



Development of a Quantitative UPLC-ESI/MS Method for the Simultaneous Determination of the Chitin and Protein Content in Insects

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Abstract

In a context where the commercial and nutritional interest in insect chitin is always increasing, an accurate and precise method to quantify this biopolymer, especially in food/feed, is required. In addition, quantification of insect crude protein through nitrogen determination is normally overestimated due to the presence of chitin. In this work, for the first time, an RP-UPLC-ESI/MS method for the simultaneous quantification in insects of chitin, as glucosamine (GlcN), and protein, as total amino acids, is presented. The method is based on acid hydrolysis and derivatization of amino acids and GlcN with the AccQ Tag reagent. Method was optimized and validated in terms of linearity, LOD and LOQ, intraday and inter-day repeatability, and accuracy. A hydrolysed commercial chitin was selected as reference standard for calibration. The instrumental LOD and LOQ correspond respectively to a concentration of 0.00068 mM and 0.00204 mM. The intraday precision satisfied the Horwitz ratio. Data from inter-day precision showed the necessity to perform the analysis within 1 week utilizing standard calibration solutions freshly prepared. A matrix effect was observed, which suggested the necessity to use an internal calibration curve or to work in a particular concentration range of GlcN. The chitin and protein content in black soldier fly (*Hermetia illucens*) and lesser mealworm (*Alphitobius diaperinus*) were found in agreement with results obtained by independent methods. The optimized method was also tested on two different commercial food supplements, suggesting its applicability on a wide range of matrices. This newly developed method proved to be simple, more accurate, and faster if compared to methods which separately analyse chitin and protein content.

Keywords Chitin · Glucosamine · Black soldier fly · *Alphitobius diaperinus* · Edible insects · UPLC/ESI-MS · Protein · Total amino acids

Introduction

The scientific interest in polysaccharides from natural origin, in the last decade, is growing considerably thanks to their many beneficial properties observed by different studies:

possible antitumoral, antioxidant, immunomodulatory, and anti-inflammatory activities were in fact suggested for polysaccharides isolated by plants, fungi, seaweed, and animals (Yu et al. 2018). Chitin is one of the most abundant polysaccharides present in nature. It is a large and linear structural

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polymer composed of repeating N-acetyl-D-glucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose) units, β (1 \rightarrow 4) linked together, principally found in cell wall of fungi and bacteria, in crustaceans exoskeleton, in insect cuticle, and in internal structure of other invertebrates (Muthukrishnan et al. 2012). The interesting properties of chitin such as biodegradability, biocompatibility with living tissue, low toxicity, robustness, and film forming capability allow the application of this polysaccharide and its derivatives in a wide range of sectors (Martínez et al. 2014). In biomedical area, chitin derivatives, especially chitosan (chitin deacetylated form), are applied as antimicrobial, antiviral, antitumor, antioxidant, antihypertensive, and anti-inflammatory agents. The pharmacological interest potentialities of chitin as drug carrier are also important. In fact, its chemical structure offers link mechanisms which allow the carrying and controlled release of active components of drugs and non-viral genes (Janes et al. 2001; Prabakaran 2008). Thanks to its robustness, chitosan is also fruitfully used as biomaterial for tissue engineering and regenerative medicine (Kumar et al. 2004; Wan and Tai 2013; Anitha et al. 2014). Even if the mode of action is not well understood yet, it is also well known that chitinous compounds can inhibit the growth of pathogens. This antimicrobial power makes them perfect as food preservatives and against plant fungal pathogens in agriculture (Shahidi et al. 1999). Chitin potentialities are still being discovered, especially in emerging sectors, such as nanobiotechnology, in which chitin compounds can found a role in promising future applications as low-cost and biodegradable nanocomposite polymers (Khoushab and Yamabhai 2010). The constituent unit of chitin, glucosamine (GlcN), is also often added to human supplements against osteoarthritis (Reginster et al. 2001; Nakamura 2011). Chitin and derivatives also found multiple applications in food sector: the amino sugars capacity to electrostatically interact with lipids make them perfect for use as ingredients in nutritional supplements for weight loss, and human cholesterol control, to be used sparingly because of significant downsides on minerals and liposoluble vitamins absorption levels (Razdan and Pettersson 1994; Koide 1998; Muzzarelli and Muzzarelli 2006). Currently, chitin available on the market and used for industrial applications comes mainly from processing waste of crustacean and these chitinous discards can easily reach 20 million tons per year (Brigode et al. 2020). However, due to the increasing market interest for this biopolymer, new natural and sustainable sources of chitin, able to meet its growing industrial demand, are being investigated (Brück et al. 2011; Morganti and Chen 2015). In this context, insect chitin would represent an excellent alternative to the crustacean chitin. In all the insect species, chitin is present as main component of the cuticular exoskeleton, where it plays a structural and defensive role (Muthukrishnan et al. 2012; Zhu et al. 2016). Several studies carried out to evaluate the nutritional

composition of edible insects showed that the insect chitin content ranges from 11.6 to 137.2 mg per kg of dry mass (Finke 2007; Kouřimská and Adámková 2016). Chitin from insects is beginning to be considered as an advantageous alternative to chitin from other sources, thanks to the recent attention in the use of insects as sustainable protein sources for the increasing food and feed demand. Indeed, the perspective of increasing insect biomasses production for protein extraction will lead to a high availability of insect chitin (Cortes Ortiz et al. 2016). However, the chitin high molecular weight, its insolubility in water and most other solvents, and the peculiar association that occur between cuticular proteins and chitin during the hardening process of the insect exoskeleton make the biopolymer isolation and characterization very difficult (Rudall 1963; Ishimaru et al. 2016; Brigode et al. 2020). In general, chitin per se has limited applications due to its very low solubility; however, its presence in foods can have important positive/negative impacts, especially in the context of insect novel foods or feeds that are mainly based on insect meals containing also chitin. Chitin could have negative effects on food texture or, once ingested, on intestinal nutrient absorption (Shahidi and Abuzaytoun 2005). In fact, apart from all its positive features (biodegradability, biocompatibility, non-toxicity, potential prebiotic, antimicrobial activity), it is known that chitin could have antinutritional effects. An excess of chitin concentration in feed can be counterproductive (Hahn et al. 2018). So the need to have a precise and accurate method to quantify chitin is mainly related to the expected enhancement of its assumption by insect-based food/feed.

One of the most challenging problem is the quantification of chitin in insect material and other biomasses. Many direct and indirect methods, used to measure the chitin fraction in insects, bear indeed some limitations. Weighing the chitin residue after removal of lipids, proteins, and minerals is a very time-consuming approach. An indirect gravimetric determination of insect chitin based on the measurement of the acid detergent fibre (ADF) has been described by Finke (2007), but this analysis is specific for other type of compounds (non-acid-labile) and the total amino acids need to be subtracted from the insoluble residue obtained, hampering the determination precision. Another indirect approach generally used in biological samples is measuring acetyl groups released from chitin after acid hydrolysis (Finke 2007; Hahn et al. 2018; Han and Heinonen 2021). In addition to being a long analysis, the polymer initial acetylation degree is to be exactly known, otherwise the final value obtained cannot be considered accurate (Finke 2007; Hahn et al. 2018; Han and Heinonen 2021). Direct methods are generally preferred: the chitin monomer GlcN released after an acid treatment of the sample (Zhu et al. 2005) can be determined by colorimetric (Frey et al. 1994; Bierstedt et al. 1998; Chen and Chiou 1999; Nitschke et al. 2011), capillary electrophoresis

(Jáč et al. 2008; Volpi 2009), or chromatographic (Zhu et al. 2005; Han and Heinonen 2021) (gas or liquid chromatography) methods. Indeed, to date, quantifying chitin through the GlcN released after hydrolysis in strong acid environment is the best way to get fairly accurate chitin values in the insect samples. Anyway, to improve the capacity of measuring chitin correctly, the direct determination methods are subjected to continuous optimizations (Sanches-Silva et al. 2012; Hahn et al. 2018). However, some weakness points remain: the long analysis times, the large use of chemical agents, the insufficient sensitivity of the method, and the need to remove the sample impurities or protein fraction are the biggest limitations (Han and Heinonen 2021). Chitin has also been previously evaluated by determining the total nitrogen content and subtracting the protein contribution determined by total amino acid analysis (Caligiani et al. 2018). However, also this method is quite long, and provides only an indirect measure of chitin content.

The uncertainty in chitin content can also affect the protein determination when made through nitrogen-measuring methods (Dumas or Kjeldahl), since the nitrogen-containing chitin molecules lead to an overestimation of the protein content.

In view of all the factors that may hinder advance towards a correct molecular characterization of insects, and other chitin-containing biomasses, a faster and more precise quantification method of insect chitin, and proteins, is needed. This work aims to develop and validate a UPLC/ESI-MS method for simultaneous quantification of amino acids and GlcN, released from insect proteins and chitin, respectively, after total acid hydrolysis. To the best of our knowledge, this is the first time that a method is developed with the aim to simultaneously determine GlcN and total amino acids by using UPLC/ESI-MS.

Material and Methods

Chemicals

Kjeldahl defoamers and catalyst were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit and AccQ Tag™ were obtained from Waters (Milford, MA, USA). DL-norleucine, amino acid standard mixture, chitin from shrimp shell (practical grade), chitin from shrimp shell (purified powder), D-(+)-glucosamine hydrochloride, D-(+)-N-acetylglucosamine, and D-(+)-galactosamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of the practical grade and purified powder chitin from shrimp shell was determined according to the Kjeldahl analysis based on the measurement of chitin nitrogen. Assuming a fully acetylated chitin, the conversion from nitrogen content to amount of chitin was made using the specific nitrogen conversion factor calculated for chitin 14.5 (Caligiani

et al. 2018). The purity was found to be $89.3 \pm 2.4\%$ for the commercial practical grade chitin and $101.5 \pm 1.5\%$ for the commercial purified powder chitin. All the other solvents, salts, acids, and bases were of analytical grade and purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

Insect and Food Supplement Samples

Black soldier flies (BSF, *Hermetia Illucens*) at prepupae stage were obtained from a colony reared in the laboratory of Applied Entomology of the University of Modena and Reggio Emilia (Italy) since 2016, established starting from larvae purchased from CIMI srl (Cuneo, Italy).

Lesser mealworm (*Alphitobius diaperinus*, AD) larvae were provided by Protifarm (Ermelo, the Netherlands). Both insect species were killed by freezing at $-20\text{ }^{\circ}\text{C}$. Frozen insects were ground for 2 min with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Staufen, Germany). After grinding, samples were immediately used for the analysis or stored at $-20\text{ }^{\circ}\text{C}$ until use. Additionally, two different commercial dietary supplements, containing polymers of GlcN, were bought on the market.

Sample Preparation

Hydrolysis of Insect and Food Supplement Matrices

The workflow indicating the procedure for the analysis of GlcN and amino acids is shown in Fig. 1. Total amino acids and GlcN were respectively released from protein and chitin fraction of analysed samples by strong acid hydrolysis following the procedure described by Caligiani et al. (2018), with some modifications. Approximately, 500 mg of ground insect samples (BSF prepupae and lesser mealworm larvae) or 30 mg of the commercial dietary supplements were weighed in Pyrex glass tubes, directly mixed with 6 mL of HCl 6 N and hydrolysed at $110\text{ }^{\circ}\text{C}$ for 23 h. At the end of hydrolysis, 7.5 mL of 5 mM norleucine in HCl 0.1 N, used as internal standard for total amino acid determination, was added after cooling of the sample. The hydrolysates were then filtered using a Buchner filter and brought up to volume of 100 mL with deionized water. Then, 450 μL of hydrolysate solution was taken and mixed with 7 μL galactosamine 46 mM. The solution was brought up to 500 μL with deionized water. The hydrolysates containing the two internal standards were stored at $-20\text{ }^{\circ}\text{C}$ until derivatization.

Derivatization

The derivatization of the hydrolysed sample was necessary to detect total amino acids and GlcN content by reversed

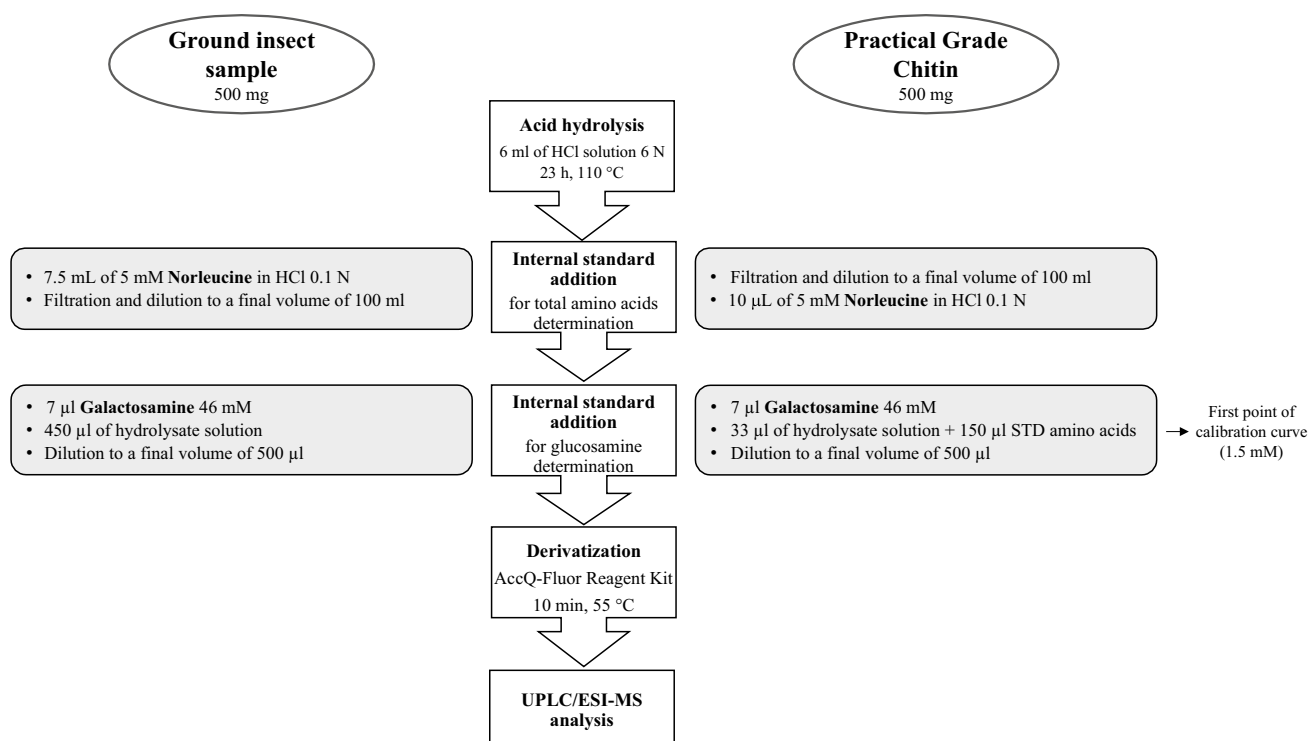


Fig. 1 Workflow indicating the procedure for insect samples and calibration curve preparation

phase UPLC. It was performed according to the method described by Leni et al. (2019). Seventy microlitres of borate buffer, 10 µl of hydrolysed samples or standard solutions, and 20 µl of reconstituted AccQ Tag reagent (Waters Co., Milford, U.S.A.) were mixed and kept at 55 °C for 10 min. To complete the derivatization, the mixture was diluted with 200 µl of Milli-Q water and mixed well. After dilution, the solution was put into an LC vial for UPLC-ESI/MS analysis.

Calibration Curve Preparation for Amino Acids and Glucosamine Quantification

The workflow indicating the procedure for calibration curve is shown in Fig. 1. For the amino acid and GlcN determination, external calibration was performed, as previously reported by Caligiani et al. (2018), with some modification. 500 mg of practical grade chitin ($89.3 \pm 2.4\%$ purity) were weighed in Pyrex glass tubes, directly mixed with 6 mL of HCl 6 N and hydrolysed at 110 °C for 23 h. The hydrolysates were then filtered using a Buchner filter and brought up to volume of 100 mL with deionized water. Then, different amounts of amino acids standard mixture (2.5 mM) and hydrolysate solution (22.6 mM) were taken and mixed with 7 µl galactosamine 46 mM and 10 µl norleucine 5 mM. The solution was brought up to 500 µl with deionized water to obtain five concentrations 1.5, 1, 0.1, 0.05, and 0.01 mM

and 1.5, 1, 0.75, 0.5, and 0.25 for amino acids and GlcN, respectively. The hydrolysates were stored at -20 °C until derivatization.

UPLC-ESI/MS Analysis of Amino Acids and Glucosamine

For GlcN and amino acid derivatives, separation was carried out by UPLC/ESI-MS analysis, and performed by using a Waters ACQUITY Ultra Performance LC system with an Acquity BEH C18 column (1.7 µm, 2.1×150 mm). The chromatographic conditions were the same already described by Caligiani et al. (2018) for amino acids determination in insect samples with some modifications: mobile phase was composed by $\text{H}_2\text{O} + 0.2\% \text{CH}_3\text{CN} + 0.1\% \text{HCOOH}$ (eluent A) and $\text{CH}_3\text{CN} + 0.1\% \text{HCOOH}$ (eluent B). Gradient elution was performed: isocratic 100% A for 7 min, from 100% A to 75.6% A by linear gradient in 21 min and plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 5 µl, column temperature 35 °C, and sample temperature 18 °C. Detection was performed by using Waters SQ mass spectrometer: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N₂): 100 l/h, desolvation gas flow (N₂): 650 l/h, full scan acquisition (m/z 100–2000), and scan duration 1 s.

Optimization and Validation of UPLC-ESI/MS Method for Chitin Quantification

Preparation of Insects Spiked Samples

Five different samples containing 500 mg of ground whole BSF prepupae were spiked with different amounts of standard practical grade chitin to obtain final added concentrations of GlcN of 1.5, 1, 0.75, 0.5, and 0.25 mM. The experiment was carried out in triplicate. Spiked samples were then subject to the same hydrolysis and derivatization protocol described in the paragraphs “Hydrolysis of Insect and Food Supplement Matrices” and “Derivatization.” These samples were used to build an internal calibration curve, utilized to obtain the real amount of chitin in the prepupae sample, corresponding to the X intercept value, and also for the evaluation of an eventual matrix effect, by comparing the internal calibration with the external calibration curve. The global recovery rate was calculated by comparing the determined amount of chitin in the spiked samples to the amount added. The amount of chitin, expressed as chitin % DM, was calculated by using the weighted mass (g) of insect sample and the added mass (g) of practical grade chitin, assuming 100% release of GlcN after hydrolysis.

Validation of N-Acetyl Glucosamine, Glucosamine, Galactosamine, Chitin, and Amino Acid Standard Working Solutions

For chitin quantification, 4 different standard solutions were tested: (i) a standard GlcN hydrochloride solution prepared by dissolving 50 mg of GlcN hydrochloride in HCl 0.1 M up to a final volume of 100 mL, reaching a final concentration of 2.3 mM, (ii) a standard solution of GlcN subjected to acid hydrolysis, (iii) a standard solution of GlcNAc subjected to acid hydrolysis, and (iv) practical grade chitin after acid hydrolysis. Solutions (ii), (iii), and (iv) were prepared by dissolving 50 mg of corresponding analyte in 6 mL of hydrochloride acid solution (6 N) and hydrolysed (23 h, 110 °C) following the same conditions set out for sample hydrolysis (see paragraph “Hydrolysis of Insect and Food Supplement Matrices”), then brought to volume of 100 mL with distilled water. All these stock solutions were diluted with Milli-Q water, in order to obtain from each one, five working solutions having 1.5, 1, 0.75, 0.5, and 0.25 mM final concentration of GlcN. Before making the individual working solutions up to volume, to each one 7 µl of galactosamine hydrochloride (46 mM) were added as standard, prepared by adding 1 mL of HCl 0.1 M to 10 mg of galactosamine. These solutions were used to determine the linearity and accuracy of the method. The hydrolysed stock solution of practical grade chitin was then also used to build the external calibration curve for the quantitative determination of chitin,

for the determination of the limit of detection (LOD), and for the determination of the limit of quantification (LOQ).

Linearity and Accuracy

The linearity of the method was tested on the four different standards reported in paragraph “Validation of N-Acetyl Glucosamine, Glucosamine, Galactosamine, Chitin, and Amino Acid Standard Working Solutions,” all potential candidates to be used as external reference standards in the analysis. Five concentration levels (1.5, 1, 0.75, 0.5, and 0.25 mM expressed as GlcN concentration) were obtained for each standard: pure GlcN hydrochloride, GlcN hydrochloride subjected to hydrolysis protocol, GlcNAc subjected to hydrolysis protocol, and practical grade chitin standard subjected to the hydrolysis protocols. Two replicates were performed per each concentration of each standard. Seven microlitres of galactosamine hydrochloride 46 Mm were added to each solution. A pure chitin hydrolysed standard ($101.5 \pm 1.5\%$ purity) solution was prepared as a control sample and used to check the accuracy of each calibration. Regression was performed on the ratio of peak areas of analyte and internal standard versus analyte concentration added. The goodness of fit was determined by means of Mandel test at the 99% significance.

Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification were calculated utilizing the S/N ratio methods, based on the determination of the peak-to-peak noise (Apostol et al. 2009). LOD and LOQ were calculated as the concentrations of chitin producing after hydrolysis a recognizable GlcN peak with a signal-to-noise ratio of, respectively, 3.3 and 10. LOD and LOQ were determined in pure standard solution of hydrolysed chitin (practical grade).

Repeatability and Effect of Time on GlcN-AccQ Tag Yield Under Derivatization

Repeatability was evaluated as intraday and inter-day precision. Intraday repeatability of the method was calculated for six different concentration levels, using the spiked samples prepared according to the procedure explained in paragraph “Preparation of Insects Spiked Samples.” The results of intraday precision were compared with Horwitz predicted intra-laboratory precision (PRSD) by the calculation of Horwitz ratio (HORRAT) (Horwitz and Albert 2006).

Inter-day repeatability was instead calculated in a 4 weeks' interval of time on two sets of four replicates of calibration standard (practical grade chitin) and BSF prepupa in two different storage conditions (before and after

derivatization protocol). Briefly, replicates of 30 mg of practical grade chitin and of 500 mg of BSF prepupae were subjected to hydrolysis protocol as reported in the paragraph “[Hydrolysis of Insect and Food Supplement Matrices](#).” Ten microlitres of each hydrolysed solution were then subjected to reaction with AccQ Tag. After derivatization, the mixtures were inserted into an LC vial for UPLC-ESI/MS analysis and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. The rest of each prepared sample was stored underivatized at $-20\text{ }^{\circ}\text{C}$ and derivatized immediately before the analysis. All samples were analysed every week for consequent 4 weeks. The results of inter-day precision were compared with Horwitz predicted intra-laboratory precision (PRSD) by the calculation of Horwitz ratio (HORRAT) (Horwitz and Albert 2006), too. Data from inter-day precision were also used to provide information about the stability of GlcN and GlcN-AccQ derivative during the time.

Data Analysis

All data were acquired and processed by the software MassLynx 4.0 (Waters, Wilmslow, UK). For amino acids, quantitative analysis was set up by external calibration using D/L-norleucine as internal standard.

Chitin amount was calculated using hydrolysed practical grade chitin as standard for external calibration and galactosamine as internal standard. In the chromatographic analysis, GlcN and galactosamine are present in the two anomeric forms, alpha and beta. To calculate the chitin content, the sum of the areas of each couple of anomer peaks was considered. Due to the use of chitin concentration in the calibration curve, the values obtained are directly expressed as chitin, even if the analytical target is GlcN. Data on chitin content were obtained as triplicate analysis of independent hydrolysed samples and are presented as mean \pm SD.

Results and Discussion

Development of Analytical Method for the Simultaneous Quantitative Determination of Total Amino Acids and Glucosamine in Insect Samples

Choice of the UPLC-ESI/MS Conditions

The UPLC-ESI/MS method conditions chosen for the simultaneous determination of total amino acids and chitin in insect samples were the same used to quantify total amino acids in BSF prepupae (Caligiani et al. 2018). This method, combined with separated determination of tryptophan and sulphurated amino acids, is widely adopted to determine the full amino acid profile of food samples and the sum of amino

acids can be used also to determine accurately the total protein content in insects (Caligiani et al. 2018). Despite GlcN from chitin is in principle detectable in the method conditions as AccQ Tag derivatives, its content in insect samples was never determined simultaneously with the amino acid content by an UPLC-ESI/MS chromatographic method. So, the aim of this work was to optimize and validate the analytical method for amino acid analysis also to directly evaluate the chitin content (via GlcN detection) of insect samples. As a first step, it was necessary to assess the suitability of the UPLC-ESI/MS parameters in separating it. For this purpose, standard derivatized GlcN hydrochloride dissolved in HCl 0.1 N was analysed. As for amino acids, the derivatization reaction was essential to make GlcN visible through chromatographic analysis. Being an amino sugar, in fact, GlcN presents an amino group that react well with the amino acid derivatization reagent (AccQ Tag), as already reported by Díaz et al. (1996). From the UPLC-ESI/MS analysis, as already observed in other GlcN retention studies (Sanches-Silva et al. 2012; Han and Heinonen 2021), it was possible to observe the separation of GlcN-AccQ Tag in the two anomers, alpha and beta, with retention times respectively of about 3.3 and about 6.2 min. The most intensive precursor ion of the GlcN peaks was m/z 350.2 corresponding to the derivatization product between GlcN and AccQ Tag. The GlcN peaks were found in the insect samples with the same characteristic m/z and the same retention times of the standard, together with the derivatized amino acids, and no interferences were observed with the peaks of amino acids-AccQ Tag, ensuring the applicability of the method for the simultaneous determination of GlcN and total amino acids derivatives in insects (Fig. 2).

Choice of the Internal Standard

While 5 mM norleucine in HCl 0.1 already selected by the method described by Caligiani et al. (2018) was maintained as internal standard for the amino acid determination, it was necessary to choose an internal standard for the quantitative analysis of GlcN. In literature, L-cysteic acid, L-norleucine, D-galactosamine, and α -aminobutyric acid are the molecules most widely used as internal standard in the chromatographic methods for the analysis of GlcN (Hagen 1993; Díaz et al. 1996; Flannery et al. 2001; Geradets et al. 2011; Liu et al. 2013). However, to obtain more precise and reliable measurement of chitin in the sample, an internal standard as close as possible to the analyte should be chosen and added in a similar concentration. Due to its easily availability on the market and very similar molecular structure to GlcN, the amino sugar galactosamine was chosen as internal standard. Galactosamine was added after hydrolysis and before derivatization protocol, generating two anomeric signals not interfering with the

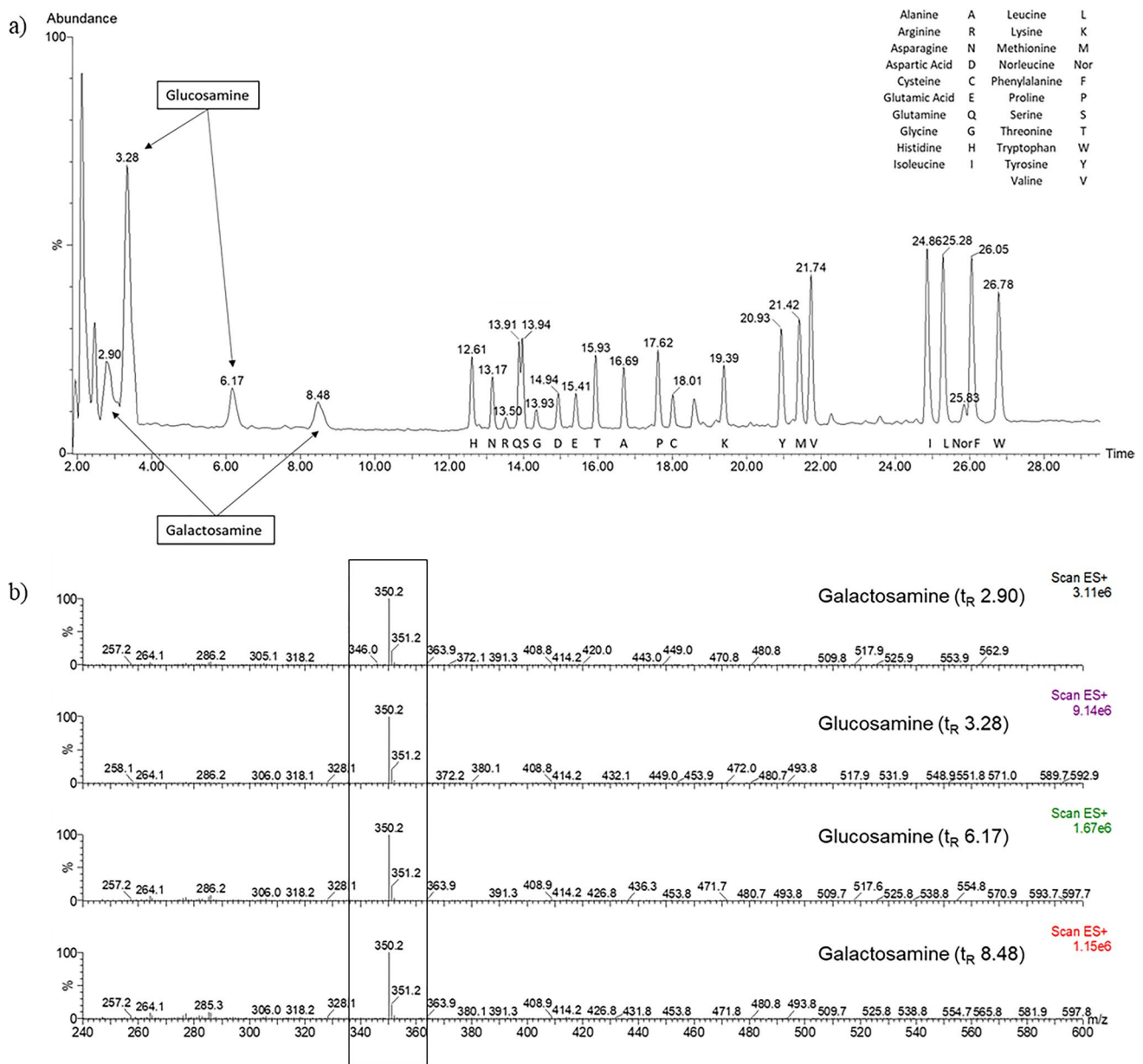


Fig. 2 a UPLC–MS chromatogram of glucosamine, galactosamine (internal standard), and amino acids in black soldier fly prepupa sample, **b** MS full scan spectra of glucosamine and galactosamine, show-

ing the characteristic ions corresponding to the derivatization product between glucosamine/galactosamine and AccQ Tag (m/z 350.2)

GlcN peaks (retention time respectively of about 2.9 and about 8.5 min, Fig. 2a). The mass spectra of galactosamine are identical to that of GlcN, allowing to use the same precursor ion for quantification (m/z 350.2, Fig. 2b).

Method Validation

The UPLC-ESI/MS method for the simultaneous quantification of total amino acids and GlcN was subjected to validation in terms of linearity, detection and quantification

limits, precision, and accuracy following the recommendations of the International Conference on Harmonization-Validation of analytical procedures: Text and Methodology (GUIDELINE, ICH 2005).

Linearity

Three potential external reference standards (GlcN, GlcNAc, and chitin) were selected for chitin quantification, as reported in paragraph “Validation of N-Acetyl

Glucosamine, Glucosamine, Galactosamine, Chitin, and Amino Acid Standard Working Solutions.” Linearity was tested for GlcN hydrochloride, GlcN hydrochloride hydrolysed in the same conditions of the method, hydrolysed GlcNAc, and hydrolysed chitin, in the concentration range 0.25–1.5 mM, expressed as GlcN concentration. The linear regression equations are reported in Table 1, demonstrating a good linearity for all the tested standards, except for hydrolysed GlcN, supposedly for the fastest and most unpredictable degradation of the free monomer in so strong hydrolysis conditions.

In order to select the one allowing the most accurate quantification of chitin in insect samples, each calibration curve was tested for the quantification of a purified chitin sample (purity of $101.5 \pm 1.5\%$). Results are reported in Table 1. By observing the recovery results, it was found that only using the calibration line built with hydrolysed practical grade chitin as external standard, the recovery of the control sample was total ($103 \pm 4\%$). This result could be easily explained by the fact that the two samples, being both polymers, degrade during hydrolysis in a similar way, allowing a more accurate measurement of the analyte. A lower recovery value of the control sample was instead found using the other curves, built by using as reference standard the non-hydrolysed GlcN, hydrolysed GlcN, and hydrolysed GlcNAc. The lowest recovery was found to be $61 \pm 3\%$, when using for calibration GlcN nonsubjected to hydrolysis. As explained, with this method, chitin content was determined as amount of GlcN released from chitin fraction of the samples after strong acid hydrolysis. The conditions of this step may affect the release of GlcN from insect chitin, also causing its partial degradation. For this reason, even if in general GlcN is one of the main external references used to quantify chitin, its use can expose to an incorrect quantification, and in particular an underestimation of chitin content of the insect sample due to a possible GlcN degradation during acid treatment of the sample only. The degradation of glucosamine in strong acidic systems has been recently reported, accounting to a 30% after 16 h in conditions similar to ours (D’Hondt et al. 2020). A higher, but not optimal recovery percentage

of the chitin control sample, respectively of $87 \pm 5\%$ and $91 \pm 5\%$, was found by using the acid-treated standard monomers of GlcN and GlcNAc. This was probably due to a different behaviour of the standards (monomers) respect to chitin during acid hydrolysis. In fact, as reported by D’Hondt et al. (2020), free monomers GlcN and GlcNAc, compared to whole biopolymer chitin, under the same condition of acid hydrolysis (extended periods and high temperature) have a different behaviour. The chitin polymer in a biological matrix could have a gradual, and supposedly incomplete release of GlcN, which degrades differently respect to the pure GlcN standard under the same hydrolysis conditions, leading to a final incorrect estimation of the sample chitin fraction. Consequently, the only way to obtain accurate chitin quantification is by using as reference standard a polysaccharide which during hydrolysis has an analogous behaviour respect to the chitin present in the sample.

The commercial practical grade chitin subjected to hydrolysis was therefore used as standard and to quantify chitin content in insect samples and to evaluate the limit of detection and quantification, the precision, and the recovery of the analytical procedure. This obviously implies that all the quantitative results are obtained directly in terms of chitin content, being chitin the external standards, even if the actual analyte detected is GlcN.

The linearity of the method was also tested in matrix, using an insect sample (BSF prepupae) spiked with different amount of practical grade chitin, as reported in “**Preparation of Insects Spiked Samples.**” The spiked samples were then subjected to hydrolysis. The experiment was carried out in triplicate. The linear regression equation obtained for the calibration curve in matrix was $y = 0.3605x + 0.014$ with a correlation coefficient > 0.96 , demonstrating the linearity of the methods also in matrix. Calibration curve in matrix was also used to calculate the real starting concentration of chitin in the prepupa sample and, by comparing its slope with that one of external calibration line, to evaluate the presence of a matrix effect. These aspects are deeply described in paragraph “**Evaluation of Hydrolysis Condition.**”

Table 1 Linear regression equations and R^2 of the different standards tested and the purified standard chitin values (%) respectively recovered using each standard curve

Standard	Regression equation	R^2	Recovery Chitin determination in purified chitin (%) using the corresponding standard calibration curve
Hydrolysed practical grade chitin	$y = 0.5316x + 2.00556E-05$	0.9946	103 ± 4
GlcN	$y = 1.1836x - 0.0019$	0.9922	61 ± 3
Hydrolysed GlcN	$y = 0.7821x - 0.0003$	0.9382	87 ± 5
Hydrolysed GlcNAc	$y = 0.7811x - 0.0014$	0.9938	91 ± 5

Evaluation of Hydrolysis Condition

The main novelty and aim of this work were to quantify total chitin in insects together with total amino acids. The release of amino acids from protein requires strong acid (HCl 6 N), long times (23 h), and high temperature (110 °C). In these conditions also chitin is hydrolysed releasing GlcN that can be analysed in UPLC-ESI-MS simultaneously with the amino acids. However, as reported by D'Hondt et al. (2020), for the determination of chitin content is recommended a hydrolysis time not more than 10 h to avoid a monomer degradation during the procedure. More precisely, 4–6 h and 6–8 h were the optimal hydrolysis times respectively for the whole insect and for commercial chitin. These mentioned hydrolysis times are clearly less harsh than those used in this work. Therefore, to be sure that the long hydrolysis time here applied for amino acid release were also suitable for obtaining GlcN from chitin, a BSF sample, analysed in triplicate, was hydrolysed for 6 h at 110 °C in 6 mL of HCl 6 N. Also, practical grade chitin reference standard was hydrolysed in the same conditions of the samples and used to prepare the five working solutions to build the external calibration and quantify chitin amount. Data obtained show that the content of chitin determined after 6 h ($2.4 \pm 0.1\%$) and 23 h ($2.9 \pm 0.5\%$) of hydrolysis was not significantly different when the calibration is made by treating the chitin standard in the same conditions of the sample. These preliminary data suggested that the method normally used to release amino acids from protein is also suitable for chitin hydrolysis.

Recovery Test

Due to the absence of a “blank” insect sample, a recovery test was carried out in matrix on BSF prepupa samples. To obtain the initial analyte concentration in the sample, an internal calibration line was built by adding to ground BSF prepupa samples different amounts of practical grade standard chitin to obtain five levels of concentration of GlcN, as described in

paragraph “Preparation of Insects Spiked Samples” and it was compared with an external calibration line of the same standard chitin (Fig. 3). Utilizing the equation of the internal calibration, the real GlcN concentration of the sample was obtained at $y=0$, graphically represented by the concentration at the intercept of the calibration line with the x axis. This value was found to be 0.97 mM in the sample being analysed, corresponding to $9.8 \pm 0.3\%$ of chitin on insect dry mass. This result can be considered in line with chitin values found by the validating method in the no spiked prepupae and with the data reported in literature regarding the average chitin content in BSF prepupae (Caligiani et al. 2018; D'Hondt et al. 2020; Soetemans et al. 2020) where it always remains around 8–10% on DM.

Once found this starting concentration value, the recovery of chitin was performed by using the spiked insect samples mentioned above. After adding the different practical grade chitin weights to the samples, they were subjected to hydrolysis and derivatization protocol and the monomer recovery was determined by using UPLC-ESI/MS. Results are reported in Table 2.

As it is possible to observe from Fig. 3 and from the recovery results (Table 2), the higher is the amount of chitin in the sample, the lower the recovery of the monomer. According to the validation criteria (DIR 2001/22/CE), the recovery should remain between 80 and 100%, and the corresponding concentrations range allowing an acceptable chitin determination goes from 0.25 to 1 mM, expressed as GlcN. This is probably related to a matrix effect, evidenced also from the comparison of both calibrations, internal and external. In fact, the angular coefficients of the two calibration curves are different, suggesting the need to analyse samples in the specific range allowing more than 80% of recovery. Alternatively, it could be necessary to make for each analysis an internal calibration.

LOD and LOQ

The instrumental limit of detection and limit of quantification were calculated utilizing the S/N ratio methods, based

Fig. 3 Calibration curves with linear regression equations and correlation coefficient (R^2) generated by plotting peak areas versus chitin concentration (0.25–1.5 mM, expressed as glucosamine concentration after hydrolysis) of a practical grade chitin reference standard in BSF prepupa matrix; **b** practical grade chitin reference standard (A, area; SI, internal standard; CONC, concentration)

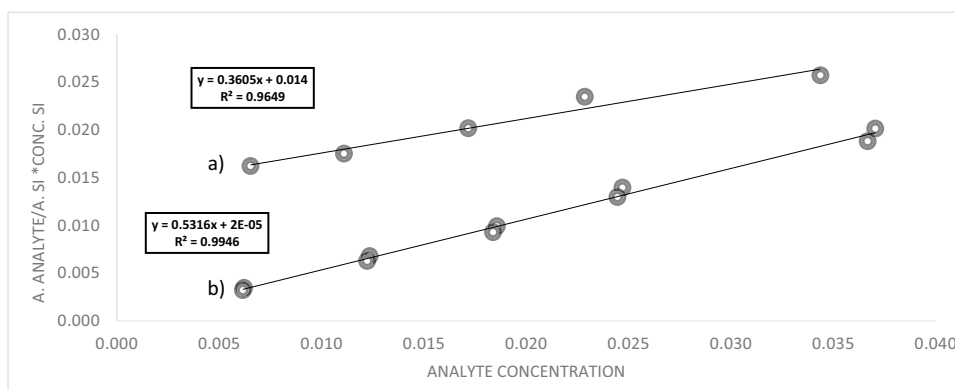


Table 2 Recovery of GlcN by UPLC-ESI/MS method after acid hydrolysis of chitin with 6 M HCL for 23 h under 110 °C

Amount of commercial practical grade chitin spiked (mg)	Amount of GlcN expected in the sample (mg) ± SD*	Amount of GlcN calculated in the sample (mg) ± SD	Recovery (%) ± SD**
26.8	44.9 ± 0.3	32 ± 4	71 ± 9
17.9	35.7 ± 0.8	29 ± 1	82 ± 5
13.4	31.2 ± 0.5	25 ± 3	81 ± 9
8.9	26.4 ± 0.6	21.9 ± 0.5	83 ± 4
4.4	22.7 ± 0.2	21 ± 3	90 ± 15

*% derived from the real chitin starting amount calculated with the in matrix line plus the chitin amount added

**Calculated chitin percentage vs expected

on the determination of the peak-to-peak noise (Apostol et al. 2009). LOD and LOQ were therefore calculated as the concentrations of practical grade chitin producing after hydrolysis a recognizable GlcN peak with an S/N ratio of, respectively, 3.3 and 10. At least, with the conditions of this method, the limit of detection (S/N ratio of 3.3) and the limit of quantification (S/N ratio of 10) values corresponded respectively to a concentration of 0.00068 mM and 0.00204 mM of chitin, corresponding, according to the present method, to a final concentration in a solid sample of 0.04% and 0.11%, respectively.

Repeatability

Repeatability was calculated at first as intraday precision (intermediate precision) on a BSF prepupae sample spiked with five chitin concentration levels (0.25, 0.5, 0.75, 1, and 1.5 mM expressed as GlcN concentration).

Samples were prepared using the same procedure (described in paragraph “[Preparation of Insects Spiked Samples](#)”) and equipment, in the same laboratory, and were analysed in triplicate within 1 day. The results of precision, expressed in Table 3, were compared with Horwitz predicted intra-laboratory precision (PRSD), calculated as $0.66 \times 2 \times c - 0.1505$, where c is the concentration level expressed as a mass fraction. A HORRAT value of less than 2 commonly indicates satisfactory precision results. From the repeatability results obtained (Table 1) is possible to observe that the Horwitz equation is always satisfied, also in the presence of different concentrations of chitin. It is

Table 3 Intra-day repeatability of the method, calculated on BSF prepupa spiked with different chitin levels

Chitin level (mM expressed as GlcN)	Intraday repeatability (RSD %)	Horwitz ratio for intraday repeatability
0.25	3.02	0.9
0.5	1.54	0.4
0.75	0.53	0.1
1	3.12	1.0
1.5	2.87	0.9

therefore possible to affirm that the method has a good intraday precision in the range of concentration considered.

Due to the possible lability of the GlcN and derivatized GlcN monomer, it was also interesting to evaluate whether the precision of the method was maintained over time and when it could no longer be defined as acceptable. Therefore, repeatability was also calculated as inter-day precision on a period of 4 weeks by analysing once a week a sample of hydrolysed practical grade chitin and a sample of hydrolysed BSF prepupae, prepared using the same procedure, in the same laboratory. One set of hydrolysed practical grade chitin and BSF prepupae was stored at -20 °C (acid environment), executing the derivatization ex novo every week, just before the analysis. A second identical set of hydrolysed practical grade chitin and BSF prepupae was instead derivatized with the AccQ Tag and stored at -20 °C for the following analyses. The quantification of GlcN of each set of samples was done by preparing every week an independent and fresh practical grade chitin calibration line. The results, reported in Table 4, were compared with Horwitz predicted intra-laboratory precision (PRSD).

The first evidence shown from the results is that the recovery and the chitin quantification followed the same trend during the time, whether the GlcN was derivatized at the moment or remained derivatized throughout the entire course of the experiment (4 weeks). This result suggests that the derivatization step is not the main factor affecting the stability of the analyte. On the other side, results suggest that the stability of GlcN overtime in the hydrolysed practical grade standard of chitin is different from the stability of the monomer in the real hydrolysed prepupae sample. In fact, comparing the two samples (Table 4), it can be noted that the GlcN degradation in the standard chitin is faster than in the real prepupa sample. In the insect sample, the recovery of the analyte remains identical in the first 2 weeks of the experiment, while for the standard chitin, already after the first week, it drops to a recovery around 60%. This result suggests that the stability over time of the monomer is related to the matrix. As reported by D’Hondt et al. (2020), in fact, the GlcN behaviour mostly depends on

Table 4 Inter-day repeatability of the method, calculated on practical grade chitin and BSF prepupa in two different storage conditions

Week	Practical grade chitin (stored after derivatization)		Practical grade chitin (derivatized immediately before the analysis)	
	Recovery (%)	RSD%	Recovery (%)	RSD%
1	109 ± 5	30.9	97 ± 4	32.2
2	63 ± 2		65 ± 2.2	
3	58 ± 1		55 ± 1.5	
4	50 ± 2		49 ± 1.3	
Week	BSF prepupae (stored after derivatization)		BSF prepupae (derivatized immediately before the analysis)	
	Quantification (% on WW)	RSD%	Quantification (% on WW)	RSD%
1	2.7 ± 0.2	24.7	2.7 ± 0.2	21.6
2	2.7 ± 0.5		2.2 ± 0.5	
3	1.8 ± 0.4		1.8 ± 0.3	
4	1.7 ± 0.3		1.7 ± 0.3	

the sample characteristics, such as its chitin content or, in the case of a biological sample, the presence of other molecules potentially affecting the analyte protection. In this regard, the absence of other substances in the commercial standard probably leads to a faster degradation of the free analyte during the storage. Instead, the lower amount of chitin and the presence of other components in the insect body bring to a reduced degradation during the time of the analyte. Hence, the less quantity of GlcN released, and it is bound with the other compounds probably protect it during a long-term storage from an excessive degradation.

Finally, the precision parameter, evaluated through RSD% calculated on the average of the quantification and recovery values found during the time in the two different matrices, suggests that in any case the analysis should be performed immediately after the samples preparation to obtain more precise results and prepare always a fresh hydrolysed practical grade chitin standard solution to build the external calibration, avoiding possible errors due to the matrix and time of storage.

Method Application

Amino Acids and Chitin Content in Insects and Food Supplements

After optimization and validation, the quantitative method developed was applied for the simultaneous determination of total amino acids and chitin in insect samples and then extended to other food matrices containing both polymers of GlcN and proteins. Specifically, two insect species (*Hermetia illucens* and *Alphitobius diaperinus*) were considered, based on their large use in the developing edible insect industry. The results related to amino acids and chitin content in the two insect samples, obtained on three replicates and reported as average percentage ± DS on dry weight,

are showed in Table 5. The amino acids content found with our method falls perfectly in the range of 37–56% from the literature (Bosch et al. 2014; Sánchez-Muros et al. 2014; Spranghers et al. 2017). Regarding chitin content, the new method reports an average chitin amount in BSF prepupae of 9.4% ± 0.8 dry matter basis. If compared with the chitin amount obtained from indirect chitin quantification methods, this value is quite different. For example, if theoretically calculated as reported Caligiani et al. (2018), i.e., by difference from the protein content, the chitin content in BSF prepupa is found higher than ours, ranging from 11.7 to 14.6%. If calculated following the method of Liu et al. (2012) (based on the weight of the residue obtained after the total elimination of protein and mineral), the chitin amount calculated is lower, around 6–7% (Spranghers et al. 2017). These differences are not so evident when the chitin percentage obtained with our simultaneous method is compared to the value obtained using direct quantification method. The chitin content in our samples results in fact in accord with the result of Caligiani et al. (2018) and D'Hondt et al. (2020) which used for the analyte quantification respectively GC–MS and LC analysis. Regarding the *Alphitobius diaperinus* larva, as a whole, the amino acids

Table 5 Total amino acid and GlcN (as equivalent chitin) content of black soldier fly prepupae (expressed as g/100 DM) compared with other matrices containing amino acids and chitin (*Alphitobius diaperinus* larvae, supplement 1 and supplement 2). The results are the mean of triplicate analysis

	Chitin (%DW)	Total amino acids (%DW)
<i>Hermetia illucens</i> prepupae	9.4 ± 0.8	43.9 ± 0.7
<i>Alphitobius diaperinus</i> larvae	7 ± 1	53 ± 1
Supplement 1	13 ± 2*	15 ± 1
Supplement 2	5.3 ± 0.1*	87 ± 3

*Expressed as chitin equivalents

and chitin amount obtained from the simultaneous method are coincident with those reported in literature. The chitin value only results slightly higher respect the results found in previous works. In the works of Leni et al. (2019) and Han and Heinonen (2021), for example, the chitin percentage is lower (around 4.5%) as compared to ours. Despite all, the discordances found are negligible considering that the differences in diets, rearing conditions, larval stage, etc., can affect the compositional properties of the same insect species (van Huis et al. 2013). With this new method is therefore possible to avoid the errors which occur when indirect methods are used and to obtain the same accurate results of direct quantification methods, but analysing two main insects' components simultaneously, dramatically reducing the analysis times. In order to extend the applicability and evaluate the efficiency of this developed quantitative method also on others matrix, two different commercial supplements containing GlcN in the form of GlcN hydrochloride or sulphate, chondroitin sulphate (supplement 1 and supplement 2) together with a protein source were analysed. The samples were prepared using the same conditions applied for insect samples. However, because the external calibration curve for the quantification of chitin is built using practical grade standard chitin, the results obtained are directly expressed as chitin amount. Hence, analysing samples containing free GlcN or other GlcN-containing polymers and not chitin, it is necessary to take in consideration that the final values of GlcN, using this calibration, will be expressed as chitin equivalents. The effectiveness of the method also on these commercial matrices could result very attractive for the pharmaceutical field, making possible to quantify faster and precisely amino acids and GlcN and assess the veracity of what is indicated on the label. The results obtained from the two samples analysed, shown in Table 5, were therefore compared with the values declared by the company on the label. According to the label, the supplement 2 is almost totally composed by amino acids, while GlcN amount is present in a very little percentage (around 3%). Regarding supplement 1, it was labelled as having 15% of GlcN and 12% of proteins. Despite the limitation of using chitin as external calibration, after the comparison of our results with the labels, it was possible to confirm the agreement between the values found by UPLC-ESI/MS and what declared on label by the producers, confirming the efficiency of the method also on non-biological matrices.

Conclusions

In this work, a UPLC-ESI/MS method already used for the total amino acid determination in insect matrix was optimized and validated also for the simultaneous quantification of chitin. The

acid hydrolysis carried out on the insect sample indeed allows not only the release of amino acids from the protein fraction but also of the GlcN from chitin. The method was thus adjusted in order to detect also free GlcN after hydrolysis, from which is possible to derive the insect chitin content. This newly developed UPLC-ESI/MS method proved to be simple and fast, and no fundamental modifications were needed as compared to the analysis of amino acids. During the validation tests, the method showed a good linearity, precision, recovery, and limits of quantifications for chitin. Moreover, from the results obtained from the analysis, a precise and accurate determination of insect protein and chitin fraction was obtained. The need to have a precise and accurate method to quantify chitin in insects is mainly related to the expected enhancement of its assumption by food/feed especially in the growing context of insect novel foods and insect-based feeds, which are mainly based on insect meals containing also chitin. Chitin presence in foods has important positive/negative impacts (possible prebiotics or, on the other hand, antinutritional factor) and its correct quantification is the basis to develop a correct risk assessment.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval This article does not contain any studies with humans or animals.

Consent to Participate Not applicable.

Competing Interests Anna Valentina Luparelli declares that she has no conflict of interest. Giulia Leni declares that she has no conflict of interest. Andrea Fuso declares that she has no conflict of interest. Clara Pedrazzani declares that she has no conflict of interest. Sara Palini declares that she has no conflict of interest. Stefano Sforza declares that he has no conflict of interest. Augusta Caligiani declares that she has no conflict of interest.

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References

- Anitha A, Sowmya S, Sudheesh Kumar PT, Deepthi S, Chennazhi KP, Ehrlich H, Tsurkan M, Jayakumar R (2014) Chitin and chitosan in selected biomedical applications. *Prog Polym Sci* 39:1644–1667. <https://doi.org/10.1016/j.progpolymsci.2014.02.008>
- Apostol I, Miller KJ, Ratto J, Kelner DN (2009) Comparison of different approaches for evaluation of the detection and quantitation limits of a purity method: a case study using a capillary isoelectrofocusing method for a monoclonal antibody. *Anal Biochem* 385:101–106. <https://doi.org/10.1016/j.ab.2008.09.053>
- Bierstedt A, Stankiewicz BA, Briggs DEG, Evershed RP (1998) Quantitative and qualitative analysis of chitin in fossil arthropods using a combination of colorimetric assay and pyrolysis-gas chromatography-mass spectrometry. *Analyst* 123:139–145. <https://doi.org/10.1039/a704799a>
- Bosch G, Zhang S, Oonincx DGAB, Hendriks WH (2014) Protein quality of insects as potential ingredients for dog and cat foods. *J Nutr Sci* 3. <https://doi.org/10.1017/jns.2014.23>
- Brigode C, Hobbi P, Jafari H et al (2020) Isolation and physicochemical properties of chitin polymer from insect farm side stream as a new source of renewable biopolymer. *J Clean Prod* 275:122924. <https://doi.org/10.1016/j.jclepro.2020.122924>
- Brück WM, Slater JW, Carney BF (2011) Chitin and chitosan from marine organisms. *Se-Kwon Kim Mar Biomater Isol Charact Appl CRC-Press*. New York 11–23. <https://doi.org/10.1201/EBK1439816035>
- Caligiani A, Marseglia A, Leni G et al (2018) Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin. *Food Res Int* 105:812–820. <https://doi.org/10.1016/j.foodres.2017.12.012>
- Chen W, Chiou RYY (1999) A modified chemical procedure for rapid determination of glucosamine and its application for estimation of mold growth in peanut kernels and koji. *J Agric Food Chem* 47:1999–2004. <https://doi.org/10.1021/jf981049f>
- Cortes Ortiz JA, Ruiz AT, Morales-Ramos JA et al (2016) Insect mass production technologies. *Insects as Sustain Food Ingredients* 153–201. <https://doi.org/10.1016/b978-0-12-802856-8.00006-5>
- D'Hondt E, Soetemans L, Bastiaens L et al (2020) Simplified determination of the content and average degree of acetylation of chitin in crude black soldier fly larvae samples. *Carbohydr Res* 488:107899. <https://doi.org/10.1016/j.carres.2019.107899>
- Díaz J, Lliberia JL, Comellas L, Broto-Puig F (1996) Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection. *J Chromatogr A* 719:171–179. [https://doi.org/10.1016/0021-9673\(95\)00372-X](https://doi.org/10.1016/0021-9673(95)00372-X)
- Finke MD (2007) Estimate of chitin in raw whole insects. *Zoo Biol* 26:105–115. <https://doi.org/10.1002/zoo.20123>
- Flannery MB, Stott AW, Briggs DEG, Evershed RP (2001) Chitin in the fossil record: identification and quantification of D-glucosamine. *Org Geochem* 32:745–754. [https://doi.org/10.1016/S0146-6380\(00\)00174-1](https://doi.org/10.1016/S0146-6380(00)00174-1)
- Frey B, Vilariño A, Schüpp H, Arines J (1994) Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. *Soil Biol Biochem* 26:711–717. [https://doi.org/10.1016/0038-0717\(94\)90263-1](https://doi.org/10.1016/0038-0717(94)90263-1)
- Geraedts MCP, Troost FJ, Fischer MAJG et al (2011) Direct induction of CCK and GLP-1 release from murine endocrine cells by intact dietary proteins. *Mol Nutr Food Res* 53:476–484. <https://doi.org/10.1002/mnfr.201000142>
- GUIDELINE, ICH Harmonised Tripartite et al (2005) Validation of analytical procedures: text and methodology. Q2 (R1) 1.20:05
- Hagen SR (1993) High-performance liquid chromatographic quantitation of phenylthiocarbamyl muramic acid and glucosamine from bacterial cell walls. *J Chromatogr A* 632:63–68. [https://doi.org/10.1016/0021-9673\(93\)80026-5](https://doi.org/10.1016/0021-9673(93)80026-5)
- Hahn T, Roth A, Febel E et al (2018) New methods for high-accuracy insect chitin measurement. *J Sci Food Agric* 98:5069–5073. <https://doi.org/10.1002/jsfa.9044>
- Han X, Heinonen M (2021) Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin. *Food Chem* 334:127577. <https://doi.org/10.1016/j.foodchem.2020.127577>
- Horwitz W, Albert R (2006) The Horwitz ratio (HorRat): a useful index of method performance with respect to precision. *J AOAC Int* 89:1095–1109. <https://doi.org/10.1093/jaoac/89.4.1095>
- Ishimaru Y, Tomonari S, Matsuoka Y et al (2016) TGF- β signaling in insects regulates metamorphosis via juvenile hormone biosynthesis. *Proc Natl Acad Sci U S A* 113:5634–5639. <https://doi.org/10.1073/pnas.1600612113>
- Jáč P, Los P, Spáčil Z et al (2008) Fast assay of glucosamine in pharmaceuticals and nutraceuticals by capillary zone electrophoresis with contactless conductivity detection. *Electrophoresis* 29:3511–3518. <https://doi.org/10.1002/elps.200800068>
- Janes KA, Calvo P, Alonso MJ (2001) Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv Drug Deliv Rev* 47:83–97. [https://doi.org/10.1016/S0169-409X\(00\)00123-X](https://doi.org/10.1016/S0169-409X(00)00123-X)
- Khoushab F, Yamabhai M (2010) Chitin research revisited. *Mar Drugs* 8:1988–2012. <https://doi.org/10.3390/md8071988>
- Koide SS (1998) Chitin-chitosan: properties, benefits and risks. *Nutr Res* 18:1091–1101. [https://doi.org/10.1016/S0271-5317\(98\)00091-8](https://doi.org/10.1016/S0271-5317(98)00091-8)
- Kouřimská L, Adámková A (2016) Nutritional and sensory quality of edible insects. *NFS J* 4:22–26. <https://doi.org/10.1016/j.nfs.2016.07.001>
- Kumar MNVR, Muzzarelli RAA, Muzzarelli C et al (2004) Chitosan chemistry and pharmaceutical perspectives. *Chem Rev* 104:6017–6084. <https://doi.org/10.1021/cr030441b>
- Leni G, Caligiani A, Sforza S (2019) Killing method affects the browning and the quality of the protein fraction of black soldier fly (*Hermetia illucens*) prepupae: a metabolomics and proteomic insight. *Food Res Int* 115:116–125. <https://doi.org/10.1016/j.foodres.2018.08.021>
- Liu L, Liu Y, Shin HD et al (2013) Microbial production of glucosamine and N-acetylglucosamine: advances and perspectives. *Appl Microbiol Biotechnol* 97:6148–6158. <https://doi.org/10.1007/s00253-013-4995-6>
- Martínez JP, Falomir MP, Gozalbo D (2014) Chitin: a structural biopolymer with multiple applications. In: *eLS*. <https://doi.org/10.1002/9780470015902.a0000694.pub3>
- Morganti P, Chen HD (2015) From the circular economy to a green economy. Note 1. Chitin nanofibrils as natural by-products to manage the human and environment ecosystems. *J Appl Cosmetol* 33:101–113
- Muthukrishnan S, Merzendorfer H, Arakane Y, Kramer KJ (2012) Chitin metabolism in insects. In: Gilbert LI (ed) *Insect molecular biology and biochemistry*. Academic Press, p 193–235. <https://doi.org/10.1016/B978-0-12-384747-8.10007-8>
- Muzzarelli RAA, Muzzarelli C (2006) Chitosan as a dietary supplement and a food technology agent. In: Biliaderis CG, Izydorczyk

- MS (eds) *Functional Food Carbohydrates*. Francis and Taylor, Orlando, FL, pp 215–248
- Nakamura H (2011) Application of glucosamine on human disease—osteoarthritis. *Carbohydr Polym* 84:835–839. <https://doi.org/10.1016/j.carbpol.2010.08.078>
- Nitschke J, Modick H, Busch E et al (2011) A new colorimetric method to quantify β -1,3-1,6-glucans in comparison with total β -1,3-glucans in edible mushrooms. *Food Chem* 263–273. <https://doi.org/10.1016/j.foodchem.2010.12.149>
- Prabaharan M (2008) Review paper: Chitosan derivatives as promising materials for controlled drug delivery. *J Biomater Appl* 23:5–36. <https://doi.org/10.1177/0885328208091562>
- Razdan A, Pettersson D (1994) Effect of chitin and chitosan on nutrient digestibility and plasma lipid concentrations in broiler chickens. *Br J Nutr* 72:277–288. <https://doi.org/10.1079/bjn19940029>
- Reginster JY, Deroisy R, Rovati LC et al (2001) Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. *Lancet* 357:251–256. [https://doi.org/10.1016/S0140-6736\(00\)03610-2](https://doi.org/10.1016/S0140-6736(00)03610-2)
- Rudall KM (1963) The chitin/protein complexes of insect cuticles. *Adv in Insect Phys* 1:257–313. [https://doi.org/10.1016/S0065-2806\(08\)60177-0](https://doi.org/10.1016/S0065-2806(08)60177-0)
- Sanches-Silva A, Ribeiro T, Albuquerque TG et al (2012) Ultra-high pressure LC determination of glucosamine in shrimp by-products and migration tests of chitosan films. *J Sep Sci* 35:633–640. <https://doi.org/10.1002/jssc.201100855>
- Sánchez-Muros MJ, Barroso FG, Manzano-Agugliaro F (2014) Insect meal as renewable source of food for animal feeding: a review. *J Clean Prod* 65:16–27. <https://doi.org/10.1016/j.jclepro.2013.11.068>
- Shahidi F, Abuzaytoon R (2005) Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. *Adv Food Nutr Res* 49:93–13. [https://doi.org/10.1016/S1043-4526\(05\)49003-8](https://doi.org/10.1016/S1043-4526(05)49003-8)
- Shahidi F, Arachchi JKV, Jeon YJ (1999) Food applications of chitin and chitosans. *Trends Food Sci Technol* 10:37–51. [https://doi.org/10.1016/S0924-2244\(99\)00017-5](https://doi.org/10.1016/S0924-2244(99)00017-5)
- Soetemans L, Gianotten N, Bastiaens L (2020) Agri-food side-stream inclusion in the diet of *alphitobius diaperinus*. Part 2: impact on larvae composition. *Insects* 11:190. <https://doi.org/10.3390/insects11030190>
- Sprangers T, Ottoboni M, Klootwijk C et al (2017) Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *J Sci Food Agric* 97:2594–2600. <https://doi.org/10.1002/jsfa.8081>
- van Huis A, Van Isterbeeck J, Klunder H, Mertens E, Halloran A, Muir G, Vantomme P (2013) Edible insects: future prospects for food and feed security. *FAO forestry paper No. 171*. <http://www.fao.org/docrep/018/i3253e/i3253e00.htm>
- Volpi N (2009) Capillary electrophoresis determination of glucosamine in nutraceutical formulations after labeling with anthranilic acid and UV detection. *J Pharm Biomed Anal* 49:868–871. <https://doi.org/10.1016/j.jpba.2009.01.006>
- Wan ACA, Tai BCU (2013) CHITIN — a promising biomaterial for tissue engineering and stem cell technologies. *Biotechnol Adv* 31:1776–1785. <https://doi.org/10.1016/j.biotechadv.2013.09.007>
- Yu Y, Shen M, Song Q, Xie J (2018) Biological activities and pharmaceutical applications of polysaccharide from natural resources: a review. *Carbohydr Polym* 183:91–101. <https://doi.org/10.1016/j.carbpol.2017.12.009>
- Zhu KY, Merzendorfer H, Zhang W et al (2016) Biosynthesis, turnover, and functions of chitin in insects. *Annu Rev Entomol* 61:177–196. <https://doi.org/10.1146/annurev-ento-010715-023933>
- Zhu X, Cai J, Yang J, Su Q (2005) Determination of glucosamine in impure chitin samples by high-performance liquid chromatography. *Carbohydr Res* 340:1732–1738. <https://doi.org/10.1016/j.carres.2005.01.045>

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