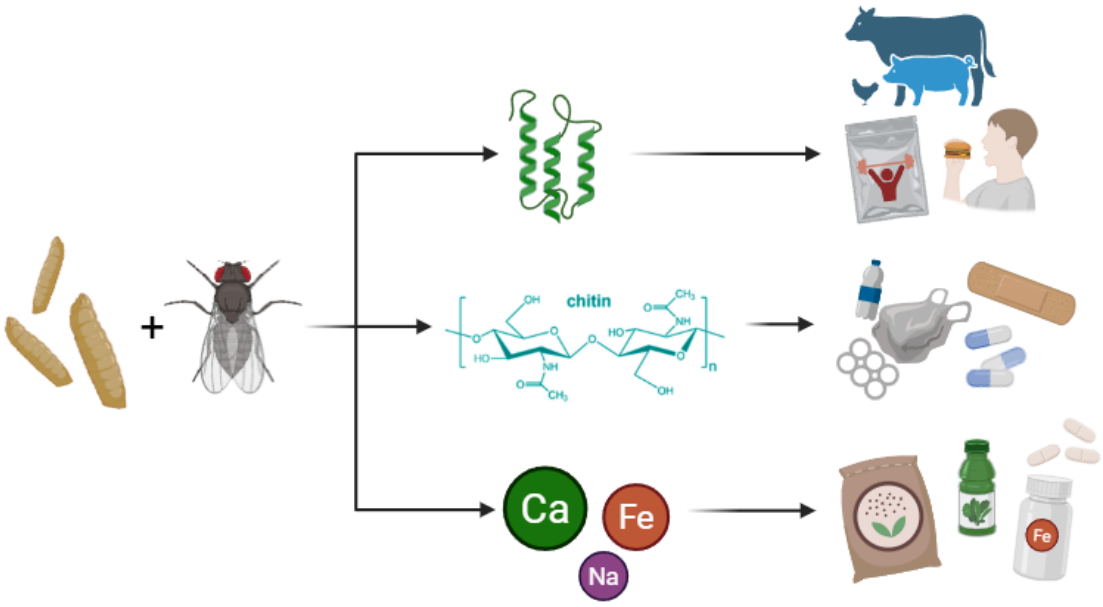
Optimization Towards a Multi-Product Biorefinery Concept for Chitin Extraction from Black Soldier Fly Biomass

Master Thesis

Emma Bjeldbak

2024/2025



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Abstract:

The demand for sustainable resources is increasing due to global resource depletion and increasing environmental challenges. The aim of this project is to optimize the extraction process of chitin from black soldier flies, larvae and frass, by implementing a biorefining approach to recover other valuable compounds such as proteins and minerals during the process A conventional chemical extraction effectively extracted minerals and produced high purity chitin, but yielded protein extracts that were highly degraded and contaminated. Therefore milder alkaline deproteinization methods were applied to the extraction process, and the majority resulted in the extraction of intact protein, but simultaneously lead to a decrease in chitin purity. Implementing a two step deproteinization yielded a successful extraction of high quality chitin, proteins and minerals. In an attempt to minimize the use of chemicals, enzymatic protein extraction was employed. Results suggested that intact proteins were extracted using chitinase, and that they had a different protein profile, compared to the one seen when using mild alkaline treatments.

Preface

Aalborg University, June 1, 2025

This project is written as part of a master thesis by a student from the Department of Chemistry and Bioscience at Aalborg University. The project was prepared during the period September 2024 to June 2025. I would like to direct extended gratitude toward supervisors Simon Gregersen and Torsten Nygård Kristensen for constructive guidance throughout the project.

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List of Abbreviations

BSF	Black soldier fly
GlcNAc	N-acetylglucosamine
GlcN	Deacetylated glucosamine
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
NaOH	Sodium hydroxide
H_2O_2	Hydrogenperoxid
NaClO	Sodium Hypochlorite
SO_2	Sulfur dioxide
DD	Degree of deacetylation
MES	2-(N-morpholino)ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HPAEC-PAD	High-performance anion-exchange chromatography With pulsed amperometric detection
EA	Elemental analysis
FTIR	Fourier-transform infrared spectroscopy
AAS	Atomic absorption spectroscopy
COS	Chitooligosaccharides
CP	Crude protein
Ca	Calcium
K	Potassium
Na	Sodium
Mg	Magnesium

Introduction

The demand for sustainable resources is increasing in both interest, but more importantly in global importance [1]. It is predicted that the global natural resource consumption, will increase with 60 % by 2060, when compared to 2020. This increase is after material use has already more than tripled over the last 50 years. Some of the resources in question is crops for food, fossil fuels, non-metallic minerals, as well as land and water [2]. When combining this resource depletion with the rapidly increasing environmental challenges the need for sustainable and efficient solutions are critical [3]. In response to the growing demand for sustainable resources, the process of biorefining has emerged. It is a process that transforms biomass resources into a broad range of products, with the goal of optimizing the use of all components in the material [4]. Sustainable biomaterials are among some of the valuable outputs of biorefining. It is a fast growing field in research, due to the increase in environmental challenges. These materials are characterized by being eco-friendly, biodegradable, and derived from renewable resources, which aligns with general efforts being made to minimize environmental impact and promote green technologies [3]. Furthermore they can easily be incorporated into the human body, since they are biocompatible to the living tissues, making them highly applicable in the biomedical field. Everything from functional performance, environmental impact from production, to strategies for recycling or disposal, is thought of when developing sustainable biomaterials. This is done in order ensure that environmental benefits are achieved throughout the whole life cycle [5] [1].

1.1 Chitin

Chitin is the second most abundant polymer on the earth after cellulose, and is considered an important biomaterial. It is used in multiple industries such as agriculture, food and cosmetics, but most of all the biomedical and pharmaceutical industry [6]. The use of chitin across multiple different industries can be seen in figure 1.1. Both chitin and its deacetylated derivative chitosan are biocompatible, biodegradable, and non-toxic biopolymers. Furthermore they are antimicrobial and hydrating agents, thus having a wide variety of applications in the biomedical field, such as tissue engineering, drug and gene delivery, wound healing, and stem cell technology, making both chitin and chitosan highly desirable compounds [7]. Chitin is a natural polymer, that is synthesized by many types of organisms such as insects or crustaceans, but also by lower invertebrates like nematodes and mollusks. It has also been found in many types of microbes like fungi and algae, but the process of chitin biosynthesis has been lost in the early evolution of deuterostomes, and it is therefore not present in vertebrates [8] [9].

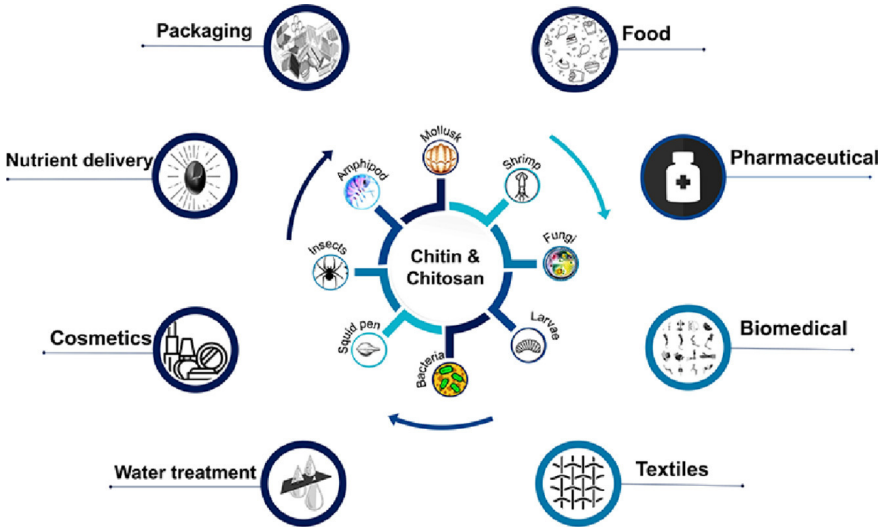


Figure 1.1: An overview of common sources of chitin and its deacetylated derivative chitosan, as well as the multiple industries in which the polymers are used [10].

In nature the polymer occurs as crystalline microfibrils, and is generally used as a structural element in organisms [7]. In some types of fungi, chitin is used as the main structural constituent, instead of cellulose, which stabilizes the cell wall. In invertebrates it makes up the fibrillar element of e.g. cuticles, cuttlebones and shells, which make these parts mechanically resilient. All organisms that produce chitin have a conserved cellular machinery, which converts sugars into chitin chains. The chains can then be transferred into the extracellular space, where they are assembled into microfibrils, and organized within the extracellular matrix [8].

It is estimated that approximately 10^{10}–10^{12} tons of chitin are synthesized annually by chitin-producing organisms worldwide [11]. Most of the chitin produced today is extracted from byproducts from the fishing industry, such as shells of shrimps, prawns, crabs and lobsters. Combining the production of chitin and chitosan, it is valued at around US$10 billion on the global market in 2023. The production is expected to reach US$27 billion by 2030, with an expected Compounded Annual Growth Rate of 15.3% during these years, due to the increasing demand across several industries [12]. New applications are constantly being found for chitin and chitosan, and therefore alternative sources are needed and are already starting to emerge. New or underutilized materials are being explored as a sustainable chitin source, and especially insects has shown great potential as an alternative to crustaceans [11].

1.2 Structure and Properties of Chitin and Chitosan

The pure form of chitin is a linear homopolymer of N-acetylglucosamine (GlcNAc), which is linked by β-1,4 glycosidic bonds. However chitin is almost never found in its pure form in natural sources. Instead it often contains both GlcNAc and varying amounts of deacetylated glucosamine (GlcN) units, making it a heteropolymer [8]. The polymer occurs in three different crystalline forms α-, β-, and γ-chitin, which differ in orientation of the microfibrils. The α-chitin is stacked in an anti-parallel orientation, with hydrogen bonds binding the chains together. This makes it the most stable of the structures, and is therefore also the most abundant form in hard structures such as the shell of crustaceans. The polymers of the β-chitin are stacked in a parallel fashion, whereas the γ-chitin has both parallel and anti-parallel orientations. These two crystalline forms are often found in softer structures such as cuttlefish or cocoon fibers [13]. The three crystalline structures of chitin can be seen in figure 1.2.

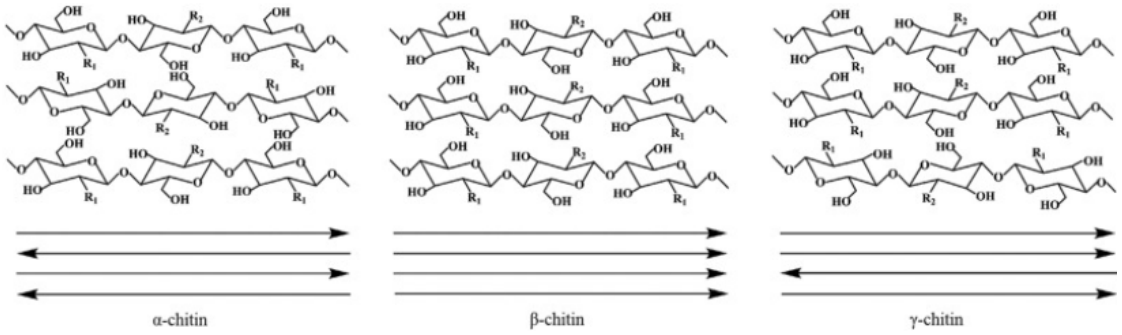


Figure 1.2: The three different crystalline structures of chitin, α-, β-, and γ-chitin. This figure shows the intermolecular hydrogen bonding between chitin chains. The R refers to the N-acetyl groups on the glucosamine units [14].

The derivative chitosan is made by partial deacetylation of chitin, which results in the removal of the acetyl groups on the chitin chain [13]. However depolymerization also occurs during the deacetylation process, leading to changes in the molecular weight of chitosan [15]. As a general rule of thumb, chitin has 0-15% degree of deacetylation (DD), whereas chitosan has between 75-80% [13]. The polymeric structure of chitin, and its extensive intermolecular hydrogen bonding, makes it insoluble in water and most organic acids [16]. It is possible to solubilize chitin in concentrated acids, such as hydrochloric acid (HCl) or sulfuric acid (H_2SO_4), but this could also result in depolymerization and to a lesser extend deacetylation of the polymer [17]. The derivative chitosan is however soluble in most diluted acids, due to its lower molecular weight and the free amino groups, as a results from deacetylation. Chitosan resembles the chemical structure of cellulose, and the only difference is the amine group in the C-2 position of chitosan, instead of the hydroxyl group of cellulose. The structures of chitin, chitosan, and cellulose can be seen in figure 1.3.

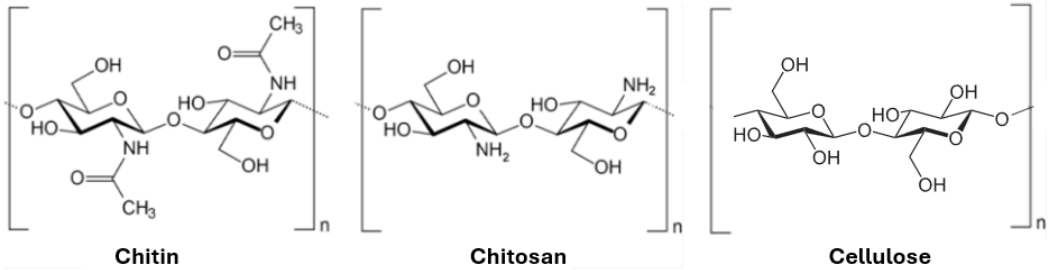


Figure 1.3: The chemical structures of pure chitin, its derivative chitosan, and cellulose. This figure shows the similarities between the three polymers with the only difference being the functional groups. Chitin: $-NHCOCH_3$, chitosan: $-NH_2$ and cellulose: -OH. [18]

Due to this change in functional groups, chitosan can obtain positive ionic charges under acidic conditions. This makes it possible for the polymer to chemically bind with negatively charged molecules, such as lipids, metal ions, proteins, and macromolecules. These excellent properties makes it the most useful derivative of chitin, and has resulted in an increasing commercial interest [11]. As seen in the figure above, chitin and chitosan both contain hydroxyl and amine groups, which are reactive functional groups, making them responsive to chemical modifications. Multiple modifications have already been proven useful, such hydroxylation, phosphorylation and graft copolymerization. This chemical modification ability makes it possible to control the polymers physicochemical properties and gives them a wide range of applications [15].

1.3 Insects as an Alternative to Crustaceans

Approximately 6.1 million tonnes of crustaceans are caught each year for human consumption, and 40-50% of this material is discarded as waste [19]. Crustacean shells are an optimal source of chitin, containing 13–42% of the polymer, as well as 30–40% proteins, and 30–50% minerals [20]. However this source is limited by things like seasonal availability, location on coastal areas, and therefore also transport costs. As mentioned before, chitin is structurally present in a lot of different organisms, like in the exoskeleton of insects, which could be of interest [21]. Whole insects contain 5–25% chitin, 30–60% protein, 10–25% lipid, and 2–10% minerals, and since 900,000 out of 1.3 million known species are insects, they are the most abundant class of organisms in the world [11]. Insect farms are developing worldwide for the production of animal feed and waste management. The farms are generating side streams, which consists of chitin-rich materials, that could be used instead of discarded. Therefore farmed insects could potentially be a more sustainable source of chitin, as it is easily available, adaptable and resistant to many types of pathogens [21].

The exoskeleton of both crustaceans and insects consist of chitin chains, with 18-25 chitin units, surrounded by structural protein molecules. This makes up the chitin-protein fibers, which are usually about 50-250 nm thick. Calcium carbonate is another component that is also present in both organisms, but found in much larger quantities in crustacean exoskeletons. Here it is present primarily in amorphous form, which is embedded in the chitin-protein fibers. Over time the calcium carbonate transforms into calcite, the most stable form of this mineral, which strengthens the exoskeleton and makes it hard and rigid. Small amounts of the calcium carbonate grow along the chitin-protein fibers, in the form of calcite crystals, further strengthening the structure [22]. An example of the chitin-protein fibers in shrimps, can be seen in figure 1.4. The exoskeleton of insects are not as mechanically resistant as that of crustaceans, due to the lower amount of calcium carbonate in the structure. Here it is mainly the structural proteins and the highly crystalline α-chitin that make up bearing structure of the exoskeleton [23].

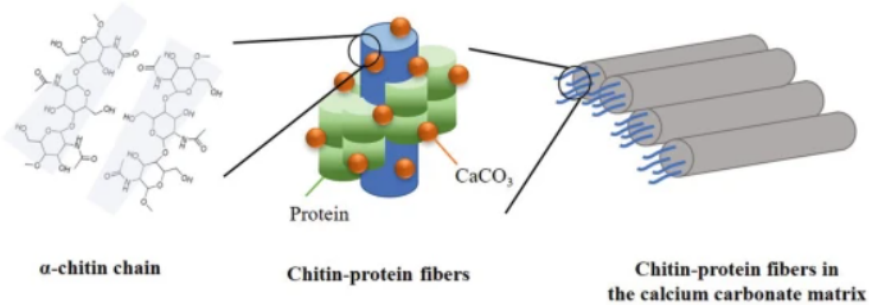


Figure 1.4: The structure of the chitin-protein fibers, found in the exoskeleton of shrimps. The α-chitin chains form rigid crystalline structures, that binds to structural proteins, forming the chitin-protein fibers. Calcium carbonate molecules are embedded within, and grows along the fibers, both contributing to strengthening the structure. [22]

Insects are not only rich in chitin, but proteins as well, containing up to 50 %, indicating that they also could play an important role as an alternative protein source [11]. Approximately 26 % of the global greenhouse gas emission is directly or indirectly related to food, with 16 % of this being due to animal-related products like meat, fish and dairy. Due to the rapid growth in population size, the demand for animal-related products will increase accordingly, leading to additional environmental contamination [24]. Alternative protein sources are necessary to sustainably feed the global population as it increases, as well as to lower the emission of greenhouse gasses. Multiple different alternative protein sources has been investigated like plant-based proteins, algae, mycoproteins, bacterial proteins and of course insects. Some plant-based proteins have already been implemented in our day to day life, such as soy, and are growing in popularity. Protein from insects is not as popular for human consumption, and even though it is normal to consume them in large parts of the world, adding insects to the human diet remains a sensitive subject [24]. However due to the rapidly increasing demand of ingredients for livestock feed, insect protein has gained recognition in this field. It has proven to be a great source of protein in feed, that can reduce the environmental footprint of livestock production, compared to fish and soy. [25]. Insects can easily be farmed using plant feedstock, in the form of food waste, as a growth medium.

Furthermore the overall process requires minimal water and produces less greenhouse gas than livestock. This suggests that insects could be a sustainable and economically favorable source of protein that can be used in both animal feed and for human consumption [24].

Insects also contain a substantial amount of minerals with some of the most common being iron, zinc, potassium, sodium, calcium, magnesium, and copper. Depending on the insect, they have varying mineral content. As an example the the large caterpillar of the moth *Gonimbrasia belina* called *mopani* has shown to contain up to 77 mg of iron and 14 mg of zinc per 100 g of dry matter [26]. These are some of the essential minerals that are critical for human health, since they are important for various physiological and biochemical processes. Minerals are one of the seven essential nutrient groups that the human body needs. The body itself cannot synthesize minerals, emphasizing the importance of a daily intake [27]. While insects are being farmed across the world, due to their protein content, a broader potential for insect farming could be considered. The mineral composition of the farmed insects can be controlled via their feed, meaning that the nutritional value of the final product can be predetermined [26]. This could offer an interesting opportunity for nutrient enhancement, which allows for insect-based products to meet specific requirements.

1.4 The Black Soldier Fly

Hermetia illucens, also known as black soldier flies (BSF) have great potential as both alternative food and feed sources. They have a high nutritional value due to their amino acid composition as well as their mineral, vitamin, and polyunsaturated fatty acid content [27]. BSF are being reared by around 80% of the European insect farms, due to its bioconversion ability [21]. The BSF larvae can rapidly feed on decaying organic waste and transform it into a high-value biomass containing proteins and lipids. This biomass is mainly used for animal feed like the insect itself, but can also be used in other industries such as cosmetics and energy [28]. The BSF is a large fly of around 13-20 mm, believed to have originated in America, but can now be found worldwide. It is primarily adapted to the tropic and tempered regions, although it has learned to tolerate a broad range of temperatures. The fly is considered a non-pest and is not associated with disease transmission, as they cannot bite, sting or even feed during its short life span of 5-8 days. This is due to the absence of mouthparts, stingers, and digestive organs. A BSF life cycle is approximately 45 days and can be divided into 5 stages: egg, larval, prepupal, pupal, and adult stage, which can be seen in figure 1.5. A female BSF lays between 500 and 900 eggs each, that hatch after around 4 days, depending on the season. The next stage is the larval, which can be subdivided into 6 instars. The larvae start feeding immediately after hatching and can survive on a wide range of organic matter, from animal manure to decaying fruits and vegetables. During the 6th instar the larvae undergo melanisation to become prepupae, which results ind a darker coloring of the cuticles. During the prepupae stage they stop feeding, empty out their digestive system, and migrate to dry places where they become pupae. After 8 days the adult fly emerges. During this last stage they only feed on water and solely relies on the fat stored during its larval stage. In nature it lives remotely from

humans and does not harm crops or pollute the environment, at odds with many other types of insects [29]. Chitin can be extracted from most of the BSF life stages, from larvae and pupae to adult flies, since all stages contain varying amounts of chitin. It is difficult to determine the exact chitin content of the different life stages, since it varies depending on the study. Difference in the extraction method or growth medium can affect the chitin yield of the analyzed source, but pupae generally show the greatest chitin yield of approximately 25%, followed by larvae at 10% and flies at 6% [21]. The chitin content of BSF frass has also been extensively researched, since it is easily harvested during the farming process. It is a byproduct from the larvae, and is a mixture of sheddings, larvae excreta and leftover growth material. Frass is thought to contain lower amounts of chitin but have a general high nutritional value, making it a material of interest [30].

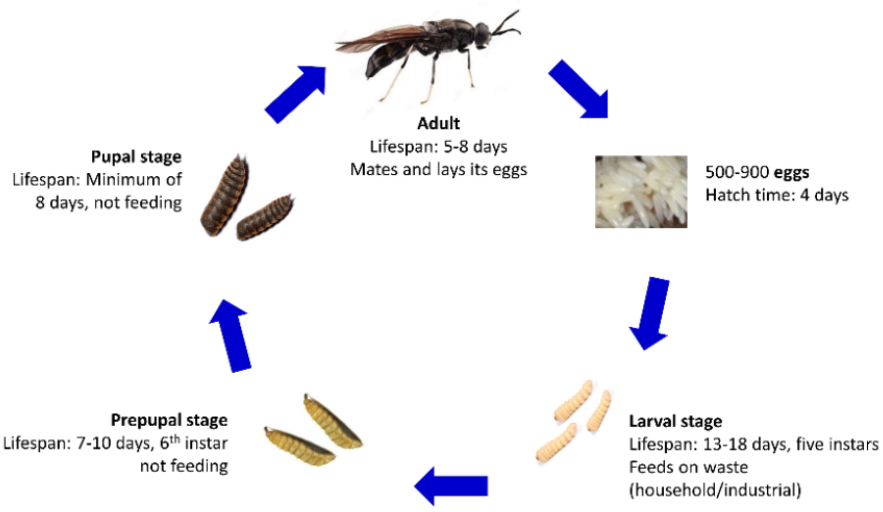


Figure 1.5: The life cycle of the black soldier fly. The cycle starts and ends with the adult flies, that lays between 500-900 eggs. The eggs hatch into larvae, which pass through six instars, the last being the prepupal stage. This stage is followed by the pupae stage, where the flies emerges and repeat the cycle [29].

1.5 Extraction of Chitin

Both biological and chemical extraction methods, or a combination of the two, have been shown to be effective in extracting chitin. However the chemical method is the most commonly used in industrial production, since it is both productive and practical [16]. The purpose of the extraction is to remove minerals and organic materials like lipids, proteins and pigments. The removal of these compounds is essential, since they have an effect on the purity of the final chitin product. As an example, when using crustaceans, the removal of proteins is especially important for chitin used in biomedical applications. Some people are allergic to shellfish, thus a high purity of the extracted chitin is not only preferred, but crucial [18]. The removal of these compounds is normally done in four separate steps, with demineralization and deproteinization being the two primordial steps. The chemical chitin extraction method is outlined in figure 1.6.

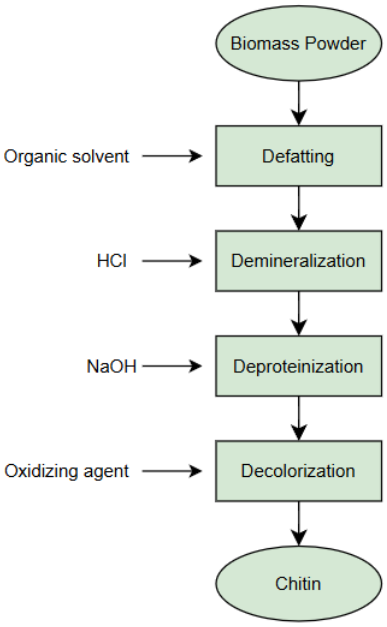


Figure 1.6: The chemical extraction process of chitin. The process includes four main steps: defatting using an organic solvent, demineralization with hydrochloric acid (HCl), deproteinization with sodium hydroxide (NaOH), and decolorization using an oxidizing agent. The final product of the process is pure chitin.

Before the extraction a preliminary treatment is often implemented to remove coarse organic waste from the biomass, of which chitin is extracted. Here the raw material is cleaned using demineralized water and is afterwards dried and ground. Grinding the material is an important step, as this increases the surface area, which allows the solutions used during the extraction, to better react with the material [16]. The first step in the extraction is defatting, where lipids are removed. A conventional chemical method used for defatting is a solid-liquid extraction, where the material is homogenized in an organic solvent, like hexane [31]. The next step is usually demineralization. Here the minerals, mainly calcium carbonate, are removed using hydrochloric acid (HCl). The calcium carbonate undergoes a reaction, that produces water, carbon dioxide and calcium chloride, which is highly water soluble. the majority of the other minerals found in the shell of crustaceans react similarly, which makes the acidic treatment very effective. The deproteinization step is next, and here the proteins need to be solubilized, which is done by increasing the pH. Sodium hydroxide (NaOH) is normally used, since it also has the ability to disrupt the chitin protein-complex. However harsh alkaline treatments can also cause depolymerization of the polymer, which affects the integrity of the chitin chains, but makes it easier for the proteins to be released to the solution [32]. The last step is the decolorization, since the exoskeleton of many organisms contains pigments. The pigments can be removed by using oxidants like hydrogen peroxide (H_2O_2), sodium hypochlorite (NaClO), or sulfur dioxide (SO_2). However it is not always important to remove pigments from the final chitin product [11].

The chemical extraction process has proven to be effective on both crustaceans and insects like BSF, but it is also lengthy and uses harsh chemicals to remove the unwanted structures, which are naturally present in the exoskeleton [11]. New methods for chitin extraction could be of interest, to minimize the extraction time, use of harsh chemicals and maybe increase the chitin yield in the process. Furthermore proteins and minerals, are discarded during the chitin extraction process, since they are seen as impurities. However both are valuable compounds that can be used for human consumption or in animal feed. This is of great importance when trying to meet the nutritional demands of a growing population, while focusing on environmental sustainability [33]. It would therefore also be relevant to incorporate a biorefining approach where these valuable compounds are also extracted during the chitin extraction process. If this is possible without compromising the chitin quality, it could increase the overall resource efficiency and potentially the economical viability of insect rearing.

Thesis Statement

The growing demand for chitin, due to its wide variety of applications across industries, has resulted in the search for other alternative and sustainable sources of the polymer. Today crustaceans are the main source of commercial chitin, and though it remains effective, it is limited by factors such as seasonal availability and location on coastal areas. However chitin is also structurally present in the exoskeleton of insects, the most abundant class of organisms in the world. Among the species of insects, the BSF presents as an ideal candidate, since they are reared in 80% of the insect farms across Europe, due to its bioconversion ability. Furthermore chitin-rich material is found in most of its life stages, from larvae to adult flies, making it a promising and sustainable source of chitin. The BSF also has a general high nutritional value, due to their protein, mineral, vitamin, and polyunsaturated fatty acid content. The aim of this project is therefore to establish a baseline extraction protocol and test its efficiency on adult BSF, as well as larvae and frass. The focus will then be to implement a biorefining approach to recover other valuable compounds such as proteins and minerals during the process to maximize resource use and improve the overall sustainability.

Is it possible to extract other valuable compounds such as proteins and minerals during the chitin extraction process of adult black soldier flies, larvae and frass? What effect does implementing less harsh treatment methods and enzymes have on the yield and quality of the extracted chitin, proteins and minerals?

As such, the project aims to:

- Establish and conduct a baseline extraction method for chitin in adult BSF, larvae and frass, using a well-established chemical extraction method based on literature.
- Determine the yield of chitin, proteins and minerals from the baseline extraction.
- Characterize the purity and/or the composition of the extracted elements.
- Optimize the process by implementing less harsh chemical treatments and enzymes.
- Analyze if the changes in the process has an effect on the yield and quality of the extracts.

Materials and Methods

The equipment and chemicals used during this project are listed in appendix A, with model and manufactures, to ensure reproducibility. Furthermore all of the raw data from the different analyses was processed in R.

3.1 Rearing of Black Soldier Flies

Eggs from the previous generation were harvested. They were weighed, and 200 g were transferred to a container with 500 g of growth medium (3.3 g/L dry yeast, 5 g/L malt, 66 g/L alfalfa flour, 133 g/L wheat bran). The container was covered with a cloth, to keep hatching larvae inside, and placed in a controlled climate cabinet with a temperature of 37 °C and a humidity at 60 %. After hatching, the larvae were weighed every 3 days, so their growth could be followed. When the larvae were deemed big enough for relocation, they were transferred to a bigger container with 1.8 kg of growth medium. after 10 days another 1.8 kg of medium was added to the container. It takes approximately a month from the eggs hatch, to adult BSF emerges under the chosen conditions.

3.2 Sample Selection

3.2.1 Frass

The frass was gently removed from the top of the container with growing larvae, to minimize the amount of growth medium in the sample selection. It was then transferred to a plastic bag, weighed, and frozen to -80 °C, until further processing and analysis.

3.2.2 Larvae

The larvae were harvested before reaching the pupal stage. The time of harvest was determined based on their size and color, as the light brown color of the larvae turns darker when entering the pupal stage. When the larvae began to darken they were removed from the growth medium and gently cleaned using dH_2O, before being transferred to a plastic bag. The bag was weighed and the larvae were then euthanized via freezing at -80 °C and kept there until further processing and analysis.

3.2.3 Adult Flies

Flies that remained alive after the last egg retrieval, were euthanized by freezing the fly cages at -20 °C. The flies were removed from the cage and transferred to a plastic bag. They were weighed and frozen to -80 °C until further processing and analysis.

3.3 Baseline Extraction

3.3.1 Sample Preparation

Frass, larvae, and flies were thawed, weighed, and cleaned using 70% ethanol, followed by a rinse with dH_2O, in order to remove gross contamination. The material were then placed in a drying oven at 60 °C for 24 h. Afterwards the dried material was weighed and ground to a fine powder.

3.3.2 Defatting

1 g of the dried material and 10 mL of 97% hexane was added to 50 mL Greiner tubes. The tubes were placed in an ice bath and the samples were homogenized at 11,000 rpm for 5 min using an ULTRA-TURRAX homogenizer (Bie and Bentsen A/S). Afterwards the samples were centrifuged at 2540 xg, for 5 min, at room temperature before being filtered through a coffee filter. The process was repeated, and the remaining pellets were gently dried with nitrogen gas.

3.3.3 Demineralization

Minerals were removed from the material by adding 20 mL of 1 M HCl to the defatted samples. The samples were vortexed and placed on a rotation incubator for 1 h at room temperature. They were then centrifuged at 2540 xg, for 5 min, at room temperature and filtered through a coffee filter. If there were traces of the pellet on the filters, they were removed and added back to the Greiner tubes. The supernatant containing the minerals was stored at -20 °C until further analysis.

3.3.4 Deproteinization

The proteins were removed next by adding 20 mL of 1 M NaOH to the demineralized samples. The samples were vortexed, before they were placed on a rotation incubator over night at 80 °C. They were then centrifuged at 2540 xg, for 5 min, at room temperature and filtered through a coffee filter. If there were traces of the pellet on the filter, they were removed and added back to the Greiner tubes. The remaining pellets were neutralized, to a pH of 7, using HCl and filtered once more. The supernatant containing the proteins was divided into two 50 mL Greiner tubes. One tube was stored at -20 °C until further analysis and the other was freeze dried for 2 days.

3.3.5 Decolorization

Lastly pigments were removed by adding 20 mL of 10% H_2O_2 to the deproteinized samples. The samples were vortexed and placed on a rotation incubator for 4 h at 80 °C. They were then filtered through a coffee filter, and the chitin pellets were removed from the filter and added back to the Greiner tubes. The pellets were neutralized, to a pH of 7, using 1 M NaOH and filtered once more, followed by a thorough rinse with dH_2O directly on the filter. The cleaned pellets were removed from the filter and added to new Greiner tubes. They were then freeze dried over night and weighed.

3.4 Process Optimization of the Baseline Extraction Method

The main focus of the process optimization was the deproteinization step, while the rest of the experiment was carried out as in the baseline extraction. As part of the optimization, the deproteinization and demineralization steps were reversed, with deproteinization now performed first. Furthermore a variety of milder deproteinization methods were implemented, which are summarized in table 3.1. Due to insufficient amount of biomass, biological triplicates were not made during this process.

Method	Sample ID	Agent	Time	Temperature	pH
1.	1M-ON-80	NaOH	ON	80 °C	12.50
2.	1M-ON-25	NaOH	ON	25 °C	12.50
3.	pH9-1h-25	NaOH	1 h	25 °C	9.00
4.	pH9-2h-25	NaOH	2 h	25 °C	9.00
5.	pH9-4h-25	NaOH	4 h	25 °C	9.00
6.	pH9-ON-25	NaOH	ON	25 °C	9.00
7.	pH9/1M	NaOH	1 h/ON	25/80 °C	9.00/12.50

Table 3.1: Overview of the seven different deproteinization methods performed during the optimization process. Sample IDs were created to distinguish between the methods throughout the project. The IDs are a summary of experimental parameters used during each method.

3.5 Enzymatic-Assisted Protein Extraction

The enzyme chitinase was also introduced to the deproteinization step, replacing NaOH, in order to try and minimize the use of chemicals in the extraction process. The optimal temperature for chitinase activity was known to be 37 °C, but other parameters was unknown [34]. Previous to the to the enzymatic deproteinization, an enzyme assay was performed to determine the optimal enzyme-to-substrate ratio and pH. This process is described in section 3.6. An overview of the methods used during the enzymatic-assisted protein extraction and the chosen parameters can be seen in table 3.2.

Method	Sample ID	Agent	Time	Temperature	pH
8.	pH5-1h-37	Chitinase	1 h	37 °C	5.00
9.	pH5-2h-37	Chitinase	2 h	37 °C	5.00
10.	pH5-3h-37	Chitinase	3 h	37 °C	5.00

Table 3.2: Overview of the enzymatic facilitated deproteinization methods, with different reaction times. Sample IDs were created to distinguish between the methods throughout the project. The IDs are a summary of experimental parameters used during each method.

Enzymatic facilitated deproteinization was performed by adding 260 mg dried non-defatted material and 1.3 mL 0.25 U/mL chitinase in sodium acetate buffer (pH 5) to 15 mL Greiner tubes. Three identical solutions were incubated on a rotation incubator at 37 °C for 1, 2, and 3 hours respectively. The solutions were centrifuged at 2540 xg, for 5 min, at room temperature, and filtered through a coffee filter. The protein extracts were stored at -20 °C until further analysis.

3.5.1 Buffer Control

To isolate the protein extraction effect of chitinase, deproteinization was performed using sodium acetate buffer without the enzyme. This experiment was conducted by adding 260 mg dried non-defatted material and 1.3 mL sodium acetate buffer (pH 5) to 15 mL Greiner tubes. Two solutions were incubated on a rotation incubator at 37 °C for 1 and 3 hours. The solutions were centrifuged at 2540 xg, for 5 min, at room temperature, and filtered through a coffee filter. The protein extracts were stored at -20 °C until further analysis.

3.6 Chitinase Assay

A stock solution of the enzyme was made by solubilizing 5 units of freeze dried chitinase in 1 mL 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.976 % w/v MES, 5 % glycerol (pH 6)). An enzyme dilution sequence was made, with the first concentration being 1 U/mL, followed by eight 1:2 dilutions. 10 mg of commercial chitin and 500 μL of each enzyme dilution was added to 1.5 mL Short Thread Vials. The vials were placed on a rotation incubator for 90 min at 37 °C. After the incubation the samples were transferred to 1.5 mL Eppendorf tubes, and centrifuged at 14,100 xg for 5 min. Standards, samples, and a blank were prepared for analysis in a 96-well microplate, using the Megazyme D-Glucosamine Assay Kit, according to the manufacture guideline. The absorbance was measured in triplicates at 340 nm using an infinite M1000 microplate reader (Tecan).

3.6.1 pH Optimum Determination

The optimal pH for chitinase was tested by preparing buffers with a concentration of 50 mM and a pH ranging from 4 to 8. The buffers were sodium acetate (0.060 % w/v sodium acetate and 0.257 % w/v acetic acid, (pH 4)), sodium acetate (0.260 % w/v sodium acetate and 0.110 % w/v acetic acid, (pH 5), MES buffer (pH 6 and 7) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (11.92 % w/v HEPES (pH 8)). 10 mg of commercial chitin and 500 μ L of 0.25 U/mL enzyme solution, in the different buffers, were added to 1.5 mL Short Thread Vials. The vials were placed on a rotation incubator for 90 min at 37 °C. The samples were then transferred to 1.5 mL Eppendorf tubes, and centrifuged at 14,100 xg for 5 min. The samples were prepared for analysis in a 96-well microplate, using the Megazyme D-Glucosamine Assay Kit, and measured in triplicates at 340 nm on an infinite M1000 microplate reader (Tecan), as previously described.

3.7 Characterization of the Chitin Samples

3.7.1 Bright-Field Microscopy

To get a visual representation of the chitin extracts, they were analyzed using microscopy. A drop of water, and a small amount of the freeze dried chitin extracts were added to slides, and cover glasses were added on top. The slides were placed on the Axiolab light microscope (Zeiss), and the 10x magnification was chosen for analysis. Images were captured through the microscope's lens.

3.7.2 Elemental Analysis

Elemental analysis (EA) was also performed on the freeze dried chitin extracts. This was done in order to determine the percentage of carbon and nitrogen in the samples, which can give insight into the purity. To validate the accuracy of the elemental analyzer 3 blank tinfoil capsules and 3 acetanilide standards were made. 1 mg of acetanilide was added to the first standard, 2.5 mg to the second and 3.5 mg to the third. Then 3-4 mg of freeze dried samples was added to tinfoil capsules. Additionally samples composed of raw insect biomass were included for comparison and these were prepared the same way as the chitin samples. The difference between the weight of the empty capsule and the capsule containing the sample was noted, so the exact amount of sample added was known. The capsules containing the samples were then added to a rack, and inserted into the FlashSmart elemental analyzer (Thermo Scientific). The flow of oxygen and helium was turned on at a pressure of 5 bar. The temperature was set to 950 °C in the left reactor (combustion furnace), 400 °C in the right reactor (reduction furnace), and the oven was set to 50 °C. The helium carrier flow was 140 mL/min, the helium reference flow was 100 mL/min and the oxygen flow was 250 mL/min. The run time for each sample took 7 min.

3.7.3 Fourier-Transform Infrared Spectroscopy

The functional groups present in the extracted chitin were identified using fourier-transform infrared spectroscopy (FTIR). The sample holder was cleaned using ethanol (70 %) and the Tensor II FTIR spectrometer (Bruker) was blanked, by recording the background noise, with no sample loaded. A small amount of the freeze dried chitin samples was added to the sample holder, and the probe, containing the attenuated total reflectance crystal, was lowered onto the sample. 66 scans were performed on each of the samples, and the final spectrum was made based on the average of these scans. Triplicates were measured for each sample. The absorbance measurements from the FTIR was also used to calculate the DD according to [35] as:

$$DD\% = 100 - (31.92 * (A_{1320}/A_{1420}) - 12.20) \tag{3.1}$$

Where A represents the absorbance at wavenumber 1320 cm^{-1} and 1420 cm^{-1}. Both of these values are characteristic for chitin.

3.7.4 Acid Hydrolysis

Acid hydrolysis was performed on a fraction of the freeze dried chitin extracts, in preparation for spectrophotometry and high-performance liquid chromatography (HPLC). This step was necessary to break down the insoluble chitin into soluble monomeric units of N-acetyl glucosamine. 5 mg of the dried chitin samples was mixed with 150 μL of concentrated sulfuric acid (95-97%), and placed on a rotation incubator over night at 30 °C. The solutions were then diluted with ELGA water, to a concentration of 1 N, and autoclaved for 1 hour at 120 °C and 15 psi. When cooled they were neutralized using 1 M NaOH. 1 mL of the solutions was transferred to 1.5 mL Eppendorf tubes for the HPLC analysis and the remaining was saved for the spectrophotometry analysis.

3.7.5 Spectrophotometry

The concentrations of N-acetyl-D-glucosamine in the acid hydrolyzed chitin samples were measured using spectrophotometry. The acid hydrolyzed chitin samples, as well as a standard and a blank were prepared for analysis in a 96-well microplate, using the Megazyme D-Glucosamine Assay Kit, and measured in triplicates at 340 nm on an infinite M1000 microplate reader (Tecan), as previously described.

3.7.6 High-Performance Liquid Chromatography

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a Thermo Scientific HPLC system. This analysis was performed in order to determine the the N-acetyl-D-glucosamine recovery yield from the acid hydrolysis, and to asses the presence of glucose contamination in the samples. 1 mL of acid hydrolyzed samples, was filtrated using a 0.22 μm syringe filter, and added to 1.5 mL Short Thread Vials. The vials were then inserted into the autosampler. The column used for sample separation was a Dionex CarboPac

PA20 IC column (150 x 3 mm). The sample injection volume was 25 μL, and it was separated using the eluent (1 M sodium acetate), with a flow rate of 0.4 mL/min and a pressure of approximatly 2440 psi, for 45 min. Triplicates were made for each sample, and a calibration curve for both glucose and glucosamine hydrochloride was prepared.

3.8 Characterization of the Protein Extracts

3.8.1 UV Absorption Spectroscopy

UV Absorption Spectroscopy was used to determine the protein concentration in the protein extracts. The concentration was estimated using the method A280 and 1A=1mg/mL. A blank was run on the DS-11 FX Nanodrop machine (DeNovix) using 2 μL of the solvent used for the protein extraction. The NanoDrop was then cleaned with a paper towel, and 2 μL of the sample was added. The concentration, in mg/mL, was measured, as well as the 260/280 ratio. Triplicates were made for each sample.

3.8.2 Fluorescence Quantitation Assay

For a more sensitive determination of the protein concentration, a fluorescence quantitation assay was performed. Qubit test tubes were labeled, and 190 μL of working solution (Qubit Protein Reagent 1:200 in Qubit Protein Buffer) was added to each tube. 10 μL of the three standards was added to 3 of the tubes, and 10 μL of the protein samples were added to the remaining. The samples were then vortexed and incubated for 15 min at room temperature. The protein concentrations were measured using the protein assay on the Qubit 4 fluorometer (Invitrogen).

3.8.3 SDS-PAGE

SDS-PAGE was performed in order to visualize the protein profile of the extracted protein. 12 μL of the protein extracts was added to 1,5 mL eppendorf tubes. Then 20 μL of 40 mM DTT sample buffer (10% SDS, 50% glycerol, 250 mM Tris HCl, 0,5% bromophenol blue and 3.85% 1 M DTT) was added to each of the tubes containing the samples. The tubes were put in a heating block for 10 min at 95 °C, and was afterwards centrifuged at 14.100 xg for 1 min. 8 μL of Pierce Protein Marker (26610)(Thermo Sceintific), and 15 μL of each of the samples was loaded to the 4-20% polyacrylamide gel. Electrophoresis was run at 160 V, 3 A and 150 W for 50 min. The gel was removed from the chamber, covered with a coomassie brilliant blue InstantStain solution, and placed on an orbital shaker over night. The staining solution was then removed and replaced by dH_2O. Pictures were taken of all gels on the ChemiDoc MP Imaging System (BioRad).

3.8.4 Elemental Analysis

EA was also performed on the freeze dried protein extracts, to determine the crude protein (CP) content and to asses the purity of the protein extracts. The samples, standards and a blank were prepared as previously described. The capsules containing the samples were added to a rack, and inserted into the FlashSmart elemental analyzer (Thermo Scientific). The same EA parameters was used as previously mentioned.

3.8.5 Dry Matter Determination

4 mL of each protein extract was transferred to foil trays and dried in an oven at 70 °C overnight. The foil trays were weighed before and after the extracts were added and dried, and the difference in weight was used to determine the dry matter content of the extracts. Based on the dry matter determination and the CP from the EA, the protein recovery yields were determined, using the following equation:

$$Protein\% = (DM_{Extract} * CP_{Extract}) / (DM_{Biomass} * CP_{Biomass}) \tag{3.2}$$

Where DM is the dry matter content.

3.9 Characterization of the Mineral Extracts

3.9.1 Atomic Absorption Spectroscopy

The mineral content of calcium, potassium, sodium and magnesium was measured at their respective wavelengths of 422.7, 404.4, 330.2 and 202.6 nm. The PinAAcle 900F atomic absorption spectrometer (PerkinElmer) was calibrated by making a six point calibration curve for each of the minerals, with a minimum R^2 of 0.97. The detection range for the minerals were 0.092-4 mg/L for calcium, 7.8-350 mg/L for potassium, 1.7-80 mg/L for sodium, and 0.19-9 mg/L for magnesium. No sample preparation was needed for the mineral extracts. However if the concentration was too high to measure at the given wavelength, the samples were diluted using 1 M nitric acid.

3.9.2 Complete Acid Digestion of Raw Insect Biomass

In order to determine the total mineral content of flies, larvae and frass, acid digestion was performed on the material. 0.5 g of ground material was mixed with 5 mL nitric acid (67-70%) in Teflon PTFE vessels. After 30 min evaporation in a fume hood, the vessels were sealed and inserted into the Multiwave 7000 microwave digestion system (Anton Paar), with a starting pressure of 40 bar. The samples were then heated to 220 °C for 10 min, with a maximum pressure of 140 bar. They were afterwards cooled down to 80 °C, with a pressure release rate of 10 bar/min. The standards were diluted before measured on the spectrometer and measured in triplicates.

3.10 Statistical Analysis

One-way ANOVA was performed on the biological triplicates from the baseline extraction method, to determine if there could be seen a significant difference between sample types (flies, larvae and frass). Furthermore a two-way ANOVA was performed on the technical triplicates from the experiments where alternative deproteinization methods were used. This was done in order to compare the effects of both method and sample type. The analysis was only performed on technical triplicates that were independently prepared and thus not repeated measurements. A Post-hoc comparisons was performed using Tukey's HSD test and a significance level of $p < 0.05$. It was assumed that the data met the criteria of normality and homogeneity of variance.

Results and Discussion

In this chapter the results from the different extraction methods will be presented and discussed, and the quality of the chitin, protein, and mineral extract will be evaluated. Additionally the overall efficacy of the baseline extraction will be presented, and the optimization methods, as well as the enzymatic deproteinization will be assessed and compared. An overview of the experimental process can be seen in figure 4.1.

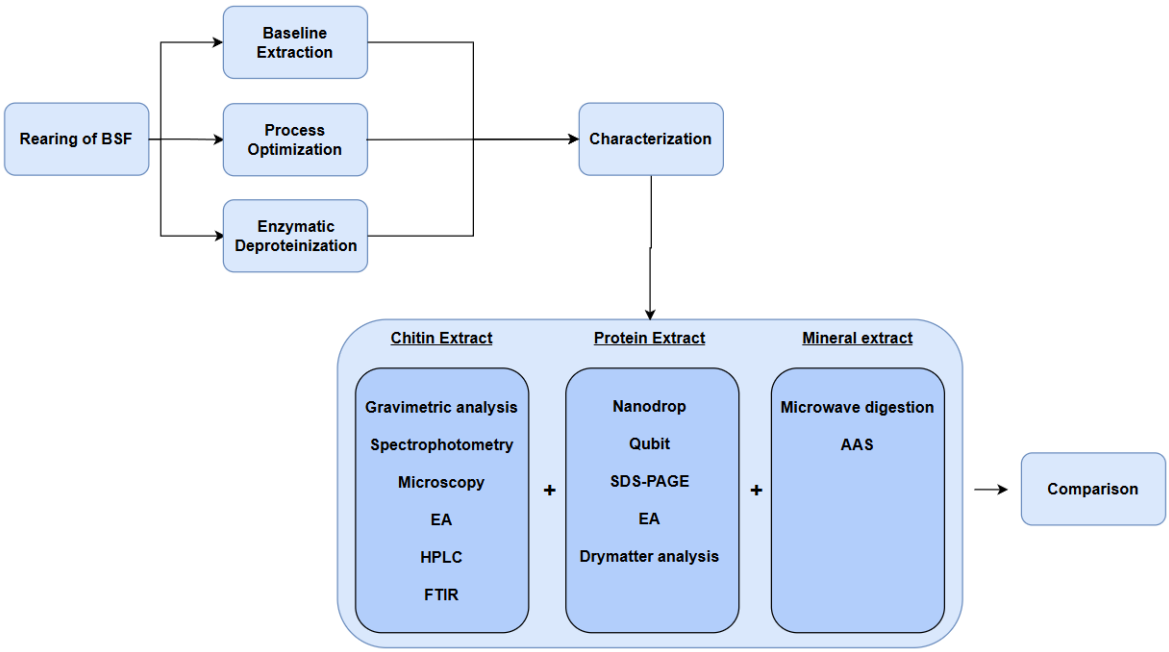


Figure 4.1: Workflow of the experimental process of chitin, protein and mineral extraction from black soldier flies, larvae and their frass. The process begins with rearing of the flies and larvae, and harvesting of the frass. A baseline extraction, followed by process optimization and enzymatic facilitated deproteinization was performed. The extracts from each process were characterized using multiple different analysis methods and the results from each method were compared.

The process was started by rearing BSF and harvesting the flies, larvae and frass. The baseline extraction method was carried out, to establish the efficacy of the industrially used chemical extraction on the different BSF materials. Efforts were made to optimize the process, focusing on resource optimization and the extraction of high quality, chitin, proteins and minerals. Furthermore enzymatic facilitated protein extraction was performed in order to try and minimize to use of chemicals, and to see if it was possible to use chitinase to break the chitin-protein matrix in the insect exoskeleton. A range of analytical techniques were used to characterize the chitin, protein and mineral extracts. The results will be compared, to determine which method or methods are most efficient and produces the highest quantity and quality extracts.

4.1 Analysis of the Chitin Extracts from the Baseline Extraction

4.1.1 Gravimetric Analysis

In order to determine the efficiency of the baseline extraction, the chitin yield of the extracts was determined gravimetrically following freeze drying of the extracted chitin samples. The results can be seen in table 4.1.

Gravimetric Chitin Yield [%]		
Flies	**Frass**	**Larvae**
4.12 ± 0.14^{B}	35.64 ± 2.24^{A}	3.39 ± 0.59^{B}

Table 4.1: The mean gravimetric yields of the biological triplicates of chitin extracted from black soldier flies, larvae and their frass, using the baseline extraction method. The table includes the calculated standard deviations. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

As previously mentioned, it is difficult to find sources describing the exact chitin content of the different BSF stages, since this varies depending on the extraction method used. However a study, implementing a similar extraction method, as the baseline extraction, has shown that the gravimetric yield can vary depending on the type of BSF material, with adult BSF, larvae and sheddings at approximately 6 %, 10 % and 31 % chitin respectively [36]. Compared to these data the determined yield of the flies and larvae are low. This could be due to small variations between the extraction method used in this project, and the one used in the study. The most notable difference is that less steps were implemented in the extraction from the study, as fat and pigments were not removed from the material. As more steps are added to the extraction, like in this project, there is an increasing risk of loosing some of the sample during the process. Part of the sample got stuck to the homogenizer when defatting and residues would also be present on the coffee filters during the many filtration steps, all of which could results in a lower chitin yield. Furthermore the mean yield of the adult flies, at 4.12 % chitin, is slightly higher compared to that of the larvae at 3.39 % chitin, although the difference is not statistically significant. Based on literature, larvae generally contains more chitin than adult flies. However there could be differences in the chitin-protein structures between these life stages, that may alter the efficiency of the chitin extraction. It is also possible, that the larvae samples contain residual moisture after the 24 hour drying process, since they have a higher moisture content than adult flies [37]. This could result in an overall lower yield, as the amount of starting dry matter would be lower compared to what was expected.

The chitin yields of the frass samples are significantly higher compared to both flies and larvae. However these results are also higher than those of pure sheddings at 31 % chitin, which is not expected, as frass is a mixture of sheddings, larvae excreta, and leftover growth material [30]. The frass samples are therefore expected to contain lower amounts of chitin than what is found in pure sheddings, since part of the sample consists of material that might not contain chitin, like

the larvae growth medium. The high gravimetric chitin yields of the frass samples could suggest, that the extracted chitin may contain traces of non-degraded contaminants, possibly stemming from the growth material.

4.1.2 Spectrophotometry

The chitin extracts were also investigated using a spectrophotometry assay, due to its higher accuracy, and to account for the possibility of contamination from non-degradable material in the samples. The chitin yields of the samples determined by spectrophotometry can be seen in table 4.2.

Spectrophotometric Chitin Yield [%]				
	Batch 1	**Batch 2**	**Batch 3**	**Overall**
Flies	1.60 ± 0.63	1.91 ± 0.18	1.69 ± 0.39	1.73 ± 0.16^{B}
Frass	0.25 ± 0.11	0.27 ± 0.09	0.44 ± 0.16	0.32 ± 0.10^{A}
Larvae	1.60 ± 0.60	1.64 ± 0.63	1.32 ± 0.50	1.52 ± 0.17^{B}

Table 4.2: The mean spectrophotometric yields of chitin extracted from black soldier flies, larvae and their frass, using the baseline extraction method. The table includes the mean yields from the technical triplicates from each batch, and the overall yields from the biological triplicates, as well as the calculated standard deviations. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

The flies show a slightly higher mean yield of 1,73 %, although not significant, compared to the larvae of 1.52 %, which was the same tendency seen in the gravimetric determined yields. However the frass samples have a significantly lower mean chitin yield of 0.32 %, which is opposite to what was seen in the previous results. This supports the suspicion of non-degraded contamination in the samples, possibly stemming from the larvae growth material. Generally the analysis shows much lower chitin yields compared to the gravimetric results, which could indicate the presence of non-degraded contamination across all samples, suggesting that the baseline extraction method was inefficient at extracting pure chitin. Another explanation could be that the chitin extracts contain impurities that interfere with the spectrophotometric assay, resulting in an underestimation of the yields. The assay used for this analysis is a coupled enzyme assay, that quantifies D-glucosamine by measuring NADPH formation. Contaminants could bind to essential assay components, making the analysis less accurate. Lastly an acid hydrolysis of the extracted chitin samples is required, to release soluble GlcNAc. Adding more steps to an experimental process always increases the risks of error, and an incomplete acid hydrolysis could also explain the low yields. Normally the amount of GlcNAc is proportional to the amount of chitin in the sample, but if the acid hydrolysis was incomplete, the samples could contain insoluble chitin, which is impossible to detect. Despite the results, the triplicate values for each type of material is within two standard deviations of the mean, indicating acceptable reproducibility of the baseline method.

4.1.3 Bright-Field Microscopy

The extracted chitin was analyzed under a light microscope to visualize the morphology of the chitin, and to see if other structures are visibly present in the samples. Figure 4.2 shows the microscopic images of the three chitin extracts from flies, larvae, and frass.

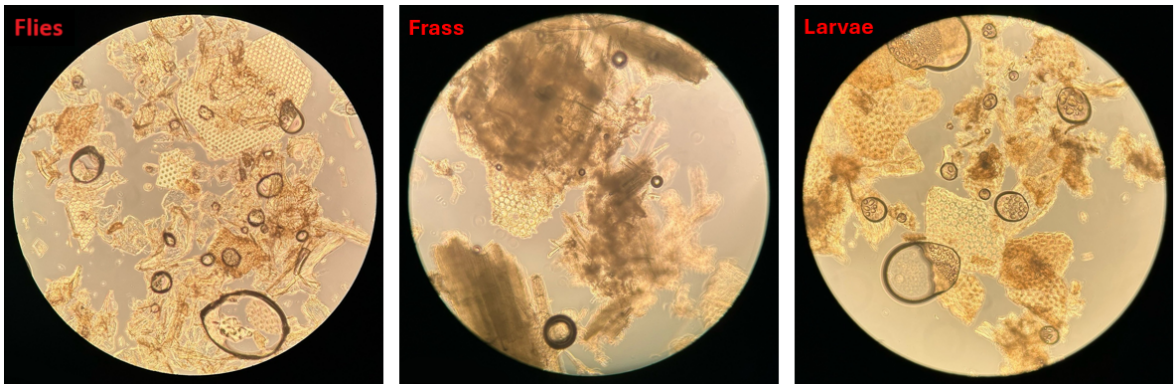


Figure 4.2: Microscopic images of chitin extracted from black soldier flies, larvae and their frass using the baseline extraction method at magnification 10x.

All three of the microscopic images show the presence of honeycomb-like structures, that is characteristic for chitin, due to well organized repetitions of hexagonal units. The intact honeycomb sheets is a result of the microfibrillar structure of the chitin-protein matrix from the exoskeleton of the insect, which could indicate that residual proteins are still present in the chitin extracts [38]. However it should be noted, that the honeycomb sheets has been found in both intact insect cuticles, as well as in purified chitin [39]. The preservation of the microfibrillar structure could therefor also be a sign of high structural integrity of the samples. When looking at the microscopic image of the frass sample large rod-like structures can also be seen. These resemble structures that can be found in plant fibers like hay [40]. Based on the comparison of the gravimetric and spectrophotometric analysis it is clear that non-degraded contamination were present in the frass samples. The microscopic images further support the suspicion that the contamination is stemming from the larvae growth material, since it contains both alfalfa flour and wheat bran, both being fibrous plant material.

4.1.4 Elemental Analysis

EA was performed in order to determine the C:N ratio of the freeze dried chitin samples. This ratio can provide insight about the chemical composition of the extracted chitin samples, and thus the purity. Generally chitin has a C:N ratio of approximately 6-7, but the ratio can vary depending on the DD and the biomass from which the chitin is extracted [41] [42]. The C:N ratios of the chitin extracts can be seen in figure 4.3.

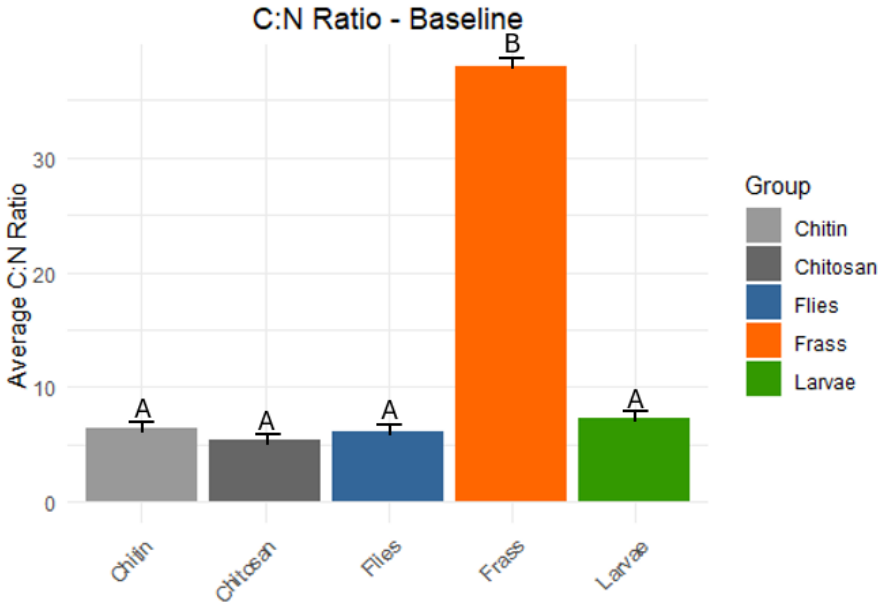


Figure 4.3: The carbon to nitrogen ratios, as determined by elemental analysis, of freeze dried chitin extracted from black soldier flies, larvae and their frass, using the baseline extraction method. Two standards from commercial chitin and chitosan can also be seen on the plot. The gray column represents the standards, blue represents the adult flies, orange the frass, and green the larvae. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

The ratios of the flies and larvae at 6.16 and 7.34 respectively is statistically similar to the commercial chitin, thus indicating pure chitin has been extracted. The table in appendix B.1, contains the C% and N% of both standards and samples. It shows that the percentage of carbon and nitrogen in chitin should be around 42 % and 6.5 % respectively, based on the commercial chitin sample. The percentage of carbon and nitrogen for both flies and larvae, are around 43-45 % and 6-7 % respectively, which roughly correlates with the expected values. The small variation could be due to differences in the chemical composition of chitin from different sources, as the commercial chitin is from shrimp. However these results generally indicate, that pure chitin has been extracted from both flies and larvae. This also rules out the suspicion of excess protein in the samples, which was suspected due to the honeycomb-like sheets seen microscopically, as residual protein would result in a higher percentage of nitrogen [43]. The mean C:N ratio of the frass samples, at 37.86 is significantly higher compared to those of both flies and larvae, as well as commercial chitin. The percentage of carbon in the frass samples, are slightly higher compared to that of the two other materials, but the percentage of nitrogen is approximately 80% lower. This significant decrease in nitrogen, results in a high mean C:N ratio, and strongly indicates contamination from a nitrogen-poor material. As previous results suggested, the frass samples are thought to contained wheat from the larvae growth medium. This correlates with the EA results, as wheat contains large amounts of nitrogen-poor components like cellulose [44]. Furthermore cellulose is very difficult to degrade, due to its compact structure and its intermolecular hydrogen bonding, which could result in it not being broken down during the extraction process. [45].

4.1.5 High-Performance Liquid Chromatography

An HPAEC-PAD analysis was performed to examine the compounds present in the extracted chitin samples, and to give insight into the effectiveness of the acid hydrolysis. The chromatograms of the acid hydrolyzed chitin extracted from flies, larvae, and frass, can be seen in figure 4.4.

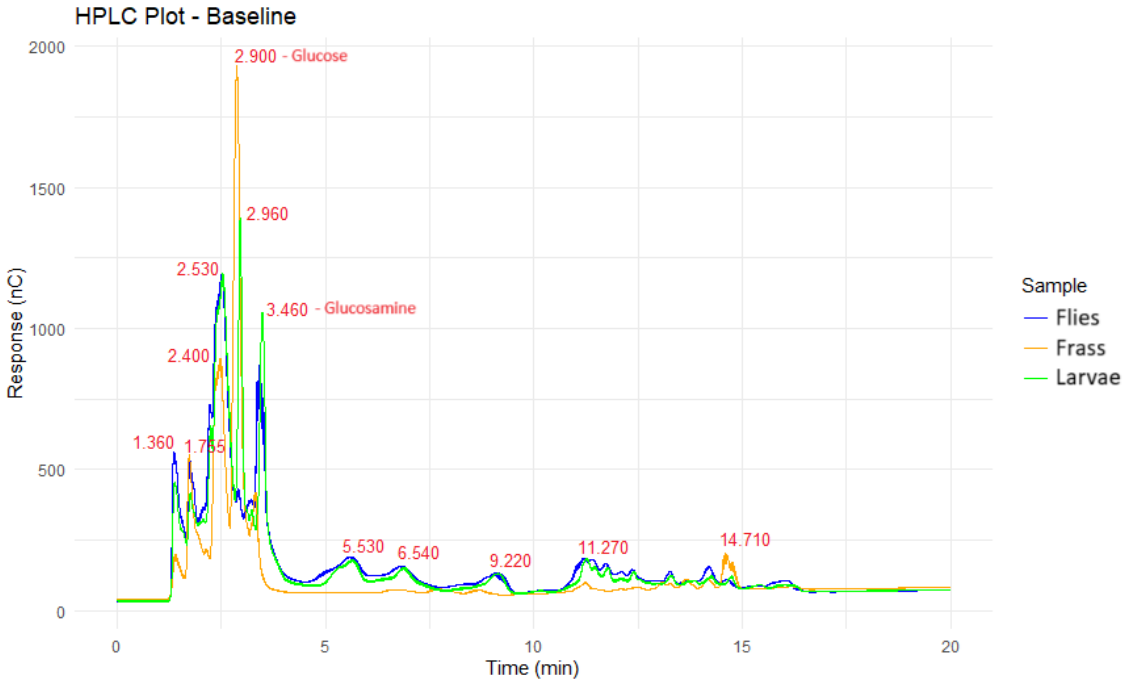


Figure 4.4: HPAEC-PAD chromatograms of acid hydrolyzed chitin extracted from black soldier flies, larvae and their frass, using the baseline extraction method. The blue chromatogram represents the flies, orange the frass, and green the larvae. The x-axis shows the retention time [minutes] and the y-axis shows the response [nC].

A general tendency can be seen across the three chromatograms, with high intensity peaks between 1 and 4 min and a multiple smaller peaks from 5 to 15 min. The peaks are not completely separated which could indicate, that multiple structures with similar retention times are present in the samples. Prior to the sample analysis, standards were made containing both glucose and glucosamine hydrochloride, to optimize the chromatographic conditions needed for the two sugars to be separable. This was done, so the suspected cellulose contamination in frass would be distinguishable from the rest of the sample. However it was not taken into account, that the acid hydrolysis may produce both GlcN and GlcNAc, if the extracted chitin samples had been partially deacetylated. Additionally the samples could contain chitooligosaccharides (COS) with varying degrees of depolymerization of both structures. This would also apply to the contaminant if it is in fact cellulose. This would result in a lot of peaks that could possibly overlap, since GlcN COS elutes almost at the same time as GlcNAc [46]. It is therefor difficult to detect contamination and to determine the exact glucosamine recovery yield from the hydrolysis, as was the purpose of the analysis. Furthermore the majority of the standards showed that glucose and glucosamine

hydrochloride elutes around 2.90 and 3.50 min respectively. However some of the later run standards, showed lower retention times, which further complicates peaks assignment. An example of standards with different retention times can be found in appendix C.1. Assuming that the majority of the standards are reliable, the large peaks at 2.90 and 2.96 min in the frass and larvae samples respectively, could be an indication glucose. Previous results did not show signs of nitrogen-poor contaminants in the larvae sample, but the larvae does feed on the growth material, that is suspected to contaminate the frass sample. This could result in residual glucose-containing compounds being present in the larvae digestive system. The flies however, shows almost no signal around that retention time, possibly due to the flies inability to feed. The frass sample has the highest signal at the supposed retention time of glucose, suggesting that these samples have the highest content of glucose containing compounds. Furthermore the larvae and flies also show peaks around the suspected retention time of monomeric glucosamine at 3.46 min, which is absent in the frass, further indicating that these samples are highly contaminated and that the chitin content is limited. This overall correlates with the result seen in the spectrophotometric analysis and the EA.

Studies have shown that when using HPAEC the elution order of COS is different compared to other oligosaccharides, where retention times increases with increasing degree of depolymerization. However COS with the highest degree of depolymerization elutes first followed by lower degree of depolymerization, except for the monomer, that can elute differently depending on the chromatographic conditions. The reversed elution order of COS, is because the net negative charge of the molecule decreases as degree of depolymerization increases [47]. This would also explain why the peak of the glucosamine monomer is seen in the middle of the peak pattern. Even without determining the glucosamine recovery yield, the results suggest, that the hydrolysis has produced COS of varying degrees of depolymerization, indicating an incomplete hydrolysis. This suspicion is further supported by the low spectrophotometric determined chitin yields.

4.1.6 Fourier Transform Infrared Spectroscopy

An FTIR analysis was made to determine the functional groups and the DD of the chitin extracts. This was done, as the HPLC results could indicate that both GlcN and GlcNAc was present in the samples, suggesting that deacetylation of the chitin had occurred. FTIR spectra can be divided into two groups: Group frequencies and molecular fingerprint frequencies. The group frequencies depicts the functional groups in a molecule, and is often seen above 1500 cm^{-1}. The fingerprint frequencies are seen below 1500 cm^{-1} and is characteristic of the molecule as a whole. Some of the peaks below 1500 cm^{-1} can be used to determine the DD, but visually they are more unreliable since functional groups sometimes also absorb in the lower area. The main focus will therefore be on the peaks above 1500 cm^{-1} [48]. FTIR spectra were made for commercial chitin and chitosan ($\geq$ 75% deacetylated), derived from shrimp shells, as well as glucose. The standard FTIR spectra can be seen in figure 4.5.

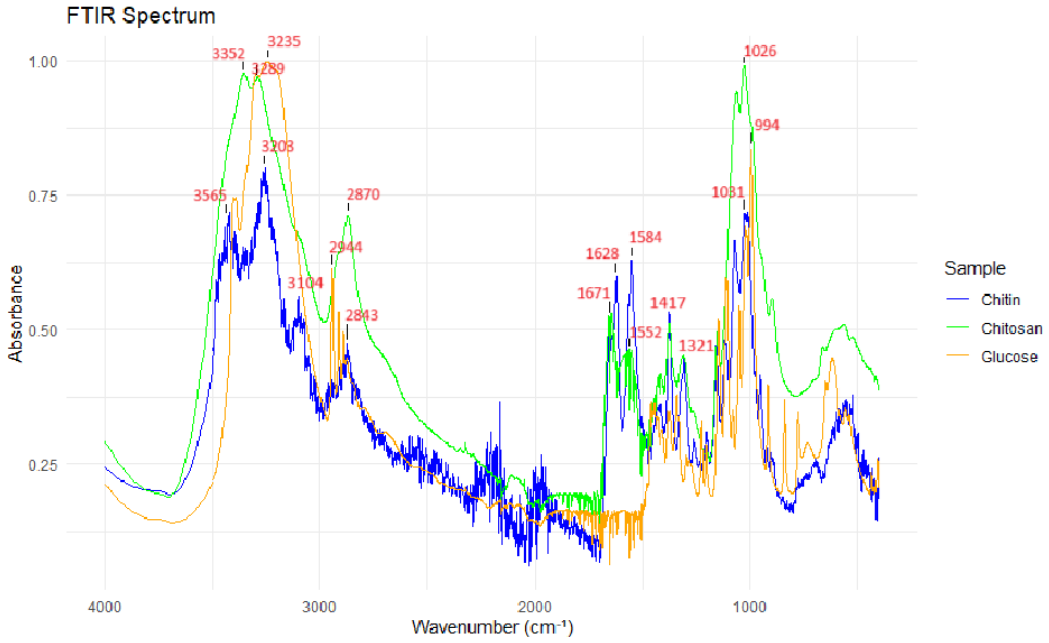


Figure 4.5: FTIR spectra of commercial chitin, chitosan and glucose. The blue graph represents the chitin, green the chitosan, and orange the glucose. The x-axis shows the wavenumber [cm^{-1}] and the y-axis shows the absorbance.

The standards will be used to visually asses the peak patterns of the chitin extracts from flies, larvae and frass, using different extraction methods. Figure 4.5 shows that the three samples have slightly different peak patterns, indicating visual differences in the functional groups. The commercial chitosan with a known DD at 75 % was also used as validation of the chosen equation 3.2 for DD calculation of the extracted chitin. The calculation resulted in a mean DD of 76 %, closely aligning with the expected value of chitosan. The DD of the chitin extracted from flies, larvae, and frass using the baseline extraction method, can be seen in table 4.3.

Degree of Deacetylation [%]		
Flies	**Frass**	**Larvae**
65.53 ± 0.92^{A}	73.35 ± 0.75^{B}	67.56 ± 0.42^{A}

Table 4.3: Calculated mean degree of deacetylation (DD) of the chitin extracted from black soldier flies, larvae and their frass using the baseline extraction method. The table includes the calculated standard deviations based on technical triplicates. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

As suspected based on the HPLC results, all of the samples show high DD values above 50%, indicating a partial deacetylation of the chitin samples. This is not necessarily a disadvantage, since chitosan also has a wide variety of applications across different industries [42]. However if fully

N-acetylated chitin is the target compound, these results indicate that the baseline extraction method is not well-suited for extraction of chitin from insects. Deacetylation and depolymerization of chitin can occur under alkaline conditions at high temperatures (> 80 °C), which is similar conditions used during deproteinization [49]. It is suspected that a partial deacetylation could have taken place during this step in the extraction process. The problem could possibly be solved by implementing milder chemical treatments during the extraction [18]. The frass samples show a significantly higher DD value with a mean of 73.35 % compared to flies and larvae at 65.53 % and 67.56 %, which could indicate that more of the chitin extracted from frass has been converted into chitosan. However the DD is highly dependable on purity, and if contaminants, such as cellulose, were present in the frass samples, it could interfere with the 1320/1420 ratio. The peak at 1320 cm^{-1} is called the amide III peak and can be due to both C-N stretching and N-H bending [50]. Cellulose does therefore not absorb light at that wavenumber. However like chitin, cellulose absorbs light near 1420 cm^{-1}, since this band is due to CH_2 bending [51]. Both of these trends would result in an increase in the 1320/1420 ratio and thus an increase in the calculated DD. This leads to a less reliable determination of the DD value of chitin extracted from frass.

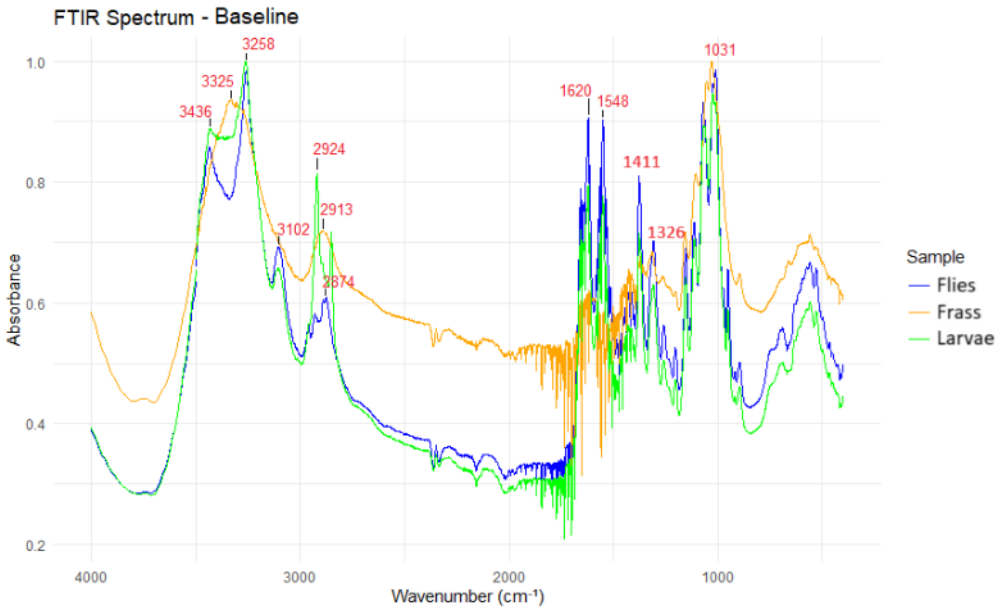


Figure 4.6: The averaged FTIR spectra from the biological triplicates of freeze dried chitin extracted from black soldier flies, larvae and their frass, using the baseline extraction method. The blue spectrum represents the flies, orange the frass, and green the larvae. The x-axis shows the wavenumber [cm^{-1}] and the y-axis shows the absorbance.

Figure 4.6 shows the averaged FTIR spectra from the biological triplicates of chitin extracted from the flies, larvae, and frass. The spectra from flies and larvae, follow approximately the same peak pattern as the chitin standard in figure 4.5. They both show two distinct peaks at approximately 3436 cm^{-1} and 3258 cm^{-1}, which is due to O-H and N-H stretching respectively, and is a clear indication of chitin. O-H stretching occurs in the hydroxyl groups of the sugar backbone in the

glucosamine units, while N-H stretching happens in the amide group, making it distinguishable from other carbohydrates like cellulose. When looking at the spectrum from the larvae, a more pronounced overlap can be seen between the two peaks, compared to what can be seen for the flies. The difference in separation of the two peeks could be due to structural differences of the chitin extracted from different sources. However this could also be due to a presence of glucose, as it has more hydroxyl groups, resulting in a more distinct O-H stretching peak [52] [42]. This also correlates with the HPLC results, that suggested that more glucose was present in larvae. Both flies and larvae also exhibits a small peak at 3102 cm^{-1}, due to N-H symmetrical stretching and the C-H stretching peak at 2924/2874 cm^{-1}. The C-H peak from the larvae samples looks considerably different compared to that of the flies. As C-H stretching also occurs in glucose, the difference in these peaks could also be attributed to the higher levels of glucose in the larvae samples. The peaks at 1620 cm^{-1} and 1548 cm^{-1} corresponds to C=O stretching (amide I) and N-H bending/C-N stretching (amide II), which is normally associated with proteins, but are also characteristic for chitin [42]. Furthermore clear peaks can be seen at 1411 cm^{-1} and 1326 cm^{-1}, indicating a reliable calculated DD for both flies and larvae. Lastly the peak at 1031 cm^{-1} is most likely due to C-O-C stretching, which happens inside the β-D-glucopyranose ring [42].

The FTIR spectrum from the chitin extracted from frass, does not show the same peak pattern as the chitin or chitosan standards. The spectrum consists of one broad peak around 3325 cm^{-1}, which indicates the lack of N-H stretching. Furthermore the C-H stretching peak can be found at 2913 cm^{-1}, but the amide I and II peaks are not present. This pattern is consistent with the glucose standard. The presence of glucose-related peaks, further supports the presence of contamination in the chitin extracted from frass [52]. It was expected to see a clear 1420 peak on the spectrum due to glucose contamination. However neither a clear 1420 or 1320 peak can be seen on the spectrum, which could be due to overlapping signals, baseline noise, or glucose contamination. Either way the poor resolution of these bands, would undoubtedly result in uncertainty in the calculated DD of the frass samples. Generally there are no peaks associated with nitrogen, which could suggest, that glucose masks the presence of chitin, since the microscopic results showed structures that could indicate chitin was actually present in the frass samples. The C-O-C stretching peak is also present at 1031 cm^{-1}. However this is expected as both cellulose and chitin consists of β-D-glucopyranose rings.

Overall the results from the chitin analysis, suggest a low quality of the chitin extracted from frass. There was found clear signs of non-degraded and nitrogen-poor contaminants in the samples, most likely due to residual wheat from the larvae growth medium. The results also suggest that high quality chitin was extracted from flies and larvae. However the calculated DD values indicated that the chitin had been partially deacetylated, possibly due to the harsh alkaline treatment used during deproteinization. Lastly the exact chitin yield of the three different sources remains uncertain, since the the gravimetric determined yields could be over estimated, while the spectrophotometric determined yields are underestimated. However it is thought that the gravimetric yields provide the most realistic yield estimation for the flies and larvae.

4.2 Analysis of the Protein Extracts from the Baseline Extraction

4.2.1 UV Absorption Spectroscopy and Fluorescence Quantitation Assay

The concentration and 260/280 contamination ratio of the protein extracts, were measured using a NanoDrop spectrophotometer. Furthermore a Qubit fluorometric assay was performed for a more selective and sensitive detection of proteins. For the NanoDrop, technical triplicates were made for each of the biological triplicates. The mean protein concentration from NanoDrop as well as single measurements from Qubit be seen in table 4.4. The 260/280 ratio from the UV absorption spectroscopy can be found in appendix D.

Protein Concentration [mg/mL]				
Nanodrop	**Batch 1**	**Batch 2**	**Batch 3**	**Overall**
Flies	34.15 ± 0.78^{a}	32.62 ± 1.07^{a}	34.83 ± 0.89^{a}	33.87 ± 1.15^{C}
Frass	18.65 ± 0.84^{a}	19.87 ± 0.12^{b}	18.49 ± 0.16^{a}	18.99 ± 0.76^{A}
Larvae	20.75 ± 0.23^{a}	20.80 ± 0.17^{a}	20.87 ± 1.56^{a}	20.81 ± 0.06^{B}
Qubit	**Batch 1**	**Batch 2**	**Batch 3**	**Overall**
Flies	0.153	0.166	0.165	0.161 ± 0.007^{B}
Frass	0.129	0.131	0.131	0.130 ± 0.001^{A}
Larvae	0.209	0.204	0.202	0.205 ± 0.003^{C}

Table 4.4: The protein concentrations [mg/mL] of the protein extracts from black soldier flies, larvae and their frass, obtained using the baseline extraction method. The table shows the mean concentration from the technical triplicates from UV absorption spectroscopy (NanoDrop), and single values for fluorescence quantitation assay (Qubit).The overall mean of the biological triplicates, as well as the standard deviations can be seen for both methods. Different lower case letters indicate significant differences between the batches. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

The protein concentrations, determined using the NanoDrop spectrophotometer, showed values ranging from 18.42 mg/mL to 34.83 mg/mL, with flies having a significantly higher concentration compared to larvae and frass. However the 260/280 ratios in appendix D indicate, that all samples show high levels of contamination, with the flies having the highest mean ratio of 1.41 compared to the larvae at 1.33 and frass at 1.22. This could indicate, that the overall concentration pattern across the different materials, is due to difference in contamination levels and not the amount of protein extracted. A 260/280 ratio of 0.6 is ideal, when analyzing proteins and elevated values could indicate DNA or RNA contamination [53]. It is highly likely that both DNA and RNA are present in the protein extracts, since no attempt was made to remove it from the samples, prior to the extraction process. Furthermore this indicates, that the protein concentrations are most likely

overestimated, since nucleic acids will contribute to the overall absorbance of the samples [54]. Overall no significant difference can be seen between the measurements from the three biological replicates (Batch 1–3) for each sample type, indicating good reproducibility of the extraction process. It should however be noted that some variability was observed within the technical triplicates, resulting in a high SD value, which could indicating measurement inconsistencies.

When looking at the protein concentrations, determined using Qubit, they are lower compared to those determined using NanoDrop. This was expected since Qubit is much more selective, and a lot of contaminants, such as nucleic acids, does not interfere with the measurements. However the results are much lower than expected, indicating that the efficiency of the Qubit assay might have been affected. The method is insensitive to free amino acids, which means that protein is not detected if degraded [55]. Protein degradation could be a possibility, due to the harsh chemical treatments used in the baseline extraction method. The low values indicate, that the Qubit assay does not give a true representation of the protein concentration in the samples, but that they might be underestimated. However the results could suggest that a significant difference in protein concentrations can be seen across the sample types, with larvae having the highest mean value of 0.205 mg/mL. Furthermore both protein analyses indicated, that the frass samples has the lowest protein concentrations. It is difficult to determine what the protein content of frass ideally should be, since it is a mixture of multiple things. The protein content would vary depending on the ratio of sheddings, larvae excreta, and growth material, but also depending on what type of growth material was used. However it would make sense, that frass has lower protein concentrations, since the larvae would eat and absorb most of the proteins available in the growth material, leaving behind a nutrient-depleted byproduct.

4.2.2 SDS-PAGE

SDS-PAGE was used to assess the protein profiles of the three different protein extracts. This allows for visualization of the molecular weight distribution and can give insight into the relative abundance, based on the intensity of the bands. Figure 4.7 a) displays the protein profile of the three protein extracts and b) displays the protein profile of flies throughout the baseline extraction.

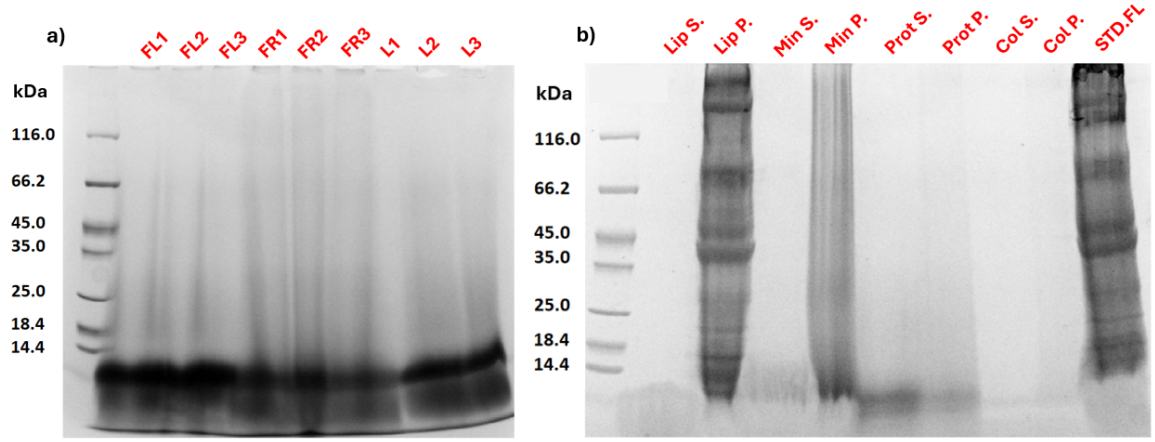


Figure 4.7: a) The protein profile of the biological triplicates of the proteins extracted from black soldier flies (FL), frass (FR) and larvae (L), using the baseline extraction method. b) The protein throughout the baseline extraction of flies and a reference of unprocessed flies (STD.FL). Lip. represents the samples taken during the defatting, Min. the samples from the demineralization, Prot. the deproteinization and Col. the decolorization. The S. and P. refers to supernatant and pellet during the purification steps respectively. Normalized sample load volume was used for all of the liquid samples, but not for the solid.

Figure 4.7 a) shows a big smear at the bottom of each lane. This indicates that the extracted proteins have been extensively degraded during, or prior to protein extraction, which correlates with the low protein content measured by Qubit in table 4.4. The possibility of protein degradation is not a concern, when the main focus of the process is to extract pure chitin, and the proteins are only thought of as contaminants. Intact proteins are however preferred if the extracted proteins are intended for use, as degradation results in the loss of protein functionality, possibly resulting in reduced nutritional quality [56]. Based on the gel it is difficult to determine if there is a difference in the relative abundance of proteins between flies, larvae, and frass. However the smears seams to be slightly less pigmented in the frass samples compared to those of the flies and larvae, which does correlate with the tendencies seen in the protein concentration result.

Based on the results above, another baseline extraction was performed on the flies, larvae, and frass. A small sample was taken from the pellet and supernatant from each step in the extraction. This was done in order to determine the fate of the proteins throughout the process. Figure 4.7 b) is an example of this analysis of the flies. The gels for frass and larvae can be found in appendix E.1, and show the same tendencies. The bands on the gel indicates, that an intact protein profile is present in the pellet during the defatting step. Furthermore it suggests that no proteins are lost during this step, since no visible bands are present in the supernatant containing the lipids. When looking at the supernatant from the demineralization, a weak smear can be seen at the bottom of the gel, indicating the presence of degraded protein in the mineral extract. The remaining demineralized pellet, appears as a smear throughout the lane, which strongly indicates DNA contamination in this fraction. This suggests that the acidic treatment results in lysis of the nucleus and the release of DNA, thus obscuring the protein profile in the demineralized pellet [57]. The

lane containing the protein extracts shows no sign of DNA contamination, indicating that the DNA might have been denatured during the alkaline conditions of the deproteinization [58]. This could also result in some of the DNA being extracted with the proteins, which is supported by the 260/280 ratios from the NanoDrop measurements. The protein supernatant shows the same smear at the bottom of the lane as was seen on gel a), as expected, which indicates that the proteins are degraded during the deproteinization step. A faint smear can also be seen in the bottom of the lane containing the deproteinized pellet, indicating that not all of the proteins have been extracted. Two similar, but faint smears can be seen for both the supernatant and the remaining pellet from the decolorization. This could indicate that traces of degraded protein is present in the extracted chitin. However EA showed no sign of protein contamination, suggesting that there might only be a very small amount of protein present in the final chitin extracts, that cannot be detected using EA.

4.2.3 Elemental Analysis

The CP% of the raw biomass was determined in order to compare the protein content of flies, larvae and frass. It was also determined for the protein extracts to asses the extent of contamination. CP% was determined based on the nitrogen content estimation from EA, and a protein factor of 4.43, that is specific for BSF larvae [59]. Table 4.5 displays the CP% in the protein extracts, as well as the in the raw biomass of flies, larvae and frass. Furthermore the percentage of carbon and nitrogen in the samples can be found in appendix B.1.

CP%					
	Batch 1	**Batch 2**	**Batch 3**	**Overall**	**Raw Biomass**
Flies	5.64 ± 0.65	5.46 ± 0.27	5.48 ± 0.007	5.53 ± 0.10 ^{C}	45.05 ± 0.35 ^{C}
Frass	1.77 ± 0.14	1.59 ± 0.17	1.99 ± 0.11	1.78 ± 0.20 ^{A}	12.89 ± 0.58 ^{A}
Larvae	3.11 ± 0.46	3.69 ± 0.47	3.70 ± 0.54	3.50 ± 0.34 ^{B}	31.52 ± 0.35 ^{B}

Table 4.5: The mean crude protein content (CP %) of the protein extracts from black soldier flies, larvae and their frass, obtained using the baseline extraction method. The table shows the mean CP % based on the technical triplicates from the protein extracts and the overall mean CP% based the biological triplicates. Additionally the mean CP% of the raw biomass based on technical triplicates can also be seen. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

The EA results of the raw biomass show, that the flies have a significantly higher percentage of CP, followed by larvae and then frass as expected. Generally adult flies has been shown to contain up to 58 % CP, whereas larvae can range from 38 % to 46 % [60]. Both flies and larvae are in the lower end of the maximum reported CP %. However the amount of protein in insects can vary depending on factors like diet and developmental stage, indicating that the results can still be considered acceptable. It should also be noted that although larvae has a lower CP% compared to the adult

fly, this does not necessarily mean that they contain less protein per organism. It is likely due to differences in the overall composition, as larvae typically contain higher levels of lipids, minerals, and other biological materials, resulting in an overall lower CP% [37]. The frass shows a significantly lower percentage of CP than flies and larvae, which is expected, since frass is known to contain mostly undigested feed residues and exoskeletal material. This is also supported by the lower percentage of carbon in the frass samples at 38.79 %, compared to the flies at 50.58 % and the larvae at 51.54 %. Since cellulose is high in both hydrogen and oxygen, this would result in a lower overall percentage of both carbon and nitrogen in the samples [61].

The CP% of the extracted protein samples were generally lower, but followed the same trend from the raw biomass, where flies have a significantly higher CP% with a mean of 5.09 %, compared to larvae and frass at 3.50 % and 1.78 % respectively. This indicates, that a lot of other nitrogen-poor compounds might be present in the extracts. Due to the harsh chemical treatments used during the extraction process, depolymerization could have occurred leading to traces of chitin or chitosan in the samples [49]. Like for cellulose, this would result in a lower percentage of both carbon and nitrogen, which correlates with the low mean C% and N% of 22.44 % and 4.77 % for the flies, as well as 15.09 % and 2.85 % for the larvae. Protein is thought to have a nitrogen content of 16 %, which is much higher compared to what can be seen in the samples [43]. Furthermore the proteins were extracted using NaOH, that introduces non-nitrogenous residues, like salts, to the extracts, which could also reduce the overall percentage of nitrogen. Again the frass has a significantly lower CP% compared to both flies and larvae, indicating the largest amount of nitrogen-poor contaminants. This could suggest, that residual cellulose from the growth medium is not only present in the chitin extracts, but the protein extracts as well. The large amount of contamination results in a mean percentage of carbon and nitrogen of 14.80 % and 1.42 % respectively. The overall results suggests that although proteins were extracted, other components are also present in the samples.

4.2.4 Protein Yield

In order to find a more precise estimate of the deproteinization efficiency, the protein recovery yield of the samples were calculated based on the CP% and the dry matter analysis. Figure 4.8 shows the protein yields% of the biological replicates of flies, larvae, and frass.

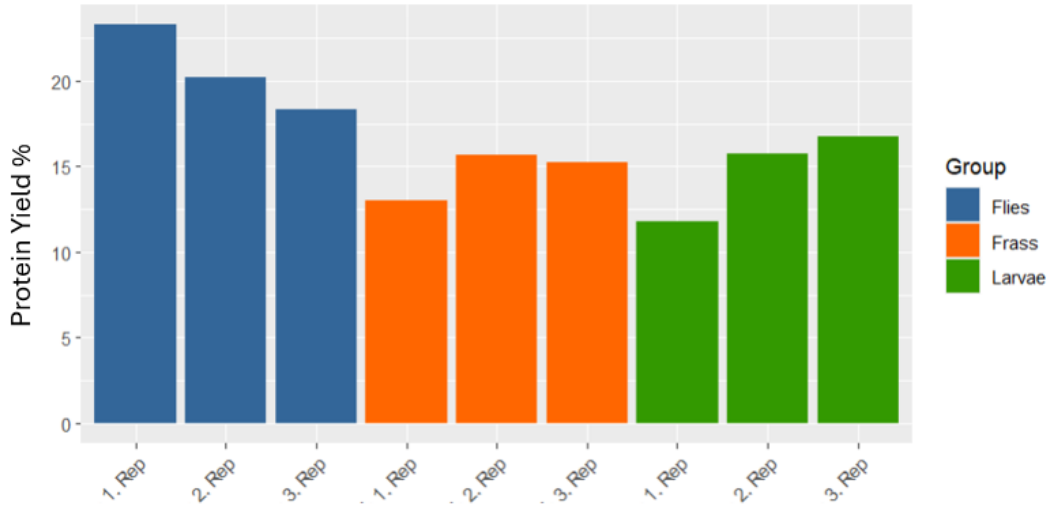


Figure 4.8: Protein recovery yield (%), based on the percentage of crude protein and dry matter content of black soldier flies, larvae and their frass. The yield is presented for each of the biological triplicates from the baseline extraction method. The blue column represents the flies, orange the frass, and green the larvae.

The highest protein yields were observed in the flies, where the values range from 18 % to 23 %. The yields in the frass and larvae samples are lower, but close in value and ranging from 13 % to 16 % and 11 % to 17 % respectively. This indicates, that these two types of material would be equally efficient for protein extraction, using the baseline extraction method. However compared to the expected protein yield of insect, in the range 30–60 %, these values are low. Although this could indicate, that not all of the proteins were extracted during the deproteinization step, it is more likely due to extensive contamination of the protein extracts, as suggested by the CP content. Furthermore the figure shows that the protein yields vary across replicates, suggesting inconsistency in extraction efficiency and or the amount of contamination in the samples, indicating low reproducibility of the deproteinization.

The overall protein results shows that the baseline extraction method is not completely effective at extracting proteins. The combined analyses indicate, that not all of the proteins were extracted during the deproteinization step. Based on the SDS-PAGE some of the protein appears to be removed during the decolorization step, but a small amount may still be present in the final chitin product, although not at levels that were detectable in the chitin analysis. Furthermore the harsh chemical treatments used during deproteinization produces degraded protein, which limits their further use. Lastly when analyzing the protein extracts it was seen that they contain large amounts

of contaminants, possibly in the form of both DNA, sugars and salts, which would be difficult to remove from the extensively degraded protein extracts. This overall strongly suggests, that the baseline extraction cannot be used for extraction of pure intact proteins.

4.3 Analysis of the Mineral Extracts from the Baseline Extraction

4.3.1 Atomic Absorbtion Spectroscopy

The amount of calcium, potassium, sodium and magnesium in the mineral extracts was measured using AAS. The minerals were chosen based on which were reported present in BSF, as well as on the detection abilities of the AAS instrument [62]. The results can be seen in figure 4.6. Furthermore an acid digestion was performed on raw flies, larvae and, frass, to identify the total mineral content in the different types of biomass. These values can be found in appendix F.1. It should be noted, that sample preparation of the mineral extracts and the raw biomass were different. Therefore the values are only used for identifying trends.

Mineral Content [mg/g]			
	Flies	**Frass**	**Larvae**
Ca	0.64 ± 0.11^{A}	0.94 ± 0.17^{A}	19.34 ± 1.80^{B}
K	5.09 ± 0.56^{B}	0.45 ± 0.21^{A}	8.20 ± 1.35^{C}
Na	2.41 ± 0.31^{A}	419.23 ± 21.87^{B}	2.14 ± 0.69^{A}
Mg	2.71 ± 0.32^{B}	0.46 ± 0.11^{A}	4.09 ± 0.64^{C}

Table 4.6: The mean mineral content [mg/g] of the mineral extracts from black soldier flies, larvae and their frass, obtained using the baseline extraction method. The table shows the mean amount of calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) based on the biological triplicates. Different uppercase letters indicate significant differences across the three materials (ANOVA + Tukey, $p < 0.05$).

The calcium content is significantly higher in the larvae compared to the flies and frass, with values up to 21 mg/g. This is expected, since calcium plays a critical role in multiple biological functions during larval growth, including enzyme activity and the formation of structural components like the exoskeleton [63]. The calcium content of frass and flies are both near or below 1 mg/g, which is lower than expected. Literature shows that frass has a calcium content around 13 mg/g, with most of this probably stemming from sheddings [64]. This could suggest that the majority of the frass consists of excrement and growth material, which also correlates with the low chitin yields of the samples. There is however no literature describing the mineral composition of adult flies, most likely because few application has been proposed for this life stage, making it difficult to determine the accuracy of the results. When looking at the determined total calcium content in the three different types of biomass, the same trend can be seen, with larvae having

a significantly higher calcium content compared to flies and frass. However 51.18 mg/g is really high even for larvae [62]. The high values could be due to the high mineral concentration of the raw biomass, since a complete acid hydrolysis was performed, resulting in the samples exceeding the optimal detection range of the instrument, potentially contributing to inaccurate readings.

A significant difference can be seen in the potassium content across all three types of material. The larvae has the highest potassium content, measured from 6.87 mg/g to 9.57 mg/g. The flies contains lower amounts in the range of 4.49-5.59 mg/g, while the frass only contains up to 0.67 mg/g. This could indicate that most of the potassium is absorbed and retained by the larvae during their growth and not excreted. This correlates with potassium having multiple different functions, such as moving nutrients in, and waste products out of cells [65]. Potassium is one of the main minerals found in insects, with values around 11 mg/g [62]. However the potassium values in the raw biomass are much higher across all three samples ranging from 60-86 mg/g, also suggesting measuring limitations of the spectrometer. Furthermore the technical triplicates shows high SD values, which reduces the reliability of the statistical analysis and strongly suggests instrumental errors have occurred.

The flies and larvae generally show lower amounts of sodium in the range of 1.52-2.88 mg/g, compared to the expected value of larvae, at approximately 5 mg/g [62]. This is the same trend as was seen in potassium, which could indicate that the demineralization was not fully successful, and that only some of the minerals were extracted using 1 M HCl. However significantly high sodium values can be found in the frass compared to flies and larvae, with values up to 439.09 mg/g. This is unnaturally high, but generally indicates high amounts of salts in the frass. This, as well as the values from the raw biomass again being high across all three samples, and ranging from around 46 mg/g to 51 mg/g, could therefore further support the suspicion of the instrument having a detection limit.

The amount of magnesium in the larvae and flies are roughly in accordance with the expected magnesium value of 3 mg/g, described in literature [62]. However results suggested that a significant difference in magnesium content could be seen across all three types of material, with frass containing the lowest amount, with a mean vale of 0.46 mg/g. This is the same trend as was seen for potassium, which could suggests that magnesium is also retained in the insects. This correlates with magnesium having a lot of important functions, like aiding in the production of energy and protein [66]. However this trend is not in accordance with the raw biomass values, where the frass has a significantly higher magnesium content of 15.44 mg/g compared to that of flies and larvae at 6.00 and 5.58 mg/g respectively. Again this could be due to instrumental errors.

4.3.2 Overall Evaluation of the Baseline Extraction Method

The overall analysis shows, that high purity chitin can be extracted from flies and larvae, using the baseline extraction method. However the results also suggests, that the extracted chitin had been partially deacetylated, implying that the extracts contain a mixture of chitin and chitosan. This highlights the need for a milder chemical treatment during deproteinization, as this step is thought to be the primary cause of deacetylation. The protein analysis indicated that the deproteinization also could cause depolymerization of the chitin, resulting in some of the polymers being solubilized and co-extracted with the proteins, which would lead to a reduced chitin yield. Additionally the harsh alkaline treatment also resulted in protein degradation, which minimizes the potential use and makes it harder to isolate the protein fraction in the extracts. Furthermore the chitin extracted from the frass, exhibited a low purity, most likely due to cellulose contamination from the larvae growth material. Protein extracts from the frass also showed higher levels of nitrogen-poor contamination. Despite this, the determined protein yields suggested that proteins could still be extracted from the material. The mineral analysis revealed, that essential minerals (calcium, potassium, sodium and magnesium) could be partially extracted from the three materials. However the frass only appeared to contain large amounts of sodium, indicating a less versatile mineral composition compared to flies and larvae. Due to the overall poor quality of the frass, it was excluded from further analysis. Finally the results suggest that the harsh deproteinization step is the main challenge of the baseline extraction method, when wanting to extract intact proteins, making this the main focus of the process optimization. Reducing the use of harsh chemical treatments could possibly enhance product quality, but also increase the resource efficiency and contribute to a more environmentally sustainable extraction process.

4.4 Analysis of the Chitin Extracts from the Optimization Process

4.4.1 Gravimetric Analysis

During the optimization process the sequence of demineralization and deproteinization was reversed, with deproteinization performed first. Furthermore milder deproteinization methods were implemented, in terms of both temperature and concentration of NaOH, and hence the pH. The gravimetrically determined chitin yields of the seven different methods tested can be seen in table 4.7.

Gravimetric Chitin Yield [%]			
Method	**Sample ID**	**Flies**	**Larvae**
1.	1M-ON-80	4.71	3.60
2.	1M-ON-25	9.74	6.72
3.	pH9-1h-25	24.38	19.20
4.	pH9-2h-25	21.50	16.80
5.	pH9-4h-25	17.10	23.20
6.	pH9-ON-25	43.94	25.54
7.	pH9/1M	4.48	2.30

Table 4.7: The gravimetric yields of chitin extracted from black soldier flies and larvae, using different deproteinization methods. The sample ID refers to the pH, reaction time and temperature used during each of the seven deproteinization methods.

The chitin yields seems to vary drastically depending on the method used for deproteinization. The first method showed some of the lowest yields of 4.71 % and 3.60 % for flies and larvae respectively. These values are slightly higher than the yields seen in the baseline, where the mean yield of flies and larvae was 4.12 % and 3.39 % respectively. The only experimental change in the first method, was that the deproteinization was performed before demineralization. This could indicate that the sequence of the two steps might have a slightly positive effect on the overall chitin yield. The yields for both flies and larvae are generally increasing from method 2-6. However the values in method 4 for larvae and method 5 for flies, deviate from that pattern. Since no biological triplicates were made, it is difficult to determine whether the variations reflect actual biological differences, or if it is due to experimental variability. Most of the values from method 2-6 are higher than the expected chitin content of both flies and larvae at 6 % and 10 % respectively. As an example method 3 shows a 4 times higher chitin yield in the flies compared to the expected value, which strongly suggests co-extraction of other compounds in the chitin extracts [36]. Furthermore the chitin yields of the flies are generally higher than that of the larvae, as was also seen

during the baseline. This tendency is attributed to the suspicion of residual moisture in the larvae material, suggesting that it was not completely dried. This would result in a lower starting dry matter content of the larvae, explaining the reduced chitin yield [37]. Method 7 uses a combination of mild and harsh chemical treatments during deproteinization, and produces yields similar to the ones seen in method 1. The yields are however a little lower with values of 4.48 % and 2.30 % for flies and larvae respectively, which could suggest that a small amount of chitin is lost when implementing two deproteinization steps, but due to a lack of biological triplicates this is merely speculations. Overall the results suggests that when using milder deproteinization methods the purity of the final chitin extract decreases. However method 1 and 7, results in similar chitin yields as seen in the baseline extraction method.

4.4.2 Spectrophotometry

The chitin yields were also determined using a spectrophotometry assay. Technical triplicates were made for each of the seven samples and the mean yields as well as the calculated SD can be seen in table 4.8.

Spectrophotometric Chitin Yield [%]			
Method	**Sample ID**	**Flies**	**Larvae**
1.	1M-ON-80	2.98 ± 1.13	1.96 ± 0.76
2.	1M-ON-25	8.30 ± 3.13	3.03 ± 1.14
3.	pH9-1h-25	1.23 ± 0.35	1.12 ± 0.43
4.	pH9-2h-25	0.86 ± 0.32	1.07 ± 0.42
5.	pH9-4h-25	1.89 ± 0.71	0.89 ± 0.33
6.	pH9-ON-25	1.31 ± 0.49	1.45 ± 0.56
7.	pH9/1M	4.89 ± 1.84	2.12 ± 0.79

Table 4.8: The mean spectrophotometric yields of chitin extracted from black soldier flies and larvae, using different deproteinization methods. The table includes the mean yields from the technical triplicates and the respective standard deviations from each of the seven optimization methods. The sample ID refers to the pH, reaction time and temperature used during each method.

The chitin yields from the first method are 2.98 % and 1.96 % for flies and larvae respectively, which is higher than what was seen in the baseline extraction, where flies and larvae had a mean yield of 1.73 % and 1.52 % respectively. This further suggests that performing deproteinization before demineralization has a positive effect on the amount of chitin extracted. Method 2 where 1 M NaOH and lower temperatures are used, produces the highest yields for both flies and larvae at 8.30 % and 3.03 % respectively. The results indicate that this deproteinization method is the the

most effective for chitin extraction in terms of yield. Unlike the gravimetric results, method 3-6 produces the lowest yields, which could indicate, that these methods are not as effective in extracting chitin from insect material. However the gravimetric results suggested that these extracts contain contamination, which could interfere with essential components in the glucosamine assay, resulting in an underestimation of the yields. Method 7 where two deproteinization steps are used produces the second highest chitin yields out of the seven optimization methods, with values of 4.89 % and 2.12 % for flies and larvae respectively. This is inconsistent with the trend seen in the gravimetrically determined yields, where method 7 showed the overall lowest values. The combination of these results could suggest that method 7 produced chitin with a higher purity, compared to the other methods used during the optimization process. All of the technical triplicate values are within 2SD of their respective sample means. However some of the SD values are much higher than expected, as these triplicates are repeated measurements and not independently prepared, which could suggest instrumental errors have occurred.

4.4.3 Elemental Analysis

In order to determine if implementing milder deproteinization methods results in a lower chitin purity, the C:N ratio of the extracts was determined. The results can be seen in figure 4.9 and their respective percentages of carbon and nitrogen can be found in appendix B.3.

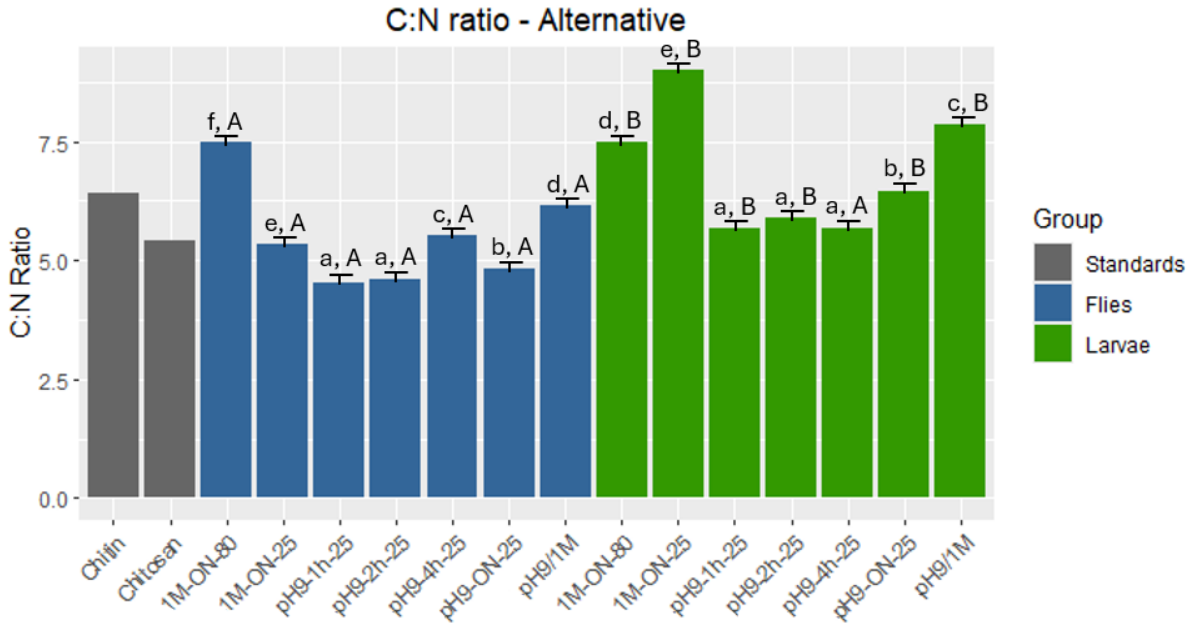
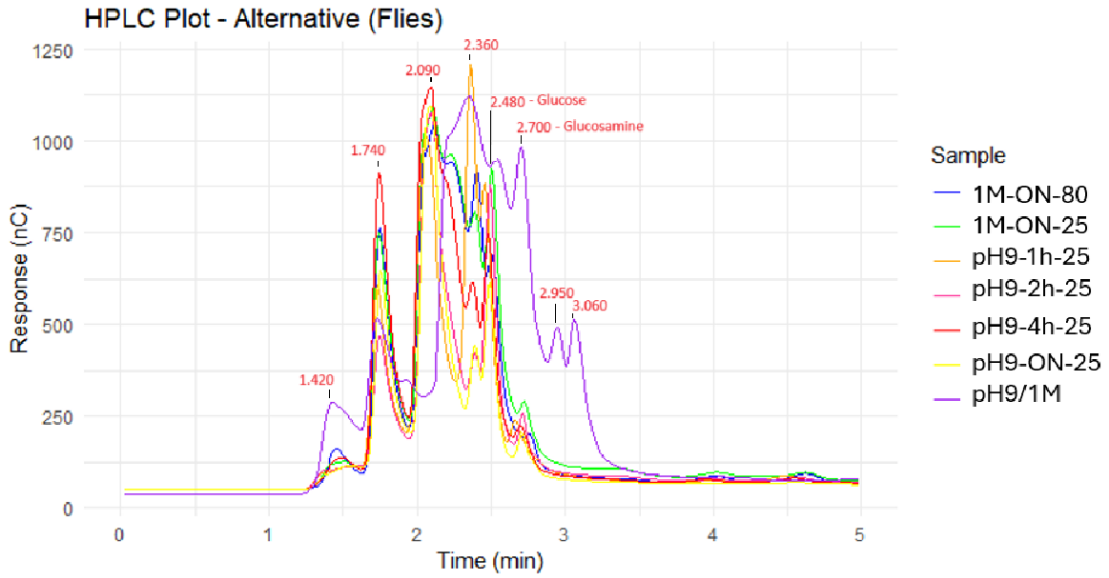


Figure 4.9: The carbon to nitrogen ratios of the chitin extracted from black soldier flies and larvae using different deproteinization methods, as well as two standards from commercial chitin and chitosan. The gray column represents the standards, blue represents the adult flies, and green the larvae. The sample ID refers to the pH, reaction time, and temperature used during each method. Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material. (ANOVA + Tukey, $p < 0.05$).

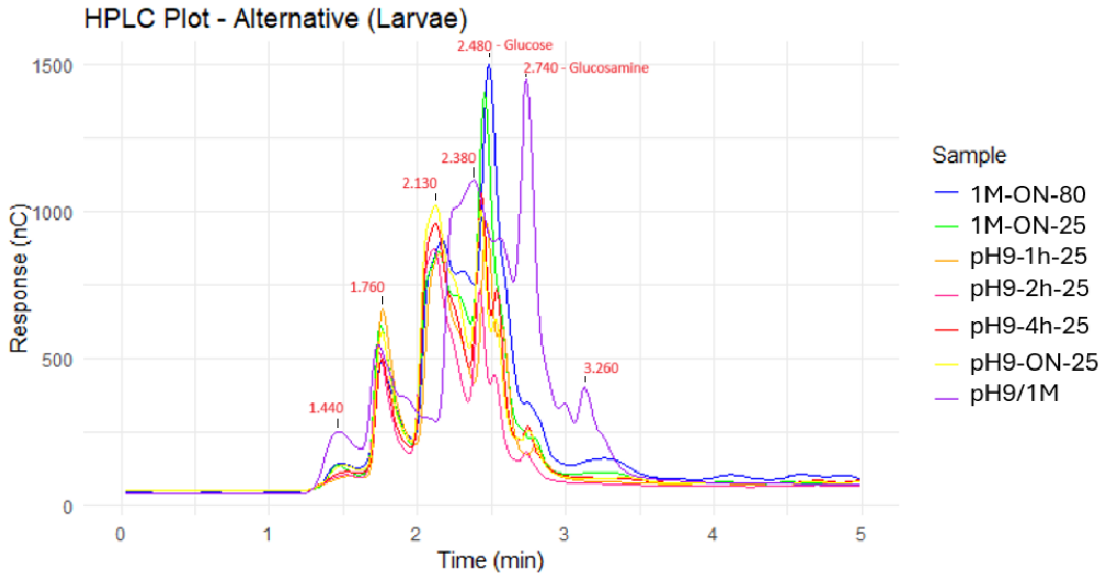
Figure 4.9 shows that most of the C:N ratios varies significantly based on the deproteinization method used. Milder deproteinization methods generally have a lower C:N ratio compared to when using a harsh chemical treatment. Chitin extracted using a milder alkaline treatment at 25 °C over night (method 6) and a double deproteinization (method 7) has C:N ratios closest to the expected value of 6-7, in both flies and larvae [41]. Some of the other ratios are either higher or lower than the expected value, which generally suggest lower purities. The table in appendix B.3, shows how the percentage of both carbon and nitrogen also fluctuates across the samples. In all of the methods, except the one where two deproteinization steps are implemented (method 1-6), the percentage of carbon is much higher than the expected value for pure chitin at 42 %, with values ranging from 55 % to 70 %. Furthermore most of the samples with high percentage of carbon also shows high percentage of nitrogen, with values ranging from around 9 % to 13 %, compared to the expected value of around 6.5 %. This is a strong indication of protein contamination in the samples, since proteins are rich in both carbon and nitrogen, resulting in an increase in both. The percentage of nitrogen is highest in the samples where milder alkaline treatments are used (method 3-6), where a NaOH solution of pH 9 is used for deproteinization. This suggests that a milder alkaline treatment is not as effective in protein removal as 1 M NaOH. Furthermore this could support the suspicion of residual protein in the chitin extracts, interfering with the glucosamine assay, and causing low chitin yields in the spectrophotometry analysis. For both flies and larvae the method with two deproteinization steps (method 7) is the only one producing chitin extracts with percentage of carbon and nitrogen around the expected values, with 45.84 % and 7.47 % respectively for flies and 47.24 % and 6.02 % for larvae. This suggests that the use of two separate deproteinization methods does not affect the overall purity of the chitin samples, compared to the other methods used during the optimization process. In all of the chitin samples, except the one where a mild deproteinization method was run for 4 hours, there is a significant difference in the C:N ratios between the flies and larvae, suggesting variations in either purity or chemical composition between the two materials. However further analysis is needed to determine if this is due to differences in residual protein content, chitin structure, or efficiency of the deproteinization process between the two materials.

4.4.4 High-Performance Liquid Chromatography

Again HPLC was performed in order to give insight into the effectiveness of the acid hydrolysis. A cropped version (0-5 min) of the HPAEC-PAD chromatograms, that shows the peak pattern from the seven different optimization methods, can be seen in figure 4.10. a) depicts the flies and b) the larvae. The full chromatograms can be found in appendix C.2.



(a)



(b)

Figure 4.10: HPAEC-PAD chromatograms of acid hydrolyzed chitin extracted from a) black soldier flies and b) larvae, using different deproteinization methods. Method 1 (blue), method 2 (green), method 3 (orange), method 4 (pink), method 5 (red), method 6 (yellow) and method 7 (purple). The x-axis shows the retention time [minutes] and the y-axis shows the response [nC].

As seen in the baseline extraction, high intensity peaks are present at lower retention times, and as expected they are not completely separated. Previous results have suggested that this is probably due to an incomplete acid hydrolysis resulting in GlcN and GlcNAc COS of different degrees of depolymerization. However a shift in retention time can be seen when comparing the chromatograms from the baseline extraction and the optimization process. The peaks generally have a lower retention time in the optimization process, which is not expected since the same chromatographic conditions were used across all samples. A similar shift in retention time was observed in the standards, seen in appendix C.1. Here the glucose peak shifts from 2.90 min to 2.28 min and the glucosamine peak from 3.52 min to 2.66 min. The standard with the higher retention times was run at the same time as the the baseline extraction, while the one with the lower retention times was analyzed at the same time as the samples from the optimization process a month later. This could suggest that a system or column variability has occurred during the period between the runs. Other samples have been analyzed on the HPLC during the intervening period, which could have affected the column. Furthermore the solvents were refilled, which also could have contributed to a shift in retention time. Based on the standards and the peak pattern from the baseline extraction an estimate of the glucose and glucosamine peaks has been made and can be seen on figure 4.10.

Overall the flies and larvae samples show distinct differences in peak patterns, which was also seen during the baseline. In addition to biological differences between the insects, the choice of deproteinization also produces noticeably different chromatograms. Generally similar deproteinization methods produces similar peak patterns, with the blue and green chromatograms following similar patterns as well as the orange, pink, red and yellow chromatograms forming another pattern. The purple chromatogram is notably the most different, suggesting that this method may have distinctly different composition of depolymerized COS compared to the other samples. However it could also look like a shift in retention time has occurred in this sample, making it difficult to determine if the peak pattern is different, compared to the others or if it is a result of variations in the HPLC system.

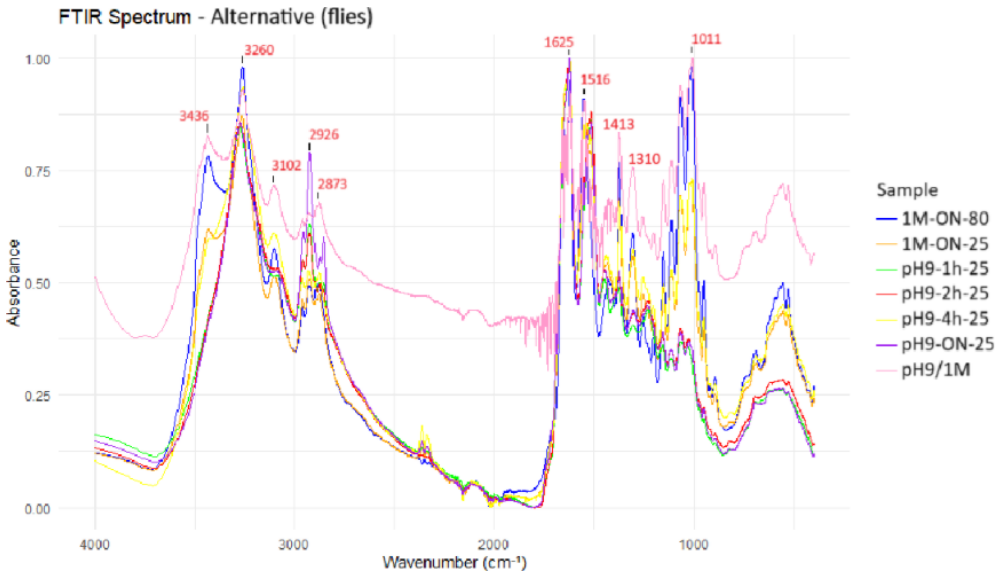
4.4.5 Fourier Transform Infrared Spectroscopy

The DD of the extracted chitin was determined using the FTIR analysis to asses if the alternative deproteinization methods also causes deacetylation of the chitin. Table 4.9 shows the DD values and the SD based on technical replicates of the chitin extracted from flies and larvae during the optimization process.

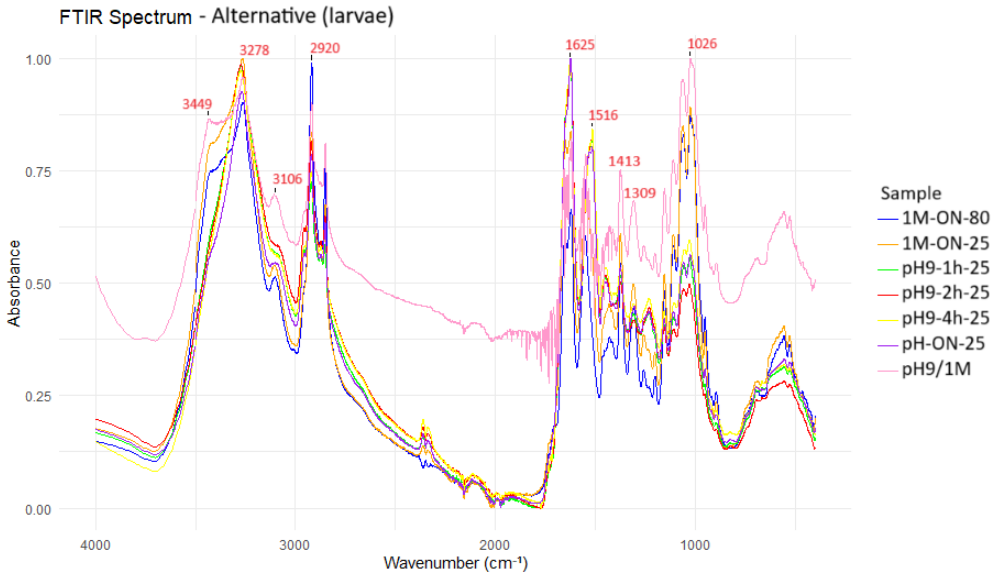
Degree of deacetylation [%]			
Method	**Sample ID**	**Flies**	**Larvae**
	Baseline	$65.53 \pm 0.92^{a,A}$	$67.56 \pm 0.0.43^{a,A}$
1.	1M-ON-80	$76.88 \pm 0.19^{c,A}$	$77.46 \pm 0.19^{c,A}$
2.	1M-ON-25	$79.93 \pm 0.20^{d,B}$	$77.33 \pm 0.67^{c,A}$
3.	pH9-1h-25	$82.69 \pm 0.27^{e,A}$	$82.04 \pm 0.40^{d,A}$
4.	pH9-2h-25	$82.50 \pm 0.08^{e,A}$	$82.47 \pm 0.20^{d,A}$
5.	pH9-4h-25	$79.95 \pm 0.32^{d,A}$	$81.66 \pm 0.11^{d,B}$
6.	pH9-ON-25	$82.38 \pm 0.26^{e,A}$	$81.67 \pm 0.41^{d,A}$
7.	pH9/1M	$71.86 \pm 0.69^{b,A}$	$71.66 \pm 1.21^{b,A}$

Table 4.9: Calculated mean degree of deacetylation (DD) of the chitin extracted from black soldier flies and larvae, using different deproteinization methods. The table shows the mean DD of each method, including the calculated standard deviations based on technical triplicates. Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$)

All of the samples have high DD values above 70 %, indicating that the majority of the chitin has been deacetylated, and thus converted into chitosan. As was seen in the baseline method there is no significant difference in the DD values of the flies compared to the larvae. However all of the DD values for both flies and larvae are significant higher compared to the baseline. This increase in DD could be expected for the chitin extracted using two deproteinization steps (method 7), as deacetylation is more prone to occur during longer alkaline treatment. However it was also expected that using a milder chemical treatment during deproteinization, like in method 3-6, would result in lower DD values. Still the results suggest that most of the chitin extracted using the milder deproteinization methods have a significantly higher DD than when using harsh chemical treatments. Previous analyses of the chitin extracted during the optimization process, suggested that protein contamination is present in these extracts. This results in a decrease in purity, which could affect the accuracy of the DD calculation. Both protein and chitin absorbs light around the amide III peak at 1320 cm^{-1}. It was therefore expected that protein contamination would lead to an underestimation of the DD. However the FTIR analysis from the baseline method has shown that contamination of the chitin extracts generally causes a poor resolution of these peaks, resulting in uncertainty in the calculated DD. Therefore it could be suspected that the values for the samples with signs of contamination, are unreliable.



(a)



(b)

Figure 4.11: FTIR spectra from the freeze dried chitin extracted from a) black soldier flies and b) larvae, using different deproteinization methods. Method 1 (blue), method 2 (orange), method 3 (green), method 4 (red), method 5 (yellow), method 6 (purple) and method 7 (pink). The x-axis shows the wavenumber [cm^{-1}] and the y-axis shows the absorbance.

Figure 4.11 a) and b) shows the FTIR spectra of the chitin extracted from flies and larvae during the optimization process respectively. The FTIR spectra for both flies and larvae show multiple different peak patterns, depending on which deproteinization method is used during extraction.

All of the samples show a distinct N-H stretching peak at 3260 cm^{-1} for flies and 3278 cm^{-1} for larvae. However only the samples from method 1, 2 and 7, where 1 M NaOH was used for deproteinization, show the peak from O-H stretching at 3436 cm^{-1} for the flies and 3449 cm^{-1} for the larvae. Method 3-6, where milder deproteinization methods were applied, are missing the O-H stretching peak. This is a clear indication of extensive protein contamination in the samples. Proteins produces a strong N-H stretching peak, that can partially or entirely overshadow the O-H signal [67]. Furthermore when looking at the larvae there is a bigger overlap between the O-H and N-H peaks, compared to what is seen in the flies. A similar tendency was seen in the baseline extraction, which is thought to be due to excess glucose in the larval digestive system. All of the samples across both flies and larvae exhibits the N-H symmetrical stretching peak at 3102/3106 cm^{-1} and a C-H stretching peak at 2900 cm^{-1}, which suggests that chitin is still present in the contaminated samples. The amide I and amide II peaks are also present across all samples at 1625 cm^{-1} and 1516 cm^{-1} respectively, which is characteristic for both proteins and chitin, along with the C-O-C stretching peak at 1011/1026 cm^{-1} [42]. Additionally the chitin extracted using milder deproteinization methods does not show clear peaks around 1420 cm^{-1} and 1320 cm^{-1}, which would explain the unexpected increase in the DD values. Overall the FTIR data supports the previous result, that indicates protein contamination in the samples where milder deproteinization methods were applied (method 3-6).

The overall chitin results from the optimization process suggests that method 3-6, where milder deproteinization treatments were implemented, was not effective in extracting high quality chitin. All of the analyses indicated that the chitin from these methods contained protein contamination. Additionally the change in sequence of depolymerization and demineralization also resulted in chitin extracts with slightly lower purity compared to the baseline method. Lastly the results showed that a two step deproteinization can be implemented, without having a negative affect on the quantity or quality of the extracted chitin.

4.5 Analysis of the Protein Extracts from the Optimization Process

4.5.1 UV Absorption Spectroscopy and Fluorescence Quantitation Assay

UV absorption spectroscopy and a fluorescence quantitation assay was used to determine the protein concentration of the protein extracts from the optimization process. The protein concentrations can be seen in table 4.10, and the 260/280 ratio from the UV absorption spectroscopy can be found in appendix D.

Protein Concentration [mg/mL]						
Nanodrop	**Sample ID**	**Flies**	**Larvae**	**Qubit**	**Flies**	**Larvae**
	Baseline	$33.87 \pm 1.15^{c,B}$	$20.81 \pm 0.06^{b,A}$		0.16	0.21
1.	1M-ON-80	$55.41 \pm 0.82^{h,B}$	$37.85 \pm 0.87^{c,A}$	**1.**	0.34	0.31
2.	1M-ON-25	$49.98 \pm 1.53^{g,A}$	$47.81 \pm 0.77^{d,A}$	**2.**	21.60	0.41
3.	pH9-1h-25	$29.88 \pm 0.60^{d,A}$	$48.50 \pm 1.14^{d,B}$	**3.**	3.24	4.20
4.	pH9-2h-25	$38.77 \pm 0.89^{f,A}$	$53.26 \pm 0.19^{e,B}$	**4.**	3.64	2.26
5.	pH9-4h-25	$39.61 \pm 1.21^{f,A}$	$54.16 \pm 0.34^{e,B}$	**5.**	4.04	4.96
6.	pH9-ON-25	$36.26 \pm 0.22^{e,A}$	$48.40 \pm 0.98^{d,B}$	**6.**	3.12	4.90
7.	**pH9**/1M	$26.13 \pm 0.45^{b,A}$	$38.29 \pm 1.11^{c,B}$	**7.**	3.33	4.47
7.2.	pH9/**1M**	$22.56 \pm 0.14^{a,B}$	$15.22 \pm 0.39^{a,A}$	**7.2.**	0.37	0.44

Table 4.10: The protein concentrations [mg/mL] of the protein extracts from black soldier flies and larvae, using different deproteinization methods. The table shows mean values of technical triplicates measured using UV absorption spectroscopy (Nanodrop) and single values for fluorescence quantitation assay (Qubit). Method 7 uses two separate deproteinization methods producing two protein extracts. 7 is the first extraction where NaOH of pH 9 was used at 25 °C and 7.2 is the extraction where 1 M of NaOH is used at 80 °C. Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$).

When looking at the protein concentrations determined using NanoDrop, the flies show a significantly higher value than the larvae when using a harsh deproteinization method at 80 °C (1 and 7.2). However when implementing milder treatments (method 3-7), the values are significantly higher for larvae ranging from around 48 mg/mL to 54 mg/mL, indicating that more protein is extracted from the larvae compared to the flies, when using these methods. It should however be noted that the 260/280 ratios are high for both flies and larvae ranging from 1.18 to 1.76, suggesting that the protein extracts contain extensive DNA contamination, as was also seen in the baseline method. The lowest 260/280 ratio was found in the last method (7.2), which is the protein sample that was extracted during the second deproteinization in method 7, indicating that this sample contains the lowest amount of contamination. This was expected since the majority of the contamination would have been removed during the first deproteinization step. Although the results may be overestimated due to DNA contamination, the analysis suggests that more protein has been extracted from larvae when using milder deproteinization methods, and that the overall purity of these extracts is slightly higher compared the flies.

In the Qubit assay, the deproteinization method 1, 2 and 7.2 shows much lower protein concentrations compared to the other samples, with the exception of the flies method 2 with a concentration of 21.60 mg/mL. The low concentrations could be an indication of the proteins from these methods being degraded, like was seen in the baseline method, as harsh alkaline treatments were used during these methods. The much higher value from method 2 in the flies, is thought to be due to protein aggregation, as it was visible in the sample. This can lead to uneven distribution of protein in the sample, resulting in an over-or underestimation of the protein concentration, meaning that this measurement is unreliable. The concentrations from method 3-6 are generally 10 times higher than of those where harsh chemical treatments are used, indicating that intact proteins may have been extracted. Furthermore the values are generally higher in the larvae samples with the exception of method 4, indicating that more protein has been extracted from the larvae compared to the flies. Both the NanoDrop and Qubit results suggest that protein has been extracted from both deproteinization steps in method 7, further indicating that not all of the protein is extracted when using milder alkaline treatments, as was expected based on the overall chitin analysis.

4.5.2 SDS-PAGE

SDS-PAGE was used to visualize the protein profiles of the extracted protein, as previous results suggest that the milder extraction may have extracted intact proteins. The two SDS-PAGE gels in figure 4.12 depict the protein profile of the protein samples extracted using different deproteinization methods. gel a) represents the flies and b) represents the larvae.

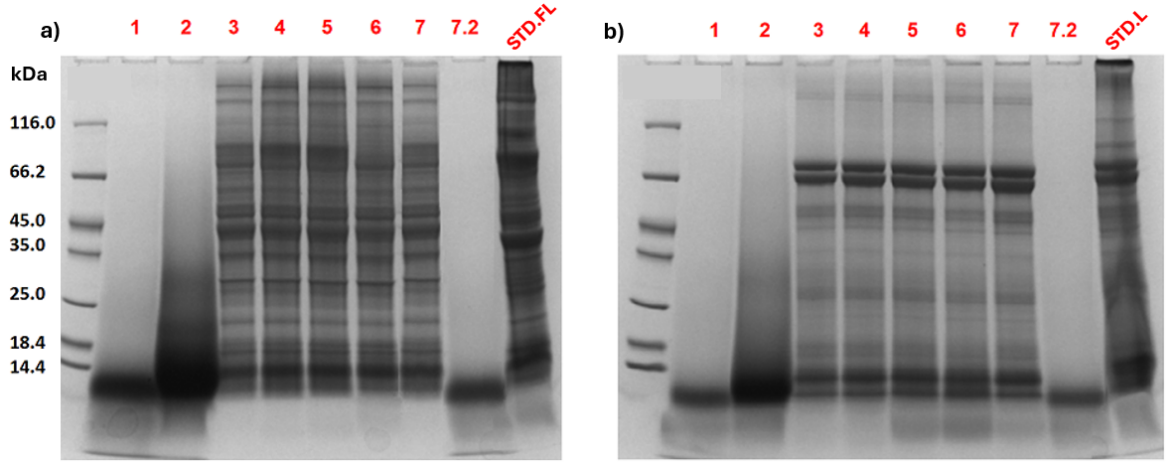


Figure 4.12: Gels displaying the protein profile of the protein extracted from a) black soldier flies and b) larvae, using different deproteinization methods. The number 7 refers to the first protein extraction in method 7, where NaOH of pH 9 was used at 25 °C, and 7.2 refers to the second extraction where 1 M of NaOH was used at 80 °C. References of untreated flies (STD.FL) and larvae (STD.L) can be seen in the last lane on both gels. Normalized sample load volume was used for all of the liquid samples.

The same overall tendencies can be seen on the two gels. Method 1 and 2 where harsh alkaline conditions were used, produce degraded protein, which indicates that changing the sequence of demineralization and deproteinization did not have visual effect on the integrity of the protein samples, compared to the baseline method in figure a) 4.7. However a longer smear can be seen in method 2, suggesting that lowering the temperature during deproteinization from 80 °C to 25 °C, results in a less complete degradation of the protein. For both flies and larvae, method 3-7 show protein profiles similar to the untreated references. This indicates that despite previous results suggesting that not all of the protein have been extracted, the protein that is extracted, is intact. A smear at the bottom of lane 7.2 correlates with the protein concentration results, indicating that proteins are extracted during both deproteinization steps in method 7. This further suggests that implementing two separate steps could be effective if both protein and chitin are to be successfully extracted during this process. The combined results from the analysis of the chitin and protein extract, indicates that by implementing a milder alkaline treatment first, intact proteins can be extracted. Afterwards residual protein can be removed using a harsher treatment, resulting in a pure chitin extract.

The SDS-PAGE also reveals that the protein profiles of larvae and flies are very different. In larvae two strong bands are observed at approximately 66.2 kDa. However the protein profile of the flies seems to be more complex and suggests a more diverse protein composition. On figure a) 4.12, slightly more intense bands can be seen in for method 4 and 5, which correlates with the higher protein concentration from Qubit. Besides from that nothing indicates that longer deproteinization results in more protein being extracted.

4.5.3 Elemental Analysis

The CP% of the protein extracts was calculated in order to determine if using milder deproteinization methods would result in less nitrogen-poor contamination. The results can be seen in table 4.11, and the respective percentages of carbon and nitrogen is found in appendix B.4.

CP%			
Method	**Sample ID**	**Flies**	**Larvae**
	Baseline	$5.53 \pm 0.10^{a,B}$	$3.50 \pm 0.34^{a,A}$
1.	1M-ON-80	$9.78 \pm 0.22^{b,A}$	$6.73 \pm 0.73^{b,A}$
2.	1M-ON-25	$9.44 \pm 0.35^{b,A}$	$6.99 \pm 0.36^{b,A}$
3.	pH9-1h-25	$20.29 \pm 5.31^{d,B}$	$14.64 \pm 0.43^{c,A}$
4.	pH9-2h-25	$16.06 \pm 0.75^{c,A}$	$14.68 \pm 0.51^{c,A}$
5.	pH9-4h-25	$16.87 \pm 1.50^{c,A}$	$14.76 \pm 2.34^{c,A}$
6.	pH9-ON-25	$16.25 \pm 1.01^{c,A}$	$14.93 \pm 0.70^{c,A}$
7.	**pH9**/1M	$23.57 \pm 1.02^{d,A}$	$19.14 \pm 0.99^{d,A}$
7.2	pH9/**1M**	$9.42 \pm 1.99^{b,A}$	$5.53 \pm 0.85^{b,A}$

Table 4.11: The mean crude protein content (CP) based on technical triplicates of the protein extracts from black soldier flies and larvae, obtained using seven different deproteinization methods. The number 7 refers to the first extraction in method 7, where NaOH of pH 9 was used at 25 °C, and 7.2 the second extraction where 1 M of NaOH was used at 80 °C. Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$).

A significant increase in CP% can be seen in the optimization process, compared to the baseline. This could suggest that more protein is extracted when performing deproteinization before demineralization, indicating that some proteins might be extracted during the demineralization. However since previous results suggested that not all of the deproteinization methods was equally effective in extracting protein, the increase in CP% is most likely due to a decrease in the presence of nitrogen-poor compounds, indicating lower sample contamination. The protein extracted using harsh alkaline treatments (method 1,2 and 7.2) has significantly lower CP% values compared to those extracted using milder methods. As shown in the baseline method, using a harsh alkaline deproteinization resulted in more protein being extracted, but also produced a more contaminated sample, due to the presence of deproteinized chitin, causing a decrease in the overall CP%. These results indicate that using milder alkaline treatments lowers the risk of depolymerization of chitin, making it less likely to be extracted with the proteins. This also correlates with the higher

percentages of carbon and nitrogen in these samples in the range of 39-56% and 10-12% respectively, compared to values as low as 15 % and 3% from the baseline extraction method. Furthermore less amount of salt would also be present in these extracts, since a lower concentration of NaOH is used, further increasing the CP%. Additionally when comparing the flies and larvae there is no significant difference between the CP%, except for the method where a mild deproteinization was performed for 1 hour. The majority of the results therefore suggest that the methods are equally effective in extracting protein from both types of material.

4.5.4 Protein Yield

The protein recovery yield was calculated based on the dry matter content and the CP%. Figure 4.13 depicts the protein yields% of the extracts from the optimization process, for both the flies and larvae.

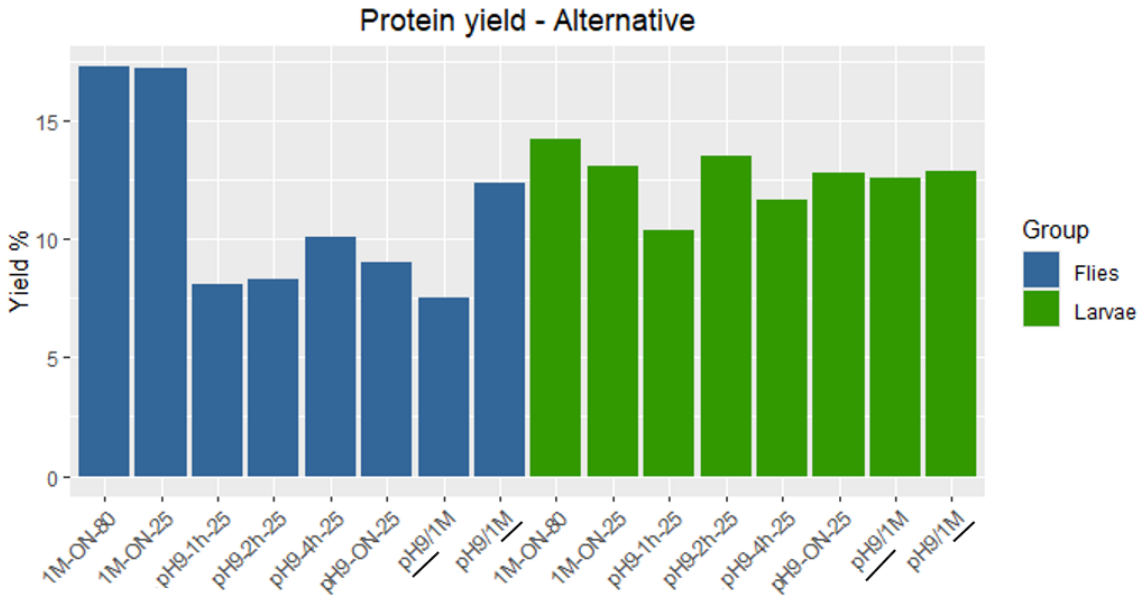


Figure 4.13: Protein recovery yields (%) of the protein samples extracted from black soldier flies and larvae based the optimization process. The blue column represents the flies, and green the larvae.

The plot shows that flies has the highest protein recovery yields when using harsh deproteinization methods, both reaching a yield around 17%. The remaining flies show lower yields, ranging between 5–12%, which further indicates that milder alkaline treatments are less effective in extracting the proteins. As previously mentioned, this is also supported by protein being extracted during both deproteinization steps in method 7. However in the larvae samples the difference between harsh and milder deproteinization methods are less distinct. The harsh deproteinization methods (1 and 2) still show some of the highest protein yields of 14% and 13% respectively, but the milder methods (3-6) have protein yields in the range of approximately 9-13 %, indicating a

more consistent protein extraction performance. The protein yields from method 1 and 2 is notably higher for flies compared to larvae, which is consistent with the trend seen in the baseline extraction. In contrast, when using milder treatments, the protein yields are generally higher for larvae than for flies. This could suggest that using milder alkaline treatments is not as effective for protein extraction of adult flies as it is for larvae, and that a more aggressive treatment is needed to break down the chitin-protein matrix in flies.

The overall protein results suggests that changing the sequence of the demineralization and deproteinization could have a slightly positive effect on not only the quantity, but also the purity of the extracted protein. However results also show that even though the extracts contain lower amount of contamination, the extracted proteins are still degraded. Additionally it was seen that implementing milder alkaline treatments does not remove all of the protein from the material. However the purity of the extracts is significantly higher and the proteins that are extracted are intact. Lastly the results indicate that using a two step deproteinization method can be used to first extract intact proteins and then remove residual protein, resulting in both high quality protein and chitin extracts.

4.6 Analysis of the Mineral Extracts from the Optimization Process

4.6.1 Atomic Absorption Spectroscopy

The mineral content was measured across all of the samples from the optimization methods in order to determine if changing the sequence of demineralization and deproteinization in the process as well as implementing different deproteinization methods have a significant effect on the mineral extraction. The mean values based on the technical triplicates, as well as the SD can be seen in table 4.12.

Mineral Content [mg/g]					
Flies					
Method	**Sample ID**	**Ca**	**K**	**Na**	**Mg**
	Baseline	$0.64 \pm 0.11^{f,A}$	$5.09 \pm 0.56^{a,A}$	$2.41 \pm 0.31^{a,A}$	$2.71 \pm 0.32^{a,A}$
1	1M-ON-80	$1.34 \pm 0.03^{g,A}$	$3.25 \pm 1.58^{a,A}$	$88.57 \pm 10.07^{d,B}$	$2.73 \pm 0.10^{a,A}$
2	1M-ON-25	$0.42 \pm 0.01^{d,A}$	$3.32 \pm 2.25^{a,A}$	$98.71 \pm 7.60^{d,B}$	$2.53 \pm 0.63^{a,A}$
3	pH9-1h-25	$0.33 \pm 0.01^{b,A}$	$6.19 \pm 1.37^{a,A}$	$36.82 \pm 3.35^{b,A}$	$2.14 \pm 0.42^{a,A}$
4	pH9-2h-25	$0.42 \pm 0.01^{d,A}$	$5.21 \pm 0.69^{a,A}$	$50.13 \pm 1.76^{c,A}$	$1.80 \pm 0.26^{a,A}$
5	pH9-4h-25	$0.38 \pm 0.01^{c,A}$	$6.51 \pm 2.83^{a,A}$	$58.37 \pm 1.87^{c,B}$	$2.02 \pm 0.91^{a,A}$
6	pH9-ON-25	$0.49 \pm 0.01^{e,A}$	$5.33 \pm 1.63^{a,A}$	$58.17 \pm 1.61^{c,B}$	$1.75 \pm 0.74^{a,A}$
7	pH9/1M	$0.29 \pm 0.01^{a,A}$	$3.03 \pm 0.93^{a,A}$	$100.38 \pm 4.05^{d,B}$	$1.75 \pm 0.76^{a,A}$
Larvae					
	Baseline	$19.34 \pm 1.80^{c,B}$	$8.20 \pm 1.35^{b,B}$	$2.14 \pm 0.69^{a,A}$	$4.09 \pm 0.64^{b,B}$
1	1M-ON-80	$11.32 \pm 0.29^{a,B}$	$4.89 \pm 0.09^{a,A}$	$41.17 \pm 1.52^{c,A}$	$1.79 \pm 0.19^{a,A}$
2	1M-ON-25	$18.91 \pm 0.68^{c,B}$	$6.01 \pm 1.55^{b,A}$	$53.26 \pm 1.75^{e,A}$	$3.33 \pm 0.75^{a,A}$
3	pH9-1h-25	$12.89 \pm 0.98^{a,B}$	$7.12 \pm 1.39^{b,A}$	$35.09 \pm 1.14^{d,A}$	$2.02 \pm 0.76^{a,A}$
4	pH9-2h-25	$16.96 \pm 0.62^{a,B}$	$7.44 \pm 0.55^{b,A}$	$38.88 \pm 1.21^{d,A}$	$2.73 \pm 0.52^{a,A}$
5	pH9-4h-25	$17.02 \pm 0.94^{b,B}$	$8.24 \pm 0.80^{b,A}$	$42.08 \pm 0.52^{c,A}$	$2.53 \pm 0.39^{a,A}$
6	pH9-ON-25	$15.71 \pm 0.04^{b,B}$	$7.46 \pm 0.56^{b,A}$	$26.18 \pm 1.90^{b,A}$	$1.92 \pm 0.45^{a,A}$
7	pH9/1M	$13.13 \pm 2.20^{a,B}$	$4.17 \pm 0.40^{a,A}$	$42.98 \pm 1.44^{c,A}$	$2.53 \pm 0.43^{a,A}$

Table 4.12: The mean mineral content [mg/g] based on technical triplicates of the mineral extracts from black soldier flies and larvae, obtained during the optimization process. The table shows the amount of calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) measured in each of the seven samples where different deproteinization methods have been used. The standard deviations (SD) are included in the table and a value 0.01 indicates a very low SD. Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$).

As seen in the baseline, the calcium content is significantly higher in larvae, with values up to 17 mg/g, compared to flies with the highest value around 1 mg/g. However the majority of the samples shows that a significant decrease in calcium content can be seen in the optimization methods compared to the baseline, where flies and larvae had a mean calcium content of 0.64 mg/g and 19.34 mg/g respectively. This indicates that performing the demineralization after deproteinization lowers the amount of calcium extracted, and that a small fraction of the minerals could have been extracted during deproteinization. Furthermore the results indicate that even though the amount of calcium in the samples varies across deproteinization methods, there is no clear correlation between method and calcium content.

Opposite the calcium values there is no significant decrease in potassium content, when compared to the baseline. This could suggest that performing depolymerization before demineralization might not have a significant effect on all of the measured minerals, indicating that it effects the minerals differently. However the true effect of changing the extraction sequence can be difficult to determine without biological triplicates. Although not significant, but a slight increase can be seen when implementing milder deproteinization methods, which could suggest that more potassium might be removed during the deproteinization when using harsher alkaline treatments. Additionally the results suggested that all of the methods used in the optimization process were equally effective in extracting potassium from both types of material.

Both the flies and larvae show significantly higher amounts of sodium across all of the samples, compared to the baseline. Even though the raw biomass show similarly high values of around 46 mg/g and 51 mg/g for flies and larvae respectively, litterateur suggests that the sodium content in larvae is around 5 mg/g [62]. This large increase in sodium is most likely due to leftover salt from NaOH used during the deproteinization. This suggests that if deproteinization is performed prior to demineralization, a cleaning step should be implemented in between, unless there is interest in recovering the sodium. Furthermore the majority of the results suggests that a significantly higher sodium content was extracted from the flies compared to the larvae.

Like potassium, there is no significant decrease in the magnesium content, when compared to the baseline, where flies and larvae had a mean magnesium content of 2.70 mg/g and 4.09 mg/g respectively. Furthermore the statistical analysis indicates that the amount of magnesium extracted is independent on both deproteinization method used in the process and the material it is extracted from.

The majority of the results from the mineral analysis suggests that the change in deproteinization methods does not have a significant effect on the demineralization. However the results are ambiguous, emphasizing the need for biological triplicates to determine the exact trend across deproteinization methods. This is further supported by not all of the technical replicate values being within 2SD of the mean, suggesting that the ASS instrument not always produces consistent results.

4.6.2 Overall Assessment of the Process Optimization Compared to the Baseline Extraction Method

Results from the optimization process generally showed that implementing milder deproteinization methods, presents both advantages and disadvantages. The chitin analyses suggested that these methods did not fully remove proteins from the insect material, leading to chitin extracts with protein contamination. The protein analyses supported the suspicion of milder alkaline treatments being less effective for protein removal. However the results also showed that the proteins that were extracted appeared intact and that the samples had reduced levels of nitrogen-poor contamination. This indicated that chitin was less depolymerized and therefore also less likely to be extracted during deproteinization, compared to the baseline method. Furthermore it was seen that when the sequence of deproteinization and demineralization was reversed, while still using 1 M of NaOH, the chitin also exhibited higher protein contamination, compared to the baseline method. This indicates that when demineralization is performed first, some of the proteins may be removed during the acidic treatment. The SDS-PAGE from the baseline, visualizing the protein profile throughout the extraction, supported this interpretation, as it showed traces of proteins in the mineral extract. However changing the sequence of demineralization and deproteinization seems to have a slightly positive effect on both the yield and purity of the extracted protein. Overall these findings suggest that a milder deproteinization results in increasing quality of the protein extracts, but simultaneously reduces the purity of the chitin drastically. The mineral analysis showed a slight decrease in calcium recovery during the optimization process compared to baseline extraction, suggesting that a fraction of the minerals may be lost during the deproteinization step. Additionally all of the samples exhibit high amounts of sodium due to excess salt from NaOH.

Implementing two separate deproteinization steps to the process showed promising results. The gravimetric analysis suggested that the chitin yields from this method, was comparable to the yields from the baseline extraction, indicating that adding a second step does not result in a decrease in chitin content. Furthermore the purity of the chitin was high, and no traces of protein was found in the samples, in contrast to the chitin extracted using the other optimization methods. The protein analyses revealed that when implementing two deproteinization steps it allows for intact proteins to be extracted during the first step, with low levels of contamination, compared the baseline extraction method. This results in proteins, that is more suitable for industrial applications and is easier to isolate. The second step removes the remaining proteins, which leads to a high quality chitin product. Furthermore the mineral analysis showed that no significant decrease was seen in the amount of extracted essential minerals, compared to the baseline method, indicating that this method is just as effective in the extraction of minerals. Overall this method does not focus on reducing the use of chemicals, and therefore does not propose a more environmental sustainable process. However it does align with the biorefining principles, as it shows the best results in terms of resource optimization since both high quality chitin, proteins and minerals have been successfully extracted.

Generally results from both the baseline extraction and the optimization process suggest that more chitin was extracted from the flies, compared to the larvae. Furthermore the protein analyses showed that more protein was extracted from the flies when using harsh chemical treatments, compared to the larvae. However more protein was extracted from larvae when implementing milder treatment methods. This suggests that flies may contain a more stable chitin-protein matrix and harsher treatments are needed for disruption. The mineral analysis suggested that both flies and larvae contained traces of all analyzed essential minerals. The most notable difference was the significantly higher calcium content in larvae compared to flies. Generally results suggest that both flies and larvae could be a good source of chitin, protein and minerals. Additionally frass could also be of interest if the primary goal is protein and mineral extraction.

4.7 Results from the Chitinase Assay and Optimal pH Determination

In order to minimize the use of chemicals in the extraction process, chitinase was implemented during deproteinization. An enzyme assay was made to determine the best enzyme to substrate ratio, with commercial chitin being used as the substrate. The chitin concentration was kept constant with varying enzyme concentrations, since it is difficult to weigh a precise amount of chitin due to its low density. The enzymatic reaction was run for 90 min and afterwards the production of GlcNAc was measured using the glucosamine spectrophotometry assay. The optimal parameters, except for the temperature of 37 °C, was unknown, and the other reaction parameters were chosen to be pH 6 and 90 min [34]. The measured amount of GlcNAc produced from the enzymatic hydrolysis of chitin at different enzyme concentration can be seen in figure 4.14

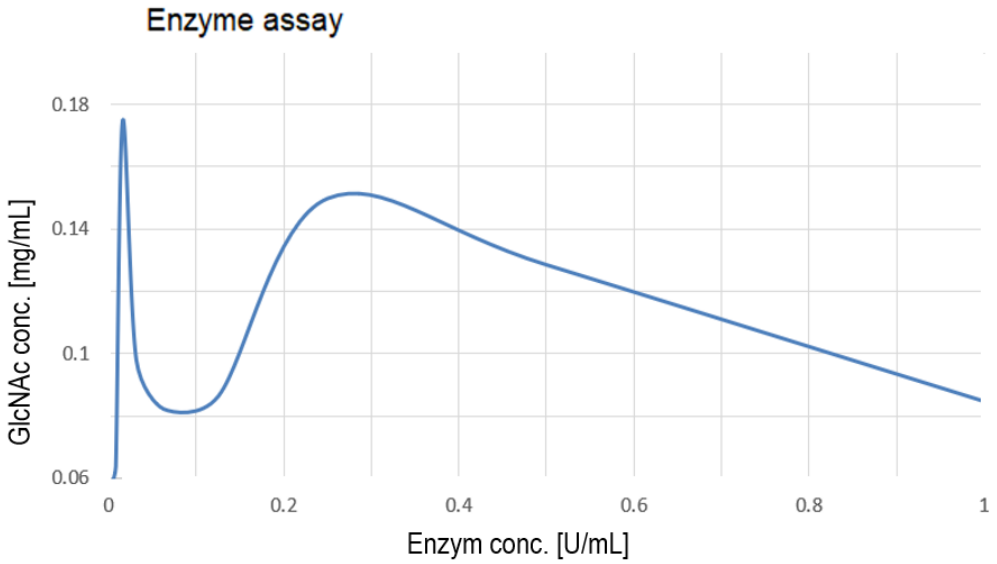


Figure 4.14: N-acetyl-D-glucosamine (GlcNAc) formation during enzymatic hydrolysis of chitin with increasing chitinase concentration. The x-axis shows the chitinase concentration [U/mL] and the y-axis shows the GlcNAc concentration [mg/mL].

The graph in figure 4.14 shows an unexpected pattern of GlcNAc formation. As in kinetic assays, it is expected to see an increase in the GlcNAc concentration as the enzyme concentration increases, since more enzyme would result in more active sites. Instead the graph exhibits two early peaks before declining. Several factors could explain a decrease in product concentration at increasing enzyme concentrations, like product inhibition, substrate depletion or enzyme instability [68]. However these results could also be due to the properties of chitin, since it is highly insoluble in aqueous solutions [16]. This may lead to reduced accessibility of the substrate, thus limiting the reaction efficiency, and causing an unexpected product formation pattern at varying enzyme concentrations. This is further supported by the overall low GlcNAc concentrations in the assay. Based on the product description of chitinase one unit of chitinase will produce 1 mg of GlcNAc from chitin per hour at pH 6 at 25 °C. During the experiment the highest enzyme concentration was 1 U/mL chitinase in 0.5 mL. This is equivalent to 0.5 units, which resulted in a GlcNAc concentration of 0.08 mg/mL, corresponding to a total yield of 0.04 mg. This is notably lower than the expected yield of 0.5 mg GlcNAc that is expected under similar conditions. Since the initial peak on the graph appears anomalous, the enzyme concentration chosen for further analysis is 0.25 U/mL resulting in an enzyme to substrate ratio of 0.0125 U/mg.

4.7.1 Optimal pH Determination

An experiment was also conducted to determine the optimal pH for chitinase activity. The experiment was performed with the previous determined enzyme to substrate ratio of 0.0125 U/mg, at 37 °C, with pH ranging from 4 to 8. The results can be seen in figure 4.15.

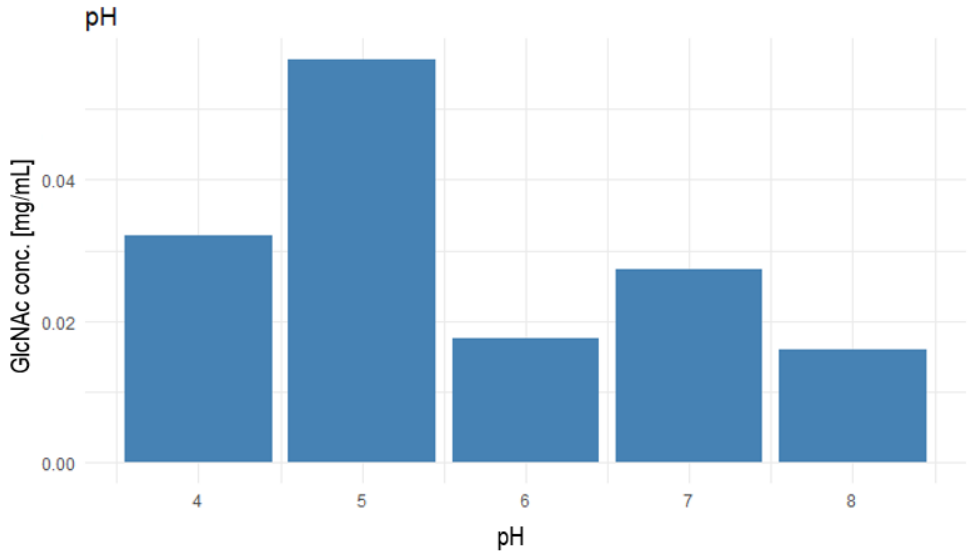


Figure 4.15: N-acetyl-D-glucosamine (GlcNAc) formation during the enzymatic hydrolysis of chitin with a constant enzyme concentration of 0.25 U/mL, E/S ratio of 0,0125 U/mg, temperature of 37 °C, and varying pH values from 4 to 8. The y-axis shows the GlcNAc concentration [mg/mL].

Based on figure 4.15 it is clear that a pH of 5 resulted in the highest concentration of GlcNAc. However as seen in the enzyme assay, the general GlcNAc concentration is low across all of the samples, with values below 0.1 mg/mL, which indicates an overall limited reaction efficiency. Similar to the enzyme assay the GlcNac concentration pattern is also irregular. Typically enzymes have a well-defined pH optimum with a symmetrical decrease in activity on either side, which is not the trend in this analysis [69]. This could further suggest general unreliable results during the determination of optimal chitinase parameters. However based on these results a pH 5 was chosen for the protein extraction with chitinase.

4.8 Analysis of the Enzymatic-Assisted Protein Extraction

4.8.1 UV Absorption Spectroscopy and Fluorescence Quantitation Assay

Enzymatic facilitated deproteinization was performed using chitinase with an enzyme to substrate ratio of 0.0125 U/mg, a pH of 5, a temperature at 37 °C, and three separate reaction times at 1, 2 and 3 hours. The protein concentrations can be seen in table 4.13 and the 260/280 ratio from the UV absorption spectroscopy can be found in appendix D.

Protein Concentration [mg/mL]						
Nanodrop	**Sample ID**	**Flies**	**Larvae**	**Qubit**	**Flies**	**Larvae**
8.	pH5-1h-37	58.47 ± 0.40 ^{a,A}	88.53 ± 1.60 ^{a,B}	**8.**	3.04	3.38
9.	pH5-2h-37	62.21 ± 1.20 ^{a,A}	88.14 ± 1.75 ^{a,B}	**9.**	3.10	3.46
10.	pH5-3h-37	64.10 ± 4.98 ^{a,A}	91.06 ± 1.37 ^{a,B}	**10.**	3.10	3.62

Table 4.13: The concentrations [mg/mL] of the protein extracts from black soldier flies and larvae, obtained using enzymatic facilitated deproteinization. The table shows mean values of technical triplicates measured using UV absorption spectroscopy (Nanodrop) and single values for fluorescence quantitation assay (Qubit). Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$).

The protein concentrations determined using NanoDrop, are significantly higher for larvae in the range of 88.14-91.06 %, compared to the flies at 58.47-64.10 %. This indicates that more protein is extracted from the larvae when using chitinase. Furthermore the slightly lower 260/280 ratio of the larvae samples ranging form 1.39 to 1.43, compared to the flies that ranges from 1.82 to 1.90, suggests that the extracted protein from larvae is also more pure compared to the flies, which was the same tendency seen when using milder chemical deproteinization methods. Furthermore the results could suggest that more protein is extracted at longer the reaction times. However the statistical analysis indicates that the increase seen with increasing reaction time is not significant for neither flies nor larvae. When comparing these protein concentrations to the ones from the optimization process where milder extraction methods were used these values seems overall

higher. This could indicate that more protein was extracted when using chitinase compared to when using chemical extraction methods. However as previously mentioned the low sensitivity of the NanoDrop machine could result in an overestimation of the protein concentration, due to DNA contamination.

The results from the Qubit assay show lower protein concentrations, compared to the NanoDrop as expected. When comparing these values to those in the range of 2.26-4.96 mg/mL from the optimization process where milder chemical deproteinization methods were used, these values are within the same range overall. This does not correlate with the NanoDrop, but since Qubit can be considered more accurate method, it suggests that the milder chemical deproteinization methods might be equally effective, as the enzymatic.

4.8.2 SDS-PAGE

SDS-PAGE was performed to visualize the protein profile of the proteins extracted when using enzymatic-assisted deproteinization. Figure 4.16 below depicts the protein profile of the enzymatic extracted protein for both flies and larvae.

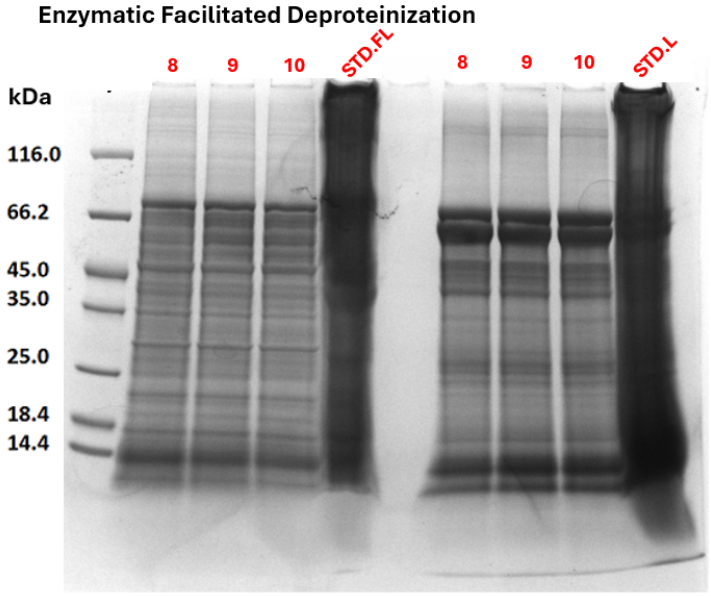


Figure 4.16: Gel displaying the protein profile of the protein extracted from black soldier flies and larvae, using enzymatic facilitated deproteinization. Three different enzyme reaction times were used and method 8 had a reaction time of 1 h, method 9 2 h, and method 10 3 h. References of untreated flies (STD.FL) and larvae (STD.L) are found in lane 5 and 10 respectively. Normalized sample load volume was used for all of the liquid samples.

The gel shows that intact proteins have been extracted from both flies and larvae, as expected based on the Qubit results. The SDS-PAGE results also correlates with the statistical analysis of the UV absorbance results, and indicate that no significant increase in protein concentrations, can be seen with increasing reaction time. The protein profile for both flies and larvae is similar to the respective protein profiles seen in the optimization process. However the top part of the profile, above 66.2 kDa, is much weaker for the enzymatic extracted protein samples, suggesting that proteins with a higher molecular weight are not extracted, when using chitinase. This could indicate that chitinase is not effective in extracting proteins with higher molecular weight. However a more likely explanation is that these proteins are not extracted due to a lower solution pH of 5 compared to a minimum of pH 9 used during the chemical deproteinization methods. The proteins that are not extracted could have an isoelectric point close to 5, resulting in the need for a solution with a higher pH to solubilize these proteins [70]. Furthermore chitinase should be visibly present around 30 kDa, but the exact placement cannot be detected due to the protein profiles of the insects [34].

4.9 Analysis of the Control Protein Extraction

4.9.1 UV Absorption Spectroscopy and Fluorescence Quantitation Assay

A control deproteinization was made using sodium acetate buffer at pH 5, without chitinase to ensure that the observed protein extraction was due to enzymatic activity and not the buffer conditions. The protein concentrations can be seen in table 4.14, and the respective 260/280 ratios from the UV absorption spectroscopy can be found in appendix D.

Protein Concentration [mg/mL]					
Nanodrop	**Flies**	**Larvae**	**Qubit**	**Flies**	**Larvae**
Control 1 h	$28.47 \pm 0.18^{b,A}$	$45.05 \pm 0.39^{a,B}$	**Control 1 h**	0.73	1.04
Control 3 h	$26.96 \pm 0.13^{a,A}$	$48.53 \pm 0.63^{b,B}$	**Control 3 h**	0.76	1.04

Table 4.14: The protein concentrations [mg/mL] of the protein extracts from black soldier flies and larvae, obtained using sodium acetate buffer at pH 5. The table shows mean values of technical triplicates measured using UV absorption spectroscopy (Nanodrop) and single values for fluorescence quantitation assay (Qubit). Different lowercase letters indicate significant differences among the deproteinization methods within the specific material. Different uppercase letters indicate significant differences within the methods across material (ANOVA + Tukey, $p < 0.05$).

Both protein concentration analyses suggests that proteins have been extracted when using sodium acetate buffer without chitinase. However compared to the results from the enzymatic-assisted deproteinization the measured values are generally lower, indicating that more protein is extracted when chitin is present in the reaction. Additionally the same tendencies from the enzymatic deproteinization can be seen here where larvae show significantly higher values at 45.05-48.53 %, compared to the flies in the range of 26.96-28.47 mg/mL by NanoDrop A280, indicating

more protein is extracted from the larvae. This is further supported by the Qubit assay. As expected the 260/280 ratios also show that the larvae protein extracts contain slightly less contamination with values of 1.46 compared to the flies at around 1.96. Furthermore the results could indicate that more protein has been extracted when reaction time increases. However the results are contradictory, making it difficult to draw reliable conclusions without additional data.

4.9.2 SDS-PAGE

SDS-PAGE was made to see if the extracted proteins had the same protein profile as was seen when chitinase was present in the reaction. Figure 4.17 shows the protein profile of the control deproteinization run for 1 and 3 hours for both flies and larvae. For comparison the protein extracts obtained using method 3 (pH9-1h-25) and 8 (pH5-1h-37) are included on the gel.

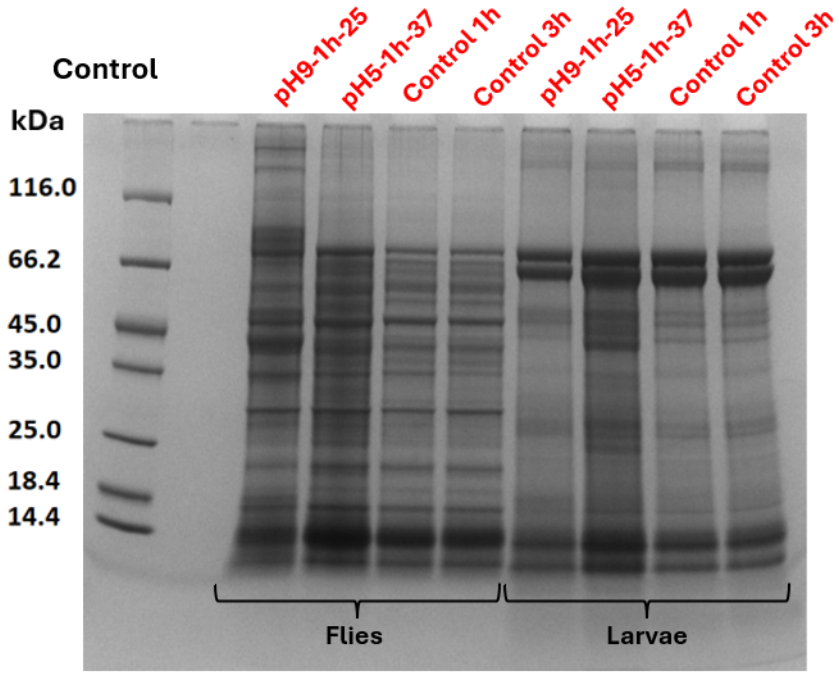


Figure 4.17: Gel displaying the protein profile of the protein extracted from black soldier flies and larvae, using sodium acetate buffer at pH 5, as well as method 3 (pH9-1h-25) and 8 (pH5-1h-37) for comparison. Normalized sample load volume was used for all of the samples.

Based on the gel it is clear that proteins have been extracted using sodium acetate buffer without chitinase. As expected based on the protein concentration analyses, the protein profiles from the controls also suggest that less protein has been extracted, when chitinase is not present in the reaction, as the bands are weaker for both flies and larvae. Furthermore these results clearly show, that less protein with a molecular weight above 66.2 kDa is also extracted during deproteinization with only sodium acetate buffer. This is especially seen when looking at the protein profile from the flies, as these has a higher content of proteins with higher molecular weight compared to the

larvae. The absence of proteins with higher molecular weight when using sodium acetate buffer both with and without chitinase, further supports that this is due to the lower pH of 5, and that a higher pH is needed to extract the full protein profile.

4.9.3 Overall Evaluation of Enzymatic-Assisted Deproteinization

The overall results from the enzymatic deproteinization experiment generally showed that proteins can be extracted from both flies and larvae using chitinase. However protein was also extracted when using sodium acetate buffer, without chitinase, suggesting that the extraction of protein is not solely due to chitinase but also the conditions in the buffer. However it was also seen that using a solution with a lower pH of 5 compared to the chemical deproteinization methods that uses solutions with a minimum pH of 9, resulted in an incomplete extraction of the full protein profile. The SDS-PAGE from the control showed that protein extraction using a solution of pH 9 was more effective in extracting the full protein profile, while a pH 5 did not extract proteins with a higher molecular weight. This indicates that an additional step would be required to extract the larger proteins from the insects, as an incomplete extracted protein profile might reduce the functional diversity and value of the overall protein product.

Optimization of the enzymatic facilitated protein extraction could be performed in order to improve efficiency of the process, since the analyses indicates that chitinase alone might not be optimal for deproteinization. However it should be noted that while enzymatic facilitated protein extraction might offer a more environmental sustainable solution compared to the chemical methods, the method might not be subtitle for industrial applications due to the cost of commercial chitinase [34]. The enzyme is significantly more expensive than any types of chemical reagents like NaOH or HCl. This results in the enzymatic approach being less attractive for large scale processes, making the chemical methods the preferred choice.

Conclusion

A well-established chemical extraction method was used to establish a baseline for the extraction of protein, minerals and chitin from BSF material. The results showed that this method was effective in extracting high purity chitin from flies and larvae. However it could be seen that frass was not suitable for chitin extraction, as this material contained high amounts of non-degraded cellulose contamination from the larvae growth medium. During the baseline extraction method, 1 M NaOH was used in the deproteinization step, which resulted in deacetylation and deproteinization of the chitin, as well as degradation of the proteins. This indicated that a milder deproteinization would be beneficial in order to increase resource efficiency of the process. During the process optimization milder deproteinization methods were implemented, both in terms of concentration of NaOH and temperature. However the use of milder deproteinization methods were less effective in removing proteins, resulting in protein contaminated chitin extracts. On the other hand these methods were effective in extracting intact proteins and reduced the amount of nitrogen-poor contamination in the samples, likely due to less extensive depolymerization of the chitin. In order to combine the benefits from the baseline and optimization process, two separate deproteinization steps were implemented. This resulted in the successful extraction of both high quality chitin and intact protein. Furthermore results showed that this process was equally effective in extracting minerals as the baseline extraction method. Additionally an enzymatic-assisted deproteinization step was also explored in order to minimize the use of chemicals in the extraction process. Results showed that this method also extracted intact proteins. However due to the lower pH of 5 in the solution, high molecular proteins with an isoelectric point close to that pH was not extracted. Generally it can be concluded that by using two separate chemical deproteinization steps, it is possible to successfully implement a biorefining approach in the extraction process of chitin from both flies and larvae, as both high quality chitin, proteins and minerals were extracted.

Perspective

Some of the experimental data is a reflection of both time and resource limitations. Especially the overall analysis of the HPLC data is uncertain, due to unexpected shifts in retention time between runs. It would have been of interest to analyze chitin samples from both the baseline method and the optimization process, spiked with glucosamine to help identify the peak corresponding to monomeric glucosamine. This would further make it possible to determine the exact glucosamine recovery yield from the acid hydrolysis, thus getting a better understanding of the hydrolysis efficiency. Furthermore the lack of flies and larvae material resulted in no biological triplicates were made during the process optimization or during the enzymatic facilitated deproteinization. This leads to a general uncertainty of the results, as it is difficult to determine whether variations are due to actual biological trends or random fluctuations.

If more time had been available,it could have been interesting to explore other variables during the extraction method. As an example hexane could have been replaced with another less toxic organic solvent like ethanol. Literature shows that ethanol can be equally effective as hexane for the defatting of insect biomass [71]. The use of ethanol could be advantageous as it is a bio-based solvent that, unlike hexane, has low toxicity and a much lower environmental impact. This could contribute to the overall environmental sustainability of the process. Additionally alternative methods for sample separation would be of interest, since a lot of material is lost during the multiple filtration steps. Vacuum filtration was initially tested before to the experiment, but the material clogged the filters, preventing an effective separation of the samples. Centrifuging the samples prior to filtration helps minimize the loss of sample and maybe centrifugation at higher rcf could be even more effective. This is especially important near the end of the extraction process, where pure chitin is difficult to separate, due to its low density.

The results generally suggest that the sequence of demineralization and deproteinization has an effect on the quality of the extracts. When performing demineralization first, results indicate that some degraded protein is present in the mineral extracts. However when performing deproteinization first a lower amount of minerals are being extracted, indicating that some of the minerals are present in the protein extracts. This further suggests the importance of alternative purification steps, since the harsh chemical treatments lead to a higher degree of contamination in the extracts. It would therefor also be interesting to measure the protein concentration in the mineral extract and the mineral content in the protein extracts, to understand the extend of contamination.

Additionally the majority of research focuses on the BSF larvae due to their high nutritional content and because they are easy to farm [62]. However the flies are relatively understudied, as they have an overall lower mass, resulting in a lower biomass content per individual. Further investigation could be of interest especially of the protein content, as the SDS-PAGE suggested that

flies and larvae has completely different protein compositions. Applying proteomics could offer insight into the protein composition, like identification of primary proteins and the amino acid distribution of the flies. The same could be of interest for the different growth stages of the larvae and maybe the pupal stage, to detect how the protein composition changes during the BSF life cycle. This could be relevant in order to determine the functionality of the proteins, as understanding the proteins similarities and differences could help identify which are better suited for specific industrial applications.

This project demonstrates that implementing a two step chemical deproteinization method in the extraction process of chitin shows promising results, as high quality proteins, minerals and chitin was extracted using this method. These findings could support the increasing focus of insects as a sustainable alternative protein source, with the potential to extend their use from only protein, to the addition of essential minerals and valuable biomaterials like chitin. Since BSF are already being reared in over 80 % of European insect farms today, and there could be a strong potential for implementing a biorefining approach like this into already existing infrastructures. The main focus of the current BSF insect farms is the bioconversion ability of BSF larvae. However implementing targeted extraction of proteins, minerals and chitin does not interfere with this process, as the extraction would take place afterwards, which would further improve resource efficiency. However implementing this at scale would give rise to several challenges, one of which is chemical waste management. The use of multiple different chemicals during this process would require meticulously planning of how to handle, dispose of, or maybe even how to recycle these reagents. Furthermore the overall cost effectiveness should be evaluated in order to ensure economic sustainability.

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Appendix A

Materials and Chemicals

A.1 Materials

Materials	Manufactor
Weight	Mettler Toledo
Foil trays	Plus Pack
Greiner tubes 50 mL, 15 mL	Sarstedt
Eppendorf tubes 1.5 mL, 2 mL	Sarstedt
Qubit tubes 0.5 mL	SSI Bio
Oven	Binder
Climate chamber	Binder
Blender	IKA
Homogenizer	Bie and Berntsen A/S
Centrifuge	Sigma
MiniSpin plus	Eppendorf
Coffee filters	Duni
Vortex 2	IKA
HLC Heating-TermoMixer	Ditabis
Pipette tips 20 mm, 200 mm, 1000 mm	Sarstedt
Heating block	Hettich Benelux
Soft tin containers	Thermo Scientific
Syringes 1 mL	Nipro

Syringe filter, nylon 0.22 microm	VWR
Short thread vial	Microlab Aarhus A/S
Short thread cap	Microlab Aarhus A/S
Tubes 11.5 mL	Sarstedt
Teflon PTFE vessels	Anton Paar
Slides	Menzel-Glaser
Cover glasses	Hounisen
Light microscope	Zeiss
PHM220 Lab pH meter	MeterLab
Mini-Protean Tetra Cell	BioRad
PowerPac HC High-current power supply	BioRad
HS 260 basic shaker	IKA
ChemiDoc Imaging System	BioRad
Atomic Absorbtion Spetrometer PinAAcle 900F	PerkinElmer
Multiwave 7000	Anton Paar
Qubit 4 Fluorometer	Invitrogen
DS-11 FX Spectrophotometer/ Fluorometer	DeNovix
Lyovapor L-200	Buchi
Vapour-Line Autoclave	VWR
Microplate Reader	Tecan
FlashSmart Elemental Analyzer	Thermo Scientific
TENSOR II FTIR Routine Spectrometer	Bruker

Dionex AS-AP autosampler	Thermo Scientific
Dionex ICS-6000 EG eluent generator	Thermo Scientific
Dionex ICS-6000 DC Detector/Chromatography Compartment	Thermo Scientific
Dionex ICS-6000 DP dual pumps system	Thermo Scientific
Dionex CarboPac PA20 IC column (150 x 3 mm)	Thermo Scientific

Table A.1: Table of materials used in the project.

A.2 Chemicals

Method	Chemicals	Manufactor
Rearing (Growth medium)	Dry Yeast	Lesafre
	Malt	Harboe
	Wheat bran	DLG
	Alfalfa flour	Equsana
Sample Preparation	Ethanol 70%	VWR
Defatting	Hexane 97%	VWR
Demineralzation	Hydrorgen cloride 37%	Sigma-Aldrich
Deproteinization	Sodium hydroxide pellets	Merck
Decolorization	Hydrogen peroxide 10%	Matas
Acid hydrolysis	Sulfuric acid 97%	J.T.Baker
Qubit	Qubit Protein reagent	Thermo Fisher Scientific
	Qubit Protein buffer	Thermo Fisher Scientific
	Standard 1, 2 and 3	Thermo Fisher Scientific
Spectrophotometry	D-Glucosamine Assay Kit	Megazyme
HPLC	D-(+)-Glucose	Sigma-Aldrich
	D-Glucosamine hydrocloride 98+%	Thermo Scientific
	1 M Sodium Acetate High-grade	Pre-made
	1 M Sodium hydroxide High-grade	Premade
FTIR	Chitin	Sigma-Aldrich
	Chitosan	Sigma-Aldrich

SDS-PAGE	DTT	Pre-made
	Sample buffer	Pre-made
	Running buffer	GenScript
	Protein ladder	Thermo Sceintific
	SurePAGE, Bis-Tris, gels	GenScript
	InstantStain (Coomassie blue)	Kem-En-Tec Nordic A/S
Atomic Absorption Spectroscopy	Nitric acid 65%	Merck
	PlasmaPure nitric acid 67-70%	SCP Science
	Calcium standard	Merck
	Potassium standard	Sigma-Aldrich
	Sodium standard	Merck
	Magnesium standard	Merck
Chitinase Assay	Nitric acid 65%	Merck
	Calcium standard	Merck
	Potassium standard	Sigma-Aldrich
	Magnesium standard	Merck
Elemental analysis	Acetanilide	OEA Labs

Table A.2: Table of chemicals used in the project. Both demineralized water and ELGA water has been used throughout the entire experiment.

Appendix B

Elemental Analysis Data

B.1 Percentage of carbon and nitrogen as well as the C:N ratios of the chitin extracted using the baseline extraction

	C%	N%	C:N
Chitin	41.70	6.49	6.43
Chitosan	37.72	6.99	5.41
Flies 1. Rep	44.32	7.17	6.19
Flies 2. Rep	43.75	7.06	6.20
Flies 3. Rep	44.32	7.39	6.08
Frass 1. Rep	45.5	1.09	41.99
Frass 2. Rep	45.44	1.31	34.64
Frass 3. Rep	45.10	1.23	36.95
Larvae 1. Rep	43.66	6.09	7.17
Larvae 2. Rep	45.00	6.02	7.48
Larvae 3. Rep	44.39	6.04	7.36

Table B.1: The percentage of carbon and nitrogen as well as the C:N ratios of the chitin extracts from black soldier flies, larvae and their frass, obtained using the baseline extraction method. The table shows the values for each of the biological triplicates.

B.2 Percentage of carbon, nitrogen and crude protein as well as the C:N ratio of the protein extracted using the baseline extraction

	C%	N%	C:N	CP%
Standard Flies	50.58	10.17	4.97	45.05
Flies 1. Rep	22.19	4.73	4.70	5.64
Flies 2. Rep	22.39	4.68	4.77	5.46
Flies 3. Rep	22.73	4.90	4.65	5.48
Standard Frass	38.79	2.91	13.33	12.89
Frass 1. Rep	14.26	1.41	10.18	1.77
Frass 2. Rep	15.03	1.40	10.73	1.59
Frass 3. Rep	15.09	1.44	10.51	1.99
Standard Larvae	51.54	7.11	7.246	31.51
Larvae 1. Rep	15.18	2.70	5.65	3.11
Larvae 2. Rep	13.94	3.04	4.59	3.69
Larvae 3. Rep	16.16	2.82	5.74	3.70

Table B.2: The percentage of carbon, nitrogen, and crude protein as well as the C:N ratios of the protein extracts from black soldier flies, larvae and their frass, obtained using the baseline extraction method. The table shows the values for each of the biological triplicates.

B.3 Percentage of carbon and nitrogen as well as the C:N ratios of the chitin extracted during the optimization process

Method	Sample ID	C%	N%	C:N
Flies				
1	1M-ON-80	55.23	7.38	7.48
2	1M-ON-25	60.36	11.28	5.35
3	pH9-1h-25	59.38	13.08	4.54
4	pH9-2h-25	58.99	12.79	4.61
5	pH9-4h-25	56.85	10.72	5.54
6	pH9-ON-25	62.06	9.47	6.45
7	pH9/1M	45.84	7.47	6.14
Larvae				
1	1M-ON-80	56.85	5.23	10.86
2	1M-ON-25	57.88	6.42	9.02
3	pH9-1h-25	61.55	10.83	5.68
4	pH9-2h-25	70.18	11.92	5.88
5	pH9-4h-25	59.91	10.53	5.69
6	pH9-ON-25	61.06	9.47	6.45
7	pH9/1M	47.24	6.02	7.84

Table B.3: The percentage of carbon and nitrogen as well as the C:N ratios of the chitin extracts from black soldier flies and larvae obtained during the optimization process.

B.4 Percentage of carbon, nitrogen and crude protein, as well as the C:N ratio of the protein extracted during the optimization process

Method	Sample ID	C%	N%	C:N	CP%
Flies					
1	1M-ON-80	37.74	6.85	5.51	9.78
2	1M-ON-25	38.44	6.91	5.57	9.44
3	pH9-1h-25	50.57	11.66	4.34	20.29
4	pH9-2h-25	52.94	12.06	4.39	16.06
5	pH9-4h-25	54.61	11.92	4.58	16.87
6	pH9-ON-25	52.66	11.57	4.55	16.25
7	**pH9**/1M	38.73	11.27	3.44	23.57
7.2	pH9/**1M**	25.31	5.49	4.62	9.42
Larvae					
1	1M-ON-80	29.11	4.77	6.11	6.73
2	1M-ON-25	31.63	5.47	5.80	6.99
3	pH9-1h-25	53.34	10.77	4.96	14.64
4	pH9-2h-25	53.65	10.97	4.89	14.68
5	pH9-4h-25	54.79	10.49	5.22	14.76
6	pH9-ON-25	55.54	10.91	5.09	14.93
7	**pH9**/1M	42.70	10.75	3.97	19.14
7.2	pH9/**1M**	18.61	3.37	5.52	5.53

Table B.4: The percentage of carbon, nitrogen, and crude protein as well as the C:N ratios of the protein extracts from black soldier flies and larvae obtained during the optimization process. he number 7 refers to the first extraction in method 7, where NaOH of pH 9 was used at 25 °C, and 7.2 the second extraction where 1 M of NaOH was used at 80 °C

Appendix C

HPLC

C.1 HPLC standards

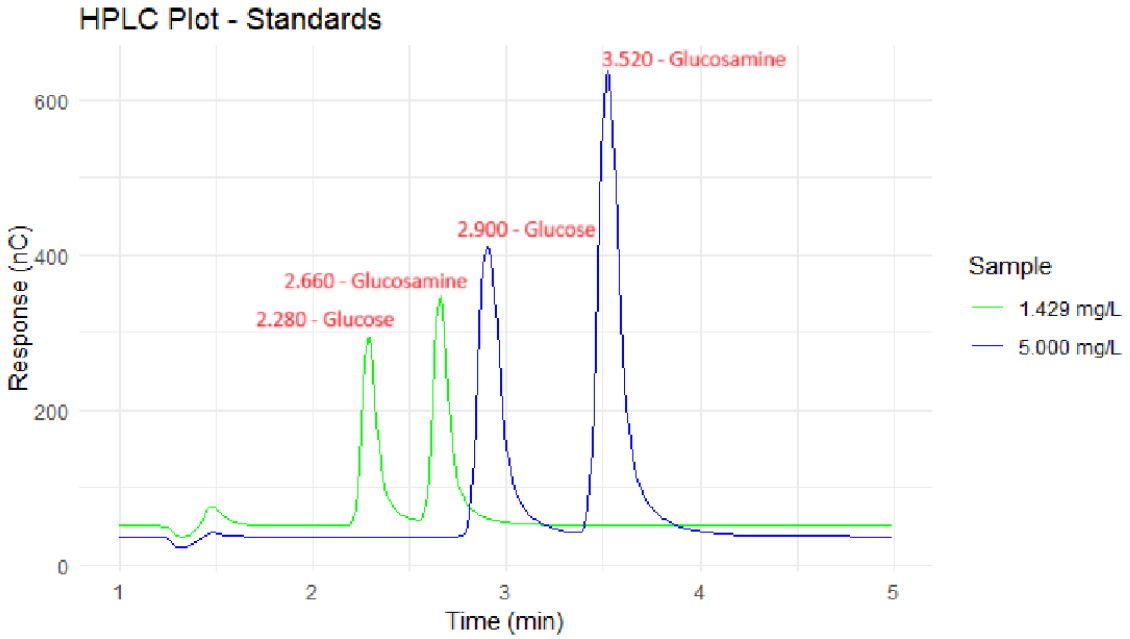
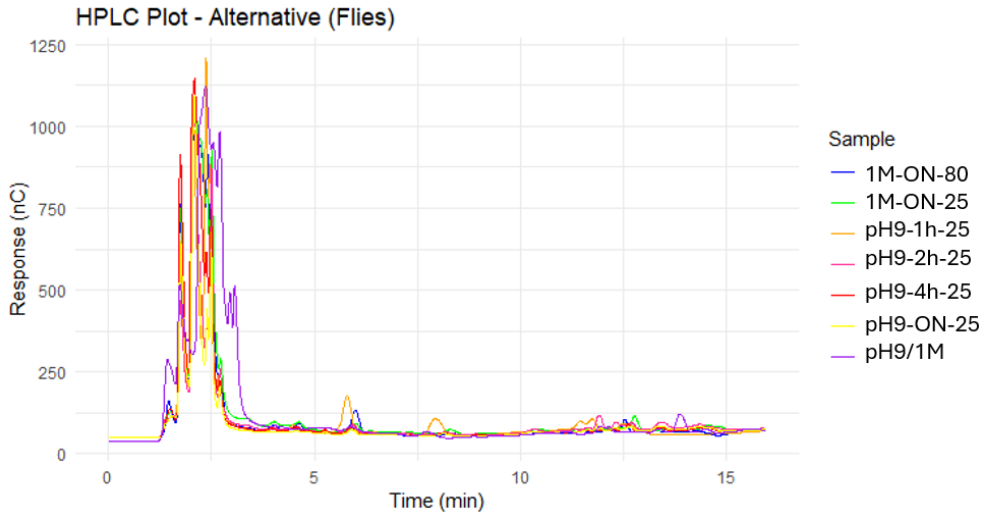
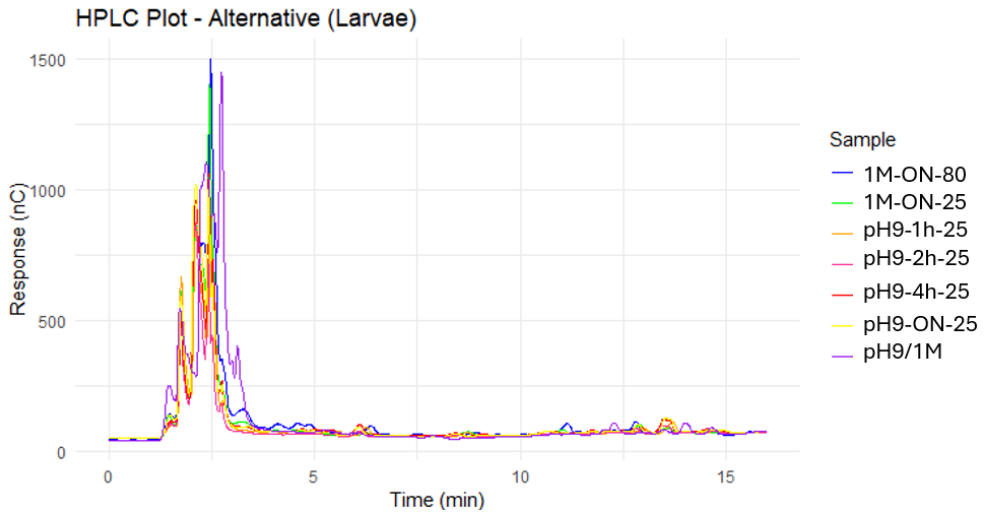


Figure C.1: HPAEC-PAD chromatograms of glucose and glucosamine standards. The green and blue chromatograms represents the glucose and glucosamine standards of 1.429 mg/L and 5.000 mg/L respectively. The x-axis shows the retention time [minutes] and the y-axis shows the response [nC].

C.2 Full HPLC graphs from the optimization process



(a)



(b)

Figure C.2: HPAEC-PAD chromatograms of acid hydrolyzed chitin extracted from a) black soldier flies and b) larvae, using different deproteinization methods. Method 1 (blue), method 2 (green), method 3 (orange), method 4 (pink), method 5 (red), method 6 (yellow) and method 7 (purple). The x-axis shows the retention time [minutes] and the y-axis shows the responce [nC].

Appendix D

260/280 ratios from UV Absorption Spectroscopy

260/280 ratio			
Baseline Extraction	**Flies**	**Larvae**	**Frass**
1. Replicate	1.40	1.29	1.35
2. Replicate	1.42	1.37	1.08
3. Replicate	1.41	1.32	1.24
Optimization process	**Flies**	**Larvae**	
1M-ON-80	1.62	1.21	
1M-ON-25	1.71	1.25	
pH9-1h-25	1.76	1.18	
pH9-2h-25	1.63	1.21	
pH9-4h-25	1.60	1.21	
pH9-ON-25	1.66	1.25	
pH9/1M	1.70	1.28	
pH9/**1M**	1.46	1.33	
Enzymatic Deproteinization	**Flies**	**Larvae**	
pH5-1h-37	1.90	1.39	
pH5-2h-37	1.85	1.43	
pH5-3h-37	1.82	1.42	
Control Deproteinization	**Flies**	**Larvae**	
Control 1 h	1.95	1.46	
Control 3 h	1.97	1.46	

Table D.1: The UV absorption spectroscopy 260/280 ratios from the baseline extraction, optimization process and the enzymatic facilitated deproteinization.

Appendix E

SDS-PAGE

E.1 SDS-PAGE of the protein profile throughout the baseline extraction of frass and larvae

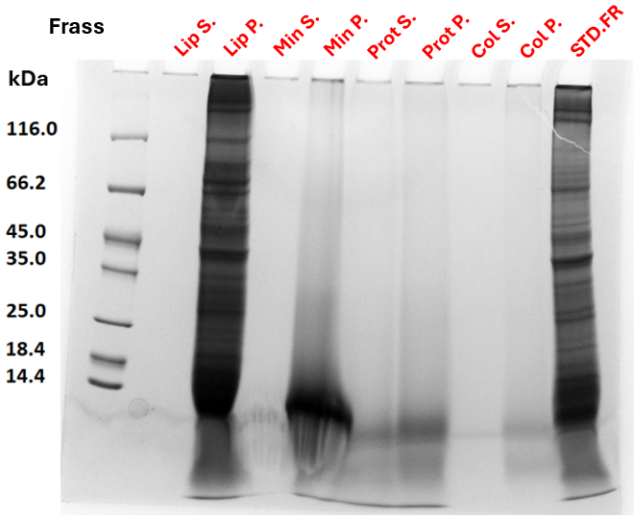


Figure E.1: Gel displaying the protein profile throughout the baseline extraction of frass and a standard from unprocessed frass (STD.FR). The S. and P. refers to supernatant and pellet respectively, during the purification steps.

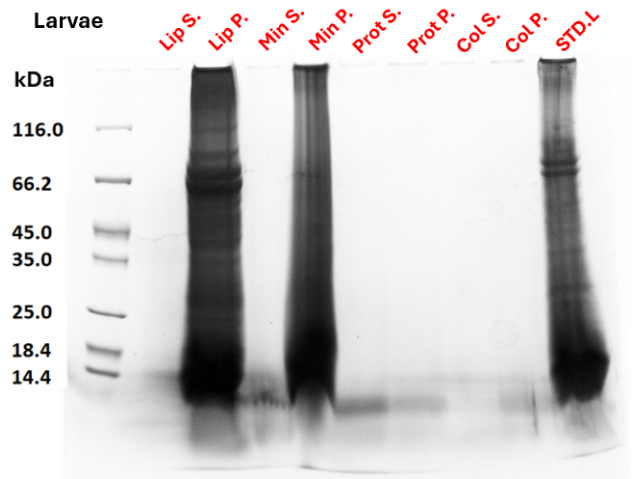


Figure E.2: Gel displaying the protein profile throughout the baseline extraction of larvae and a standard from unprocessed larvae (STD.L). The S. and P. refers to supernatant and pellet respectively, during the purification steps.

Appendix F

Atomic Absorption Spectroscopy

F.1 Mineral analysis of raw flies, frass and larvae material

Mineral Content [mg/g]			
	Flies	**Frass**	**Larvae**
Ca	1.61 ± 0.22^{A}	7.03 ± 0.44^{A}	51.18 ± 6.95^{B}
K	60.46 ± 13.36^{A}	71.29 ± 33.66^{A}	86.66 ± 18.54^{A}
Na	46.39 ± 0.72^{A}	48.43 ± 3.91^{A}	50.96 ± 1.65^{A}
Mg	6.00 ± 2.97^{A}	15.44 ± 2.66^{B}	5.58 ± 1.27^{A}

Table F.1: The mineral content [mg/g] of fully acid hydrolyzed black soldier flies, larvae and frass. The table shows the amount of calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) measured in each of the materials.