on the squid pen for because the amount of sample was limited and all squid sample was purified to obtain enough material for the characterization analysis.

When comparing the two chitin quantification approaches, differences were observed, although the general trends were similar. The first method may underestimate the chitin content due to 1) partial deacetylation (because deproteinization is performed at 80°C and involves NaOH) during deproteinization leading to losses of acetate and 2) losses of chitinous material during the extensive filtering- washing process. The second method, on the other hand, was performed on the original sample and other sources of acetic acid may result in an overestimation of acetate and thus also of the chitin content. The observation that method 1 resulted in a higher chitin content compared to method 2, could point towards an incomplete purification process for some samples. Especially for the sheddings, the chitin content differed significantly (31.1 % versus 23.7%). An additional singular trial was performed for the sheddings with a more extensive deproteinization process (15 repeats instead of 12, method 1) and a decreased chitin content of 26.6 % was measured which is more in line with the result of the second method. These findings suggest that the sheddings of the BSF were more challenging to clean compared to the other samples. As method 2 (glucosamine determination) was identified as a more accurate chitin quantification approach, further discussion will be based on these values. The high chitin content in sheddings and cocoons (± 23 %) was expected since these samples consist only of exoskeletons without flesh. Prepupae and pupae were richer in chitin (11%) compared to the larvae (8%), pointing towards increased production of chitin during these stages (the weight in dry matter of the larvae and pupae was similar), potentially for increased protection during the delicate pupate-phase. No literature was found to support this hypothesis. The chitin content of the larvae was in line with the range reported in literature which was between 2 and 11 % [234],[232],[41]. This large variation is explicable by different quantification methods but also by the rearing conditions which impact larval weight & length and the larval stage at harvest. A slightly higher chitin content of prepupae (11 % versus 6-9 % [186],[33]), and sheddings and cocoons (23 % versus 20 % [232]) was measured compared to literature. The chitin

content of the insect's shells (cocoons and sheddings) was very similar to the chitin content of the crustacean shells (around 25 %, before purification) [119], [235].

Table 6-1 Dry matter of BSF and control samples and chitin content in non-purified & purified samples, n=3.

		Whole biomass		Purified chitin samples
	DM (%)	**% chitin (method 1)**	**% chitin (method 2)**	**% chitin (method 2)**
BSF larvae	29.5 ± 0.3	9.5 ± 0.6	7.8 ± 0.3	96.3 ± 3.7**
BSF prepupae	51.5 ± 1.1	9.1 ± 0.02	10.9 ± 0.7	107.2 ± 4.4**
BSF pupae	60.9 ± 0.9	10.3 ± 0.7	10.7 ± 0.1	97.8 ± 2.0**
BSF shedding	92.3 ± 0.5	31.1 ± 0.3	23.7 ± 1.9	86.4 ± 1.4**
BSF cocoon	94.2 ± 0.9	23.8 ± 1.5	22.4 ± 0.9	103.2 ± 3.1**
BSF flies	60.5 ± 3.1	5.6 ± 0.4	8.4 ± 1.9	96.7*
Squid pen	90.5 *	35.5 *	nd	107.5*
Shrimp chitin	93,5 ± 0,1	nd	nd	97.6 ± 2.0

Method 1: gravimetric based; Method 2: glucosamine-measurement based; * single measurement, ** duplicate measurement, nd: not determined

6.3.2 Characterization of purified chitin

All samples underwent the same extraction procedure, except for commercial shrimp shells that were extracted by the supplier with an unknown method. The chitin content of all samples was above 95 % (Table 1) confirming clean samples, except for the sheddings where a lower value of 86 % confirmed the hypothesis that chitin from this source was more difficult to purify. All samples were characterized using different methods to study the chitin more in detail.

6.3.2.1 FTIR

Every organic bound has a characterized resonation frequency in the infrared spectra and thus FTIR can be used to study the molecular structure by producing a spectrum showing at which wavelengths infrared was absorbed by the sample. For chitin, characteristic wavelength at 3480-3450 cm^{-1} (O-H stretch); 1660,1627 cm^{-1} or

1656 cm^{-1} (C=O stretch); 1560 cm^{-1} (N-H bend, C-N stretch); 1420 cm^{-1} (CH2 ending and CH3 deformation); 1380 cm^{-1} (CH bend, CH3 symmetrical deformation); 1020 cm^{-1} (C-O-C asymmetric stretch in phase ring) and 890 cm^{-1} (CH rind stretch) have been reported repeatedly and are independent of the source (shrimp, prawn, crab, lobsters, silkworm or grasshopper) [143],[236],[125],[237]. Figure 6-2 shows the FTIR spectra for the shrimp, squid and BSF samples. Two regions from 3500 to 2800 cm^{-1} and especially in the fingerprint region from 1700 to 500 cm^{-1} showed high absorbance. Some samples also exhibited some negative absorbance from 2300 till 1900 cm^{-1} which can be explained by the variance in concentration of atmospheric water and carbon dioxide. The similarity of the spectra suggests the same quality except for two additional absorbance bands at 2920 cm^{-1} and 2850 cm^{-1} for the BSF samples. This may indicate an impurity in the sample, perhaps a compound that is also responsible form the slight coloration of the sample. The characteristic absorbance bands for chitin were all present in the spectrum of the BSF samples.

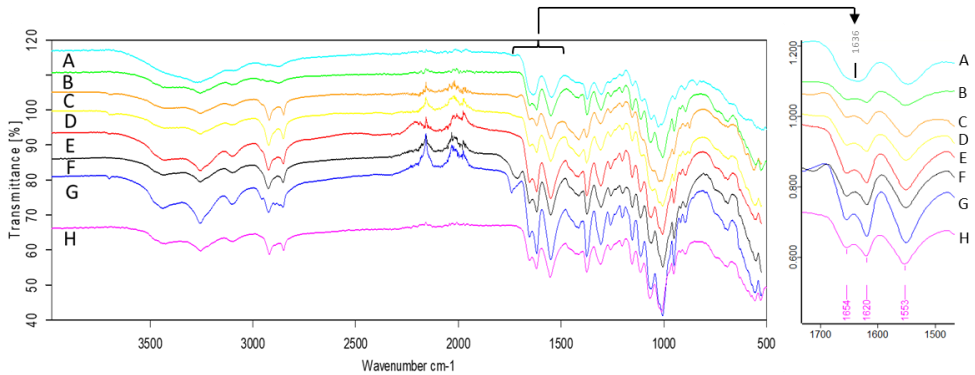


Figure 6-2 FTIR spectrum of controls and BSF samples: A) squid, B) commercial shrimp, C) larvae, D) prepupae, E) pupae, F) cocoon, G) fly, H) sheddings.

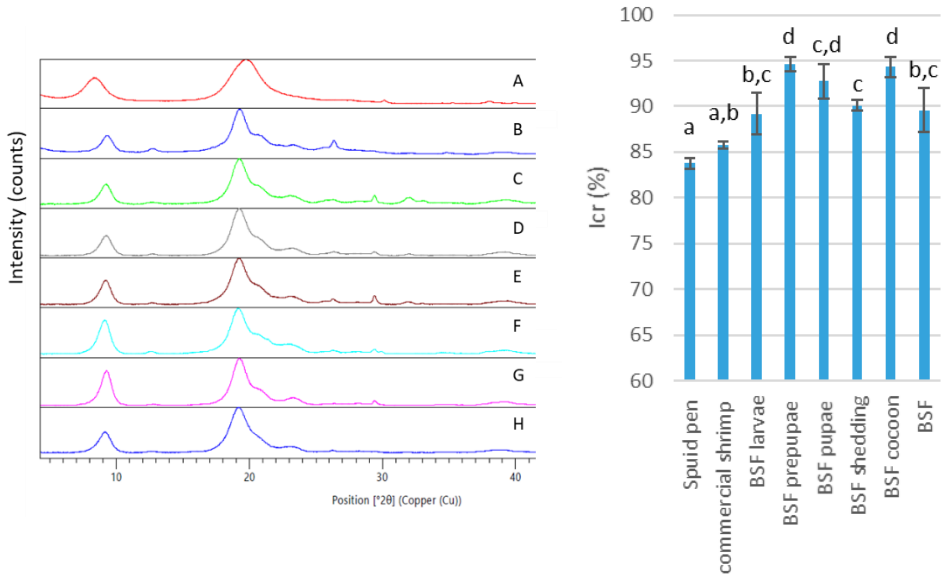
FTIR spectroscopy is also commonly used to identify the crystalline form (α or β) of chitin as differences in hydrogen bonding are detectable. The carbonyl group of α-chitin is involved in two hydrogen bonds, one intramolecular (between the carbonyl group and -CH2OH) that has an absorbance at 1620 cm^{-1}, and one intermolecular (between -NH- and the carbonyl group) at 1660 cm^{-1}. ß-chitin only exhibits one signal

at 1650 cm^{-1} as a result of the weaker intramolecular hydrogen bond [238][101]. In the current case, squid pen is known to contain β-chitin and exhibited a band at 1636 cm^{-1}. Commercial shrimp chitin showed two absorbance bands which were expected since shrimp is al well known α-chitin source. The samples of the BSF all showed two bands at 1654.08-1652.64 cm^{-1} and 1619.69 -1618.32 cm^{-1} and thus were identified as α-chitin. Most insects that are studied in published work were identified as α-chitin, for example, the *Melolontha melolontha* [106], different grasshoppers [125],[141], the potato beetle [139] and crickets [146]. Waśko et al. (2016) also found that the larvae and flies of the BSF contained α-chitin [145].

6.3.2.2 XRD

XRD involves the diffraction of X-rays by a crystal structure in the sample and thus allows the study of the atomic and molecular structure of the crystal. Figure 6-3a visualizes the XRD spectra of all chitin samples. The insect samples all have similar peaks between 4 and 25° (around 9.4°; 13.0°; 19.3°; 20.8°, 23.2°, and 29.5°) and the same peaks were found in the commercial shrimp sample (9.4°; 12.9°; 19.4°; 20.9° and 23.6°). The spectra of squid pen, however, showed only two, more broad peaks at 8.4° and 19.7° indicating that squid pen is less crystalline than the others. The results of the current study were in line with published data regarding the shrimp chitin and squid pen. Considering the BSF samples, Waśko et al. (2016) found similar peaks for the BSF cocoons and flies (at 9°, 19°, 22°, 24°, and 30°). Figure 6-3b illustrates the crystallinity index (Icr) for all samples. Commercial chitin had a lower Icr than the insect samples and was only slightly higher compared to squid chitin. This result was not expected but could be explained by the differences in extraction. For instance, Gbenebor et al. (2017) reported a shift between 79 % and 87 % on the same sample but purified differently [239]. The BSF samples and the squid pen were extracted the same way and a significant increased Icr of the BSF samples was observed. This was predicted since squid pen is ß-chitin which has a lower crystallinity. Significant differences between the BSF samples were also detected (P=

6.98 E-07). The prepupae and cocoons were significantly more crystalline (94 %) than the chitin originating from the fly, the sheddings and the larvae (89%). Kurita (2001) and Kaya et al. (2015) already reported different Icr for different α-chitin species of crustacea and grasshoppers (Icr from 63 % to 73 %) [120],[125] and the current results suggest that Icr-values can also vary throughout the life stages.



(a) (b)

Figure 6-3 (a) XRD spectrum of controls and BSF samples: A) squid, B) commercial shrimp, C) larvae, D) prepupae, E) pupae, F) sheddings, G) cocoon, H) fly; (b) Crystallinity index of the samples, n=3.

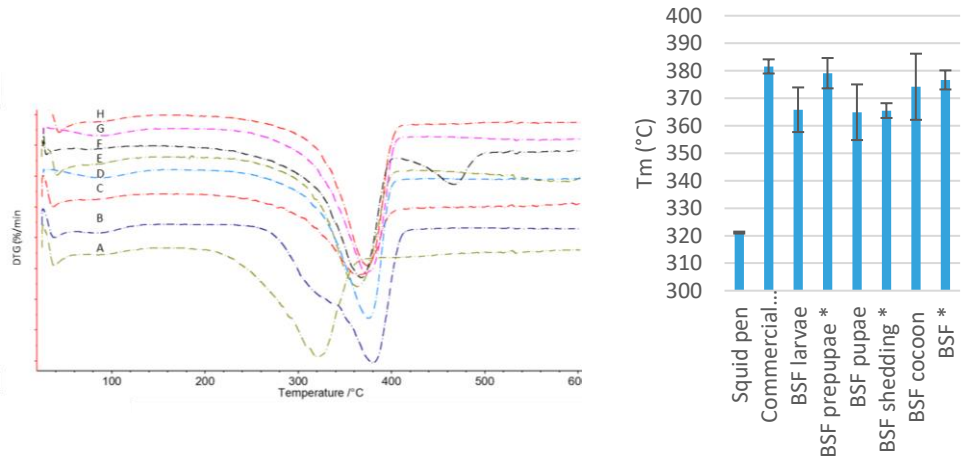
XRD is also suitable for the identification of the chitin formation. Jang et al. (2004) stated that α-chitin exhibits four sharp crystalline peaks at 9.6; 19.6; 21.1 and 23.7° whereas β-chitin only has two broader peaks at 9.1 and 20.3° [238]. Based on the results, the insect samples were identified as α-chitin. Nevertheless, some small differences were detected between the BSF samples, even though they were all characterized as α-chitin. For example, the shoulder of peak 19.6° (at 21.1°) became less pronounced with the sample of the fly. This peak indicates a small decrease in crystallinity for fly chitin. Kaya et al. (2014) also found small differences in the

location of peaks between α-chitin of different insect species [240]. The same is notable in the study with 7 grasshopper species where the extraction method was kept constant. The authors suggested that not only the isolation method but also the species impact the crystallinity [125],[240].

6.3.2.3 Thermogravimetry analysis (TGA)

A thermogravimetric analysis reports the thermal characteristics of the sample and the degradation temperature of a more crystalline sample will be higher. The first derivative of the TGA curve (DTG) for the samples is given in Figure 6-4a. Previous studies reported two mass losses for chitin samples, one representing water loss and one for the thermal decomposition of chitin [101]. A similar profile was measured in the current study for all samples where a very small peak was detected at 60°C (water loss) and a high mass loss around 370°C. The samples were dried in the oven at 105°C, explaining the small peak at 60°C. Compared to the others, squid chitin decomposed at a much lower temperature, which is in accordance with a lower crystallinity for ß-chitin. Small deviations in the profile involve a shoulder at 336°C for commercial shrimp chitin, as well as a second mass loss at 467°C for BSF sheddings. Both indicate the presence of impurities. In respect to the shedding, a 56 % weight loss was recorded at 365 °C (chitinous material) and 16 % was further burned at 467 °C. This confirms the presence of impurities as was observed by the chitin quantification (86 %). The degradation temperature (Tm) of the samples, defined as the temperature at the greatest rate of change in the TGA curve, is reported in Figure 6-4b. Squid pen chitin was degraded around 320°C and the BSF samples and shrimp between 360°C and 390°C. No significant differences in Tm were measured between the insect samples of the different life stages (P=0.73, Anova Excel). However, BSF larvae (P=0.03) and sheddings (P=0.02) did have a decreased thermal stability compared to commercial chitin. A comparison of data generated in different studies is challenging as Tm varies with the extraction method and species. For example, Jang et al. (2004) reported a Tm around 367°C for α-chitin

(shrimp) and 362°C for β-chitin (squid)[238] whereas Sagheer et. al (2009) measured a Tm of 326°C for α-chitin (crab) and 303°C for β-chitin (cuttlefish) [101]. Nevertheless, α-chitin has a more stable structure than β-chitin, resulting in a higher degradation temperature [101] which is also measured in the current study. Regarding insect chitin, other insects also contained α-chitin and exhibited a Tm between 360 °C and 390 °C [125], [106], [141], [139], [240]. The current study did not show significant differences between the life stages of the BSF regarding the Tm, which is in line with the findings of Kaya et al. (2016) who also found similar Tm fo the larvae, pupae, and adults of the Vaspa Crabro wasp [241].



(a) (b)

*Figure 6-4 (a) DTG curve of spectra for controls and BSF samples: A) squid, B) shrimp, C) larvae, D) prepupae, E) pupae, F) sheddings, G) cocoon, H) fly; (b) The degradation temperatures for the samples, n= 3, * duplicates.*

6.3.3 Impact on processing chitin to chitosan

All samples were subjected to a chemical deacetylation process (50 m% NaOH at 90°C) to evaluate whether the potential difference in chitin structure impacts further processing to chitosan. After 1 hour of deacetylation, a significant difference was observed between the BSF samples (P = 2.7 E-06, Figure 6-5). For BSF sheddings and the fly a DDA as low as 56 % was recorded whereas for cocoons and larvae (75

%) and prepupae and pupae (80 %) significantly higher values were reached. These results illustrate that chitin prepupae & pupae exhibited a higher deacetylation reactivity. Similarly, Kaya et al. (2014) reported different DDA-values for two life stages of the Potato beetle, being 76 % for larvae and 82 % for adult beetle chitin [139]. However, the current results were not expected since a higher crystallinity for prepupae and cocoons was found based on XRD analyses. Potentially, other characteristics such as particle size favored the deacetylation of these samples. Sheddings and flies performed poorly but TGA illustrated that these two samples contained impurities that may have influenced the deacetylation.

By prolonging the deacetylation time to 3 hours, the samples all reached a similar DDA of 89% (P=0.49). This suggests that 1) a longer reaction time made it possible to overcome the differences that influence the deacetylation, or 2) that BSF samples have a maximum reachable DDA. The latter would imply that prepupae and pupae reached this maximum DDA (89 %) earlier than the other samples. Literature data confirm that the deacetylation of insect-chitin generates chitosan with a DDA below 90 %. Even under more harsh conditions (100 °C), the chitosan obtained from the Potato beetle larvae or adults only had a DDA of maximum 82 % (3 h, 50 m% NaOH)[139]. Chae et al. (2018) performed deacetylation for 9 hours at 95 °C with 50 m% NaOH on crickets and found a DDA of 67 % [140]. The DDA reached in the current study was much higher which suggests that the BSF samples were more accessible for deacetylation.

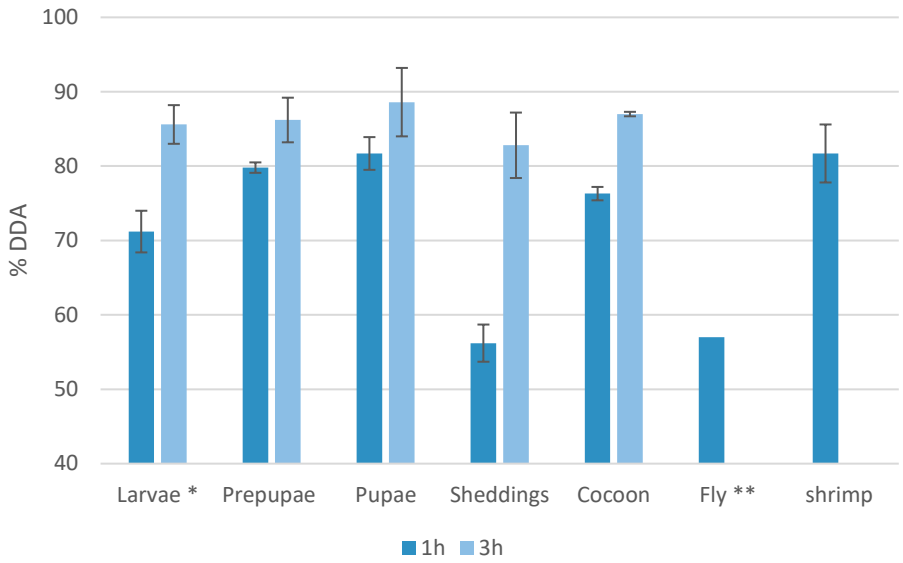


Figure 6-5 Degree of deacetylation of BSF samples after deacetylation. n= 3, duplicate, ** single analysis.*

6.4 Conclusions

Chitin from different BSF biomass samples collected along the lifecycle was purified and physicochemically characterized to identify potential differences. Based on FTIR analysis, XRD spectra and TGA, the chitin extracted from every BSF life stage was appointed as α-chitin. The FTIR spectra of the BSF samples and commercial shrimp chitin match closely. Both FTIR and TGA indicated the presence of some impurities, especially for the sheddings. Prepupae and cocoon chitin were more crystalline than chitin from sheddings, larvae, and flies with a crystallinity index of 94 % compared to 89 %. For prepupae and pupae chitin a higher deacetylation reactivity was observed, that could not be linked to crystallinity. In conclusion, chitin containing byproducts from BSF rearing was found to be a suitable source for chitin with a chitin content of 8 % to 24 %. Small differences in physicochemical characteristics between the chitin samples were observed but they did not strongly influence the further processing of chitosan leading to the advantage that all chitin-containing fraction of the BSF can be collected and processed to a homogenous product. Further

investigation is needed on the characteristics of insect-based chitosan and the suitability in crustacea chitin/chitosan-based products (listed in Chapter 1.4.4).

6.5 Acknowledgments

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Chapter 7.

PURIFICATION OF INSECT CHITIN AND THE CONVERSION TO CHITOSAN

7.1 Introduction

In general, an insect biorefinery approaches benefit from a process step that separates, chitin from proteins and lipids, especially when envisioning applications in feed or food since chitin is suspected to negatively affect protein digestibility [64] [242]. On the other hand, chitin is an interesting molecule with potential in a variety of application domains, which requires purification of the chitin and its conversion to the more soluble chitosan. For instance, in cosmetics and pharmaceutical applications pure chitin/chitosan is required as proteins can cause allergenic reactions [130].

Within the current work, the studied cascading biorefinery approach comprised a mechanical separation as the first step to separate chitin from the major part of the lipids and proteins. More specifically, insect biomass was fractionated in a liquid fraction (insect juice) and a solid fraction. As described in Chapter 3 and 4, the liquid fraction was further processed into a protein and lipid enriched fraction. The current chapter focuses on valorization of the solid fraction which comprises most of the chitin and some remaining proteins.

As insect chitin extraction and purification is a relatively new field, no engineered procedures adapted to insect-chitin were found in literature and the isolation process of crustacea is usually imitated [119]. Generally, the purification process comprises a drying and grinding step, followed by a demineralization step in acid conditions and a deproteinization step in an alkaline environment [119]. However, the composition of insects differs from that of crustacea shells, especially in protein content (between 10 % and 38 % in crustacea versus ± 36 % for the BSF) and mineral content (20-69 % in crustacea and only ± 10 % for the BSF) [119], [243], [41]. In addition, it is generally known that the efficiency of the purification process often varies depending on the source and the pre-treatment, and thus the optimal

purification process for insect chitin could differ from that of crustacea. Furthermore, the conversion of chitin into chitosan [244] requires a harsh deacetylation process involving a high alkaline treatment at high temperature. Based on a literature review related to deacetylation on insect chitosan (see '1.4.3 Conversion to chitosan ') a 50 % NaOH solution is often used.

As such, both processes (purification + deacetylation) are energy-consuming, require a high amount of chemicals including high alkaline concentration solvents and result in a lot of wastewater containing corrosive acid and alkaline residues [154],[245]. In addition, the methods do not allow recuperation of by-products such as proteins and it is a long, multistage process (see Figure 1-5 (schematic presentation of the procedure)). However, Gómez-Ríos et al. (2017) reported via a techno-economic analysis that chitosan production starting from shrimp waste is profitable. Nevertheless, they also suggested that a more cost-competitive and efficient production could be achieved by reduced water use, process intensification and commercialization of sub-products [127].

The current chapter reports on a tailored approach for purification and deacetylation of chitin from the BSF. More specifically, starting from the solid fraction after the mechanical separation of BSF biomass, different traditional crustacean purification approaches were applied. Based on the findings, a procedure tailored for insects was proposed. To the best of our knowledge, this is the first study reporting on tailoring chitin purification for insect biomass and combining it with deacetylation. During the process development, special attention was paid to 1) scalability of the process, 2) decreased use of chemicals and process water, (3) recovery of proteins present in BSF solid fraction and (4) production of chitosan with different degrees of deacetylation (DDA).

7.2 Materials and method

7.2.1 Larval processing to insect shells

Living larvae of the BSF were provided by Millibeter (Belgium). The larvae were reared on broiler feed in temperature (28-32 °C) and humidity (>60 %) controlled room. The larvae were harvested after a rearing time of 15 days and were subsequently separated from the growth medium and transported alive. Immediately after arrival, the larvae were killed by a mechanical grinding step (based on WO 2019081067 [185]). This pre-treatment simultaneously divided the larvae between a liquid and solid fraction. Both fractions were immediately stored in vacuum bags that were aerated with nitrogen and frozen at -20°C. The solids contained the exoskeletons and thus most of the chitin. The solid fraction was used as a starting point for all experiments in this study. Just before use, an aliquot of the solid fraction was defrosted in room temperature water for 10 min.

7.2.2 Traditional crustacean chitin purification approaches

Traditionally, the purification approach comprises a demineralization and deproteinization step. **Traditional demineralization** (DM-approach 1, see Table 7-1) was performed by adding 70 ml of 1 M HCl (but was diluted by the moister content of the insect sample to 0.8 M) to 15 g of defrosted BSF sample (± 5g DM) followed by magnetic shaking (100 rpm) at room temperature for 1 hour. The samples were washed over a filter cloth (Solana, PES 25/14) with demineralized water till neutral pH and the three triplicates were dried at 105 °C for 48 hours.

Three **crustacean deproteinization procedures** were implemented. Two mild deproteinization processes (DP-approach 1 and 2) at room temperature and 45°C and one harsh method at higher temperature was tested (DP-approach 3) as detailed in Table 7-1. In addition, the added value of a prolonged reaction time and a multistage approach was examined (according to DP-approach 4 and 5). The mixtures were shaken at 100 rpm (New Brunswick, Innova 42 shaker), filtered

(Solana, PA 25/14) and washed with demineralized water until neutral and dried at 105°C for 48 h (see Figure 7-1).

Table 7-1 Overview of demineralization (DM) and deproteinization (DP) approaches applied.

	Reagents (m sample on DM: m solvent)	T (°C)	Reaction time (hours)	Extra	Based on
Demineralization:					
DM-approach 1	1 M HCl (1:14)	Rt	1		[105]
DM-approach 2*	6 M HCl[1] (1:14)	Rt	0.5	Protein recovery	This study
Deproteinization (performed on residue obtained from DM-approach 2):					
DP-approach 1	1M NaOH (1:20)	Rt	6		[121]
DP-approach 2	2M NaOH (1:20)	45°C	4		[154]
DP-approach 3	1M NaOH (1:20)	80°C	1-1-…-1	Multistage (n=15)	[134][100] [101]
DP-approach 4	1M NaOH (1:20)	60°C	2,4,6 or 16		This study
DP-approach 5	1M NaOH (1:20)	60°C	2-2-2	Multistage (n=3)	This study
DP-approach 6 *	1M NaOH 30 m% NaOH (= 11.5 M) 50 m% NaOH (=19 M) (1:20)	60°C-80°C-90°C	2 2-16-2 2-2	Combined with deacetylation (multistage)	This study

1:6 M HCl was added to decrease the pH and the final concentration was 0.08 M HCl; *referred to an alternative approach, Rt = room temperature

7.2.3 Alternative demineralization

An **alternative demineralization (DM-approach 2)** was proposed based on the nitrogen solubility of the sample. The solid fraction of the BSF was diluted with water in a 1:14 (m dry sample: m water) ratio and 6 M HCl was dropwise added till pH2 was reached. The pH was checked every 10 min during mixing (100 rpm – Gerhardt laboshake) and adjusted if needed. The reaction time was decreased to 30 min based on the finding of Percot et al. (2003) that demineralization was completed after 15 min at room temperature with 0.25 M HCl [121]. A filter cloth (Solana, PES 25/14) was used to separate the solid (residue) from the filtrate. The residue was

either washed till neutral pH and dried or was further used without washing (see Figure 7-1) for deproteinization. The filtrate was freeze-dried and used for analysis or was further fractionated according to the procedure described by El-Beltagy and El-Sayed (2012) [246]. Briefly, the filtrate was centrifuged (3220 g, 30 min, 4°C) and the pellet was collected. The pH of the supernatant was adapted to pH 5 with 50 m% NaOH and after centrifugation (3220 g, 15 min, 4°C) a second pellet and supernatant were obtained). All fractions were freeze-dried and analyzed.

7.2.4 An alternative combined deproteinization/ deacetylation

Deproteinization was combined with deacetylation by mixing the wet, demineralized residue (derived from the alternative demineralization) with NaOH (1M, 30 m% or 50 m%) in the same ratio at different temperatures (60°C, 80°C or 90 °C) for different time periods (see DP-approach 6). Afterwards, the deproteinized samples were filtrated (Solana, PA 25/14) and washed with demineralized water until neutral and dried at 105°C for 48 h (see Figure 7-1).

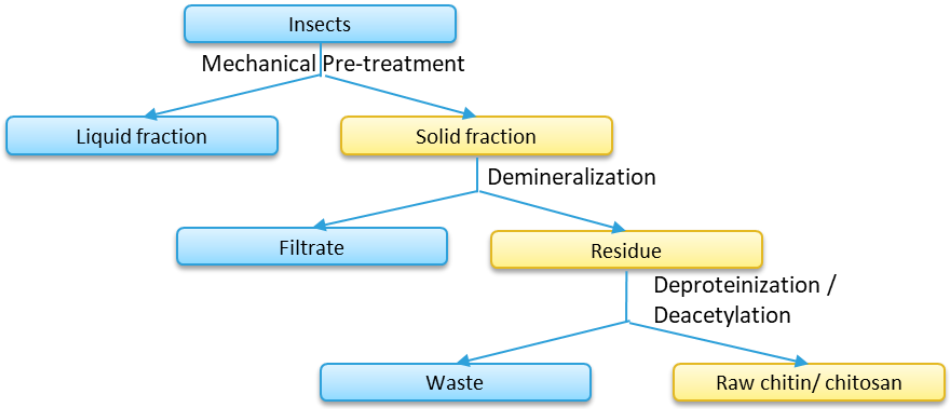

Figure 7-1 Scheme of the obtained fractions during the purification of chitin from insects

7.2.5 Analysis

Dry matter (DM) content of samples was determined gravimetrically after a drying period for 48 hours at 105 °C. All other analyses were performed and expressed on dried matter basis. Ash content was measured after incineration at 550 °C for 6

hours. A Soxhlet procedure of 6 h (diethyl ether) was employed for lipid content determination. Protein contents were calculated based on the amino acid determination according to the method described in Chapter 2. Soluble nitrogen was determined by a chemiluminescence detector (Multi N/C 3100 Analytik Jena) as described in chapter 2 and the nitrogen in solid samples was measured by the Dumas method (Elementar, vario EL cube). The pH-depended nitrogen solubility was measured at different pH-values ranging between pH2 and pH10 according to Soetemans et al. (2019) [194]. Quantification of the chitin content and the degree of deacetylation was performed as described by D'Hondt et al. (D'Hondt et al., 2019). The particle size of the obtained fractions was gravimetrically determined by shaking the samples (Fritsch Pulverisette – mortar grinder mill, amplitude 7) during 2 minutes over Retsch sieves of different mesh sizes.

7.3 Results

7.3.1 Characterization of the starting material

Insect shells were obtained after mechanical separation of the larvae in a liquid phase and a solid phase. The composition (dry matter based) of the BSF larvae and the two fractions is summarized in Table 7-2. The liquid phase represented about 55 % of the insect biomass and was rich in proteins (32 %) and lipids (36 %). The solid phase (38 % of the BSF biomass) contained almost no lipids, 42 % of protein (which corresponded to 42 % of all proteins present in the BSF larvae), and 19 % of chitin. This implies that the chitin was concentrated from 7 % in the larvae to 19 % in the solids. Only a very small amount of chitin was found present in the liquid fraction.

Table 7-2 Composition of the solid and liquid fractions after pre-treatment on dry matter basis (n =3).

	Larvae	**Liquid fraction**	**Solid fraction**	**Shrimp shells** [100]
% of larvae *	100	55,9	38,3	
% DM	29,53 ± 0,26	26,45 ± 0,42	33,16 ± 0,72	
% lipids *	19,97 ± 0,92	35,98 ± 0,77	5,56 ± 1,72	
% protein *	38,62 ± 3,73	31,7 ± 2,5	42,43 ± 2,39	34.0
% nitrogen *	6,86 ± 0,09	6,15 ± 0,09	8,77 ± 0,09	
% chitin *	8.5 ± 0,1	0,60 ± 0,02	18,63 ± 0,96	23.7
% ash *	10,42 ± 0,13	10,41 ± 0,09	9,56 ± 0,01	42.3

* Dry matter based

The high protein content in the solid fraction (42 % DM based) is explainable by chitin being embedded in a protein matrix for the formation of exoskeletons. Cuticle proteins are heavily bounded to chitin and are not easily extracted. A nitrogen solubility curve was measured to evaluate whether all proteins present in the solid phase were bounded to chitin. Some nitrogen (about 24 %) was found soluble at pH7 and a significantly increased solubility was observed at acid pH (Figure 7-2). Nitrogen could have many origins such as inorganic sources, nucleotides, free amino acids, peptides, proteins, and chitin. However, in this experiment, nitrogen sources other than chitin or proteins were not taken in to account. In addition, it is generally known that chitin is insoluble in an aqueous environment [147], leading to the hypothesis that soluble nitrogen represents soluble proteins or peptides. These proteins/peptides were likely part of the liquid phase but were separated incompletely during the mechanical step due to the viscosity of the liquid part that partially stocked to the solids.

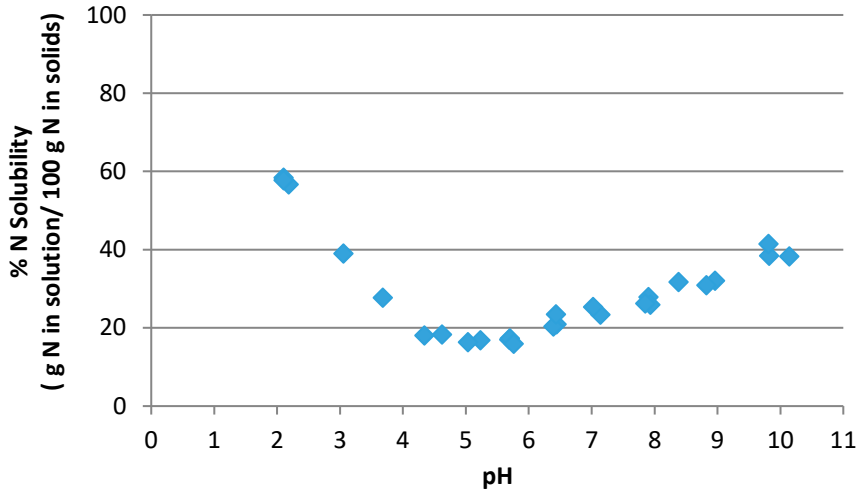

Figure 7-2 Nitrogen solubility curve of the solid phase in function of the pH.

7.3.2 Comparison of demineralization methods

The traditional demineralization (DM-approach 1) procedure was applied to the BSF solid fraction and was found a successful approach for the insect sample. A residue was obtained with 1 % remaining ash (93 % of minerals removed) and about 29 % of the biomass was dissolved in the filtrate and subsequently removed (see Table 7-3). The filtrate was not further examined since a pH lower than 0 was measured (the concentration was diluted to 0.8 M HCl because of the moisture content of the sample) and purification of the protein would lead to an excess of salt formation or the use of specialized equipment. However, based on calculations, only 6 % of the proteins would have been solubilized and present in the filtrate.

Table 7-3 Results and composition of the residue obtained after demineralization on dry matter basis (n=2).

Residue	% biomass removed	% ash	% protein	% chitin
DM-approach 1	29,14 ± 0,17	0,99 ± 0,05	56,4 ± 1,9	21,90 ± 0,62
DM-approach 2*	43,29 ± 0,28	2,96 ± 0,03	47,0 ± 1,6	33,70 ± 0,81

* alternative method

The alternative method (DM-approach 2) involved the use of 6 M HCl that was added until pH 2 was reached. Based on the amount of HCl needed and the moisture content of the insect sample, a final concentration between 0.08 and 0.1 M HCl was obtained. The demineralized residue comprised 3 % ash (82 % of minerals removed) and about 43 % of biomass was removed resulting in a residue with higher chitin content compared to the traditional demineralization (Figure 7-3). The exact reason for the increased biomass removal could not be proved, but the excess of chloride ions in the traditional method may have reduced the electrostatic repulsion between proteins, causing aggregation and precipitation and as such a lower removal via the filtrate. The particle size obtained after the alternative demineralization in the current study was similar, although a bit less fine, compared to that of commercially available chitin (sigma). The residue (obtained after demineralization) had a particle distribution of 25 % > 2 mm, 49 % between 2 and 1 mm, 17% between 1 and 0.5 mm and 9 % < 0.5 mm while commercially available chitin (sigma) had a pattern comprising 17 % > 2 mm, 29 % between 2 and 1 mm, 32 % between 1 and 0.5 mm and 26 % < 0.5 mm.

In respect to the protein-containing filtrate, a protein concentration of 39 % (= 43.4 % of the proteins in the pulp) was measured, which can be recuperated. In order to remove the added acids and formed salts, the filtrate was further fractionated to a protein-rich pellet 2 with a protein content of 71 %. An acid environment changes the ionizable residue of the proteins' amino acids and disturbs weak hydrogen bounding while promoting electrostatic repulsion. This results in increased protein solubility and thus a higher protein recovery [247] compared to neutral pH. In addition, metals that were chelated by proteins and/ or stabilized the protein-chitin matrix are solubilized by acids and therefore cause partial unfolding of proteins and destabilization of the matrix. Ionic groups that were involved in the chelation are now available for protonation and thus the electrostatic repulsion increases. El-Beltagy and El-Sayed (2012) also performed acidic isoelectric point precipitation on

shrimp shells and proteins (that still contained some techno-functional properties) from this waste source were extracted [246]. By applying the mechanical separation pre-treatment on BSF larvae followed by the alternative demineralization, 75.6 % of all proteins in the larvae were extracted and could have the potential for valorization.

7.3.3 Comparison of deproteinization methods

Firstly, two mild deproteinization procedures (DP-approach 1 and 2) were applied to the wet residues generated using the DM-approach 2. Table 7-4 shows the protein and chitin content of the resulting raw chitin sample. The generated raw chitin samples had a chitin content below 75 % and a protein content of at least 12 %. This was not in line with the findings of Bajaj et al. (2011) who reported a residual protein in the shrimp shells of 1 % after a DM-approach 2 was applied [154]. Also, Percot et al. (2003) reported a protein content lower than 2 % in the chitinous material after a DM-approach 1 [121]. These results indicate that the mild deproteinization was not sufficient for the insect samples to obtain a pure product.

Table 7-4 Results related to traditional deproteinization approaches that were performed on residues obtained from DM-approach 2.

Raw chitin sample	% Biomass removal	% Protein	% Chitin	% DDA
DP-approach 1 (mild)	58	16,5 ± 2,2	64,3 ± 4,2	30,4 ± 0,6
DP-approach 2 (mild)	63	11,9 ± 1,0	74,0 ± 5,5	31,5 ± 5,8
DP-approach 3 (harsh)	nd	3.9 ± 0.2	82,0 ± 1,1	35,8 ± 0,4

Nd = not determined

The more harsh approach (DP-approach 3) involved a multistage process where the NaOH was refreshed until it remained colorless. For the insect sample, it needed to be repeated 15 times since a light-yellow color was persistent in the last 9 repeats (see Figure 7-3a). Figure 7-3b illustrates the decreasing nitrogen content in the NaOH waste. An exponential decrease within the first 4 repeats was observed while the nitrogen removal slowed down afterwards until. After 11 repeats, the nitrogen

content in the solution was even under the detection limit (15 mg/L). The results indicated that the non-chitinous nitrogen in the BSF residue was extremely persistent and strongly bounded to chitin. An extreme amount of alkaline waste would be generated to obtain a raw chitin sample at a large scale via the DP-approach 3. Yet, only a chitin purity of 82 % was achieved.

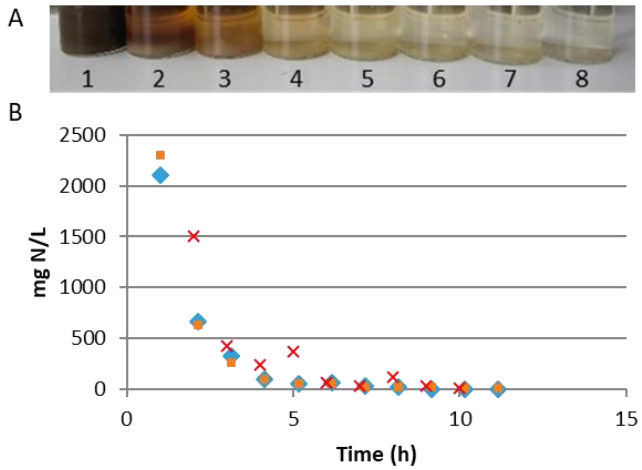

Figure 7-3 Multi-stage deproteinization, (a) solvent color after the first 8 repeats, (b), the nitrogen content in the solvents after deproteinization.

The added value of a prolonged reaction time and a multistage approach were examined more in detail to improve the economics (reduced use of chemicals) and sustainability (less waste production) of the demineralization process. In respect to the multi-stage process (Figure 7-4), the nitrogen removal realized via 3 times a deproteinization during 2 hours (DP-approach 5) increased significantly compared to a single deproteinization for 6 hours (DP-approach 4). Tolaimate et al. (2003) suggested that a multi-stage process is more efficient assuming that the first bath is needed to modify the biomass for better accessibility of the solvent [134]. Longer reaction times also increased nitrogen removal as illustrated in Figure 7-4, with 63 % and 78 % N removal, after 2 hours and 16 hours, respectively. A residual protein content of 6.7 ± 0.3 % was measured in the raw chitin after 16-hour extraction process compared to a residual protein content of 13.7 ± 1.6 % after 2 hours. In any

case, the raw chitin sample still contained proteins indicating the need for a more harsh deproteinization.

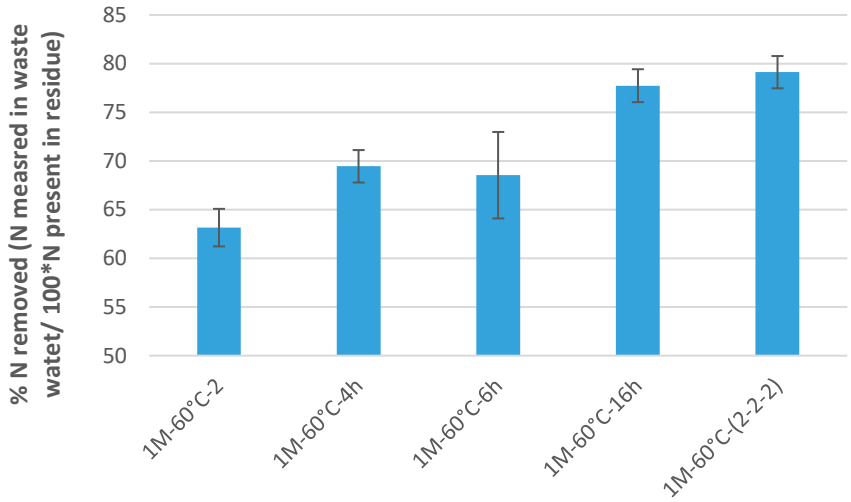

Figure 7-4 Effect of a prolonged reaction time and a multi-stage process on the nitrogen removal of the residue.

7.3.4 An alternative, combined deproteinization and deacetylation approach

Percot et al. (2003) stated that a deproteinization performed below 70 °C and with a concentration under 1M NaOH would not cause partial deacetylation [121]. Table 4 reports a DDA around 30 % after mild deproteinization while the harsh approach resulted in a raw chitin sample with a DDA of 36 %. D'Hondt et al. (219) reported a deacetylation degree of 19 % for non-processed chitin and others assume a 10 % DDA [232]. The results of the current study suggest that the BSF samples are more accessible for deacetylation and that the statement of Percot et al. does not apply to BSF chitin. For this reason, and because of the need for a more harsh deproteinization process, a combined deproteinization and demineralization approach was investigated. Table 7-5 reports the results of 13 test conditions that were applied on BSF residue generated via DM-approach 2. Seven conditions were able to achieve a high chitin/chitosan purity (> 90 %) and within these 7 conditions, different DDA values were reached. A DDA between 36 % and 40 % was obtained

when 30 % NaOH was used in either a multistage of 2-16-2 hours at 60 °C or with a single stage of 2 hours at elevated temperature. The latter would result in less chemical waste. A DDA between 50 % and 60 % was achieved with either 50 % NaOH, 60 °C for 2 hours or with 30 % NaOH, 80 °C for 2 hours. The harshest conditions (90 °C, 50 % NaOH) resulted in the highest DDA of 80 %. An amino acids analysis is needed to confirm the absence of proteins. However, some sample had a chitin content higher than 97 % and thus a high purity is expected.

Table 7-5 Chitin content and degree of deacetylation of raw chitin obtained by the alternative deproteinization/ deacetylation (n=3).

Temperature	Concentration NaOH	Time (h)	% chitin	DDA
60°C	1M	2	76,94 ± 1,65	33,27 ± 0,48
	30%	2-16-2	87,20 ± 2,49	31,78 ± 0,11
		2	76,8 ± 1,0	24,2 ± 2,4
		2-16-2	**89,99 ± 1,46**	**40,46 ± 1,79**
	50%	2	**87,01 ± 4,64**	**51,03 ± 3,98**
80°C	1M	2	76,3 ± 4,1	31,4 ± 3,5
	30%	2-16-2	84,0 ± 1,7	31,0 ± 3,4
		2	**93,24 ± 1,03**	**36,20 ± 1,30**
		2-16-2 *	**100,44 ± 0,54**	**57,93 ± 3,47**
	50%	2	86,1 ± 2,6	76,0 ± 2,5
90°C	30%	2	**97,37 ± 1,44**	**39,05 ± 1,40**
	50%	2	**96,71 ± 5,17**	**83,17 ± 0,52**
		2-2	**103,75 ± 1,53**	**87,82 ± 1,01**

* duplicate, pure fraction (> 90 %) were put in bold.

To the best of the author's knowledge, no other study was found that combined the deproteinization and deacetylation. One publication was found on obtaining different DDA's from the cricket *Gryllus bimaculatus* where the concentration of NaOH was varied. A deacetylation of 9 h at 95 °C with 50 %, 55% or 67 % NaOH resulted in a DDA of 67 %, 75% and 85 %, respectively [140]. Other publications on different insect species all resulted in a DDA between 75 and 90 % [139], [141], [142],[144], but no DDA below 75 % was reported.

7.4 Discussion

Purification of crustacean chitin usually starts by drying and grinding of the crustacean waste. Similarly, intact insects are also dried followed by grinding to obtain a powder [139],[105]. However, this means that the proteins and lipids present in the insect biomass are subjected to the degrading demineralization /deproteinization conditions and thus not usable as by-products. The authors of the current study proposed a different pre-step that involved the separation of the insect exoskeleton from the inner body. Much like the process for crustacea where the internal edible part is removed, the insects were processed mechanically. This pre-step generated multiple benefits such as (1) the opportunity to recuperate valuable products (from the inner, liquid insect biomass), (2) avoiding the need for drying and (3) grounding. Drying is an economic unfavourable step especially because of the high moisture content of the solid fraction (67 % for the solid fraction of the BSF) and the mechanical pre-step performed in the current study was able to ground fresh larvae by erupting the exoskeletons (in order to release the inner body) and further break the exoskeleton in smaller pieces. The particles generated were predominantly < 2 mm, as for commercial chitin.

A chitin purification process was tailored towards the BSF solid fraction generated after the mechanical pre-treatment process. The process consists of an adapted mineralization step, followed by a deproteinization step that was combined with a deacetylation process. As such, chitosan was simultaneously generated.

By applying the alternative demineralization method, simultaneously demineralizing and recovery of proteins were made possible. Compared to the traditional demineralization, the method led to a decrease in chemical use (the same solid: liquid ratio was upheld but the concentration of HCl in the alternative method was 0.1 M compared to 1 M in the traditional method) and a higher biomass removal (43 % instead of 29 %). All traditional deproteinizations were proven to be

insufficient for the insect samples by either generating a lot of chemical wastewater or by not achieving a high purity of chitin and still containing proteins. Bastiaens et al. (2019) reported major variations on deproteinization process parameters for temperature (25 -130°C), time (1-24 hours) and NaOH concentration (0.3 -12.5 M) that were reported in published research [119] and it seems that these harsh conditions are necessary for complete protein removal of BSF chitin. However, results did indicate an added value of a prolonged time and a multi-stage process.

A literature search revealed that after deproteinization, deacetylation is often performed [248],[249]. Both processes involve an alkaline treatment and since chitin is often merely an intermediate product, the authors combined the two processes for chitin and/or chitosan production. An alkaline treatment was proposed where the process parameters varied, and the added value of the multi-stage process and a prolonged reaction time were incorporated. Keeping upscaling in mind, process times of 2 hours or 16 hours (overnight step) were chosen, take into account warming up and cooling down periods. The concentration of NaOH was chosen in function of commercially available solutions (1 M, 30 m%, 50 m%). Different process parameters were defined to obtain high purity samples of chitin or chitosan (> 90%) without a pre-deproteinization and thus minimizing alkaline waste. However, the samples need to be evaluated on the absence of proteins. Nevertheless, chitosan with different degrees of deacetylation was obtained and this was, to the author's knowledge, the first study that aimed at developing a tailored procedure for generating different (insect-based) chitosan products. This study is needed since the properties of chitin or chitosan are extremely dependent on the DDA [102]. For example, chitin (a low DDA) is better for immobilizing enzymes and removing polycyclic aromatic hydrocarbons from petrochemical wastewater whereas chitosan (high DDA) is better in capturing Cr and promoting the growth of cells [114][250]. Some properties can be enhanced by changing the DDA, for example, a higher activity of cytotoxicity on human bladder cancer cells was

detected with a lower DDA (39 % compared to 98 %) [114], and the encapsulation of oil and oil release is better when a lower DDA (55 % compared to 61 or 85 %) is used [251]. However, the antimicrobial activity, the antioxidative activity and the chelation of metals are much better when high DDA chitosan is applied [114].

7.5 Conclusions

The present study was designed to apply different traditional crustacean purification methods on BSF samples and to propose alternative approaches tailored to the insect biomass.

The traditional demineralization was effective but a lower degree of purification was observed while the different traditional deproteinization methods were less effective on BSF samples than for crustacea. An alternative approach was proposed, comprising a milder demineralization with the recovery of proteins and harsh alkaline treatment for deproteinization and simultaneous deacetylation. This approach enabled (1) to obtain highly pure chitin/chitosan samples (> 90 %) with different degrees of deacetylation (ranging between 35 % and 80 %), (2) recuperation of the proteins (76 %) and lipids (up to 85 %) and (3) a reduction of chemical waste produced during the process. In conclusion, the BSF was found to be a good source for chitin extraction and an alternative approach was found beneficial.

7.6 Acknowledgments

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Chapter 8.

GENERAL CONCLUSION AND FUTURE ASPECTS

The establishment of an insect-based value chain is still at its early stages and the academic interest in this new agricultural sector has seen exponential growth since 2015. The findings of the thesis, which was initiated in 2016, make several contributions to the current, limited literature about the use of insects for food, feed or technical applications. Two insect species were studied, (1) the larvae of the BSF in the meantime is been referred to as the most promising insect and has received much attention, and (2) the LM which is still a relatively unstudied insect. Over the years, the research topics have moved along the different aspects of the insect-based value chain. At the beginning of the thesis, most published research was focused on optimal breeding conditions, feed trials involving intact insects and the use of insects for waste treatment or biodiesel production. In the last few years, the topics have shifted to food safety aspects, the inclusion of food by-products to the feed and the impact on the composition of insects. In addition, the use of insects as a new protein source has been discussed extensively and an increasing amount of reports are being published on fractionation, characterization, and valorization to insect-based food or feed products. However, the data is still limited and often scattered over different insect species. The thesis focused mainly on the fractionation of insect biomass to obtain insects-based raw material that could form the basis for new feed, food or other technical applications. Four research questions were drafted that supported the aim of the thesis.

1) Does a varied insect feed composition impact the composition of the insect biomass?
2) Can a biorefinery, in the true sense of the word, separate all (major) components with minimal generation of waste?
    - Would the use of organic acids improve fractionation in an acid-based biorefinery approach to obtain lipid and protein-enriched fractions?

- Is the newly developed biorefinery approach comparable to traditional defatting methods?
- Could a modified biorefinery approach to obtain chitin and chitosan from insects be more sufficient than the traditional crustacea-based purification method?

3) What are the techno-functional properties of insect proteins and what is the impact of the extraction method?
4) What is the effect of different life stages on the chitin characteristics?

The first research question was posed on the possibility of incorporating underspent side-streams in the diet of the LM without affecting the larval composition. In this way, the larvae would be able to recycle essential nutrients from side-streams which would otherwise be removed from the food-feed chain. Experimental data (chapter 2) showed that the larvae concentrated the side-stream nutrients into their own biomass and thus indirectly increased the nutritional value of the side-stream and enabled nutrient-recycling. Some fluctuations were observed in the larval protein & lipid content and the amino acid & fatty acid profile, however, no correlation to the dietary composition was observed. The result indicated that the insect composition was, within the limitations of the study, rather constant and that some flexibility regarding the feed ingredients was possible. In addition, some side-stream inclusions in wheat middling-based diets were able to generate a better larval yield and raised the nutritional value of the larvae while reducing the cost price of the LM proteins. In conclusion, the results illustrate that successful rearing of the LM on side-stream based diets is possible and that **a circular economy strategy** can be applied. In respect to circular economy, waste (side-streams) is seen as a new resource that enables the recycling of valuable compounds. The results also show that alterations of the side-stream based diet are possible throughout the year (for example due to seasonal availability) without necessarily changing the end-product. This also implies that **one extraction procedure could be applied to the**

insect biomass. In addition, the results of the current study on the LM were in contradiction to the data found on the BSF in literature. Hence, it would be an added value to perform a similar study on multiple insect species that have the potential for large-scale rearing. Further development is needed on, for example, the effect of the carbohydrate, mineral or vitamin concentrations in the diets to study the effect on the composition of the larvae and evaluate if these can change/ improve the larval yield and nutritional value. In general, a more in-depth **knowledge of the nutritional requirements of the insect species and their metabolic conversion is necessary** to define a more balanced insect feed to optimize larval rearing. In the next step, diet formulation could be made with different side-streams (that can go beyond the choice in the current study) with flexibility towards fluctuating market prices and availability and nitrogenous emissions are kept minimal by limiting overplus of proteins.

A second research question involved the fractionation of the insect biomass to generate multiple compounds for feed, food, and technical applications. The PhD study aimed to develop a biorefinery approach where all (major) components are separated & valorized and waste generation should be kept to a minimum. A two-step fractionation approach was elaborated where the insect biomass was first divided between a pulp (chitin rich) and a juice fraction followed by a chemical-based extraction process of lipids, proteins, and chitin. The first step made it possible to separate all chitin from the lipids and the study of the second step is described in chapters 3, 4 and 6.

- Starting from insect juice, a new fractionation approach based on an acid isoelectric point precipitation (AIPP) was studied for separating the lipids from the proteins in the BSF juice fraction. Within the new approach, the added value of some organic acids to improve the separation was studies. Lactic acid was most beneficial for lipid recovery while acetic acid combined with HCl or a complete inorganic treatment was best for protein extractions.

A protein-enriched fraction with a protein content between 45-57 % was generated while simultaneously creating a lipid-rich fraction with a lipid content between 75 % to 85 % lipid content. These three conditions were also applied on the LM juice (partially described in chapter 4) but no added value of the organic acids was measured. Nevertheless, acid-based isoelectric point precipitation was, up till now, not yet reported and for both insects, a protein-enriched fraction was obtained that met the demands on protein concentration for use as feed ingredients. It would be of interest to evaluate if the use of organic acids would be economically beneficial. In addition, **further optimization** of the fractionation process is required, especially towards upscaling. For example, the recycling of additives would (1) decrease production cost and (2) increase the purity of the fractions.

- The new AIPP separation technique was compared to other traditional defatting methods on LM juice and resulted in a protein-enriched fraction of 71 % protein which corresponds to a 50 % protein recovery and a lipid-enriched fraction of 86 % lipid resulting in a lipid recovery of 85 %. Compared to SFE, heat extraction or solvent-based extraction, the new method was comparable to SFE, although SFE was better regarding the protein recovery and extraction yield, yet the AIPP method was more suitable with respect to scalability and creating crude protein fractions. However, these studies were performed on lab scale and **upscaling is required** to verify the conclusions.

- The pulp fractions originating from the mechanical treatment contained proteins and chitin. Published studies on extracting chitin from insects employ the traditional purification method that is elaborated for crustacea shells. An alternative, insect-adapted method was composed and compared to the traditional method. This new method performed better in

recuperating proteins, fewer chemicals were needed, and the process steps were minimalized. Purified chitin/ chitosan (> 90 % chitin) fractions were obtained with a different degree of deacetylation ranging from 35 % to 80 %. Although it is not yet performed on an industrial scale, chitin of crustacea can also be **extracted biologically by fermentation**. It would be interesting to evaluate this approach would on insect-based biomass as it offers potential to make the process even more environmentally friendly.

In conclusion, organic acids could have an increased value in separating proteins from lipids, depending on the insect species. AIPP on insect biomass proved to be comparable with other techniques for creating crude protein and lipid fractions and a purification process for chitin/ chitosan extractions was feasible for larvae of the black soldier fly. The biorefinery approach developed in this thesis (mechanical treatment followed by fractionation of juice & fractionation of pulp) was able to recuperate 76 % of all proteins, 73 % all lipids and, 75 % of the BSF chitin. This method was capable of, in contrast to many other approaches, separate the chitin from the proteins and did not involve the use of organic solvents. It generated less chemical waste and 53 % of the larval biomass was processed to marketable fractions. Fractionation led to protein-enriched samples suitable for the **feed applications** and thus could be used to **tackle future protein shortage**. In addition, since insects can be reared in Europa, this new protein source **lowers the dependency for imported protein-rich feed**. With respect to **food applications**, consumers have a more positive attitude towards insects being incorporated as an unrecognizable food ingredient and thus a fractionation approach would make integration of insects in the food chain more feasible. In **respect to other fractions that were not suitable for feed** ingredients but still contained proteins and lipids, other applications should be considered such as bioplastics, fertilizer, textile applications or the recovery of lipids for biodiesel (one non-used fraction contained a high lipid content). However, these non-nutritional applications are less studied. Lastly, insect chitin was successfully extracted from the biomass and can be

employed for **technical applications**. The next steps in the process chain would be (1) **characterization of chitin and chitosan** towards functional properties (film properties, oil binding capacity, emulsifying, chelating of metals, …) and (2) **depolymerization of chitosan** to different sized poly- and oligomers. The latter possesses other or enhanced functional properties (antimicrobial properties, anti-thrombogenic properties, prebiotic, …) and generally have a higher market value. A wide range of possible applications was found in literate for chitin and chitosan and **further tailoring and testing to a specific application** is needed. Lastly, it would be interesting to compare chitin-based products derive from insect chitin to crustacea-chitin-derived product to investigate of the chitin/chitosan properties are different and/or could impact further product development.

The third research question is related to the techno-functional properties of proteins extracted from the LM. Measurements were performed on the protein-enriched fraction defatted by the new AIPP fractionation approach, an SFE, and heat treatment. The native protein (after the mechanical pre-step) displayed a relatively good solubility and gelling ability while no foaming properties were observed. Depending on the fractionation approach, the techno-functional properties were either increased or decreased. In conclusion, the LM proteins exhibit techno-functional properties and these can significantly change according to the fractionation approach. This offered the potential to tailor the properties. These results indicate that the proteins from the LM can have a **higher market** value since the techno-functional properties will have an added value to the food product texture and structure. In addition, **other applications** besides food and feed could benefit from techno-functional proteins. The first step towards fraction characterization was taken but **further examination of the techno-functional properties** of insect proteins is needed. The thesis only reports on the techno-functional properties of the juice and fractionated juice but also the proteins derived from the pulp fraction would be interesting to be examined and the study needs to

be completed with data on protein emulsification. Further, the impact of pH and the ionic strength on functionalities is further to be explored. Additional tests may comprise (1) a dialysis step to remove the high salt concentrations and (2) pH adjustments of all samples. The study could be further expanded by measuring the techno-functional properties of all samples at different pHs (pH 3-7 which is used in food-feed applications) and after adding NaCl to change the ionic strength since food/feed applications generally, have a higher salt concentration. Further, these experiments elaborate on the techno-functional properties of the proteins on the mesoscale but **further testing on the macroscale** is needed. Product development, as described in chapter 5, is the next step in the insect-based value chain and can start after characterization. Up till now, little research has been published on food/feed product development. Expansion to **other protein functionalities and characteristics** such as digestibility and bioactive properties is also required to facilitate insect-based product development.

The last **research question** was posed on the characteristics of chitin extracted from the black soldier fly in different life stages. Chitin extracted from the larvae, prepupae, pupae, flies, shedding & cocoons were examined and the data indicated that BSF chitin was α-chitin in all life stages (chapter 6). In addition, little differences were found on physicochemical characteristics. Further deacetylation to chitosan indicated that prepupae and pupae chitin were more reactive but after deacetylation of 3 hours, all samples had a similar DDA of 89 %. The chitin content in the collected biomass ranged between 8 % and 24 %, with sheddings and cocoons being most rich in chitin. In conclusion, the results indicated that all chitin-containing fraction of the BSF can be collected and processed to a homogenous chitosan product with a similar degree of deacetylation. The results indicate that waste-streams originating from the insect rearing industry could also be valorized. Dead insects, cocoons and shedding could serve as secondary raw materials for chitin production and thus implying a **circular economy**. The alternative extraction

method for chitin, proposed as part of this PhD study, should also be tested and adapted to these sources. Even though the deacetylation was similar for all chitin-sources, other differences in properties of chitosan should be examined. In addition, no information is published on the available amount of these chitin-rich side-streams which is needed for an economic study.

For the establishment of an insect-based value chain, the **economic viability** of the process is crucial. Therefore, a **techno-economic evaluation** would be useful for all aspects of the thesis since it could (1) be used to select other interesting side-streams for insect rearing that are economically interesting on logistics (depends on the location of the rearing farm), availability and cost price, (2) be used to evaluate the potential of different chitin-rich by-product for chitin extraction and (3) evaluate the added value of organic acids in the AIPP treatment and assist in optimizing the process parameters to upscaling. For the latter, **a pilot-scale study is required** for the economic analysis input to ensure realistic data. In view of an economically viable value chain, focus on high-end products may be considered as a priority since the yield of the fractions is already quite high. A good understanding of the exact functionalities and characteristics is necessary as well as a robust fractionation approach to guarantee the yield and quality of the end-product. Insect based high-end products could be, besides a nutritional ingredient in food/ feed, employed as functional ingredients for cosmetics, nutraceuticals or a functional ingredient in food or feed (emulsifying agent for example [57]). Chitin/chitosan offers in this perspective potential for multiple applications for example in wound healing dressings, in wastewater treatment or in cosmetic products such as shampoo or as fertilizer and plant-stimulant [133],[160],[162]. Technical applications for proteins are mainly in research phase but could also be used in cosmetics when film-forming properties are present, but also for example in glue or bioplastics [79],[82].

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- Published

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Gianotten N., Soetemans L., Bastiaens L. (2019). Agri-food Side-stream Inclusions in the Diet of *Alphitobius diaperinus* Part 1: Impact on Larvae Growth Performance Parameters. *Insects*

Soetemans L., Gianotten N., Bastiaens L. (2019). Agri-food side-stream inclusion in the diet of *Alphitobius diaperinus*: Part 2 Impact on larvae composition. *Insects*

Leni G., Soetemans L., Jacobs J., Depraetere S., Gianotten N., Bastiaens L., Caligiani A., Sforza S. (2019). Protein hydrolysates from Alphitobius diaperinus and Hermetia illucens larvae treated with commercial proteases. *Journal of insects as Food and Feed*

Conferences: oral presentation

Insecta conference 2017

Biorefinery of Insects to high-added value products: evaluation of fractionation procedures

RRB conference 2018

Insects as alternative source of chitin

Insecta conference 2019

Insects as alternative source of chitin and chitosan

Conferences: poster presentation

RRB conference 2016

Biorefinery of Insects to high-added value products: evaluation of fractionation procedures

EAPP conference 2019

Purified chitin does not impair protein and lipid digestion in monogastric animals

RRB conference 2016

1) Impact of side-stream based feed on the composition of the lesser mealworm
2) Impact of traditional defatting methods on yield and quality of the protein-enriched fraction from the lesser mealworm
3) Fractionation of insects biomass
4) Optimal rearing of *Alphitobius diaperinus* on organic side-streams → Poster award young scientist

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Annex 1: chapter 2

Table 0-1 Composition of side-stream ingredients (on dry matter basis).

	Wheat middlings	Rice bran	Rapeseed meal	DDGS	Corn gluten feed	Brewery grains
protein content (%)	16,2 ± 1,0	14,2 ± 1,8	29,7 ± 0,6	29,2 ± 3,4	16,4 ± 4,6	20,7 ± 0,4
Amino acids (g/kg)						
Arginine[E]	46,2 ± 5,3	70,4 ± 8,8	73,9 ± 11,1	108,7 ± 34,5	39,0 ± 10,8	29,9 ± 2,8
hydroxylysine	0,04 ± 0,01	0,02 ± 0,02	0,06 ± 0,10	0,03 ± 0,06	0,0 ± 0,0	0,01 ± 0,00
Lysine[E]	5,6 ± 0,7	4,8 ± 0,7	15,3 ± 1,4	6,8 ± 1,1	6,4 ± 1,7	2,7 ± 0,1
Alanine	7,5 ± 0,5	6,9 ± 1,1	14,1 ± 0,8	19,2 ± 1,8	15,7 ± 4,6	12,0 ± 0,3
Threonine[E]	5,6 ± 0,5	4,9 ± 0,9	14,6 ± 1,7	9,5 ± 0,2	6,8 ± 1,8	9,1 ± 0,1
Glycine	7,9 ± 0,9	6,2 ± 0,7	16,6 ± 0,8	11,0 ± 0,7	7,6 ± 2,1	9,5 ± 0,2
Valine[E]	7,8 ± 0,4	6,7 ± 1,7	19,5 ± 1,3	15,9 ± 1,5	8,0 ± 2,1	13,7 ± 0,3
Serine	6,7 ± 0,7	4,8 ± 0,8	7,6 ± 6,6	10,7 ± 0,2	7,5 ± 2,3	9,4 ± 0,1
Proline	9,9 ± 1,3	5,4 ± 0,5	16,9 ± 0,5	18,3 ± 0,8	15,5 ± 4,0	23,9 ± 0,4
Isoleucine[E]	4,7 ± 0,2	3,4 ± 0,5	12,4 ± 1,4	8,5 ± 0,3	5,4 ± 1,5	9,3 ± 0,0
Leucine[E]	11,5 ± 0,8	8,2 ± 0,9	23,5 ± 1,5	31,3 ± 2,7	18,0 ± 5,0	20,2 ± 0,1
Methionine[E]	3,3 ± 0,4	2,4 ± 0,4	5,1 ± 1,2	3,7 ± 1,1	0,3 ± 0,1	2,9 ± 0,3
Histidine[E]	2,7 ± 0,1	2,1 ± 0,5	7,7 ± 0,4	5,5 ± 1,6	5,9 ± 1,6	5,0 ± 0,3
phenylalanine[E]	6,9 ± 0,3	4,7 ± 0,2	13,1 ± 0,6	11,7 ± 1,9	7,0 ± 2,0	14,2 ± 0,2
Glutamate	34,7 ± 1,6	16,1 ± 2,4	59,8 ± 6,8	45,9 ± 0,7	32,5 ± 9,9	52,3 ± 2,1
Aspartate	12,2 ± 0,7	11,2 ± 1,2	23,9 ± 2,4	17,1 ± 0,5	9,7 ± 2,5	15,1 ± 0,2
Cystine	1,7 ± 0,1	1,4 ± 0,1	4,7 ± 0,2	3,1 ± 0,3	1,1 ± 0,4	3,2 ± 0,1
Tyrosine	3,7 ± 0,4	2,8 ± 0,3	8,5 ± 0,1	9,2 ± 0,7	4,4 ± 1,2	7,6 ± 0,1
Sum EAA (g/kg)	98,7 ± 6,6	107,5 ± 14,0	188,3 ± 2,2	201,7 ± 44,9	96,7 ± 26,6	107,1 ± 3,8
lipid content (%)	4,3 ± 0,1	18,7 ± 0,5	1,7 ± 0,1	14,2 ± 2,4	1,6 ± 0,1	9,8 ± 0,1
Fatty acids (mg/kg)						
C12:0	755,7 ± 11,8	0,0 ± 0,0	6,0 ± 5,7	46,9 ± 40,1	10,2 ± 3,2	15,9 ± 4,3
C14:0	306,6 ± 3,3	721,6 ± 30,4	24,4 ± 18,3	42,5 ± 39,2	9,1 ± 3,3	248,9 ± 47,9
C14:1	12,3 ± 2,0	189,2 ± 30,7	4,0 ± 2,0	8,0 ± 8,6	7,8 ± 7,6	8,6 ± 1,6
C16:0	5.213,5 ± 140,2	22.468,5 ± 646,6	677,7 ± 465,5	13.473,1 ± 2.472,6	1.708,9 ± 755,5	12.779,8 ± 4.134,3
C16:1	184,5 ± 75,6	1.049,0 ± 99,9	230,8 ± 137,2	365,6 ± 236,2	17,5 ± 7,2	1.377,4 ± 499,2
C18:0	290,9 ± 6,6	1.435,9 ± 64,2	70,2 ± 64,0	1.025,7 ± 218,6	242,8 ± 109,4	639,7 ± 168,3
C18:1	6.001,7 ± 166,7	62.398,7 ± 521,2	3.257,7 ± 2.677,1	32.120,8 ± 5.945,6	2.965,6 ± 1.220,6	5.818,5 ± 1.191,5
C18:2[E]	17.907,9 ± 605,7	53.336,2 ± 279,6	1.829,8 ± 1.060,9	59.517,4 ± 11.442,9	6.454,9 ± 2.526,7	27.469,1 ± 4.949,9
C18:3[E]	1.765,3 ± 65,6	1.764,8 ± 14,7	348,8 ± 247,5	1.456,1 ± 260,6	373,6 ± 143,9	3.020,5 ± 539,6
C18:4	0,0 ± 0,0	48,4 ± 1,2	9,0 ± 15,6	41,5 ± 13,2	6,7 ± 1,6	0,0 ± 0,0
C20:0	54,2 ± 5,0	1.098,4 ± 119,8	155,9 ± 130,8	392,9 ± 61,5	67,3 ± 29,3	127,7 ± 28,4
C20:1	270,6 ± 10,0	1.409,2 ± 298,0	81,5 ± 132,6	454,5 ± 94,0	48,0 ± 23,8	451,0 ± 105,4
C20:2	87,8 ± 13,7	285,8 ± 68,1	33,9 ± 17,5	20,4 ± 18,9	47,0 ± 3,9	
C20:5[E]	20,3 ± 17,6	0,0 ± 0,0	5,9 ± 10,1	27,1 ± 23,8	5,8 ± 5,6	93,2 ± 20,2
C22:0	223,1 ± 22,7	1.731,5 ± 406,0	125,5 ± 93,4	334,4 ± 50,0	19,3 ± 5,9	334,8 ± 87,3
C22:1	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	73,2 ± 14,0
C20:3	53,8 ± 22,8	281,7 ± 67,3	12,7 ± 9,4	126,1 ± 61,8	344,8 ± 100,8	
C22:5[E]	3,5 ± 6,1	0,0 ± 0,0	2,0 ± 3,5	0,0 ± 0,0	0,0 ± 0,0	334,4 ± 54,6
C24:0	60,0 ± 6,2	2231,6 ± 581,8	126,0 ± 139,5	217,9 ± 40,0	38,9 ± 17,8	553,4 ± 130,8
C22:6[E]	18,5 ± 17,0	207,0 ± 292,7	80,5 ± 125,0	22,8 ± 20,0	0,0 ± 0,0	109,5 ± 55,2
Sum EFA (g/kg)	19,7 ± 0,7	55,3 ± 0,6	3,04 ± 0,0	61,0 ± 11,7	6,8 ± 2,7	31,0 ± 5,6
Sum SFA	6,9 ± 0,2	29,7 ± 1,8	1,2 ± 0,9	15,5 ± 2,9	2,1 ± 0,9	14,7 ± 4,6

	Wheat middlings	Rice bran	Rapeseed meal	DDGS	Corn gluten feed	Brewery grains
Sum MUFA	6,5 ± 0,3	65,0 ± 0,9	3,6 ± 2,9	32,9 ± 6,3	3,0 ± 1,3	7,7 ± 1,8
Sum PUFA	19,9 ± 0,7	55,9 ± 0,7	2,3 ± 1,5	61,2 ± 11,8	6,8 ± 2,7	31,4 ± 5,7
% starch *	21,8	32,7		4,8	15,3	3,7
% sugar*	6,3	4,1	8,3	1,5	2,4	2,4
Total *	28,1	36,8	8,3	6,3	17,7	6,1

EAA: essential amino acids for insects, EFA: essential fatty acids, E= essential, SFE: saturated fatty acids, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acids, * according to CVB table (2018)

Table 0-2 Protein content, amino acid profile and sum of essential amino acids of the diets on dry matter basis. E essential amino acids for insects.

g/kg	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15	Diet 16	Diet 17	Diet 18
Protein content (%)	15,5±1,0	17,2 ±0,6	14,2 ±1,8	17,9 ±0,8	29,2 ± 0,6	29,2 ± 3,4	26,4±2,2	17,9±3,2	17,1±0,5	17,1±0,5	17,1±0,5	17,0±0,4	17,7±0,5	18,2±0,5	18,6±0,4	19,1±0,4	21,8±1,3	17,6±1,4
Arginine	46,2±5,3	41,0±2,9	70,4±8,8	47,4±4,5	73,9 ±11,1	108,7±34,5	82,6 ±22,4	35,9 ±7,80	41,8±2,9	42,6±3,0	43,4±3,0	44,2±3,1	41,6±2,5	42,5±2,3	43,5±2,1	44,4±2,0	61,7 ±12,6	38,2±2,5
Lysine[E]	5,6±0,7	4,7±0,5	4,8±0,7	3,6±0,2	15,3±1,4	6,8±1,1	5,5±0,8	5,2±1,1	4,7±0,5	4,6±0,5	4,6±0,5	4,6±0,4	5,0±0,5	5,3±0,5	5,6±0,5	5,9±0,5	5,1±0,5	4,9±0,4
Alanine	7,5±0,5	8,9±0,3	6,9±1,1	9,8±0,5	14,1±0,8	19,2±1,8	16,8±1,1	14,5±3,1	9,0±0,2	8,9±0,2	8,9±0,2	8,9±0,2	9,3±0,2	9,5±0,2	9,7±0,2	9,9±0,1	12,9±0,7	11,7±1,5
Threonine[E]	5,6±0,5	6,8±0,3	4,9±1,0	7,3±0,4	14,6±1,7	9,5±0,2	9,4±0,1	7,6±1,2	6,7±0,3	6,7±0,3	6,7±0,3	6,7±0,3	7,1±0,3	7,4±0,3	7,7±0,2	8,0±0,2	8,1±0,2	7,2±0,5
Glycine	7,9±0,9	8,4±0,6	6,2±0,7	8,0±0,4	16,6±0,8	11,0±0,7	10,5±0,4	8,2±1,4	8,4±0,5	8,3±0,5	8,2±0,4	8,2±0,4	8,7±0,5	9,0±0,5	9,3±0,5	9,6±0,5	9,5±0,4	8,4±0,5
Valine[E]	7,8±0,4	9,7±0,4	6,7±1,7	10,7±0,8	19,5±1,3	15,9±1,5	15,2±0,9	9,9±1,5	9,7±0,4	9,7±0,4	9,6±0,4	9,6±0,5	10,2±0,4	10,6±0,4	11,0±0,4	11,4±0,5	12,5±0,3	9,9±0,8
Serine	6,7±0,7	7,6±0,4	4,8±0,8	7,4±0,4	7,6±6,6	10,7±0,2	10,3±0,1	8,1±1,5	7,5±0,4	7,5±0,3	7,4±0,3	7,3±0,3	7,7±0,3	7,7±0,4	7,7±0,6	7,8±0,8	8,9±0,2	7,9±0,7
Proline	9,9±1,3	14,4±0,9	5,4±0,5	15,9±0,3	16,9±0,5	18,3±0,8	20,2±0,7	18,3±2,8	14,3±0,8	14,2±0,8	14,0±0,7	13,9±0,6	14,9±0,8	15,1±0,8	15,4±0,7	15,6±0,7	17,4±0,5	16,6±1,3
Isoleucine[E]	4,7±0,2	6,2±0,2	3,4±0,5	6,8±0,2	12,4±1,4	8,5±0,3	8,8±0,2	6,7±1,0	6,1±0,2	6,1±0,1	6,0±0,1	6,0±0,1	6,5±0,1	6,8±0,1	7,0±0,1	7,3±0,2	7,5±0,1	6,5±0,6
Leucine[E]	11,5±0,8	14,3±0,6	8,2±0,9	15,0±0,4	23,5±1,5	31,3±2,7	27,6±1,8	18,7±3,4	14,2±0,5	14,1±0,4	14,0±0,4	13,8±0,3	14,8±0,5	15,2±0,4	15,6±0,3	16,0±0,2	21,0±1,1	16,6±1,8
Methionine[E]	3,3±0,4	3,2±0,2	2,4±0,4	2,6±0,3	5,1±1,3	3,7±1,1	3,4±0,8	1,1±0,1	3,1±0,2	3,1±0,1	3,1±0,1	3,0±0,1	3,2±0,1	3,3±0,1	3,3±0,1	3,4±0,1	3,3±0,3	2,2±0,0
Histidine[E]	2,7±0,1	3,4±0,1	2,1±0,5	3,8±0,4	7,7±0,4	5,4±1,6	5,3±0,9	5,6±1,1	3,4±0,1	3,4±0,1	3,4±0,1	3,3±0,1	3,6±0,1	3,8±0,1	4,0±0,1	4,1±0,1	4,4±0,5	4,5±0,5
phenylalanine[E]	6,9±0,3	9,3±0,2	4,7±0,2	10,1±0,1	13,1±0,6	11,7±1,9	12,6±1,3	9,4±1,4	9,2±0,2	9,1±0,2	9,1±0,1	9,0±0,1	9,6±0,2	9,8±0,1	10,0±0,1	10,2±0,1	11,0±0,7	9,5±0,7
Glutamate	34,7±1,6	40,3±1,4	16,1±2,4	36,7±0,3	59,8±6,8	45,9±0,7	48,0±1,1	39,1±6,9	39,7±1,3	39,1±1,2	38,5±1,0	37,8±0,9	41,5±1,2	42,3±1,1	43,1±1,0	44,0±1,0	44,3±1,0	40,1±3,2
Aspartate	12,2±0,7	13,1±0,5	11,2±1,2	13,4±0,4	23,9±2,4	17,1±0,5	16,4±0,4	11,5±1,7	13,1±0,4	13,1±0,4	13,0±0,3	13,0±0,3	13,6±0,4	14,0±0,4	14,3±0,3	14,7±0,3	14,8±0,4	12,4±0,8
Cystine	1,7±0,1	2,2±0,1	1,4±0,1	2,4±0,1	4,7±0,2	3,1±0,3	3,1±0,2	1,8±0,3	2,2±0,1	2,2±0,1	2,2±0,1	2,2±0,1	2,3±0,1	2,4±0,1	2,5±0,1	2,6±0,1	2,7±0,1	2,0±0,2
Tyrosine	3,7±0,4	4,9±0,3	2,8±0,3	5,5±0,2	8,5±0,1	9,2±0,7	8,7±0,5	5,4±0,8	4,9±0,3	4,9±0,2	4,8±0,2	4,8±0,2	5,1±0,3	5,3±0,2	5,5±0,2	5,6±0,2	6,8±0,4	5,2±0,4
Sum of EAA (g/kg)	94,40± 6,31	98,47± 3,27	107,52±13,99	107,27± 7,01	185,16± 2,32	201,65±37,88	170,23±24,27	100,15±18,39	98,94± 3,24	99,38± 3,30	99,83± 3,43	100,27± 3,62	101,70± 2,89	104,70± 2,67	107,70± 2,45	110,70± 2,23	134,42±13,72	99,53± 7,57

*Table 0-3 Lipid content, fatty acid profile and sum of essential fatty acids of the diets on dry matter basis. [E] essential fatty acids, * analysis performed in duplicate, SFE: saturated fatty acids, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid*

	Diet 1	Diet 2	Diet 3 *	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15	Diet 16	Diet 17	Diet 18
Lipid content (%)	4,3±0,1	6,1±0,1	18,7±0,5	13,7±0,3	1,7±0,1	14,2±2,4	12,8±1,6	4,3±0,1	6,6±0,1	7,1±0,1	7,6±0,1	8,1±0,1	6,1±0,1	6,0±0,1	5,9±0,1	5,8±0,1	9,5±0,8	5,3±0,0
C12:0	755,7±11,8	518,3±8,9	0,0±0,0	14,4±11,5	3,1±2,6	46,9±40,1	36,6±26,4	12,1±3,4	491,5±8,7	466,3±8,6	441,2±8,6	416,0±8,7	480,2±8,3	455,3±7,8	430,4±7,4	405,6±6,9	273,2±15,9	256,1±5,3
C14:0	306,6±3,3	288,1±17,4	721,6±30,4	451,2±18,1	35,0±0,5	42,5±39,2	111,1±42,1	89,3±15,8	301,9±16,6	315,9±15,8	329,8±15,0	343,8±14,2	278,1±18,0	269,1±17,9	260,1±17,8	251,1±17,7	199,3±30,0	189,3±17,3
C14:1	12,3±2,0	11,1±1,7	189,2±30,7	75,0±21,5	5,1±0,9	8,0±8,6	8,2±5,9	8,1±4,9	16,2±2,7	21,3±4,2	26,4±5,8	31,5±7,4	10,8±1,6	10,6±1,5	10,3±1,4	10,1±1,3	9,6±2,7	9,6±1,8
C16:0	5.213,5±140,2	7.641,9±1.422,1	22.468,5±646,6	16.891,5±2.174,4	946,4±9,1	13.473,1±2.472,6	13.242,7±2.540,8	5.412,3±1.416,0	8.237,5±1.411,5	8.816,9±1.392,3	9.396,3±1.373,3	9.975,8±1.354,3	7.635,5±1.488,2	7.494,4±1.483,3	7.353,3±1.478,4	7.212,2±1.473,4	10.485,3±1.910,9	6.680,4±1.466,4
C16:1	184,5±75,6	567,3±168,8	1.049,0±99,9	1.256,5±238,1	308,4±27,5	365,6±236,2	701,8±323,5	472,4±163,8	600,8±165,4	631,6±161,0	662,5±156,7	693,3±152,3	592,7±175,7	596,8±174,5	600,8±173,3	604,9±172,2	641,4±246,0	541,9±172,7
C18:0	290,9±6,6	402,8±56,7	1.435,9±64,2	951,0±88,8	101,9±32,6	1.025,7±218,6	897,4±187,3	375,6±95,6	439,8±55,5	476,1±54,2	512,4±53,2	548,7±52,6	402,8±58,4	396,5±57,2	390,3±56,0	384,0±54,8	652,1±119,4	395,6±70,9
C18:1	6.001,7±166,7	5.942,9±489,9	62.398,7±521,2	28.270,1±3.420,7	4.803,2±29,5	32.120,8±5.945,6	23.380,1±4.164,3	3.919,9±860,4	7.701,8±588,7	9.461,2±763,1	11.220,7±974,5	12.980,2±1.203,5	5.900,0±503,9	5.860,4±499,6	5.820,7±495,2	5.781,1±490,9	14.660,4±2.239,9	4.944,8±560,3
C18:2 [E]	17.907,9±605,7	20.976,6±1.988,7	53.336,2±279,6	34.361,0±8.063,6	2.441,8±41,6	59.517,4±11.442,9	48.867,3±8.413,7	13.484,5±2.332,5	21.862,3±2.129,6	22.727,8±2.393,8	23.593,4±2.752,8	24.459,0±3.174,6	20.635,4±2.047,9	20.124,0±2.029,7	19.612,7±2.011,5	19.101,3±1.993,3	34.976,2±4.877,0	17.462,8±2.035,7
C18:3 [E]	1.765,3±65,6	2.168,2±216,8	1.764,8±14,7	2.279,9±484,1	490,4±33,4	1.456,1±260,6	1.976,0±297,0	1.259,1±204,1	2.155,1±221,0	2.139,3±227,3	2.123,5±236,5	2.107,8±248,2	2.148,4±224,2	2.106,2±223,1	2.064,0±222,1	2.021,9±221,1	2.079,2±251,1	1.743,5±211,4
C18:4	0,0±0,0	0,0±0,0	48,4±1,2	207,7±323,7	13,5±13,5	41,5±13,2	27,7±8,8	4,4±1,1	16,3±25,5	32,7±50,9	49,0±76,4	65,4±101,8	0,4±0,4	0,9±0,9	1,3±1,3	1,8±1,8	13,8±4,4	2,2±0,5
C20:0	54,2±5,0	77,8±11,4	1.098,4±119,8	593,4±80,7	231,3±3,9	392,9±61,5	304,7±47,8	87,5±18,5	117,1±6,7	156,2±8,1	195,3±13,9	234,4±20,7	85,0±11,8	90,8±11,8	96,7±11,8	102,5±11,8	191,7±29,2	83,9±10,6
C20:1	270,6±10,0	328,5±30,5	1.409,2±298,0	1.155,0±515,1	117,3±117,3	454,5±94,0	453,4±83,5	182,8±38,3	390,4±45,6	451,8±80,8	513,3±119,5	574,8±159,1	326,6±36,2	321,6±40,0	316,5±43,9	311,4±47,9	392,0±55,7	260,1±34,7
C20:2	87,8±13,7	74,7±8,8	285,8±68,1	149,8±26,1	43,9±1,6	20,4±18,9	29,2±13,2	15,7±1,3	87,8±19,5	100,9±30,3	114,0±41,0	127,1±51,8	72,6±8,1	71,1±7,7	69,7±7,2	68,2±6,7	51,7±2,4	45,0±4,1
C20:5 [E]	20,3±17,6	43,7±14,9	0,0±0,0	53,1±11,5	0,0±0,0	27,1±23,8	49,0±19,9	35,0±4,6	43,1±14,4	42,4±13,8	41,8±13,3	41,1±12,8	44,3±14,3	43,6±13,8	42,9±13,3	42,3±12,8	46,8±17,4	40,7±8,3
C22:0	223,1±22,7	259,0±24,6	1.731,5±406,0	1.189,3±435,3	176,9±28,5	334,4±50,0	334,6±60,6	124,9±29,4	330,2±19,6	401,2±52,8	472,1±88,5	543,1±124,5	259,4±25,1	257,9±24,4	256,4±23,7	254,8±23,0	297,4±42,6	195,0±27,8
C22:1	0,0±0,0	23,5±4,5	0,0±0,0	41,7±8,0	0,0±0,0	0,0±0,0	24,3±4,7	24,5±4,7	23,7±4,5	23,7±4,5	23,7±4,5	23,7±4,5	24,8±4,8	24,8±4,8	24,8±4,8	24,8±4,8	24,3±4,7	25,3±4,8
C20:4	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0	0,0±0,0
C20:3	53,8±22,8	147,2±32,2	281,7±67,3	320,7±36,3	7,7±3,6	126,1±61,8	198,7±73,7	115,3±33,7	155,8±30,7	163,7±29,1	171,7±27,4	179,6±25,7	150,9±33,4	149,3±33,2	147,8±33,0	146,3±32,8	174,6±51,1	136,7±33,7
C22:5 [E]	3,5±6,1	109,7±21,2	0,0±0,0	190,5±31,1	0,0±0,0	0,0±0,0	111,1±18,1	111,9±18,3	110,3±21,1	110,2±20,9	110,1±20,7	110,0±20,6	115,5±21,9	115,4±21,7	115,3±21,5	115,2±21,3	112,3±19,9	116,7±20,6

	Diet 1	Diet 2	Diet 3 *	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15	Diet 16	Diet 17	Diet 18
C24:0	60,0±6,2	218,3±42,9	2.231,6±581,8	1.280,4±111,4	186,0±93,2	217,9±40,0	329,4±58,5	211,0±48,1	293,3±30,0	367,1±18,2	441,0±12,6	514,9±19,5	231,3±48,1	235,5±51,1	239,6±54,0	243,8±57,0	276,7±50,7	223,6±47,1
C22:6 [E]	18,5±17,0	47,7±14,3	207,0±292,7	#DIV/0! ±#DIV/0!	112,2±112,2	22,8±20,0	51,6±23,3	36,6±18,5	58,9±24,9	69,8±35,5	80,8±46,0	91,8±56,5	52,4±12,4	55,5±10,2	58,6±88	61,7±85	50,2±15,6	43,9±16,4
Sum of EFA (g/kg)	19,7±0,7	23,3±2,2	55,3±0,6	36,9±8,5	3,0±0,0	61,0±11,7	51,1±8,7	14,9±2,6	24,2±2,4	25,1±2,7	25,9±3,0	26,8±3,4	23,0±2,3	22,4±2,3	22,9±1,9	21,3±2,2	37,3±5,1	18,5±2,4
Sum of SFA (g/kg)	6,9±0,2	9,4±1,6	30,2±1,6	21,4±2,0	1,7±0,0	15,5±2,9	15,3±2,9	6,3±1,6	10,2±1,5	11,0±1,5	11,8±1,4	12,6±1,4	9,4±1,6	9,2±1,6	9,0±1,6	8,9±1,6	12,4±2,2	8,0±1,6
Sum of MUFA (g/kg)	6,5±0,2	6,9±0,7	61,3±6,4	30,8±3,2	5,2±0,1	32,9±6,2	24,6±4,5	4,6±0,9	8,7±0,8	10,6±0,9	12,4±1,1	14,3±1,3	6,9±0,7	6,8±0,7	6,8±0,7	6,7±0,7	15,7±2,5	5,8±0,6
Sum of PUFA (g/kg)	19,9±0,7	23,6±2,3	46,4±16,5	37,5±8,3	3,1±0,0	61,2±11,8	51,3±8,8	15,1±2,6	24,5±2,4	25,4±2,6	26,3±2,9	27,2±3,3	23,2±2,3	22,7±2,3	22,1±2,3	21,6±2,3	37,5±5,2	19,6±2,3

*Table 0-4 Dry matter, protein content, amino acid profile, sum of the essential amino acids and chitin content of larvae on dry matter basis. [E] essential amino acids for humans, * analysis performed in duplicate, nd: not determined*

	Diet 1	Diet 2	Diet 3*	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15	Diet 16	Diet 17	Diet 18*

Dry matter (%)	28.3 ± 0.2	32.0 ± 0.0	nd	32.4 ± 0.1	32.6 ± 0.2	30.8 ± 0.2	23.5 ± 2.4	27.5 ± 1.0	31.6 ± 0.3	32.0 ± 0.2	32.6 ± 0.0	32.3 ± 0.2	31.2 ± 0.1	30.5 ± 0.2	31.9 ± 0.1	31.2 ± 0.3	31.2 ± 0.2	31.5 ± 0.5
Protein content (%)	48.3±2.3	37.4±1.2	46.1±1.9	43.7±2.3	49.0±1.4	37.5±3.0	39.1±0.8	38.8±1.5	37.5±2.7	37.5±1.0	43.6±2.8	38.4±0.8	41.7±0.4	41.0±0.4	47.8±1.9	39.1±0.9	39.2±1.3	38.9±1.9
Arginine	**69.7±4.2**	**39.6±2.5**	**73.2±3.0**	**62.3±10.5**	**61.3±8.0**	**46.6±5.3**	**42.7±3.0**	**44.8±4.3**	**56.1±4.8**	**46.9±3.2**	**52.2±7.7**	**50.2±3.3**	**60.2±2.5**	**57.1±3.2**	**53.4±3.0**	**49.7±3.9**	**37.3±1.4**	**39.2 ± 3.2**
Alanine	55.5±6.2	33.5±1.8	54.9±8.4	37.9±2.5	42.5±2.9	36.6±2.0	39.3±1.1	38.7±2.7	37.3±1.2	42.1±1.9	37.8±1.8	43.1±4.2	45.6±0.6	44.9±1.5	43.5±1.9	42.7±0.5	36.7±0.5	35.9±1.8
Aspartate	45.1±1.6	40.9±0.5	41.8±1.2	45.1±5.4	52.8±6.3	39.8±2.8	41.6±0.6	40.9±1.7	38.9±2.9	38.3±0.8	46.0±5.7	40.0±1.9	41.8±0.9	41.6±1.3	49.7±1.3	40.9±1.2	51.9±4.9	49.4 ± 1.3
Cystine	3.3±0.5	2.9±0.3	3.0±0.4	3.5±0.8	4.2±0.2	0.4±0.5	0.6±0.2	0.6±0.4	0.2±0.0	0.1±0.1	3.3±0.8	0.2±0.2	1.8±1.9	0.2±0.2	3.4±0.7	0.1±0.0	2.3±0.3	2.5 ± 0.2
Glutamate	76.8±3.1	60.0±0.8	73.5±3.9	64.4±8.9	82.7±10.7	58.4±3.5	56.1±0.9	55.9±1.2	55.9±4.1	55.1±0.5	67.7±9.7	57.1±0.7	59.5±0.7	59.3±1.3	71.6±2.6	58.5±1.8	54.8±1.4	56.6 ± 5.1
Glycine	29.2±1.2	20.5±0.9	27.6±0.5	23.8±1.8	28.5±0.1	23.2±1.4	22.9±0.3	22.9±1.6	23.4±1.7	25.7±1.0	24.1±1.2	26.3±2.7	25.7±0.6	25.3±1.1	25.4±0.5	24.0±1.3	24.1±0.3	23.7±0.8
Histidine[E]	19.5±2.6	14.4±1.0	17.6±0.1	16.7±0.4	19.7±1.2	14.7±1.3	16.6±0.3	16.2±0.6	15.6±1.2	16.7±0.7	17.2±0.6	16.6±1.2	17.5±0.4	17.9±1.6	18.0±0.8	16.5±0.7	17.7±0.1	17.6 ± 0.4
Hydroxylysine	0.03±0.05	0.6±0.03	0.34±0.10	0.2±0.1	0.3±0.0	6.4±10.7	0.5±0.5	0.00±0.00	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.3 ± 0.0
Isoleucine[E]	23.2±0.7	20.1±0.5	21.0±1.5	23.0±1.7	26.3±2.0	22.5±1.3	23.2±1.3	22.9±0.5	20.7±1.4	20.6±0.4	22.8±2.0	21.5±1.0	22.3±0.5	22.5±0.5	24.5±1.1	21.9±0.4	25.2±1.9	25.0 ± 0.7
Leucine[E]	41.0±1.6	31.9±0.1	40.1±0.9	36.6±1.7	42.5±2.3	32.4±1.7	32.3±0.9	31.1±1.1	35.3±2.3	35.4±0.4	36.8±1.8	36.3±0.3	37.7±0.7	38.6±1.0	38.1±2.3	37.9±0.6	37.2±1.3	37.6 ± 2.3
Lysine[E]	36.9±1.9	27.6±0.7	35.1± 0.0	32.1±1.8	37.4±2.1	20.4±17.2	31.6±1.5	32.2±1.2	4.9±0.4	4.6±0.4	32.5±1.9	4.6±1.2	5.8±0.4	5.8±1.3	33.2±0.7	5.3±0.6	1.9±0.2	2.1 ± 0.3
Methionine[E]	7.5±0.5	7.2±0.5	6.9±0.3	7.8±2.0	8.5±2.2	4.9±1.3	3.2±0.6	2.9±0.5	0.8±0.2	1.8±0.9	7.8±2.0	2.4±0.6	4.9±3.2	2.2±0.8	9.0±1.0	1.6±0.5	6.0±0.8	6.8 ± 0.1
phenylalanine[E]	22.2±1.0	19.7±0.5	19.9±0.1	20.9±2.3	25.1±2.7	17.2±1.8	20.8±0.2	20.2±1.1	21.4±2.3	22.0±0.3	22.9±2.5	21.8±1.9	22.7±1.2	22.7±1.4	25.3±1.2	22.6±0.6	22.9±0.8	23.2 ± 1.6
Proline	30.5±1.6	25.2±1.5	28.7±0.7	28.8±1.9	30.1±1.1	25.9±2.1	28.2±0.1	29.1±1.6	27.5±1.7	28.2±0.2	29.5±1.8	27.8±0.2	30.9±0.3	31.1±0.9	32.1±1.7	29.3±0.3	28.6±0.5	26.5 ± 1.2
Serine	18.8±0.5	15.7±2.5	18.6±0.2	20.2±1.9	22.3±1.3	18.2±2.0	18.4±1.0	18.8±0.7	15.7±1.2	15.7±0.2	18.6±3.1	16.3±0.5	17.9±0.3	17.9±0.3	32.1.±1.7	17.5±0.9	19.3±0.7	17.0 ± 1.1
Threonine[E]	17.9±1.7	17.9±0.9	18.6±0.6	20.3±1.8	23.9±1.9	19.8±1.6	19.9±0.3	19.9±0.9	19.1±1.3	19.1±0.3	20.2±1.9	19.5±0.3	20.9±0.1	20.5±0.6	22.3±0.6	19.9±0.8	22.4±0.9	21.3 ± 0.7
Tyrosine	29.2±1.4	31.6±1.6	21.7±0.0	32.2±2.9	37.9±3.4	21.2±1.7	29.0±0.7	26.8±0.3	35.2±4.1	34.7±4.0	36.2±2.5	34.1±0.5	37.8±0.5	37.4±0.3	40.5±2.8	36.6±1.3	34.1±0.6	35.5 ± 1.7
Valine[E]	35.9±2.3	25.5±2.3	33.6±1.2	31.2±1.9	37.4±2.8	28.1±1.5	28.2±0.4	27.9±2.1	28.5±1.7	29.8±0.8	30.5±1.7	30.4±1.1	32.2±0.9	32.7±0.3	33.8±1.3	30.9±0.5	34.42±1.9	33.6 ± 0.4
Sum of EAA (g/kg)	204.2±10.6	164.4±3.9	193.0±4.7	188.6±12.5	220.8±13.5	159.9±24.1	175.9±4.4	173.4±5.3	146.4±10.3	149.9±3.0	190.7±13.6	153.1±4.1	163.9±2.9	163.0±3.5	204.0±8.5	156.6±2.0	167.9±7.2	167.1±6.4
Chitin content (%)	4.2±0.1	6.2±0.1	4.3±0.7	5.7±0.2	6.0±0.2	5.4±0.6	4.7±0.0	5.2±0.4	5.6±0.3	5.0±0.2	5.9±0.1	5.0±0.2	5.3±0.2	5.4±0.1	6.2±0.3	5.4±0.3	5.6±0.4	4.9±0.2

*Table 0-5 Lipid content, fatty acid composition and sum of essential fatty acids of the larvae on dry matter basis. [E] essential fatty acids, * analysis performed in duplicate, SFE: saturated fatty acids, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fat, nd not determined*

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15 *	Diet 16	Diet 17	Diet 18
Lipid content (%)	26.8 ± 0.5	26.5 ± 2.1	24.6 ± 0.7	22.3 ± 0.7	14.4 ± 1.3	16.1 ± 2.6	28.0 ± 0.6	26.7 ± 1.1	22.2 ± 1.0	26.4 ± 0.5	26.0 ± 1.4	25.5 ± 0.2	23.7 ± 0.3	24.1 ± 0.5	23.7 ± 1.8	23.3 ± 0.6	24.6 ± 1.1	26.3 ± 2.2
C12:0	114.8 ± 1.2	445.9 ± 33.1	93.9 ± 5.7	76.1 ± 10.3	205.6 ± 30.8	nd	nd	nd	127.0 ± 5.1	127.3 ± 9.2	147.5 ± 10.2	106.6 ± 5.6	154.8 ± 10.1	152.6 ± 5.6	156.6 ± 14.9	107.1 ± 2.4	83.6 ± 14.7	92.5 ± 11.1
C14:0	1355.2 ± 78.9	1007.2 ± 729.9	1110.4 ± 45.1	934.5 ± 89.7	915.5 ± 137.9	610.4 ± 24.9	1115.4 ± 55.9	1127.8 ± 266.9	1165.6 ± 19.2	1293.9 ± 109.3	1282.9 ± 16.6	1206.4 ± 85.4	1345.3 ± 44.6	1335.2 ± 30.1	1417.6 ± 170.7	1214.3 ± 52.0	1026.2 ± 191.9	1064.0 ± 105.5

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15 *	Diet 16	Diet 17	Diet 18
C14:1	6.7 ± 0.8	68.5 ± 59.6	14.8 ± 10.5	65.7 ± 1.8	73.4 ± 9.4	36.5 ± 2.4	91.0 ± 10.6	101.2 ± 29.2	84.0 ± 4.1	94.4 ± 6.8	87.2 ± 25.4	82.0 ± 4.8	46.5 ± 39.4	35.9 ± 10.8	175.5 ± 152.4	93.5 ± 9.5	26.5 ± 3.3	26.6 ± 12.1
C16:0	56083.2 ± 6034.4	44790.1 ± 4487.9	36663.8 ± 1861.3	28458.6 ± 2355.3	25630.2 ± 4166.5	21091.5 ± 1320.2	43334.0 ± 2697.3	42112.6 ± 9732.7	33507.9 ± 1529.3	39150.1 ± 3136.5	41568.7 ± 1154.4	37105.7 ± 1887.1	39886.8 ± 1076.7	43943.3 ± 455.4	38453.4 ± 957.9	38459.9 ± 2468.0	36649.8 ± 8258.6	
C16:1	1163.6 ± 33.7	1876.1 ± 474.2	1408.3 ± 40.6	1904.4 ± 404.7	2235.1 ± 613.6	586.0 ± 36.4	1141.8 ± 56.1	836.2 ± 194.9	2328.9 ± 1267.4	2025.4 ± 367.3	2486.8 ± 423.9	1699.0 ± 157.1	1886.5 ± 369.3	1676.5 ± 318.1	2362.4 ± 863.5	1650.3 ± 182.5	1949.9 ± 636.4	1965.2 ± 358.5
C18:0	16853.7 ± 1982.5	7016.3 ± 1660.7	14578.9 ± 517.4	3971.4 ± 781.8	3739.4 ± 269.3	504.3 ± 113.6	6619.9 ± 428.0	8172.6 ± 2120.7	5476.6 ± 260.7	6184.3 ± 475.3	6344.1 ± 186.3	5749.8 ± 172.2	7016.7 ± 93.3	7311.3 ± 168.3	6754.1 ± 1285.1	5832.8 ± 359.9	5820.8 ± 1957.1	5848.7 ± 616.1
C18:1	85203.8 ± 2053.0	50125.9 ± 4761.5	86063.9 ± 3265.6	42715.1 ± 3604.0	38810.4 ± 5099.4	4914.6 ± 389.6	63410.8 ± 3114.3	59229.2 ± 14337.9	43408.3 ± 1586.2	51682.2 ± 4995.4	52286.9 ± 1389.3	53008.2 ± 4354.2	46342.6 ± 1558.1	50333.4 ± 1233.1	41067.7 ± 7600.5	42754.4 ± 1742.1	48422.1 ± 10241.2	53162.5 ± 4887.4
C18:2 [E]	55825.6 ± 1152.4	56465.9 ± 5639.4	66148.1 ± 1957.3	54415.2 ± 4303.8	21162.5 ± 2090.6	38401.2 ± 2295.4	85450.6 ± 3695.0	62016.7 ± 15350.2	51519.1 ± 1069.1	60888.4 ± 4676.4	57479.0 ± 2283.4	60989.6 ± 4520.4	54051.8 ± 1669.9	54792.6 ± 1355.0	36051.8 ± 30320.3	50342.8 ± 1918.6	50183.6 ± 10468.0	72319.7 ± 5283.8
C18:3 [E]	2216.5 ± 32.0	3841.4 ± 387.0	1198.1 ± 48.5	2672.6 ± 221.7	1729.9 ± 177.5	43660.6 ± 2654.1	3517.3 ± 183.5	3904.9 ± 958.7	3543.4 ± 101.0	4052.8 ± 314.1	3546.1 ± 134.8	3740.0 ± 274.7	4030.8 ± 123.9	3964.3 ± 115.9	2692.2 ± 2334.8	3854.5 ± 143.5	3296.7 ± 699.3	3477.8 ± 229.8
C18:4	33.8 ± 23.4	nd	18.1 ± 14.2	nd	nd	1031.3 ± 54.7	nd	nd	31.7 ± 0.6	30.7 ± 3.0	nd	37.9 ± 6.7	22.2 ± 19.3	22.3 ± 19.5	nd	nd	nd	nd
C20:0	804.7 ± 88.2	398.7 ± 44.2	845.3 ± 38.0	353.8 ± 31.9	301.0 ± 68.0	nd	550.8 ± 17.5	641.6 ± 201.0	330.1 ± 30.8	433.6 ± 42.5	422.4 ± 12.5	443.0 ± 47.1	378.6 ± 53.4	422.6 ± 5.4	628.9 ± 556.2	315.3 ± 18.3	402.2 ± 111.5	451.3 ± 57.2
C20:1	252.1 ± 3.6	749.0 ± 76.3	453.5 ± 19.7	870.4 ± 68.7	44.1 ± 38.4	574.0 ± 42.1	506.0 ± 36.5	370.0 ± 57.9	524.6 ± 11.9	624.1 ± 69.4	931.6 ± 81.7	649.4 ± 38.3	523.0 ± 5.4	544.6 ± 17.3	1179.8 ± 1001.3	482.7 ± 22.1	388.5 ± 97.7	524.7 ± 97.6
C20:2	0.0 ± 0.0	287.1 ± 149.9	nd	478.5 ± 12.4	144.0 ± 20.8	nd	233.0 ± 83.3	186.3 ± 80.8	275.0 ± 6.4	318.9 ± 22.5	459.3 ± 58.3	331.6 ± 27.4	241.1 ± 5.5	262.3 ± 3.6	279.3 ± 82.3	234.5 ± 7.3	328.1 ± 99.4	375.2 ± 115.5
C20:5 [E]	37.5 ± 3.8	nd	40.8 ± 1.0	nd	12.6 ± 21.9	313.6 ± 12.1	62.4 ± 108.0	46.3 ± 80.2	132.3 ± 114.6	244.5 ± 24.9	nd	225.7 ± 31.5	129.6 ± 113.5	202.6 ± 15.6	nd	199.7 ± 16.5	111.6 ± 21.2	330.3 ± 366.2
C22:0	144.9 ± 6.1	622.6 ± 89.3	201.5 ± 7.0	910.9 ± 62.5	178.2 ± 7.4	nd	331.4 ± 8.2	257.8 ± 76.0	nd	nd	738.1 ± 113.6	nd	66.4 ± 115.0	nd	444.1 ± 169.2	nd	208.1 ± 58.5	307.9 ± 74.7
C22:1	3.5 ± 6.1	nd	5.7 ± 4.9	nd	nd	216.8 ± 19.7	nd	nd	261.9 ± 92.6	268.1 ± 41.1	nd	281.1 ± 9.3	242.2 ± 21.4	260.3 ± 9.8	nd	248.4 ± 6.7	26.3 ± 7.5	40.0 ± 17.4
C20:4	nd	nd	nd	nd	nd	213.0 ± 32.1	nd	nd	7.4 ± 1.4	15.9 ± 7.1	nd	9.7 ± 2.7	7.8 ± 6.9	12.7 ± 1.4	nd	12.3 ± 1.2	nd	nd
C20:3	nd	805.2 ± 230.1	nd	283.7 ± 8.2	240.6 ± 18.9	131.0 ± 14.4	432.5 ± 12.2	466.0 ± 177.3	456.1 ± 185.8	581.5 ± 133.7	548.7 ± 108.8	715.9 ± 368.5	559.8 ± 136.8	772.9 ± 179.4	348.5 ± 44.7	760.8 ± 347.0	1884.9 ± 1777.3	893.6 ± 121.0
C22:5 [E]	nd	nd	nd	nd	17.2 ± 29.8	156.4 ± 137.5	850.5 ± 64.1	677.0 ± 118.7	516.3 ± 207.9	675.2 ± 307.2	nd	800.6 ± 144.7	286.9 ± 313.7	511.2 ± 304.7	nd	553.6 ± 194.3	38.8 ± 10.2	13.3 ± 23.0
C24:0	15.7 ± 3.7	164.0 ± 6.9	88.9 ± 0.9	172.4 ± 27.2	28.8 ± 25.0	66.5 ± 115.2	207.5 ± 179.7	nd	nd	nd	213.0 ± 18.2	nd	nd	nd	311.7 ± 371.0	nd	28.5 ± 49.4	35.8 ± 62.0
C22:6 [E]	nd	9.8 ± 17.0	nd	nd	2.4 ± 4.2	nd	nd	189.7 ± 328.5	61.9 ± 13.1	129.5 ± 14.8	nd	96.4 ± 24.5	31.7 ± 54.8	83.4 ± 72.5	74.1 ± 110.5	100.1 ± 18.3	1.3 ± 2.2	nd

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Diet 15 *	Diet 16	Diet 17	Diet 18
Sum of EFA (g/kg)	58.1 ± 1.2	60.3 ± 6.0	68.1 ± 2.3	57.1 ± 4.5	22.9 ± 2.3	44.8 ± 2.7	89.2 ± 3.6	66.0 ± 16.3	55.8 ± 1.3	66.0 ± 5.3	61.0 ± 2.4	65.9 ± 5.0	58.5 ± 1.4	59.6 ± 1.2	57.6 ± 1.7	55.1 ± 2.1	53.6 ± 11.2	73.0 ± 2.7
Sum of SFA (g/kg)	75.4 ± 8.2	54.4 ± 6.9	53.6 ± 2.4	34.9 ± 2.6	31.0 ± 4.5	27.5 ± 1.8	52.2 ± 2.7	52.3 ± 12.4	40.6 ± 1.5	47.2 ± 3.6	50.7 ± 1.3	44.6 ± 1.9	48.8 ± 1.2	53.2 ± 0.5	48.2 ± 1.4	45.9 ± 2.9	44.2 ± 10.5	45.3 ± 3.9
Sum of MUFA (g/kg)	86.6 ± 2.1	52.8 ± 5.3	87.9 ± 3.3	45.6 ± 3.5	41.2 ± 5.0	39.0 ± 2.3	65.1 ± 3.2	60.5 ± 14.6	46.6 ± 2.4	54.7 ± 5.4	55.8 ± 1.5	55.7 ± 4.5	49.0 ± 1.3	52.9 ± 1.3	44.8 ± 7.3	45.2 ± 1.9	50.8 ± 10.9	55.7 ± 5.3
Sum of PUFA (g/kg)	58.1 ± 1.2	61.4 ± 6.0	67.4 ± 2.0	57.7 ± 4.3	23.3 ± 2.3	45.4 ± 2.8	90.5 ± 3.7	67.5 ± 16.7	56.5 ± 1.5	66.9 ± 5.4	62.0 ± 2.5	66.9 ± 5.3	59.4 ± 1.2	60.6 ± 1.1	39.4 ± 32.5	56.1 ± 2.3	55.8 ± 9.5	77.4 ± 5.8