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Simplified determination of the content and average degree of acetylation of chitin in crude black soldier fly larvae samples

Els D'Hondt[a], Lise Soetemans[a], Leen Bastiaens[a], Miranda Maesen[a], Vincent Jespers[a], Bert Van den Bosch[a], Stefan Voorspoels[a], Kathy Elst*[,a]

a: Flemish Institute for Technological Research (VITO), Boeretang 200, 2400 Mol (Belgium)

els.dhondt@icats.be

lise.soetemans@vito.be

leen.bastiaens@vito.be

miranda.maesen@vito.be

vincent.jespers@vito.be

bert.vandenbosch@vito.be

stefan.voorspoels@vito.be

kathy.elst@vito.be (* Corresponding author)

Highlights

- Determination of chitin content and acetylation degree on black soldier fly larvae.
- Simultaneous determination of chitin content and acetylation degree.
- Applicable on crude samples without extensive pretreatment or derivatization.
- Assessment of total chitin content, including soft tissues.
- Determination of acetylation degree of highly acetylated chitin samples.

Abstract

Insects are considered a promising alternative protein source for food and feed, but contain significant amounts of chitin, often undesirable due to indigestibility, disagreeable texture and negative effect on nutrients intake. Fractionation strategies are thus increasingly being applied to isolate and valorize chitin separately. The analysis of chitin generally requires an intensive pretreatment to remove impurities, and derivatization to generate sufficient detector response. In this work, a liquid chromatography method, without pretreatment nor derivatization, was developed for the simultaneous determination of chitin content and degree of acetylation in non-purified samples of black soldier fly (BSF) larvae. The method is found to be more suitable, compared to traditional methods, for assessing high degrees of acetylation. For the first time, the degree of acetylation of BSF chitin (81±2%) is reported. Additionally, the chitin content of BSF soft tissues is estimated at approximately 20% of the total chitin content (8.5±0.1 %).

Keywords

Chitin content, degree of acetylation, UPLC-MS, analysis, crude samples, black soldier fly larvae, fast method

1. Introduction

Chitin is the most abundant natural polymer next to cellulose and is found in crustaceans, insects and some microorganisms. Because of their biodegradability and low toxicity, chitin and chitosan (produced via deacetylation of chitin), and derivatives thereof have a variety of applications in multiple sectors such as medicine, food, textile, cosmetics and wastewater treatment. Since Western countries nowadays show an increased interest in insects as a sustainable and local protein source for food and feed, increasing amounts of insect chitin are expected to become available on the market. This is especially expected for black soldier fly (*Hermetia Illucens*; BSF), as it is already reared commercially and is one of the only seven insect species recently allowed in aqua feed applications. [1]

Chitin is present in insects in the cuticle, gut lining, peritrophic matrix, salivary gland, trachea, eggshells and muscle attachment points. [2] Chitin in the cuticle can be relatively easy isolated and used as resource for bio-based products. Determination of the chitin content and degree of acetylation (DA) of biological samples, however, requires time intensive and high chemical consuming sample preparation to remove minerals, catechols, proteins and lipids. [3]

To quantify the chitin content in biological samples several methods are available in literature, including methods for sample clean-up prior to characterization via either NMR, spectroscopic and/or chromatographic procedures. Even though 1H-NMR and especially 13C-NMR are well suited to determine the chitin content and its acetylation degree [4], the technique is not universably available. Hence, a performant chromatographic approach is expected to provide a more accessible alternative for implementation within chitin processing facilities and laboratories. An overview of techniques to determine the chitin content by chromatographic methods is well described by Zhu *et al.*.[3] To summarize, most methods to quantify the chitin content of biological samples include 3 steps, *i.e.* (i) sample clean-up, (ii) acidic hydrolysis and (iii) derivatization of the monomer glucosamine with a fluorenyl group via

the amine function to generate an spectroscopic response. [3,5–9] Derivatization (iii) can be omitted when using mass spectrometric or refractive index detection. [10,11] The sample clean-up step (i) requires an acidic thermal treatment to remove minerals and catechols. This is followed by neutralization to subsequently subject the sample to a thermal alkaline treatment to remove proteins and ends with a neutralization step.[5,7,12,13] This lengthy procedure is not only time consuming, also generates a large amount of chemical waste even for small samples sizes. Additionally, the chitin structure is often altered by these procedures, *i.e.* partially depolymerized and deacetylated.[14,15] Crespo *et al.* already partially avoided the lengthy pretreatment in the case of crab shells, by setting up a chromatographic method suitable to determine the chitin content after removing the protein-rich soft tissue.[10] Nevertheless, to the best of our knowledge, no chromatographic methodology is currently available to achieve a fast and efficient quantification of chitin in crude biological samples such as whole BSF larvae.

Another challenge in chitin characterization concerns the accurate quantification of its DA, especially for highly acetylated chitin. Knowing and controlling the acetylation degree of chitin is very important for its properties. [16] Various methods were described in literature and discussed in a review by Kasaai. [17] Fourier-Transform Infrared (FT-IR) is the most frequently used method, but as commonly known, its accuracy is insufficient at high acetylation degrees. [18] Additionally, interpretation of FT-IR data and subsequent calculation of the acetylation degree varies largely between research groups and is affected by the presence of impurities. [19] Moreover, the extensive pretreatment procedure needed for chitin quantification affects the acetylation degree of the chitin sample, making the simultaneous determination of both parameters challenging.

The main aim of this article is to provide a simplified chromatographic method, avoiding lengthy pretreatment steps, for the characterization of chitin in biological samples, with a

prime focus on its application on the increasingly important BSF larvae. To this, a method is developed that allows the determination of the chitin content on crude, unprocessed samples, thus with many impurities such as proteins and lipids, without clean-up or derivatization steps. Moreover, the method is designed to provide simultaneous information on the content and DA of the chitin. The proposed methodology is based on acidic hydrolysis, followed by analysis of the chitin via LC-MS/MS and LC-RID. Another goal of the article is to characterize for the first time the DA of unprocessed BSF larvae chitin and to assess the proportion of chitin contained in the soft tissues of BSF larvae.

2. Materials and methods

2.1. Materials

Materials were purchased from different chemical providers, *i.e.,* HCl, NaOH, sulphuric acid 95-97 %, glacial acetic acid 100%, acetonitrile analytical grade from VWR [Radnor, Pennsylvania, US]; N-acetylglucosamine (> 99 %) from Alfa-Aesar [Ward Hill, Massachusetts, US]; glucosamine sulphate (98 %) from Sigma-Aldrich [St Louis, Missouri, US]. Chitin (from shrimp shells), and chitosan were bought on the market. The acetylation degree was determined by the supplier with an in-house method based on the measurement of picric acid sorption and desorption [20], and was reported to be 98 % for the commercial chitin and 22 % for the commercial chitosan. Fresh and living BSF larvae were kindly provided by Millibeter [Turnhout, Belgium]. The larvae used in this work were grown on crumb for 18 to 20 days.

2.2. Raw materials and their pretreatments

In this study, 3 different types of raw chitin materials were used with increasing impurity and thus also complexity. First, commercially available pure chitin and chitosan were applied as reference materials for process optimization. Subsequently, clean and crude exoskeletons of

BSF larvae were analyzed. The study was ended with the assessment of dried whole BSF larvae. The preparation and pretreatment of the different materials was done as follows.

The purchased chitin was used without any further purification, and only dried in an oven at 105 °C for 48 h and grinded in a ball mill to reduce its particle size.

To produce the clean and crude exoskeletons, fresh and living larvae of the BSF were mechanically treated to separate the exoskeletons from the liquid internal organs with a mesh size of 0.3 mm to 0.5 mm and thus removing the chitin contained in the soft tissue. The dry matter content of both fractions was determined after drying in an oven at 105 °C for 48 h. The slaying mechanical treatment of the BSF larvae was found to result in 40.6 % (*w/w*) of exoskeletons and 59.4 % (w/w) of dry intestines (not used as sample) on dry matter base. A fraction of the dried crude exoskeletons was used without further pre-treatment and is further addressed in the manuscript as “crude pellet”. The other fraction was cleaned in several successive steps based on literature methods.[21,22] The washing procedure was performed in triplicate experiments. First, the dried exoskeletons were washed twice with demineralized water (solid to water ratio 1:75; *w/w*) with intermediate filtration to remove most of non-bound proteins and lipids. To remove the remaining proteins, the water-washed exoskeletons were treated in several subsequent washing cycles at 80 °C for 1 h with 1 M NaOH until the liquid was clear (13 cycles with solid to solvent ratio of 1:50; *w/w*). After the alkaline treatment, the solids were washed with demineralized water until neutral pH, resulting in the clean exoskeletons. The 3 batches of clean exoskeletons were merged to obtain 1 homogeneous batch for the hydrolysis experiments and are further on noted as “clean pellet”. During the washing procedure, in total 77.0 ± 1.2 % (*w/w*) of impurities were removed from the crude pellets on dry weight base. Per gram of dry clean pellet produced, this lengthy cleaning procedure consumed roughly 650 mL of demineralized water in 2 steps and 2.8 L of 1 M NaOH in 13 deproteination steps of each 1 h at 80 °C.

All exoskeleton fractions, *i.e.* all replicates from the "crude and clean pellets", were dried in an oven at 105 °C and grinded in a ball mill to reduce the particle size.

Finally, also samples of whole larvae were prepared. To this, a batch of fresh larvae were oven dried at 105 °C for 48 h and grinded with ball mill to reduce their particle size.

The particle size of all samples was determined by laser diffraction (Microtrac, S3500 with Flex software). The commercial chitine and clean BSF pellet had an average particle size of 200 µm, the crude BSF pellet and grinded whole larvae was lower at 80-100 µm.

2.3. Acidic hydrolysis

The optimization of the hydrolysis was performed with either pretreated commercially available chitin or monomeric N-acetylglucosamine. In each experiment, 50 mg of the chosen substrate was transferred to heat and pressure resistant glass vials and 2.5 mL of 3 N or 6 N HCl were added. The hydrolysis was executed in closed vials under N_2 atmosphere in an aluminum block on a magnetically stirred heating plate. The reaction was performed in function of time between 0.5 h and 16 h at 90 °C and 110 °C.

For the 3 types of biological samples, *i.e.* the clean pellet, crude pellet and whole ground dry larvae, 50 mg of the dried powdered sample was combined with 5 mL 6N HCl in heat and pressure resistant glass vials. The stirred reaction at 110 °C was monitored between 2 to 16 h.

After reaction, the closed vials were immediately cooled in a laminar air flow. The reaction mixture was centrifuged to remove the residual non-dissolved material and subsequently filtered over 0.45 µm and used for chromatographic analysis as detailed in section 2.4. All experiments were executed in triplicate.

2.4. Chromatographic analysis and method validation

To quantify the chitin content of the starting raw material, the filtered hydrolysate was analyzed for its free acetate (as acetic acid), glucosamine and N-acetylglucosamine content.

The acetate concentration (as acetic acid) was determined with the method of De Sitter *et al.*[23], equally validated for other organic acids.[24] In brief, 250 µL of the filtered supernatant hydrolysate samples were partially neutralized with 750 µL of 400mM NaOH. Twenty-five µL of diluted samples were injected on an Agilent 1200 series HPLC containing an Agilent Hi-Plex H column (300 × 7.7 mm, 8µm particles) at 55 °C and an Agilent 1260 refractive index detector at 55 °C. The mobile phase was 0.01 M H_2SO_4 at a flow rate of 0.6 mL/min. The retention time of acetate (measured as acetic acid) was 15.49 minutes. Calibration was performed by injecting 11 different stock dilutions of acetic acid reference standards with a concentration ranging from 50 to 25000 mg/L and a linear order calibration curve was fitted ($R^2 > 0.999$). The limit of detection was found to be 9.6 mg/L.

Two quality control samples, with a known concentration of 12500 and 1000 mg/L respectively and independently prepared from the calibration standards, were added at the start of the series, in the middle and at the end. For extended measurement series, the measurement of both quality controls was repeated at least every 20 samples. No drift was observed during any of the analytical batches. The recovery was found to be on average 100.8 ± 3.1 % (n=34) for the quality control of 12500 mg/L and 101.4 ± 3.4 % (n=32) for 1000 ml/L.

For the determination of glucosamine and N-acetylglucosamine content, an aliquot of 0.5 mL of filtered supernatant was diluted to 10 mL with milliQ water. Then 40 µL of this diluted sample was added to 960 µL of a mixture prepared from 50 mL acetonitrile, 45 mL water and 1 mL 1 N NaOH for Ultra High-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS) analysis. Five µL of diluted and neutralized samples were injected on a Waters Quattro Premier UPLC-MS/MS system equipped with a Waters UPLC BEH HILIC 2.1 × 100 mm, 1.7 µm column at 40 °C. Chromatographic resolution of the analytes was obtained by 40 °C isothermal gradient elution using water with (A) 20 mM ammoniumformiate and 0.1% formic acid (pH = 3) and acetonitrile (B) with 0.1 % formic acid. A constant flow of 0.4 mL/min was

199 used with following gradient settings: 5-25 % A (0-3 min), 25 % A (3-4 min), 25-5 % A (4-4.1
200 min), 5 % A (4.1-7 min). The retention times of N-acetylglucosamine and glucosamine were
201 1.75 and 3.25 min respectively.

202 Peak detection and quantification was done using a triple quadrupole MS operated in
203 Multiple Reaction Monitoring mode. After electron spray ionization, the following *m/z* pairs
204 were recorded: 222 → 204 and 222 -> 168 for N-acetylglucosamine and 180 → 162 and 180 ->
205 84 for glucosamine. After confirmation of linear detector response, calibration was performed
206 by injecting stock dilutions of N-acetylglucosamine and glucosamine sulphate reference
207 standards with a concentration ranging from 0.4 to 400 mg/L and a linear (glucosamine) and
208 second order (N-acetylglucosamine) calibration curve was fitted (R^2 > 0.999). The limit of
209 quantification was found to be 4 times lower than the lowest standard, so 0.1 mg/L. The fitted
210 calibration equation was used to calculate the monomer content. To ensure reliability of the
211 analytical method, a quality assurance scheme was in place. Apart from the fit and working
212 range as mentioned above, method precision was assessed and monitored by means of
213 random replicate samples analysis. During the course of the experiments, 33 samples were
214 measured in duplicate. Variance of the results was used to assess method precision. Results of
215 n paired measurements were used to estimate the precision according to EUROCHEM/CITAC
216 Guide CG 4 [25], resulting in a precision uncertainty of 5.2 % for glucosamine (n = 108) and 5.4
217 % for acetylglucosamine (n = 11). This precision was considered fit-for-purpose to evaluate the
218 experiments. Trueness was investigated by means of repetitive analysis of spiked samples. To
219 this extent, random samples were fortified with a known amount of analyte and measured.
220 Recovery was used as a measure for trueness and was 91 ± 7 % for glucosamine and 77 ± 8 %
221 for N-acetylglucosamine. N-acetylglucosamine was shown not to be present in hydrolysis
222 samples (see 2.6 and 3.1.1), thus the low recovery did not affect the interpretation of the
223 results of this article. All glucosamine measurements were corrected with the recovery. To

ensure system stability, reference standards were inserted in the analytical sequence every 10 samples. No drift was observed during any of the analytical batches.

2.5. Fourier-Transform Infrared and elemental analysis

FT-IR spectra of commercial chitin, commercial chitosan, and clean pellet were run in Attenuated Total Reflectance mode on an ALPHA spectrometer [Bruker, Evere, Belgium] with a resolution of 4 cm^{-1} and 32 accumulations. The average DA was calculated via Eq. (1) described by Czechowska-Biskup *et al.*. [26]

$$DA\ (\%) = \frac{100}{1.33} * \frac{[\text{area } 1500-1700\text{ nm}]}{[\text{area } 2700-3600\text{ nm}]} \quad \text{Eq. (1)}$$

The carbon and nitrogen contents (mole%) were determined for the clean exoskeletons (clean pellet) and commercial chitin through elemental analysis (C/N) with a Vario micro cube [Elementar, Langenselbold, Germany]. 2.5 mg of dry sample was weighted and treated with a combustion temperature of 1150 °C and a reduction temperature of 850 °C. To convert the C/N ratio to the average DA, the formula of Xu *et al.* was used (Eq. (2)). [27]

$$DA\ (\%) = 100 * \frac{\text{C/N}-5.14}{1.72} \quad \text{Eq. (2)}$$

2.6. Calculations

The stability of the monomers N-acetylglucosamine and glucosamine under hydrolysis conditions was evaluated over time by calculating the monomers reduction, $X(t_x)$, via Eq. (3). N-acetylglucosamine, as being the prime chitin monomer, was added at an initial molar concentration $[\text{N} - \text{acetylglucosamine}]t_0$. As N-acetylglucosamine easily converts to glucosamine, both monomers were measured to evaluate the monomer stability. So, the time dependent (t_x) monomer molar concentration was determined by the sum of both glucosamine and N-acetylglucosamine. In practice, however, mostly glucosamine was observed due to the immediate deacetylation at the hydrolysis conditions.

$$X\ (monomers\ at\ tx)(\%) = 100 - 100 * \frac{[\text{sum monomers}]\text{tx}}{[\text{N}-\text{acetylglucosamine}]\text{t0}} \quad \text{Eq. (3)}$$

To account for monomer degradation during hydrolysis, the molar concentration of glucosamine measured in the hydrolyzed samples was corrected with the slope of trendline equation of monomer conversion, *i.e.* X = 1.846*t(h) (see 3.1). By applying Eq. (4), 'corrected' glucosamine values were obtained ([glucosamine]$_c$) that were used in all further calculations.

$$[glucosamine]c = [\text{glucosamine}]\text{measured} * \frac{100}{100-1.846*\text{t(h)}} \quad \text{Eq. (4)}$$

The amount of hydrolyzed chitin (mg/ml) was derived from the analytically determined molar concentrations of glucosamine, acetate and N-acetylglucosamine in the hydrolysate. The conversion to mass was performed by considering their respective molecular masses of the monomer unit as present in the chitin chain; *i.e*. 161.15, 42.04 and 203.19.

$$Hydrolyzed\ chitin\ \left(\frac{mg}{ml}\right) =$$

$$[\text{glucosamine}]\text{c} * 161.15 + [\text{acetate}] * 42.04 + [\text{N} - \text{acetylglucosamine}] * 203.19 \quad \text{Eq. (5)}$$

The fraction of sample liberated as hydrolyzed chitin as function of hydrolysis time, X(t_x), was determined via Eq. (6) with the calculated amount of hydrolyzed chitin (mg/ml) of Eq. (5) as the numerator and the amount of loaded sample (mg/ml) as denominator.

$$X\ (chitin\ at\ tx)(\%) = 100 * \frac{\text{Hydrolyzed chitin } \left(\frac{\text{mg}}{\text{ml}}\right)}{\text{Loaded sample } \left(\frac{\text{mg}}{\text{ml}}\right)} \quad \text{Eq. (6)}$$

To convert the chitin content of the pellet samples (calculated via Eq. (6)) into the chitin content of the insect, the dry weight of fractions and the loss of insect mass during pre-treatment were taken into account (see section 2.2). Eq. (7) and Eq. (8) were used for the clean and crude pellet data respectively.

$$Insect\ chitin\ content\ via\ clean\ pellet\ (\%) =$$

$$\frac{[\text{Chitin content clean pellet}] * \frac{(100-[\text{ DW cleaning loss}])}{100} * [\%\ \text{exoskeleton DW fraction}]}{100} \quad \text{Eq. (7)}$$

$$Insect\ chitin\ content\ via\ crude\ pellet\ (\%) =$$

$$\frac{[\text{Chitin content crude pellet}] * [\%\ \text{exoskeleton DW fraction}]}{100} \quad \text{Eq. (8)}$$

The average DA of the chitin, was determined by dividing the molar concentration of acetate by the corrected molar concentration of glucosamine (cfr. Eq. (4)).

3. Results and discussion

N-acetylglucosamine (chitin monomer) and commercially available crustacean chitin were first used as reference substrates in section 3.1 to develop a procedure to determine both the chitin content and average DA via hydrolysis and LC analysis. Monomer stability was evaluated under the applied conditions, and the overall method was verified for commercially available chitin by calculating the mass balance. Thereafter in section 3.2, biological samples of black soldier fly larvae with increasing amounts of impurities were subjected to hydrolysis to validate the method for insect chitin and to evaluate the effect of impurities on the accuracy of the method. All triplicate hydrolysis results generated as function of time were evaluated to develop a protocol to calculate the chitin content and average DA of crude biological chitinous samples. The results were compared to each other and literature to establish a standard procedure. Finally, the applicability of the procedure and generated results were evaluated (see section 3.3).

3.1. Hydrolysis of commercial chitin and chitin monomers

Previous experiments with commercial chitin and chitin monomers as substrate (not shown) indicated that 110 °C and 6 N HCl were the best conditions to fully hydrolyze chitin with minimal monomer degradation. Milder conditions resulted in incomplete chitin conversion while stronger conditions resulted in excessive monomer degradation. To quantify monomer degradation and allow the determination of the chitin content and average DA, N-acetylglucosamine, the chitin monomer, was subjected to hydrolysis. Subsequently, commercial chitin of known purity was hydrolyzed to construct a mass balance to verify the calculation method and determine the optimal hydrolysis time range.

294 **3.1.1. Monomer stability**

295 In first instance, N-acetylglucosamine was subjected to the hydrolysis procedure to
296 evaluate its stability. It must be noted that during hydrolysis N-acetylglucosamine was
297 immediately deacetylated to glucosamine since only glucosamine was detected in LC analysis.
298 References to monomers in all discussions below are therefore related to the detection of
299 glucosamine solely. Figure 1A shows the evolution of the monomer concentration as function
300 of time (see Eq. (3)). The linear decrease indicates a zeroth kinetic order for monomer
301 decomposition implying that the degradation rate is independent of its concentration. Figure
302 1B shows that the monomer percental conversion (secondary y-axis) is significant and
303 increases linearly at a rate of X(t) (%) = 1.846*t(h), wherein X(t) is the percentage of monomer
304 conversion/degradation as function of time. Because of significant glucosamine degradation
305 during hydrolysis, in this work it was opted to always correct the measured glucosamine
306 concentration in hydrolysis experiments with this time dependent correction factor (see Eq.
307 (4)) to calculate the chitin content.

308 **3.1.2. Commercial chitin hydrolysis**

309 When commercial chitin is used as reference substrate (Figure 1B), chitin hydrolysis
310 increases from 0 h to 8 h, remains stable for two hours and declines subsequently. When
311 monomer decomposition as function of time is considered via Eq. (4), the curve results into a
312 plateau after 8 h which indicates maximal chitin conversion whereof the average value of the
313 plateau is 95.9 ± 1.4 %. This is in line with the expected chitin content of this commercial
314 sample suggesting a complete hydrolysis. Moreover, the stable plateau indicates that
315 monomer decomposition was compensated sufficiently without overestimation of the
316 glucosamine content in the time range studied. In the discussions below and in all figures, the
317 data corrected for monomer decomposition are referred to as 'corrected' and are always

displayed with grey symbols. 'Uncorrected' data on the other hand are displayed as black symbols.

3.2. Hydrolysis of insect samples

The different types of samples of BSF larvae were subjected to hydrolysis to evaluate the performance of the analytical procedure and the quantification of the chitin content and average DA of BSF larvae chitin. As indicated above, samples with increasing amount of impurities, *i.e.* purified exoskeletons (clean pellet), crude exoskeletons (crude pellet) and whole larvae, were prepared as detailed in section 2.2. Subsequently, hydrolysis experiments were performed and the chitin content and DA of these samples were calculated via the method of this article, *i.e.* by hydrolysis and LC analysis (see section 3.2.1 and 3.2.1). At the end, based on the various observations, a simplified standard protocol is derived to characterize chitinous samples (see section 3.3).

3.2.1. Evaluation of hydrolysis results

As a first step, the release of acetate and (corrected) glucosamine was investigated in function of hydrolysis time for the various sample types. Figure 2 shows the molar release of acetate and (corrected) glucosamine, normalized per gram of dry matter. At shorter hydrolysis times all samples show a similar pattern, with first a systematic increase in glucosamine and acetate concentration indicative of the proceeding hydrolysis, followed by a stabilization related to maximal hydrolysis. The similar behavior of glucosamine and acetate as function of time is coherent with the hypothesis that released N-acetylglucosamine, the monomer unit of chitin, is instantaneously deacetylated with the formation of glucosamine and acetate, as was also observed in the monomer stability experiments (see section 3.1.1). At hydrolysis times of more than 10 h, a different behavior is observed between commercial crustacean chitin and the two insect derived pellet fractions. The corrected glucosamine remains stable for commercial chitin (see Figure 1B) but increases for the crude and clean pellet (Figure 2A). The

different behavior at long hydrolysis times is attributed to the simplified correction applied for monomer degradation. The correction model assumes that all N-acetylglucosamine is liberated at the start and then degrades during hydrolysis in a similar way as was observed for the monomer. In practice, however, the hydrolysis kinetics and the details of the N-acetylglucosamine release depend on the sample characteristics, such as for instance crystallinity and/or chitin content. The biological samples of this work are known to have crystalline regions, of about 35 % according to Wasko *et al.*. [8] Additionally, the pellet cleaning with a hot aqueous medium (see section 2.2) is known to be able to induce re-crystallization. The crystalline regions are expected to slow down hydrolysis, resulting in a delayed release of N-acetylglucosamine and hence overestimation of the loss of glucosamine due to degradation. Very similarly, as indicated above, the more "pure" samples (commercial chitin > clean pellet > crude pellet > whole insect) have an extended hydrolysis period (see Figures 1B and 2), likely related to their higher chitin content. The more pure BSF samples have a delayed release of N-acetylglucosamine which is expected to lead to inaccuracies in the correction applied at long hydrolysis times. As a result, a hydrolysis time of at most 10 h is recommended for the determination of the chitin content. Under these circumstances, the monomer degradation is estimated to be at most 20 % for the worse case in which all monomers are immediately released at the start of hydrolysis (see Figure 1A), limiting the possible errors induced by the simplified model used for correction.

3.2.2. Insect chitin content and degree of acetylation

If impurities do not interfere with the analysis, the calculated insect chitin content determined via the crude and clean pellet samples should result in a similar value. Subjecting the whole larvae to hydrolysis should lead to a higher insect chitin content considering the presence of chitin in the inner parts which were removed from the pellet samples in the first mechanical clean up step (see 2.2).

Figure 3A shows the calculated insect chitin content as a function of time for the different samples. As explained above, the hydrolysis time is restricted to 10 h to limit the inaccuracy that might be induced by the correction applied for monomer degradation. The plateau values, averaged between time intervals as well as at relevant discrete points are summarized in Table 1. In case of the whole insect, all data points result in an insect chitin content in accordance with the result of Caligiani *et al.*, *i.e.* 9 ± 1 %, obtained after hydrolysis, derivatization and gas chromatography/mass spectrometry analysis. [28] The insect chitin content calculated via the average value of the plateau is 8.0 ± 0.4 % (plateau 2 h – 8 h in Table 1). For the crude and clean pellet, the average values of the plateaus between 4 h and 10 h for are respectively 6.9 ± 0.4 % and 6.5 ± 0.6 % (Table 1) which are in accordance with each other and with literature. Spranghers *et al.* determined an insect chitin content of 5.6 % to 6.7 % [29] after applying the time consuming clean-up method of Liu *et al.* [12] which is similar to the clean-up method used in this work to produce the clean pellet. This agreement with literature for the three biological samples, supports the validity of the followed approach for the determination of the chitin content of BSF larvae. Moreover, the difference measured between the chitin content of the whole larvae and the one of the pellets, suggests a chitin content of about 1 – 2 % in the soft tissues. It is well known that, besides in the cuticle, insects also contain chitin in their gut lining, peritrophic matrix, salivary gland, trachea, eggshells and muscle attachment points. [2]

An important characteristic of the chitin concerns its DA. The acetyl group typically contributes to the formation of hydrogen bonds that can stabilize the crystalline structure of the chitin, influencing important material properties such as solubility and mechanical strength. [30] Hence, this work aimed to determine the average DA simultaneously with the chitin content. As an approach, the released acetate was measured during hydrolysis (see Figure 2), and the DA was determined as the ratio between the molar concentrations of acetate and the corrected glucosamine. Figure 3B gives an overview of the average DA for the

various samples in function of hydrolysis time. After an initial transition period, presumably related to differences in the release of glucosamine and acetate during the proceeding hydrolysis, the DA remains relatively constant between 4 h and 10 h. Averaging the plateau values within the same time ranges as used for the chitin calculation, *i.e.* 4 to 10 h for the pellets and 2 to 8 h for the whole insect, the average DA values obtained for the chitin are 78.1 ± 3.4 % for the whole insect, 78.6 ± 2.4 % for the crude pellet and 72.0 ± 3.7 % for the clean pellet (Table 1). As would be expected, the DA of the crude pellet and whole larvae are similar (78.6 ± 2.4 % *versus* 78.1 ± 3.4 %), but the one of the clean pellet is slightly lower (72.0 ± 3.7) probably related to the harsh cleaning procedure which is known to result in minor deacetylation. [14,15]

Since to the best of our knowledge, no public data are available on the average DA of BSF larvae chitin, these results could not be positioned against literature. Therefore, the method to determine DA was additionally validated for selected samples by comparison with conventional techniques, *i.e.,* FT-IR and C/N analysis. Since it is well-known that both FT-IR or C/N have limitations in the presence of impurities [18], the assessment was concentrated on the pure samples only, *i.e.* commercial chitosan for low DA, commercial chitin for high DA and the clean pellet (see Table 1). For commercial chitosan and chitin, the results were also analyzed against the supplier's specifications. To ensure an equal comparative base, for these samples a slightly different calculation method was applied. As these methodologies assume that the sample is uniquely composed of N-acetylglucosamine and glucosamine, the DA was based on the acetate analysis only and assuming a pure chitin/chitosan.

FT-IR analysis of commercial chitosan resulted in 18 %, a value in accordance with the DA result obtained via LC after 8 h of hydrolysis (20.5 % for oven dried and 20.2 % for untreated, see Table 1). These values are also in agreement with the specification of the supplier (22%) based on picric acid adsorption, indicating the validity of the LC-approach for low DA samples.

418 FT-IR analysis of commercial chitin and the clean pellet resulted in low DA values, *i.e.* 31 %
419 and 23 % respectively, which is a known drawback of the technique, *i.e.* samples with high DA
420 cannot be analyzed accurately with FT-IR. The second benchmark technique, C/N analysis,
421 resulted in very high DA values, *i.e.* 96 % and 147 %, for respectively commercial chitin and the
422 clean pellet (C/N resp. 6.79 and 7.66). Elemental analysis of commercial chitin resulted the
423 expected DA as communicated by the supplier (DA 98 %) while the clean pellet showed a value
424 that is typical for impure chitin samples (DA >> 100 %, cfr. Wasko *et al.* [8]). The DA values
425 attained in this work for the untreated commercial chitin after 6 and 8 h of hydrolysis (92.0 -
426 91.4 %, see Table 1), are in good agreement with the C/N result and the specifications
427 provided by the supplier, which supports the validity of the approach for high DA samples. In
428 case of the oven-treated commercial chitin, the LC method provides a slightly lower DA of 88%
429 as compared to the untreated sample, suggesting that the drying at 105 ^{o}C overnight might
430 influence the chitin. The slightly lower value obtained with the LC-method as compared to the
431 specifications is within the uncertainty that can be expected from the different methodologies
432 applied. For instance, it has been reported that the reference method based on picric acid
433 might be affected by the low solubility of the chitin sample, which might hamper complete
434 picric acid adsorption and result in an overestimation of the DA. [31] The full destruction of the
435 samples followed by acetic acid determination applied in this work probably ensures a more
436 fair measurement of the real DA in case of limited solubility.

437 **3.3. The standardized protocol and its applicability**

438 Based on the results obtained in section 3.2, an assessment was done to standardize
439 the procedure to determine both the content and average DA of chitin in biological samples.
440 Moreover, recommendations were derived on its overall applicability which are discussed in
441 more detail below.

When hydrolysis followed by LC analysis is used to determine the chitin content and corresponding DA, the hydrolysis times should be limited to at most 10 h to avoid the previously mentioned uncertainties related to the precise correction of monomer degradation. To determine the best hydrolysis time, LC plateau data and literature data are compared to data obtained at discrete hydrolysis times for both the chitin content and DA (see Table 1).

For the whole larvae, both 4 h and 6 h of hydrolysis gave the same chitin content and average DA as the plateau data (within the error margin of the plateau). For both crude and clean pellet, the values obtained after a hydrolysis time of 6 h and 8 h are in line with the plateau values reported in section 3.2.2. Moreover, at these times the results for the crude pellet have similar results as those from whole larvae. This indicated that for the crude and clean pellet, hydrolysis times between 6 h and 8 h seem the most suitable. Based on Figure 1B, 8 h will also be most efficient for analyzing commercially available chitin since both purified samples have a higher chitin content compared to the crude samples. Summarizing all above information, it is concluded that crude biological samples are preferably hydrolyzed for 4 h to 6 h to determine both the chitin content and DA while purified biological samples, thus with a higher chitin content, are preferably hydrolyzed for 6 h to 8 h. Based on all results, the following procedure is suggested: 50 mg of crude grinded sample is stirred during 6 h at 110 °C in 5 mL of 6 N HCl as sample preparation method prior to analysis by LC-MS and LC-RID to determine the chitin content and average DA of crude biological samples. For purified chitinous samples, the same procedure can be used, but with a hydrolysis time of 8 h.

For the first time the average DA of black soldier fly larvae chitin is reported carefully as 81.0 ± 2.1 % (6 h, crude pellet result). Since literature data are not available, further confirmation is needed in future work. Additionally, it must be noted that fresh and living larvae were used to generate the three biological samples. For the accurate determination of acetylation degree, it is important that chitin is the only source leading to the formation of

acetate. Therefore, careful storage of the samples is needed to avoid decay of the biological material when crude pellets are used. Moreover, it is recommended that for every new biological resource a comparison between crude and clean pellet is performed to exclude the contribution of non-chitin sources to acetate formation. In view of applications, the insect chitin content of 8.5 ± 0.1 % (6 h) determined by analyzing whole larvae is interesting from a biological point of view. However, when targeting chitin valorization, it is best to use the chitin content determined by measuring the crude pellet, *i.e.* 6.7 ± 0.4 % (6 h), to prevent overestimation of the revenues since chitin present in the soft tissues cannot be isolated easily. Both results imply that chitin in the soft tissues represents about 1.8 wt.% of the total dry insect which is which about 20 % of the total chitin content.

In summary, an LC method, without pretreatment nor derivatization, was developed for the simultaneous determination of the chitin content and degree of acetylation in crude samples of BSF larvae with up to 90% impurities on dry matter base. As compared to the traditional methodologies, the method avoids the labor intensive and harsh clean up procedure and allows the assessment at high acetylation degrees of acetylation. For the first time, the DA of BSF larval chitin (81±2%) is reported. Additionally, the chitin content of BSF larval soft tissues is estimated at 20% of the total chitin content (8.5±0.1 %). In further research, the generic nature of this method should be validated with other samples and resources since the effect of chitin crystallinity or other types of impurities was not evaluated.

List of Abbreviations

BSF	Black Soldier Fly
C/N	Elemental analysis
DA	Degree of acetylation
FT- IR	Fourier Transform Infrared Radiation

492 HPLC High Performance Liquid Chromatography

493 (UP)LC-MS (Ultra High Performance) Liquid Chromatography-Mass Spectrometry

494

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499 **Declarations of interest**

500 None

501

502 **References**

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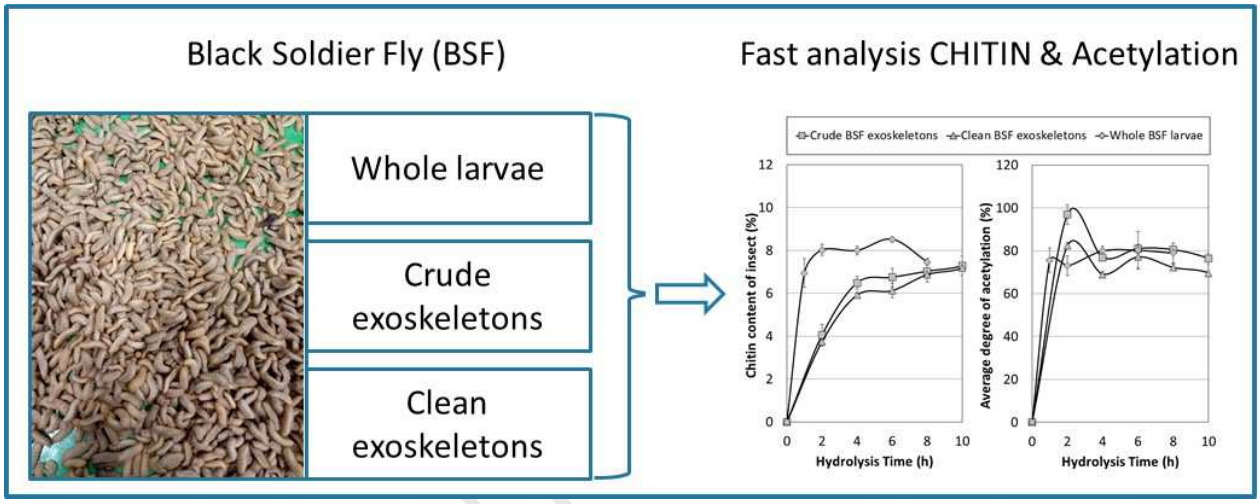
Graphical abstract



Captions

Figure 1: (A) Kinetic plot of monomer degradation and (B) hydrolysis of commercial chitin and chitin monomers with standard deviations as error bars.

Figure 2: Molar release of (corrected) glucosamine and acetate normalized per gram of dry sample as function of time during acidic hydrolysis of (A) whole larvae, (B) crude pellet and (C) clean pellet with standard deviations as error bars.

Figure 3: (A) LC determined chitin content in biological samples and (B) average degree of acetylation as function of time with standard deviations as error bars.

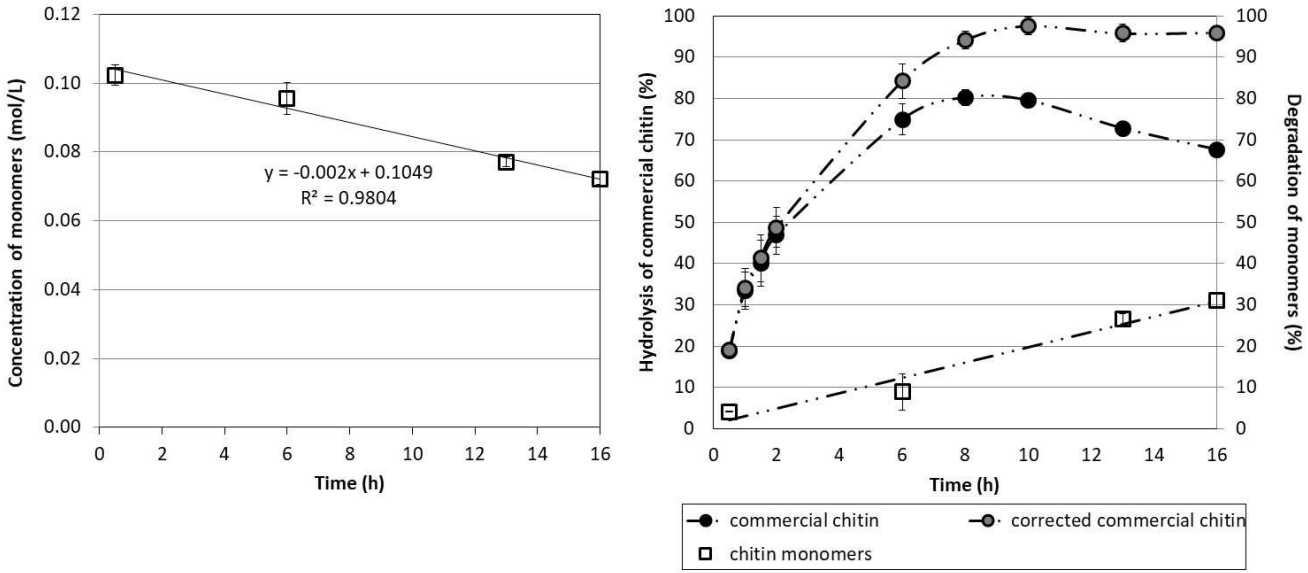
Table 1: Characterization of chitinous samples based on LC data and standard techniques for DA determination. The data were derived from the corrected glucosamine contents. * The DA the commercial chitin/chitosan was determined based assuming a 100 % pure sample to allow comparison with specifications. ^ Result for undried sample.

626 **Table 1:** *Characterization of chitinous samples with the LC method using the corrected glucosamine. Comparison of DA against standard techniques.*

627 **DA the commercial chitin/chitosan was determined assuming a 100 % pure sample to allow comparison with specifications. ^ For undried sample.*

Method →	**FT-IR**	**C/N**	**LC: Average of plateau**				**LC: Discrete hydrolysis time**			
Sample ↓	**Average DA (%)**		**Time range**	**Average DA (%)**	**Chitin content of sample (%)**	**Chitin content of insect (%)**	**Discrete time**	**Average DA (%)**	**Chitin content of sample (%)**	**Chitin content of insect (%)**
chitosan (DA<25%)	18	n.d.	n.d.	n.d.	n.d.	/	8h 10 h	20.5 ± 0.01* 20.2 ± 0.01*^	103.7 ± 1.8 101.9	/
chitin (DA>95%)	31	96	6h-8h 6h-10h **8h-10h**	n.d. n.d. **n.d.**	89.2 ± 7.0 92.0 ± 6.9 **95.8 ± 2.4**	/	6h **8h** **10h**	92.0 ± 0.03* **91.4 ± 0.01*** **88.0 ± 0.02*^** **n.d.**	84.2 ± 4.2 **94.1 ± 2.2** **97.6 ± 2.0**	/ **/** **/**
clean BSF pellet	23	147	4h-8h **4h-10h** 6h-8h 6h-10h 8h-10h	72.8 ± 4.1 **72.0 ± 3.7** 74.7 ± 3.5 73.0 ± 3.8 70.9 ± 1.8	64.1 ± 5.1 **66.3 ± 6.1** 66.0 ± 5.4 68.3 ± 5.5 71.4 ± 2.2	6.3 ± 0.5 **6.5 ± 0.6** 6.5 ± 0.5 6.7 ± 0.5 7.0 ± 0.2	4h **6h** **8h** 10h	69.0 ± 1.1 **77.1 ± 5.2** **72.2 ± 1.7** 69.6 ± 0.3	60.1 ± 0.7 **62.2 ± 3.2** **69.8 ± 1.3** 73.0 ± 0.7	5.9 ± 0.1 **6.1 ± 0.3** **6.9 ± 0.2** 7.2 ± 0.1
crude BSF pellet	n.d.	n.d.	4h-8h **4h-10h** 6h-8h 6h-10h 8h-10h	79.4 ± 2.2 **78.6 ± 2.4** 80.7 ± 0.4 79.2 ± 2.6 78.4 ± 2.9	16.6 ± 0.7 **17.0 ± 0.9** 17.0 ± 0.5 17.3 ± 0.7 17.7 ± 0.5	6.8 ± 0.3 **6.9 ± 0.4** 6.9 ± 0.2 7.0 ± 0.3 7.2 ± 0.2	4h **6h** **8h** 10h	76.9 ± 1.2 **81.0 ± 2.1** **80.4 ± 1.8** 76.3 ± 2.6	16.0 ± 0.3 **16.6 ± 0.4** **17.3 ± 0.5** 18.0 ± 0.5	6.5 ± 0.3 **6.7 ± 0.4** **7.0 ± 0.5** 7.3 ± 0.5
whole BSF larvae	n.d.	n.d.	2h-6h **2h-8h** 4h-6h 4h-8h 6h-8h	77.8 ± 4.1 **78.1 ± 3.4** 80.2 ± 0.2 79.8 ± 0.7 79.6 ± 0.9	8.2 ± 0.3 **8.0 ± 0.4** 8.3 ± 0.4 8.0 ± 0.5 8.0 ± 0.7	8.2 ± 0.3 **8.0 ± 0.4** 8.3 ± 0.4 8.0 ± 0.5 8.0 ± 0.7	2h **4h** **6h** 8h	73.0 ± 4.5 **80.0 ± 2.1** **80.3 ± 8.8** 79.0 ± 4.6	8.0 ± 0.3 **8.0 ± 0.2** **8.5 ± 0.1** 7.5 ± 0.2	8.0 ± 0.3 **8.0 ± 0.2** **8.5 ± 0.1** 7.5 ± 0.2

628 **Figures**



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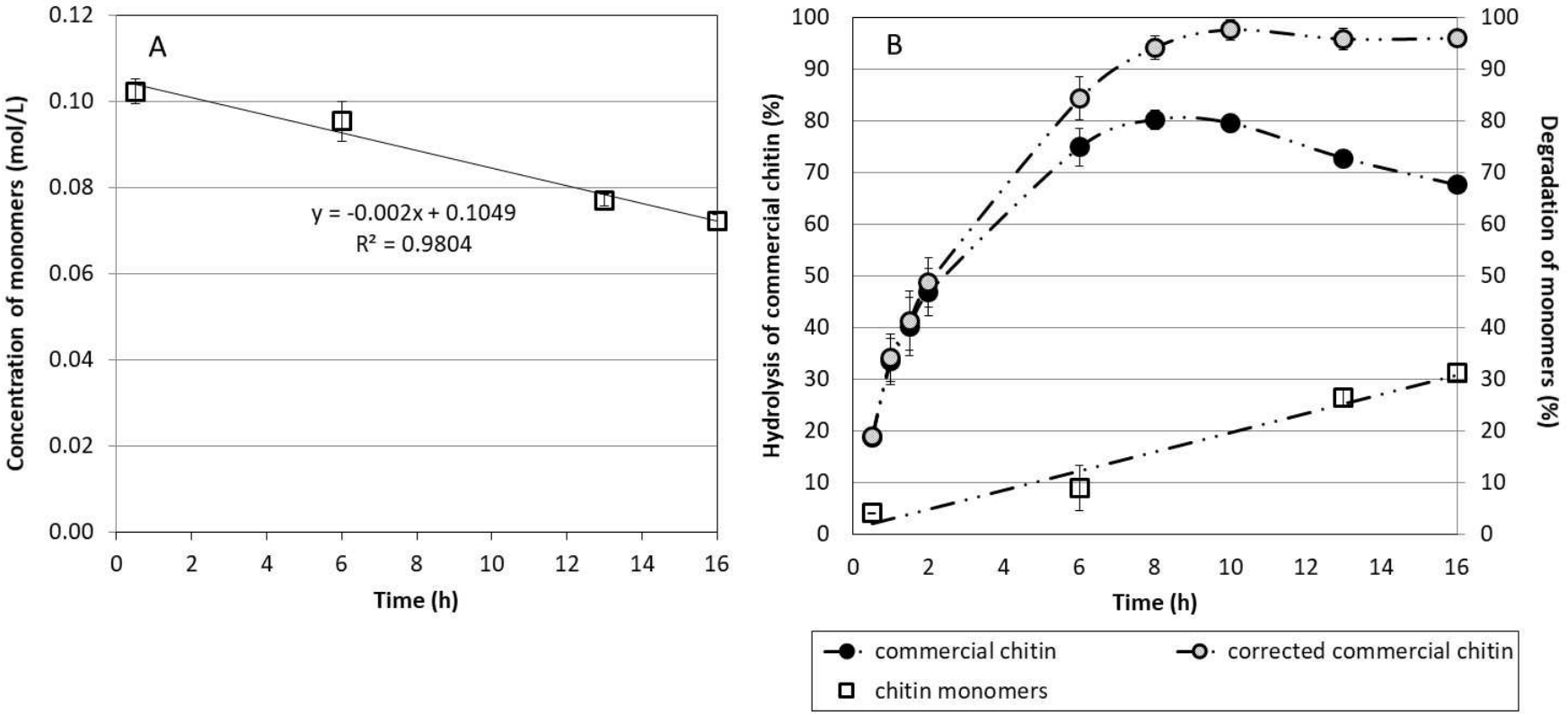

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632 *Figure 1: (A) Kinetic plot of monomer degradation and (B) hydrolysis of commercial chitin and chitin monomers with standard deviations as error bars.*

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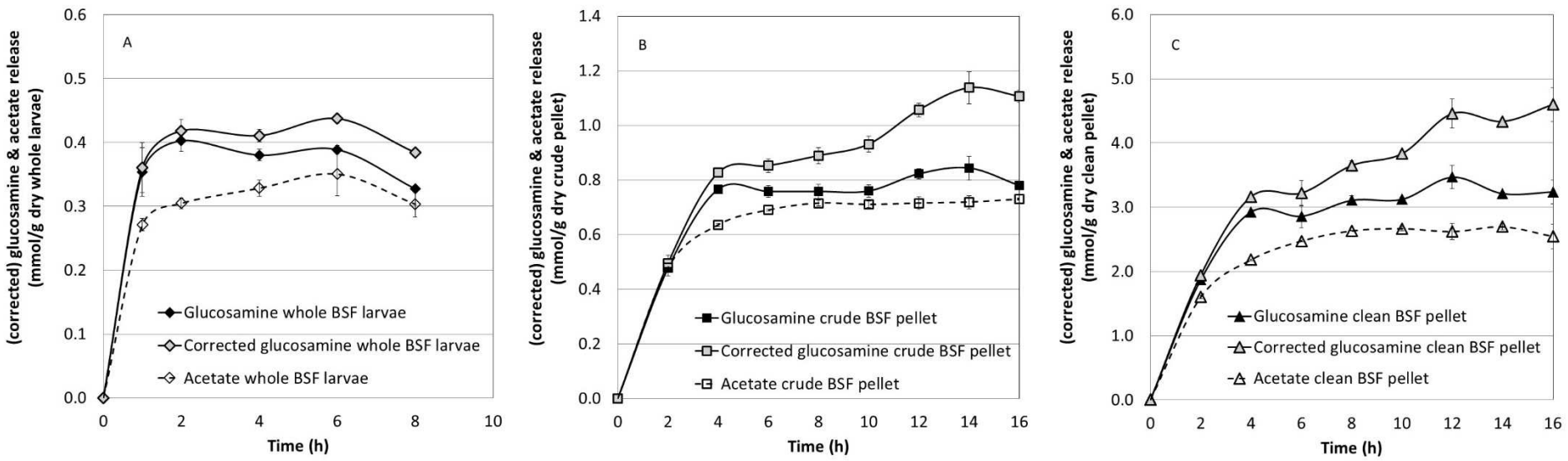

A
(corrected) glucosamine & acetate release (mmol/g dry whole larvae)
Glucosamine whole BSF larvae
Corrected glucosamine whole BSF larvae
Acetate whole BSF larvae
Time (h)
B
(corrected) glucosamine & acetate release (mmol/g dry crude pellet)
Glucosamine crude BSF pellet
Corrected glucosamine crude BSF pellet
Acetate crude BSF pellet
Time (h)
C
(corrected) glucosamine & acetate release (mmol/g dry clean pellet)
Glucosamine clean BSF pellet
Corrected glucosamine clean BSF pellet
Acetate clean BSF pellet
Time (h)

636

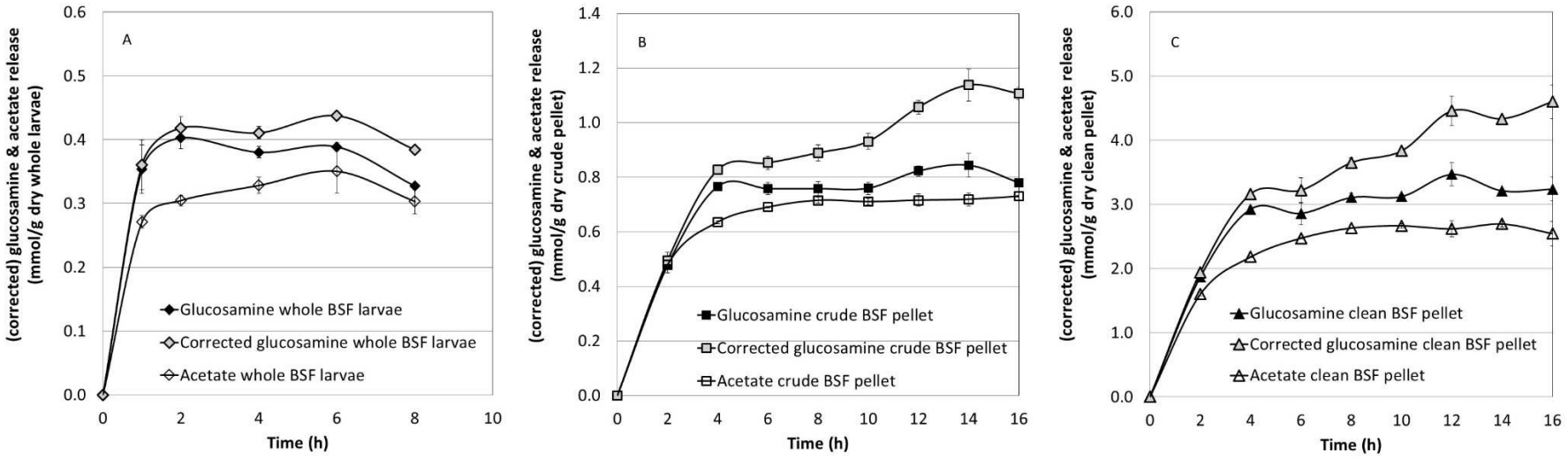
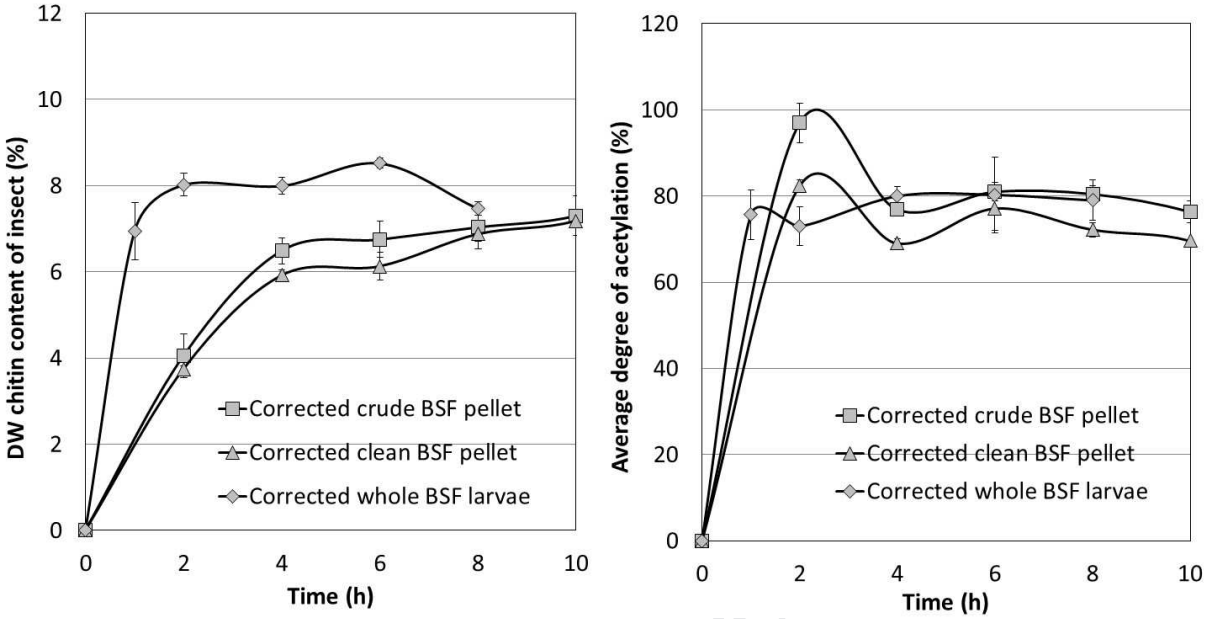
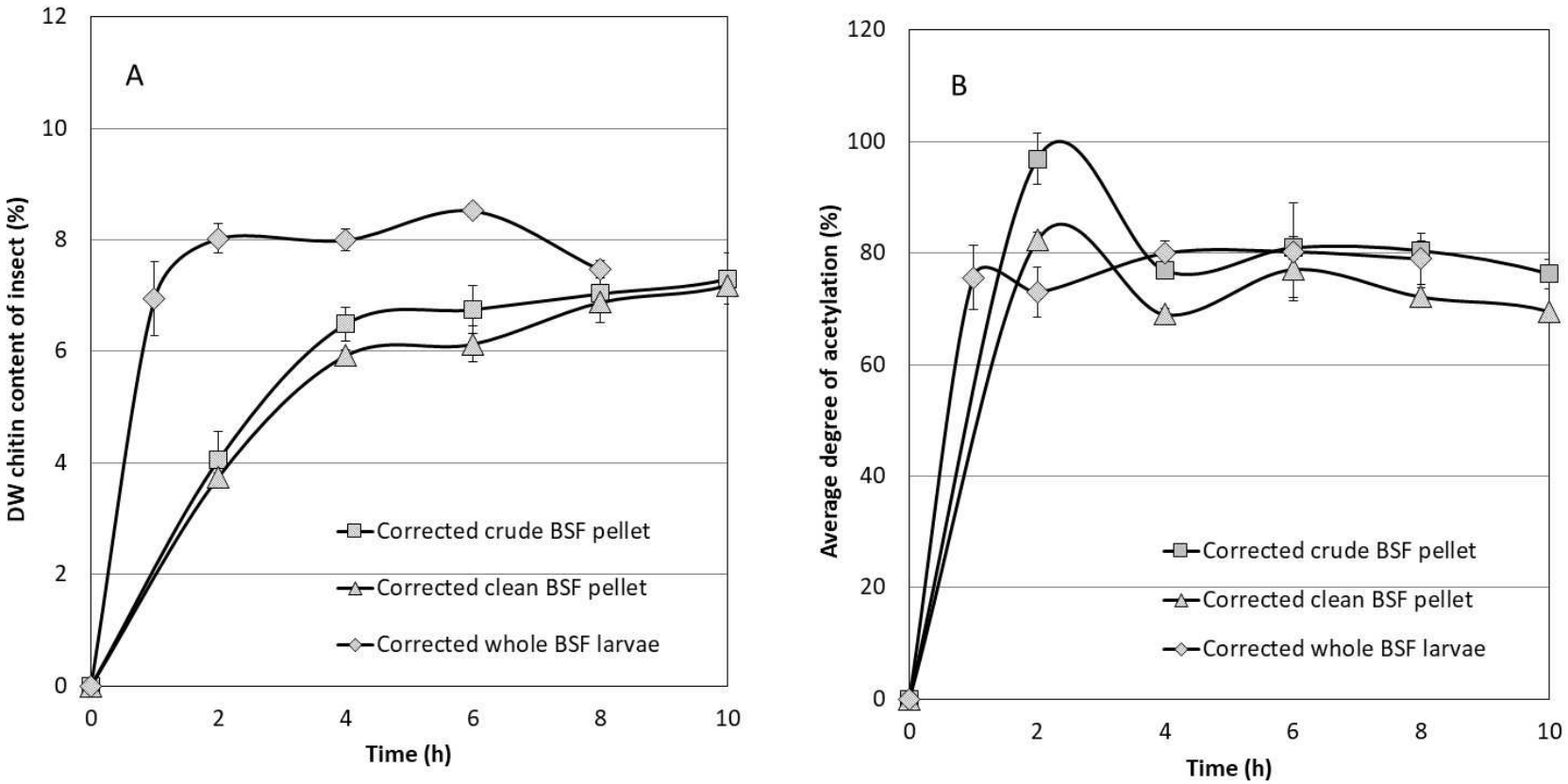


Figure 2: Molar release of (corrected) glucosamine and acetate normalized per gram of dry sample as function of time during acidic hydrolysis of (A) whole larvae, (B) crude pellet and (C) clean pellet with standard deviations as error bars.


DW chitin content of insect (%)
Time (h)
Corrected crude BSF pellet
Corrected clean BSF pellet
Corrected whole BSF larvae
Average degree of acetylation (%)
Time (h)
Corrected crude BSF pellet
Corrected clean BSF pellet
Corrected whole BSF larvae

646



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648 *Figure 3: (A) LC determined chitin content in biological samples and (B) average degree of acetylation as function of time with standard deviations as*

649 *error bars.*

650

The authors declare no conflict of interest.