Sequential extraction and characterisation of lipids, proteins,
and chitin from black soldier fly (*Hermetia illucens*) larvae,
prepupae, and pupae

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Abstract

Over the past years, several insect species have gained increased attention as feedstock for food, feed, and industrial applications. One such species is *Hermetia illucens*, whose larvae can convert low-value organic waste into valuable fat- and protein-rich biomass. Previous research on extracting their lipids, proteins, and chitin has repeatedly focused on one life stage, while in practice different life stages coexist in the same rearing batch. In this study, the feasibility of the sequential extraction of said components from the larval, prepupal, and pupal stage of *H. illucens* was investigated. Additionally, the chemical composition of the life stages and their extracts was analysed.

Following the lipid extraction with petroleum ether, insect proteins were extracted via solubilisation at pH 11.0 and precipitation at pH 4.0. This procedure delivered protein recoveries ranging between 27 – 57 % for the three life stages, with the extracts having high protein contents (85 – 98 %). After protein extraction, the residual impure chitin was treated sequentially with HCl and NaOH for further purification. No residual amino acids were detected by UPLC analysis of the purified chitin, which showed acetylation degrees of ± 90 %. Overall, it was concluded that the extraction procedure is indeed suitable for all investigated life stages of *H. illucens*, allowing for the extraction high-value biomolecules for use in industrial applications.

Keywords

Hermetia illucens, life stages, chemical extraction, lipids, proteins, chitin

Graphical abstract

Statement of novelty

Research towards *Hermetia illucens* as a sustainable raw material is growing. However, in general only one life stage of this insect species is investigated. Based on their chemical characteristics, one single extraction procedure to isolate lipids, proteins, and chitin was developed that can be applied to the larvae, prepupae and pupae of *H. illucens*. Application of the proposed extraction procedure yields high purity extracts for all three life stages with only minor differences in their composition. As such, it is not necessary to separate these life stages in industrial rearing facilities prior to downstream processing.

1. Introduction

Black soldier fly larvae (*Hermetia illucens*, BSF) are small harmless insects suitable for mass production and several agricultural and industrial applications. They have the potential to efficiently dispose of a wide variety of organic waste materials by converting it into valuable protein-rich and fat-rich biomass thanks to them being nonselective in terms of their rearing substrate and their efficient feed conversion ratio [1]. The life cycle of *H. illucens* consists of several developmental stages and includes eggs, larvae, pupae, and adults. The feeding activity of the insect is limited to the larval stage and during the last larval stage, the so-called

prepupae, they migrate from the feed source in search of a dry site at which pupation occurs [2]. At the time the larvae reach the prepupal stage the fat content reaches a maximum because they need to have enough energy reserves to sustain all their activities during the subsequent pupal and adult stages [3].

Several studies describe the protein and lipid composition of larvae and prepupae reared on different organic substrates. It is known that, depending on the rearing conditions, black soldier fly larvae contain protein and fat quantities on dry matter basis ranging from 35 to 57 %, and from 15 to 49 %, respectively [2,4–7]. Not only the amount of fat but also the fatty acid composition of the larvae depends to a great extent on the composition of the diet [8,9]. The amino acid profile of BSF larvae, however, is not subjected to such large variation when different rearing substrates are used [9,10].

Because of its good nutritional profile, insect meal shows a lot of potential for the production of compound feeds in the feed industry, more specific as a potential alternative to fish or soy meal. In this regard, black soldier fly larvae have already been fed experimentally to several animal species [11–13]. In all of the conducted studies, the black soldier fly larval meal was a suitable replacement for conventional protein and lipid sources. Apart from using insect meal, extraction of nutritional components (proteins, lipids, etc.) from *H. illucens* may result in a more refined product that can be considered as high-value feed ingredient [14]. Caligiani et al. [15] already made significant efforts on such extraction procedures for *H. illucens* prepupae yet more research is still required. Additionally, extracted biomolecules may also have applications in other industries. For instance, the resulting lipids can be used for the production of biodiesel, surfactants and cosmetics among others [16,17].

Next to proteins and lipids, black soldier fly larvae also contain the valuable fibre chitin. Chitin and its main derivative chitosan have great economic value because of their interesting properties and their numerous potential applications. These cover various industrial fields

including the biomedical, pharmaceutical, agricultural, textile, and food industry [18,19]. For example, chitin and chitosan have been shown to have antitumour, haemostatic, wound-healing, antimicrobial, and antioxidant activities [18,20–23]. Currently, commercially available chitin is extracted from crustacean waste. This crustacean waste is, however, subjected to a large variation since the amount of chitin as well as its physicochemical characteristics vary between crustacean species [24,25], while such variation in raw material is generally not desired by industry. As insects also contain chitin as a part of their cuticle, they can be considered an alternative source [26]. Although procedures for obtaining chitin and chitosan have been developed in the past, most of them have been developed for crustaceans [21,22] and only a limited number of studies have focused on insects so far [15,20,26,27]. These studies, however, have often only focused on one life stage while in industrial rearing facilities, three life stages (i.e., larvae, prepupae, and pupae) occur together in the same rearing batch. This makes it difficult to use one particular life stage for further processing. In previous research [26,27], it could be concluded that the investigated insect species were promising alternative sources of chitin and chitosan. Moreover, the extracted chitin exhibited a similar chemical structure and physicochemical properties compared to commercially available chitin derived from crustaceans.

As described in previous paragraphs, the different biomolecules (i.e., lipids, proteins, and chitin) have a lot of potential for several applications, making black soldier fly larvae, prepupae, and pupae a source of valuable biomass. Furthermore, this high-value biomass can be produced by rearing *H. illucens* on low-value waste streams with varying compositions such as restaurant waste and manure. When extracting the aforementioned biomolecules from insects, a better cost-benefit ratio can be achieved if all fractions are valorised. Thus, from an economic point of view, a sequential extraction delivering products with high purity is preferred. Since larvae, prepupae, and pupae co-occur together during rearing, it is highly

relevant to compare their chemical composition, the extraction of their biomolecules, and the composition of their extracted fractions. In this study, the feasibility of the sequential extraction of lipids, proteins, and chitin from these three life stages is investigated. The obtained biomolecules are chemically characterised to assess the composition of the extracts.

2. Materials and Methods

2.1. Pretreatment of the insects

Larvae, prepupae, and pupae of the black soldier fly (*H. illucens*) reared on supermarket waste derived from Vanheede (Belgium) were obtained from the insect rearing company Millibeter (as of February 2019: Circular Organics, Belgium), frozen and stored at -18 °C for a minimum of 24 h. Before use, all samples were lyophilised for 48 h (Vertis, Freezemobile 5SL) and ground in a mortar.

2.2. Determination of the proximate composition

The dry matter content of the fresh and lyophilised larvae, prepupae, and pupae was determined by drying in a forced-air oven (Memmert, UFB500) at 105 °C for 17 hours [28]. The ash content was determined as described in previous research by Fombong, Van Der Borght, and Vanden Broeck [29]. The organic matter was incinerated at 550 °C using a muffle furnace (Nabertherm Controller B180) until constant weight.

Lipid content was determined following Soxhlet extraction with petroleum ether (technical, boiling point 40-60 °C, VWR Chemicals) for 18 hours. Subsequently, the petroleum ether was evaporated at 50 °C using a rotary evaporator (Büchi, R-200) [30].

Total organic nitrogen was determined using the Kjeldahl method (Gerhardt, Kjeldatherm, Vapodest 20s) as described in previous research [30]. Protein content was calculated with 4.67 as the conversion factor. This factor was chosen based on previous research regarding the nitrogen-to-protein conversion factor for black soldier fly larvae [31]. All chemicals

required for the analysis (K_2SO_4 , Rectapur; $CuSO_4$, 98 %; H_2SO_4 , technical; NaOH, AnalaR NORMAPUR; H_3BO_3 , Rectapur; HCl 0.100 N, TITRINORM) were purchased from VWR Chemicals.

Chitin content was determined as described under section 2.5.

2.3. Determination of the mineral composition

To determine the mineral composition, the ashes obtained during the determination of the ash content were dissolved in 65 % HNO_3 (ultra-pure, Chem Lab) and subsequently diluted to an appropriate concentration. Calibration curves were prepared using standard solutions from certified stock solutions containing 1000 ppm of the elements investigated (Chem Lab). The content of the investigated elements was determined by ICP-OES measurements (Perkin Elmer Optima 4300™ DV ICP-OES) and the resulting spectra were processed with Winlab 32. The settings of the ICP-OES were the same as those used by Fombong et al. [29].

2.4. Protein solubility curve

Protein solubility was obtained as a function of pH as described by Del Valle and Mena [32] with slight modifications. In brief, one gram of lyophilised, ground and defatted insect biomass was dispersed in 20.0 mL of demineralised water whose pH had previously been adjusted to the desired value by addition of 0.20 M NaOH or HCl. The sample was placed on a magnetic stirrer and continuously stirred at room temperature for 60 min. The pH was verified and adjusted several times during and at the end of the period. Next, the sample was centrifuged at 4080 g for 20 min (Heraeus, Multifuge 3s) and the supernatant was collected. The protein concentration of the supernatant was determined by the Bradford assay (Sigma). Albumin from bovine serum (Sigma-Aldrich) was used as a reference.

2.5. Procedure for the sequential extraction of lipids, proteins, and chitin

The overview of the sequential extraction procedure for the valorisation of lipids, proteins, and chitin from larvae, prepupae, and pupae of *H. illucens* is shown in Fig. 1. This procedure is comparable to the first protocol used by Caligiani et al. [15], but with several distinct differences. First, lipid extraction was performed on freeze-dried and ground samples by Soxhlet extraction (see section 2.2). The remaining lipid-free solid fraction was dispersed in pH-adjusted demineralised water under conditions of maximum solubility (pH = 11.0, defined by the protein solubility curve) in a 1:10 ratio (m/v) and kept at 45 °C for 2.5 hours. Next, the solution was filtered and the solid fraction containing unpurified chitin was used for further chitin purification.

The supernatants containing the protein extract were adjusted with 1.00 M HCl solution to a pH of minimum solubility (pH = 4.0, defined by the protein solubility curve) to precipitate the proteins. Subsequently, the solution was centrifuged at 1840 g for 45 min and the pellet was immediately lyophilised thereafter. The protein recovery (i.e., the percentage of protein extracted) was calculated employing Equation 1. The protein content of the extracts was calculated as the sum of the amino acid residues as determined by the procedure described under section 2.7.

$$\text{Protein recovery (\%)} = \frac{\text{mass of freeze-dried protein extract (g)}}{\text{mass of dry insects (g)} \times \text{protein content on dry matter (\%)}} \times 100 \% \quad (\text{Equation 1})$$

The solid fraction containing unpurified chitin was treated with 1.00 M HCl solution and continuously stirred at 100 °C for 30 min to remove minerals and catechols. The impure chitin was filtrated and rinsed with distilled water until pH neutrality was reached. Further deproteinisation was performed using alkaline treatment with 1.00 M NaOH solution at 80 °C for 24 h. The chitin was separated by filtration and washed with distilled water until a neutral pH was obtained. The product was then dried at 50 °C for 2.5 h in a forced-air oven

(Memmert, UFB500). The chitin content was calculated as the ratio of dried, purified chitin to the mass of raw material corrected for lipid content, using Equation 2.

$$\text{Chitin (\%)} = \frac{\text{mass of dry, purified chitin (g)}}{\text{mass of dry, defatted material (g)} \times (1 - \text{lipid content on dry matter (\%)})} \times 100 \% \quad (\text{Equation 2})$$

2.6. Fatty acid analysis

Fatty acid methyl esters (FAMES) were prepared from the extracted lipids by esterification in a methanolic KOH solution (0.500 M, VWR) with the addition of a 20 % BF₃-MeOH solution (Sigma-Aldrich) according to Joseph and Ackman [33]. The fatty acid composition was determined with a Chrompack CP 9002 equipped with flame ionisation detector, a split/splitless injector, a CTC analytics Liquid Sampler type CTC-A200S (CP9010) and an SLB-IL111 (60 m × 0.25 mm i.d., Sigma-Aldrich) capillary column. The carrier gas was helium at a flow rate of 21 cm/s. The oven temperature was programmed, starting at 140 °C (2 min), increasing to 180 °C (8 °C/min), and subsequently to 260 °C (4°C/min). The fatty acids were identified by comparing their retention times with those of the FAME standards under the same conditions. Methyl pentadecanoate was used as an internal standard. All standards were supplied by Sigma-Aldrich.

2.7. Amino acid analysis

In order to determine the amino acid profile of the different life stages and the protein extracts, 25.00 mg of sample was subjected to acid hydrolysis in a test tube of 20 mL with screw cap in the presence of 10.00 mL 6.0 M HCl. The resulting mixtures were incubated for 22 hours at 110 °C. After cooling, the pH was adjusted with NaOH to pH 7.0 ± 0.2 and the samples were diluted to 25.0 mL with demineralised water. After centrifugation of the samples for 5 minutes at 13200 rpm with an IEC Centra-M centrifuge, they were prepared for amino acid analysis using Ultra Performance Liquid Chromatography with a Photo Diode Array detector (UPLC-PDA). It should be noted that asparagine and glutamine are

deaminated during the acidic hydrolysis, meaning that the amount of aspartic acid should be interpreted as the sum of asparagine and aspartic acid. The same also applies to glutamine and glutamic acid. For the determination of tryptophan, defatted insect samples were hydrolysed with 4.00 mL of 4.0 M LiOH for 22 hours at 110 °C, followed by cooling and adjustment of pH to 7.0 ± 0.2 with HCl.

Chromatographic separation of amino acids has been performed on an Acquity UPLC (Waters, Milford, USA), consisting of a binary solvent delivery system, sample manager, PDA detector, column heater, and an AccQ•Tag™ Ultra column (2.1 i.d. $\times$ 100 mm; Waters, USA). Sample derivatisation was realised using the Waters AccQ•Tag Ultra Chemistry Package. Gradient elution was applied according to the Waters AccQ•Tag Ultra method [AccQ•Tag Ultra Eluent A Concentrate (10 times diluted) (Waters, Milford, USA); AccQ•Tag Ultra Eluent B (Waters, Milford, USA)] using a flow rate of 0.7 mL/min and column temperature of 60 °C. For the quantification of amino acids in the sample, the system was calibrated using analytical standards of amino acids (AAS18, Sigma-Aldrich) at varying concentrations. Data processing was done using Empower 2 software.

2.8. Chitin characterisation

The extracted chitin was characterised by infrared spectrophotometry using KBr pellets (Thermo Scientific Nicolet type Avatar 360 FT-IR). Absorbance values were measured between 4000 and 400 cm^{-1} . IR spectra were recorded by accumulating 60 scans with a resolution of 2 cm^{-1} . FT-IR spectra were processed using Thermo Scientific OMNIC 7.3 software. Commercially available α -chitin from shrimp was used as a reference (Sigma-Aldrich). The degree of acetylation (AD) was determined according to Equation 3 where A_{1320} and A_{1420} are the values of absorbance at 1320 (baseline from 1276 to 1348 cm^{-1}) and 1420 cm^{-1} (baseline from 1402 to 1478 cm^{-1}) respectively [34]. The extracted chitin was also analysed for residual amino acids according to the procedure described under section 2.7.

$$AD (\%) = \frac{\frac{A_{1320}}{A_{1420}} - 0.3822}{0.03133} \quad (\text{Equation 3})$$

2.9. Statistical analysis

All measurements were performed at least three times unless specified otherwise. Results were analysed using JMP Pro 14.0.0 from SAS at a significance level of 5 %. The results obtained for the larvae, prepupae, and pupae were statistically evaluated using one-way ANOVA with Tukey HSD as post hoc test in case of equal variances. When the variances were not equal, the Welch's ANOVA with Steel-Dwass All Pairs post hoc test was used.

3. Results and discussion

3.1. Proximate composition

The results of the proximate analysis of the larvae, prepupae, and pupae of the BSF are presented in Table 1. The dry matter content of the larvae gradually increased from the larval towards the pupal stage (from 27.74 % to 39.95 %). All samples contained high levels of proteins (ranging between 31.27 % and 38.86 %) and lipids (ranging between 39.85 % and 47.65 %) but the quantities varied considerably between the different developmental stages. Larvae had the highest protein content (38.86 %) whereas prepupae had the highest lipid content (47.62 %). In the pupal stage, the lipid content declined again, presumably because the lipids were used as an energy source during metamorphosis. This decline was also observed by X. Liu et al. [3] who studied the changes in nutrient composition throughout the entire life cycle. Ash contents of the different developmental stages ranged between 8.83 % and 9.56 %. These results are similar to those found in previous research on black soldier flies [3,31]. Finally, the amount of chitin increased in parallel with the development of *H. illucens*, being lowest at the larval stage (3.85 %) and highest at the pupal stage (6.31 %).

Table 1. Proximate composition of larvae, prepupae, and pupae of Hermetia illucens. Results are means $\pm$ standard deviations of five replicates. Means in the same row with different superscripts differ significantly ($p < 0.05$).

	Larvae	Prepupae	Pupae
Dry matter [†]	27.74 $\pm$ 0.57 ^a	35.67 $\pm$ 0.41 ^b	39.95 $\pm$ 0.25 ^c
Lipids [‡]	40.96 $\pm$ 0.93 ^a	47.65 $\pm$ 0.92 ^b	39.85 $\pm$ 0.86 ^a
Protein [‡]	38.86 $\pm$ 1.70 ^a	31.81 $\pm$ 1.26 ^b	31.27 $\pm$ 0.92 ^b
Chitin [‡]	3.85 $\pm$ 0.24 ^a	4.72 $\pm$ 0.76 ^a	6.31 $\pm$ 0.41 ^b
Ash [‡]	9.56 $\pm$ 0.49 ^a	8.83 $\pm$ 0.27 ^b	9.24 $\pm$ 0.14 ^c

[†] g/100 g as is, [‡] g/100 g dry matter

The sum of lipids, proteins, chitin, and ash was for all three life stages lower than 100 %, which might be due to the presence of other components such as sugars, pigments, etc. For pupae, this was more notable as the sum is only 87 %. It might be that the used conversion factor of 4.67 for BSF [31] was too low and an underestimation of the true protein content was made. The used conversion factor was determined for BSF larvae and might be unsuitable for prepupae and/or pupae as their chitin content varies. On the other hand, using 6.25 as the conversion factor would have resulted in a sum greater than 100 %. This lower sum for the pupal life stage can also be observed in previous research [3] but the authors did not elaborate on this.

3.2. Mineral composition

The results of the mineral analysis are displayed in Table 2. The larvae, prepupae, and pupae were also a good source of mineral elements with calcium being the most prevalent mineral in all three life stages of *H. illucens*. This high amount of calcium was also observed in previous studies [3,9]. The increase in calcium from the larval to the prepupal stage might be attributed to the cuticle formation [3]. Other minerals such as potassium, phosphorous, magnesium, and

sodium also changed across the different developmental stages. These observed differences were, although statistically significant, relatively small. Trace levels of manganese, iron, zinc, and copper were also present in all three life stages, but these were not subjected to a large variation between life stages.

Table 2. Mineral composition of larvae, prepupae, and pupae of H. illucens. Results represent the mean $\pm$ SD of three repetitions. Means in the same row bearing a different superscript differ significantly ($p < 0.05$).

Element	Larvae	Prepupae	Pupae
Macro minerals [†]			
Calcium	3.565 $\pm$ 0.025 ^a	4.515 $\pm$ 0.093 ^b	4.404 $\pm$ 0.085 ^b
Potassium	0.915 $\pm$ 0.007 ^a	0.436 $\pm$ 0.007 ^b	0.611 $\pm$ 0.028 ^c
Phosphorus	0.704 $\pm$ 0.002 ^a	0.600 $\pm$ 0.009 ^b	0.631 $\pm$ 0.028 ^b
Magnesium	0.335 $\pm$ 0.004 ^a	0.349 $\pm$ 0.006 ^{ab}	0.365 $\pm$ 0.007 ^b
Sodium	0.156 $\pm$ 0.002 ^a	0.155 $\pm$ 0.004 ^a	0.172 $\pm$ 0.005 ^b
Micro minerals [‡]			
Manganese	33.5 $\pm$ 0.4 ^a	36.3 $\pm$ 0.7 ^b	37.6 $\pm$ 0.6 ^b
Iron	14.0 $\pm$ 0.2 ^a	7.1 $\pm$ 0.1 ^b	6.5 $\pm$ 0.1 ^c
Zinc	9.0 $\pm$ 0.1 ^a	9.2 $\pm$ 0.1 ^a	6.9 $\pm$ 0.1 ^b
Copper	0.7 $\pm$ 1.0	1.0 $\pm$ 0.0	1.1 $\pm$ 0.0

[†] g/100 g dry matter, [‡] mg/100 g DM

3.3.Characterisation of fatty acids

Gas chromatography analysis was performed in order to investigate the fatty acid profile of the extracted lipids of larvae, prepupae, and pupae of *H. illucens*. The fatty acid composition expressed as a percentage of total fatty acids is presented in Table 3. The total amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) are also shown in Table 3. All developmental stages of *H. illucens* contained a higher amount of saturated fatty acids (77.43 % of total fatty acids in larvae, 84.17 % in

prepupae, and 84.62 % in pupae) compared to the unsaturated fatty acids. The major fatty acid present was lauric acid (C12:0) which accounted for 55.25 % of total fatty acids in the larvae and for 64.83 % and 65.10 % in the prepupae and pupae, respectively. Although this fatty acid shows potential as a nutraceutical, the use of saturated fatty acids is generally not preferred for use in feed applications [14]. The extracted lipids can, however, still be used as a new type of feedstock to produce oleochemicals such as surface-active components. Verheyen et al. [17] investigated the use of insect fats in cosmetics, in which they concluded that fat extracted from *H. illucens* might be suitable to replace palm kernel and coconut oil for use in soaps and shower gels.

Table 3. Fatty acid profile of larvae, prepupae, and pupae of H. illucens (% of total fatty acids). Results represent the mean $\pm$ SD of three measurements. Means in the same row with a different superscript differ significantly from each other ($p < 0.05$).

Fatty acid	Larvae	Prepupae	Pupae
Lauric acid (C12:0)	55.25 $\pm$ 1.22 ^a	64.83 $\pm$ 0.69 ^b	65.10 $\pm$ 1.15 ^b
Myristic acid (C14:0)	9.99 $\pm$ 0.54	9.27 $\pm$ 0.11	9.69 $\pm$ 0.44
Palmitic acid (C16:0)	10.39 $\pm$ 0.35 ^a	8.89 $\pm$ 0.15 ^b	8.64 $\pm$ 0.30 ^b
Palmitoleic acid (C16:1)	5.07 $\pm$ 0.05 ^a	3.14 $\pm$ 0.10 ^b	2.75 $\pm$ 0.10 ^c
Stearic acid (C18:0)	1.80 $\pm$ 0.04 ^a	1.19 $\pm$ 0.05 ^b	1.20 $\pm$ 0.08 ^b
Oleic acid (C18:1)	10.42 $\pm$ 0.14 ^a	7.08 $\pm$ 0.21 ^b	6.75 $\pm$ 0.19 ^b
Linoleic acid (C18:2)	6.26 $\pm$ 0.15 ^a	4.96 $\pm$ 0.22 ^b	5.17 $\pm$ 0.09 ^b
α -Linolenic acid (C18:3)	0.82 $\pm$ 0.03 ^a	0.65 $\pm$ 0.01 ^b	0.70 $\pm$ 0.07 ^b
SFA	77.44 $\pm$ 0.34 ^a	84.17 $\pm$ 0.51 ^b	84.62 $\pm$ 0.40 ^b
MUFA	15.49 $\pm$ 0.19 ^a	10.21 $\pm$ 0.29 ^b	9.50 $\pm$ 0.28 ^c
PUFA	7.08 $\pm$ 0.16 ^a	5.62 $\pm$ 0.22 ^b	5.88 $\pm$ 0.14 ^b

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

Overall, the fatty acid profiles of prepupae and pupae were very similar but differed significantly from the larval profile. The only difference between the fatty acid composition of prepupae and pupae that was found to be statistically significant was the amount of palmitoleic acid (C16:1). As a result, the amount of MUFA is also slightly lower in the pupae. Polyunsaturated fatty acids such as linoleic acid (C18:2) and linolenic acid (C18:3) were found to be present as well. Linoleic acid was found at levels ranging from 4.96 % to 6.26 % of total fatty acids for the three developmental stages. α -Linolenic acid was present at much lower quantities at around 0.73 % of total fatty acids. α -Linolenic acid contents, however, were more comparable between life stages.

3.4. Protein solubility and separation of proteins

In order to develop a procedure for the extraction of proteins, protein solubility as a function of pH was determined for larvae, prepupae, and pupae. As shown in Fig. 2, the minimum solubility of proteins from all investigated developmental stages was found at pH = 4.0 and the maximum solubility at pH = 11.0. The resulting solubility curves of all life stages are quite similar to those found for the larval stage by Bußler et al. [35]. The marked difference in protein solubility at pH 4.0 and pH 11.0 made it feasible to extract proteins from the insect biomass by pH adjustment of the extraction solution. As there were no major differences in protein solubility, a similar approach could be used to extract proteins from all three life stages.

Using these conditions, protein recoveries of 27.58 ± 2.58 %, 56.69 ± 0.76 %, and 50.05 ± 3.36 % could be obtained for larvae, prepupae, and pupae respectively. The protein recovery from the larvae is remarkably lower than those obtained for prepupae and pupae. However, no explanation could be found for this low value. The protein content of the extracts was assessed as the sum of amino acid residues after hydrolysis, resulting in values of 95.14 ± 2.08 %, 85.21 ± 1.70 %, and 97.85 ± 1.90 % for the larval, prepupal, and pupal

extract respectively. These results imply that, although the protein recovery from prepupae is higher, the extract has a lower protein content, meaning that components other than proteins are extracted as well.

The obtained recoveries are quite low compared to the yield of 67 % found by Caligiani et al. [15] who used trichloroacetic acid in acetone to precipitate the extracted prepupae proteins. As can be seen in Fig. 2, a fraction of the proteins remains soluble at pH 4.0. Since only the mass of the precipitated proteins was used to determine the recovery, this might explain the lower value. The use of other precipitation techniques such as salting out or flocculation may increase the recovery, yet this might negatively affect the purity of the extracts. Another possible reason for the lower recovery is the different ratio of solid to liquid used. For this study a ratio of 1:10 (m:V) was used, which was higher than the ratio used by Caligiani et al. [15] as they used 40 mL of 1 M NaOH for 1 g of defatted insect pellet. The higher ratio in this study was chosen based on preferences from industry, as a higher ratio will result in a lower usage of solvents, acids, and bases; and in a reduction of the amount of waste produced for processing the same amount of raw material.

Considering the differences in the composition of the three life stages and the fact that the same extraction procedure was used; it was expected that the recovery would not be the same for all life stages. Indeed, the protein extraction from the larvae had a lower recovery, however, the extract had a high purity as it contained 95 % amino acids. Nonetheless, the proposed protein extraction procedure has proven to be suitable for larvae, prepupae, and pupae as the extracts had a high protein content and the solubility curves are highly similar. Future research should focus on increasing the protein recovery, while at the same time maintaining the high purity of the extracts.

3.5.Characterisation of proteins

The amino acid profile of the different developmental stages of *H. illucens* and the protein extracts is shown in Table 4. Aspartic and glutamic acid (including resp. asparagine and glutamine) occurred in the highest concentrations (9.76 % – 13.39 %) whereas cystine, methionine, and tryptophan occurred in the lowest concentrations (0.85 % – 2.15 %). The concentration of the other amino acids ranged from 2.65 % to 8.66 %.

Although the observed differences in amino acid composition between the three life stages of *H. illucens* are statistically significant ($\alpha = 0.05$) for all amino acids, for most of them these differences are relatively small, with the differences being much smaller between prepupae and pupae. The largest difference is observed in glutamic acid content between prepupae and pupae, being 13.39 % and 11.36 % respectively. This decrease has also been noticed in previous research but no explanation was given [3]. Noteworthy is the lysine content, which decreases from larval towards the prepupal stage but stays the same in the pupal stage. As lysine is the limiting essential amino acid for poultry and pigs [36], this decrease is not desired. It might be attributed to the melanisation process, as the side chains of lysine and cysteine will likely react with quinones [37], thus forming the dark pigments which are present in the BSF cuticle in the prepupal and pupal stage.

When comparing the extracts with their respective life stages, no major differences were observed. Some amino acids are present in a slightly lower amount in the extract compared to the full sample (e.g. glycine, alanine, proline), though other amino acids such as methionine and phenylalanine are present in a slightly higher concentration. Nevertheless, these results show that all amino acids are present in the different extracts, making them valuable as a feed additive. All essential amino acids for broilers, pigs and Atlantic salmon are present in sufficient amounts [36].

Table 4. Amino acid profile of larvae, prepupae, and pupae of *H. illucens* and their protein extracts (in g/100g protein). Results represent the mean $\pm$ SD of three measurements. Means in the same row with a different superscript differ significantly ($p < 0.05$).

Amino acid	Larvae	Protein extract larvae	Prepupae	Protein extract prepupae	Pupae	Protein extract pupae
Histidine	2.66 $\pm$ 0.13 ^a	2.85 $\pm$ 0.08 ^{ab}	3.14 $\pm$ 0.03 ^c	2.92 $\pm$ 0.11 ^{bc}	3.41 $\pm$ 0.10 ^d	2.67 $\pm$ 0.03 ^a
Serine	3.79 $\pm$ 0.09 ^a	4.11 $\pm$ 0.02 ^b	4.68 $\pm$ 0.02 ^c	4.21 $\pm$ 0.05 ^{bc}	4.72 $\pm$ 0.05 ^c	3.89 $\pm$ 0.02 ^a
Arginine	4.28 $\pm$ 0.12 ^a	4.48 $\pm$ 0.17 ^a	5.36 $\pm$ 0.04 ^b	5.49 $\pm$ 0.13 ^{bc}	5.48 $\pm$ 0.20 ^b	6.02 $\pm$ 0.05 ^c
Glycine	5.56 $\pm$ 0.10 ^a	5.00 $\pm$ 0.09 ^b	6.10 $\pm$ 0.03 ^c	4.65 $\pm$ 0.08 ^d	6.50 $\pm$ 0.12 ^e	4.37 $\pm$ 0.04 ^f
Aspartic acid [†]	9.76 $\pm$ 0.10 ^a	11.59 $\pm$ 0.19 ^b	10.52 $\pm$ 0.07 ^c	12.23 $\pm$ 0.29 ^d	10.58 $\pm$ 0.19 ^c	13.48 $\pm$ 0.26 ^e
Glutamic acid [†]	12.13 $\pm$ 0.13 ^a	10.60 $\pm$ 0.18 ^b	13.39 $\pm$ 0.12 ^c	12.15 $\pm$ 0.19 ^a	11.36 $\pm$ 0.17 ^d	11.51 $\pm$ 0.15 ^d
Threonine	4.38 $\pm$ 0.08 ^{ab}	4.42 $\pm$ 0.01 ^{ab}	4.23 $\pm$ 0.04 ^d	4.47 $\pm$ 0.05 ^{ac}	4.30 $\pm$ 0.04 ^{bd}	4.59 $\pm$ 0.02 ^c
Alanine	8.67 $\pm$ 0.20 ^a	5.03 $\pm$ 0.07 ^b	6.51 $\pm$ 0.05 ^c	5.11 $\pm$ 0.09 ^b	6.77 $\pm$ 0.25 ^c	4.51 $\pm$ 0.05 ^d
Proline	5.02 $\pm$ 0.06 ^a	4.11 $\pm$ 0.02 ^b	6.17 $\pm$ 0.01 ^c	4.77 $\pm$ 0.01 ^d	6.21 $\pm$ 0.15 ^c	4.07 $\pm$ 0.02 ^b
Cystine	1.00 $\pm$ 0.01 ^a	0.99 $\pm$ 0.05 ^{ab}	0.89 $\pm$ 0.04 ^{bc}	0.70 $\pm$ 0.03 ^e	0.83 $\pm$ 0.06 ^{cd}	0.75 $\pm$ 0.02 ^{de}
Lysine	8.24 $\pm$ 0.21 ^a	6.96 $\pm$ 0.21 ^{bc}	6.45 $\pm$ 0.08 ^c	7.26 $\pm$ 0.34 ^b	6.41 $\pm$ 0.18 ^c	8.05 $\pm$ 0.22 ^a
Tyrosine	6.22 $\pm$ 0.28 ^a	9.59 $\pm$ 0.34 ^b	6.78 $\pm$ 0.07 ^{ad}	7.66 $\pm$ 0.32 ^c	6.79 $\pm$ 0.28 ^{ad}	7.17 $\pm$ 0.11 ^{cd}
Methionine	2.15 $\pm$ 0.08 ^a	2.70 $\pm$ 0.08 ^b	1.58 $\pm$ 0.02 ^c	2.35 $\pm$ 0.09 ^a	1.65 $\pm$ 0.10 ^c	2.61 $\pm$ 0.08 ^b
Valine	6.72 $\pm$ 0.12 ^a	6.06 $\pm$ 0.05 ^{bd}	6.50 $\pm$ 0.03 ^c	6.19 $\pm$ 0.02 ^b	6.82 $\pm$ 0.14 ^a	5.84 $\pm$ 0.02 ^d
Isoleucine	4.80 $\pm$ 0.05 ^{ab}	5.09 $\pm$ 0.07 ^c	4.51 $\pm$ 0.01 ^d	4.90 $\pm$ 0.00 ^a	4.73 $\pm$ 0.02 ^b	5.16 $\pm$ 0.03 ^c
Leucine	7.66 $\pm$ 0.12 ^{ab}	8.19 $\pm$ 0.02 ^c	7.50 $\pm$ 0.02 ^b	7.89 $\pm$ 0.01 ^d	7.82 $\pm$ 0.08 ^{ad}	8.32 $\pm$ 0.05 ^c
Phenylalanine	4.87 $\pm$ 0.16 ^a	6.29 $\pm$ 0.17 ^b	4.09 $\pm$ 0.04 ^c	5.38 $\pm$ 0.22 ^d	4.05 $\pm$ 0.24 ^c	5.46 $\pm$ 0.09 ^d
Tryptophan	2.09 $\pm$ 0.14 ^a	1.94 $\pm$ 0.17 ^{ab}	1.60 $\pm$ 0.12 ^{ab}	1.67 $\pm$ 0.13 ^{ab}	1.56 $\pm$ 0.32 ^{ab}	1.51 $\pm$ 0.21 ^b

[†] Sum of asparagine and aspartate (and glutamine/glutamate) as they are deaminated during acidic hydrolysis.

3.6.Characterisation of chitin

The IR spectra of chitin extracted from *H. illucens* larvae, prepupae, and pupae and the chitin standard (α -chitin from shrimp) are shown in Fig. 3. These spectra clearly show great similarities. Based on literature regarding IR analysis of chitin [20,38], the dominant absorption bands can be assigned: 3449 cm^{-1} (ν_{OH}), 3109 cm^{-1} ($\nu_{\text{NH}}^{\text{s}}$), 2932 cm^{-1} ($\nu_{\text{CH}_2}^{\text{s}}$), 2891 cm^{-1} (ν_{CH_3}), 1662 and 1624 cm^{-1} ($\nu_{\text{C=O}}$, amide I), 1560 cm^{-1} ($\nu_{\text{C-N}}$, C-N-H + δ_{NH} , amide II), 1415 cm^{-1} (δ_{CH_2}), 1379 cm^{-1} (δ_{CH} + $\delta_{\text{C-CH}_3}$), 1315 cm^{-1} ($\nu_{\text{C-N}}$ + δ_{NH} , amide III), 1261 cm^{-1}

(δ_{NH}), 1157 cm^{-1} ($\nu_{\text{C-O-C}}^{\text{as}}$, ring), 1116 cm^{-1} ($\nu_{\text{C=O}}$), 1074 and 1027 cm^{-1} ($\nu_{\text{C-O}}$), 953 cm^{-1} (γ_{CH_3}), 897 cm^{-1} (γ_{CH}), 702 cm^{-1} (γ_{NH} , amide V) and 561 cm^{-1} ($\gamma_{\text{C-C}}$)². As pointed out in previous research [39], the absence of bands at 1540 cm^{-1} can be interpreted as a lack of protein contaminants in the extracted chitin. The amide I band was split, showing two peaks at 1662 and 1623 cm^{-1} , demonstrating that chitin from *H. illucens* was present in the α form [26]. Acetylation degrees of the extracted and purified chitin were estimated according to the method of Brugnerotto et al. [34] delivering 89 %, 90 % and 92 % for larvae, prepupae, and pupae respectively. Previous research on chitin obtained from two types of grasshoppers (*Oedaleus decorus* & *Calliptamus barbarus*) showed comparable acetylation degrees [20]. The extracted chitin was also analysed for residual proteins via amino acid analysis. No values for amino acid content were obtained as the signal of all samples was below the limit of detection of the UPLC-PDA analysis (data not shown), which confirms our FT-IR results.

3.7. Evaluation of the procedure

The extraction procedure was performed at lab scale, separately for all three commercially interesting life stages of *H. illucens* (i.e., larvae, prepupae, and pupae). However, in industrial insect rearing facilities it is difficult to separate these three life stages from each other. Therefore, it was relevant to investigate if the sequential extraction of lipids, proteins, and chitin is suitable for all three life stages and whether the extracted fractions differ from each other.

Based on the results above, it can be concluded that it is not strictly necessary to separate one particular life stage for the fractionation of BSF into lipids, proteins, and chitin. The rearing substrate will more than likely have a higher impact on the composition of the extracted fractions than the ratio larvae:prepupae:pupae in the insect batch. This assumption can be made based on previous research regarding the nutritional composition of *H. illucens* reared

on different organic waste streams [9]. The differences in nutritional composition were much larger in their study when compared to the differences across the three life stages in the present study. The influence of the substrate on the lipid-protein ratio and fatty acid profile has also been reported in the literature before [10,40].

Despite the protein extracts having a high purity (85 – 98 % amino acids), further research on this extraction is still necessary. The protein recovery might still be increased, though the purity of the extracts should not be compromised. The use of alternative solvents and other methods for the defatting step or for the separation of insect protein and chitin are all possible routes worth further research.

4. Conclusions

In the present study, the feasibility of the sequential extraction of lipids, proteins, and chitin from the larval, prepupal, and pupal stage of *Hermetia illucens* was investigated. Overall, it can be concluded that the proposed extraction procedure is indeed suitable for the extraction of lipids, proteins, and chitin from larvae, prepupae, and pupae of *H. illucens* as the resulting fractions only differ slightly in their composition. As such, it is feasible to obtain valuable biomolecules from a mixture of BSF life stages which were reared on low-value organic waste streams with varying compositions.

Although the proposed procedure has a lower protein recovery than those previously reported in the literature, the resulting protein extracts have a high protein content with values ranging from 85 – 98 %. The extracted chitin was characterised by its degree of acetylation, which was estimated at around 90 %. Additionally, no residual amino acids were detected in the extracted chitin. Future research should focus on improving the recovery of the protein extraction as well as making the process more sustainable while maintaining the high purity of the extracts.

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Fig. 1 Overview of the sequential extraction of lipids, proteins, and chitin from larvae, prepupae, and pupae of *Hermetia illucens*

Fig. 2 Solubility curve of proteins of *Hermetia illucens* larvae, prepupae, and pupae. Results are the mean three replicates. Error bars represent the standard deviation of the means

Fig. 3 Overlay of four FTIR spectra of a chitin standard (shrimp) and chitin extracted from *Hermetia illucens* larvae, prepupae, and pupae according to the extraction procedure shown in Fig. 1