Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin

Han, Xiaocui

2021-01-01

Han , X & Heinonen , M 2021 , ' Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin ' , Food Chemistry , vol. 334 , 127577 . https://doi.org/10.1016/j.foodchem.2020.127577

http://hdl.handle.net/10138/354982
https://doi.org/10.1016/j.foodchem.2020.127577

Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin

Xiaocui Han* and Marina Heinonen

Department of Food and Nutrition, University of Helsinki, Post Office Box 66, Agnes Sjöbergin katu 2, FI-00014 Helsinki, Finland

* Corresponding author. Tel.: +358 452060603.

Email address: xiaocui.han@helsinki.fi (X. Han).

ABSTRACT

A precise quantification of insect chitin is needed in order to avoid overestimation of crude protein due to chitin-bound nitrogen. An UPLC/FLR method was optimized and validated for the determination of glucosamine (GlcN) hydrolyzed from chitin in insect materials. The method was applied for quantifying the chitin content in mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus*). A baseline separation was obtained using an Acquity HSS T3 C18 column, an external calibration curve of excellent linearity, and a low limit of detection and quantification of GlcN. Even though the recovery of GlcN from spiked cricket material was slightly lower compared to that using spectrophotometric method, the UPLC/FLR method proved a sensitive and specific method of quantification of insect chitin. Chitin contents in *T.molitor* and *A.domesticus* were 4.6 ± 0.1% and 4.5 ± 0.0% on dry matter basis, respectively. Less than 0.01 % of chitin was present in insect protein-enriched fractions extracted with 0.1 N NaCl at pH 10.

KEYWORDS:

insect; chitin; N-acetyl-glucosamine; glucosamine; UPLC/FLR; spectrophotometer.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE:

Chitin (PubChem CID: 174); Glucosamine (PubChem CID: 439213); N-acetyl-D-glucosamine (PubChem CID: 439174)

ABBREVIATIONS:

ADF, acid detergent fiber; AQC, 6-aminoquinolyl-N-hydroxylsuccinimidyl carbamate; DM, dry matter; GlcNAc, N-acetyl-D-glucosamine; GlcN, glucosamine; FMOC, fluorenylmethyloxycarbonyl; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate; OPA, o-phthalaldehyde; PMP, 1-phenyl-3-methyl-5-pyrazolone; PITC, Phenylisothiocyanate; SEC/MALLS, size-exclusion chromatography with multiangle laser-light scattering; SPI, soluble protein isolation.

1. INTRODUCTION

More than 2000 insect species have been reported as edible (Jongema, 2017) with the protein content of edible insects ranging from 5% to 77% based on dry matter (DM), and 7% to 48% based on fresh weight (Huis et al., 2013; Rumpold & Schluter, 2013). Insects as such or as protein-rich isolates constitute a sustainable source of protein for different types of foods (Huis et al., 2013). Besides protein, all insects also contain the polysaccharide chitin as a component of the exoskeleton (Kramer, Hopkins & Schaefer, 1995; Nation, 2016; Roberts, 1992). Even though chitin is associated with for instance anti-microbial, anti-virus, anti-fungal, and anti-tumor activity, it may have negative effect on the digestibility of insect protein (Finke, 2007; Makkar, Tran, Heuzé & Ankers, 2014). The role of insect chitin as an allergen is controversial due to the lack of data on the association between consumption of insects and allergic effects of chitin (EFSA, 2015).

Chitin is a linear polymer composed of β (1→4) linked N-acetyl-D-glucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose) (Fig. 1) (Roberts, 1992). Pure chitin (fully N-acetylated) as a homo-polymer is rarely found in nature, because a certain degree of de-acetylation occurs, giving a structure between chitin and chitosan (polymer of glucosamine (GlcN)) (Fig. 1) (Kardas, Struzczyk, Kucharska, Broek & Dam, 2012). Insect specific chitin binds to the structure-providing/cuticle proteins of the exoskeleton, making precise measurement of chitin challenging. Moreover, chitin is a high molecular polymer and it is not soluble in water and most solvents, which adds the difficulties to chitin quantification. Using size-exclusion chromatography with multiangle laser-light scattering (SEC/MALLS) analysis molecular weight of 116000 and 312000 have been determined for dried crab shell chitin and dried squid pen chitin, respectively, with degrees of polymerization of 560 and 1580 (Funahashi et al., 2017).

To date, there is no measurement directly linked to chitin in insect materials. Available data on chitin contents in some insects are indirectly calculated from acid detergent fiber (ADF) or acetyl group (Finke, 2007; Hahn et al., 2018). In non-protein ADF measurement, chitin is gravimetrically determined as the insoluble residue remaining after extraction by an acidified liquid; the total amount of amino acids from ADF fraction is subtracted. However, the non-protein ADF is not specific to chitin but to all non-acid-labile compounds. In addition, the analytical procedures for amino acids analysis might hamper the accuracy of the chitin measurement. The acetyl group liberated from complete hydrolysis of chitin is limited to untreated insect samples because of the drastic (e.g. acidic) conditions during insect processing can result in de-acetylation. Fluorescence microscopic observation of Calcofluor Whiter stained chitin in air-classified insect fractions was used to estimate the relative amount of

chitin in insects, where chitin amount was visually compared); but accurate levels of chitin was not studied (Sipponen et al., 2018).

Chitin is also present in other materials e.g. fungi, crab and shrimp waste. In these materials, chitin is frequently quantified based on its degradation in strong acid and subsequent measurement of the produced GlcN, colorimetrically (Bierstedt, Stankiewicz, Briggs & Evershed, 1998; Chen & Chiou, 1999; Frey, Vilariño, SchÜepp & Arines, 1994; Matcham, Wood & Jordan, 1984; Ride & Drysdale, 1972), or with liquid chromatographic system (Crespo, Martínez, Hernández & Lage Yusty, 2006; Ekblad & Näsholm , 1996; López-Cervantes, Sónchez-Machado & Delgado-Rosas, 2007). In the colorimetric method, the released GlcN is deaminated to produce 2, 5-anhydromannose which is then converted to a blue complex by a reaction with 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) and ferric chloride (FeCl3); this blue complex has a max adsorption at 650 nm (Tsuji, Kinoshita & Hoshino, 1969). In liquid chromatographic system, GlcN requires pre-column derivatization at the amino group to introduce a strong chromophore for UV detection, or a fluorophore for florescence detection. Phenylisothiocyanate (PITC) (Hagen, 1993; Liang, Leslie, Adebowale, Ashraf & Eddington, 1999), o-phthalaldehyde (OPA) (Eikenes, Fongen, Roed & Stenstrøm, 2005), 1-phenyl-3-methyl-5-pyrazolone (PMP) (Aghazadeh-Habashi, Carran, Anastassiades & Jamali, 2005), fluorenylmethyloxycarbonyl (FMOC) (Zhou, Waszkuc & Mohammed, 2005; Zhu, Cai, Yang & Su, 2005) and 6-aminoquinolyl-N-hydroxylsuccinimidyl carbamate (AQC) (Díaz, Lliberia, Comellas & Broto-Puig, 1996; Wang et al., 2008) have been reported as derivative reagents for GlcN. Because GlcN has two natural stereoisomers (α and β) whose interconversion in aqueous solution is not preventable, two peaks are shown in the chromatogram (Zhou et al., 2005).

A more accurate and specific analysis of insect chitin is needed *per se* and also in order to avoid overestimation of crude protein content due to chitin-bound nitrogen (chitin-N). To address this issue, we propose to measure GlcN as hydrolyzed from chitin using an UPLC/FLR method. The aims of this study were to (1) optimize a sensitive UPLC/FLR method for the quantification of chitin, (2) validate the UPLC/FLR method for the quantitation of chitin in mealworms and crickets and their isolated protein-rich fractions, and to (3) compare results obtained from insect chitin by using the UPLC/FLR method to a spectrophotometric method modified from Tsuji et al. (1969).

2. MATERIALS AND METHODS

2.1. Materials

Freshly frozen mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus*) were purchased from Pohjolan Hyönteistalous Oy (Finland) and Entocube Oy (Finland), respectively. The mealworms were at larval stage and crickets at adult stage. Reference standard D (+)-glucosamine hydrochloride (GlcN-HCl) was purchased from Sigma-Aldrich (Steinheim, Germany). A commercial dietary supplement D (+)-glucosamine hydrochloride was purchased from Orion Corporation (Finland). HPLC grade acetonitrile (ACN), trifluoroacetic acid (TFA), triethylamine (TEA), heptane, and N-(9-fluorenylmethoxy-carbonyloxy) succinimide (FMOC-Su) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade water was purified by Milli-Q equipment (Millipore Corp., Bedford, MA, USA). All other chemicals were of analytical grade. MBTH and $FeCl_3$ were from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), potassium hydrogen sulphate ($KHSO_4$), and ammonium amidosulfonate ($NH_4SO_3NH_2$) were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of Insect Flour and Soluble Protein Isolation (SPI)

Mealworms and crickets were freeze-dried, ground into powder, and kept in desiccator under -20 °C as insect flours until use. Soluble protein isolation (SPI) was prepared by solubilizing insect flour in aqueous solution according to procedure used in isolation of plant-based proteins (Liu, Damodaran & Heinonen, 2019) with some modifications. Mealworm flour was defatted using heptane (flour: heptane = 1:10, w/v). Defatted mealworm four was dissolved in NaCl (0.1 M, flour:NaCl = 1:40, w/v), pH adjusted to 10 with 2 M NaOH, shaking overnight at 4°C. After centrifuging (27 300 × g, 5 °C, 15 min), the supernatant was collected and adjusted to pH 7, finally freeze-dried and stored at -20°C for further analysis. Cricket flour was directly mixed with 0.1M NaCl for protein extraction without defatting as according to pretesting this step was not necessary in order to improve protein isolation (Lukkari, 2018).

2.3. Chitin Hydrolysis after Removal of Protein

Protein in insect flours and SPIs were removed by alkaline hydrolysis according to Kardas et al. (2012) with modifications. Briefly, each insect flour / SPI was mixed with 0.5 M NaOH (flour: NaOH2 = 1:20, w/v) and agitated at room temperature for 0, 2 and 4 hours until the proteins were removed to a level not interfering with GlcN detection in HPLC-FLR system. After

centrifuging (27 300 × g, 5 °C, 15 min), the pellet was collected and washed with distilled water until neutral. At last the pellet was freeze-dried and stored in desiccator at room temperature as chitin flour until further use.

Chitin flour was dissolved in 6 M HCl (flour: HCl =10 mg /3 ml) and incubated at 100 °C over 6 hours, shaking every half an hour. Higher temperature of 110 °C was also applied in testing the recovery for the spectrophotometric analysis. The hydrolysate was cooled down and stored at 5 °C for further analysis.

2.4. Derivatization of GlcN with FMOC-Su

The derivatization of GlcN was performed using FMOC-Su according to Zhou et al. (2005) reporting high accuracy and precision with optimum derivatization conditions and a stable end product. Moreover, the derivatization reaction using FMOC is less time consuming in comparison to use of other reagents e.g. OPA. FMOC-Su derivatization of GlcN of both standard and sample solutions (adjusted to pH 11) were carried out with excess FMOC-Su reagent for 45 min, followed by dilution with mobile phases A/B (1/1, v/v). Each diluted sample solution was filtered through 0.2 μm film (Acrodisc GHP) into an LC vial for UPLC/FLR analysis.

2.5. Optimization and Validation of the UPLC/FLR Method

A Waters Acquity Ultra Performance LC system (Milford, MA, USA) was used for the analysis of GlcN-FMOC-Su derivatives. The integrated UPLC system consisted of a binary solvent manager, an auto-sampler, a column oven, a photodiode array detector (PDA; 210-600 nm), and a FLR detector. Waters Empower 2 software was used.

The chromatographic conditions were modified according to Ekblad & Näsholm (1996) and Zhou et al. (2005). Two reversed phase columns, a high-strength silica T3 (HSS, 2.1 mm ID × 150 mm, 1.8μm particles, ACQUITY UPLC ®, Waters) and an ethylene bridged hybrid (BEH, 2.1 mm ID × 100 mm, 1.7μm particles, Waters) column were tested at 30 °C for the separation of two isomeric GlcN-FMOC (α and β) derivatives of chitin. The mobile phase was constituted with Milli-Q Water (0.05% TFA) (mobile phase A) and ACN (mobile phase B). The gradient schedule was (a) 0-8 min, B, 27%; (b) 8-9.5 min, B, 27→100%; (c) 9.5-10 min, B, 100→27%; (d) 10-14 min, B, 27%. Flow rate ranging from 0.32 to 0.50 mL/min was tested. The absorption spectra was recorded from 210 to 400 nm; FLR was set at λex = 260 nm and λem = 330 nm.

The purity of each peak on the chromatograms from UPLC/FLR was checked by comparing the absorption spectra recorded by PDA. The comparison of the spectra and retention times of peaks between the standard and unknowns were compared to ensure the identity match. Data acquired from FLR were used for quantification of GlcN. A GlcN-HCl dietary supplement was used to optimize the UPLC/FLR method. The resolution of the two GlcN-FMOC isomers, their theoretical plate number (USP), and the peak height/shape were compared between the two columns.

Quantification of GlcN was performed by external calibration using GlcN-HCl as the reference standard. The linearity, limit of detection (LOD: signal-to-noise ratio, S/N = 3), and limit of quantitation (LOQ: 3 times of the LOD) of GlcN-HCl were determined. For linearity, the determination coefficient was used to evaluate the precision of the calibration curve (should be ≥0.999). A second GlcN-HCl standard solution was prepared as a control sample to check the precision of calibration. The control sample was injected and analyzed within each sample set. The RSD of experimental values of GlcN-HCl in the control sample obtained with using independent calibration curves for seven days was used to evaluate the accuracy and precision of the calibration. The expectation was that the difference between the average experimental value and true value of the control sample should be within 3%. Data from the control sample was also used to provide information about the stability of GlcN-FMOC derivatives. The optimized UPLC/FLR method was thereafter applied to determination of chitin in the insect samples, insect flour and SPI, prepared from both mealworms and crickets.

2.6. Recovery Test

The sample matrix (10.0 mg of cricket chitin flour) and the hydrolysis buffer (HCl 6M) were spiked with a known amount of GlcN-HCl at one level (4.0 mg), in triplicate. Chitin was hydrolyzed, derivatized, and determined by both the UPLC/FLR and the spectrophotometric method. The effect of hydrolysis temperature (100 °C and 110 °C) on recovery was tested. The recovery was calculated by comparing the determined amount of GlcN-HCl in the spiked samples to that of the amount of added GlcN-HCl.

The amount of chitin, W_{chitin} , was calculated using the mass of GlcN-HCl and the mass of the insect material applied in both HPLC/FLR and spectrophotometric methods. A factor 1.02588 was considered for the molecular weights of GlcN-HCl and GlcNAc, assuming a degree of acetylation of 100%. The amount of chitin was expressed as GlcNAc (%, DM):

$$W_{chitin}[\%] = \frac{1{,}02588 \times \mathrm{M}_{GlcN-HCl}\,[g]}{\mathrm{W}_{mass}[g]} \times 100\%$$

2.7. Spectrophotometric Determination of GlcN

The measurement of GlcN using a spectrophotometric method was performed according to Tsuji et al. (1969). In brief, the GlcN from chitin hydrolysate was deaminated with nitrous acid produced via addition of KHSO4 and NaNO2 followed by reaction with MBTH to produce a blue complex. The absorbance was measured at wavelength of 650 nm. The amount of GlcN in the insect samples (insect flour and SPIs) was calculated using external standard curve with GlcN-HCl.

3. RESULTS AND DISCUSSION

3.1. Optimization and Validation of UPLC Method

Mobile phase consisting of water (0.05% TFA) and ACN using a 14-minutes gradient programme at a flow rate of 0.5 mL/min was found optimal for the separation of GlcN-FMOC-Su derivatives with both HSS and BEH columns. The same mobile phase was also used by Zhou et al. (2005), but with a higher flow rate of 0.8 mL/min and a different column, ODS-3 100 Å, (3.2 mm ID × 150 mm, 5µm particles, Prodigy ™, Phenomenex), which consumed more ACN. In this study, the HSS column produced sharper and narrower peaks (the 2 natural stereoisomers, α and β) than those obtained by using the BEH column (Fig. 2). Moreover, the height of the GlcN-FMOC-Su α peak (Rt = 3.5 min) was about 4-fold higher than that in the BEH column (Rt=2.1 min) for an equal injection volume of the same sample solution (Fig. 2). The USP peak resolution of the HSS column was higher than that of the BEH column (2.5 vs 1.7). The theoretical plate number was 2-fold larger for the HSS column than that of the BEH column (2907 vs 1469 for GlcN-FMOC-Su α; 3768 vs 1855 for GlcN-FMOC-Su β). Under the optimal UPLC conditions, two GlcN-FMOC-Su peaks were clearly resolved within 14 min (Fig. 2), which was faster as compared to over 30 min of running time required when using HPLC (Hagen, 1993; López-Cervantes et al., 2007). As a conlusion, the HSS column was selected for the method validation and analysis of GlcN-FMOC-Su in insect materials.

The external calibration curve showed excellent linearity within the range of 0.0033-24.0 ng/inj. with the determination coefficient of more than 0.999 throughout the study period. The instrumental LOD of 0.00095 ng/inj. was 1000 times lower than that in the HPLC/UV method (3 ng/inj.) (Zhou et al., 2005) used as a standard method for dietary supplements containing glucosamine sulfate or glucosamine hydrochloride. Due to the greatly improved sensitivity of the UPLC/FLR system, it was possible to analyze samples with a GlcN as low as 0.01 g/100 g, e.g. chitin containing protein-rich isolates of insect materials. The difference between the average value and true value of the control sample obtained in seven days with the independent external calibration curves was 2.5% (RSD 5.5%) (Table 1), indicating good precision and stability of the external calibration

194 curve. Results of the control sample also showed that once formed, the GlcN-FMOC-Su derivatives were stable at 5°C for at
195 least seven days.

196 The recovery of GlcN-HCl added to cricket chitin flour after hydrolysis at one level (4 mg/10 mg) and determined by using
197 UPLC/FLR was increased from 60.8 ± 6.8% to 75.8 ± 9.3% due to lowering the incubation temperate from 110 °C to 100 °C
198 (Table 2). Higher recovery (82.3 ± 5%) has been reported for commercial chitin from crab shells hydrolyzed with 6 M HCl at
199 100 °C for 13 hours (Crespo et al., 2006). According to Crespo et al. (2006), acid treatment at a relatively higher temperature
200 result in breakdown of GlcN and thus decrease the recovery. Degradation of the chitin chain to form oligosaccharides has been
201 reported to occurre during the first few minutes of the acid hydrolysis, followed by further degradation of the oligosaccharides
202 to produce GlcNAc, and finally to yield GlcN and acetic acid (Einbu & Varum, 2007; Hackman, 1962). Thus, the hydrolysis
203 of the chitin and the recovery pf GlcN are dependent on the HCl concentration, temperature, and incubation time (Crespo et
204 al., 2006; Einbu & Varum, 2007; Hackman, 1962).

205 The insect matrix had no effect on the recovery of GlcN-HCl, because a similar GlcN-HCl recovery (74.6 ± 9.1%) was obtained
206 for the hydrolysis buffer (Table 2). The spectrophotometric method resulted in a higher recovery (92.2 ± 4.0%) of GlcN-HCl
207 added to the deproteinized cricket chitin flour as compared to the recovery from the UPLC/FLR method (Table 2). Overall, the
208 amount of chitin in all insect material analyzed resulted in a slightly higher value with using the spectrophotometric method as
209 compared to the values with using the UPLC/FLR method. This can be explained with the relatively higher GlcN-HCl recovery
210 in the spectrophotometric method than in the UPLC/FLR method. The reason for the relatively lower recovery of the
211 UPLC/FLR method might be due to an incomplete derivatization of GlcN by FMOC-Su. The amount of FMOC-Su was always
212 excessively added compared to GlcN (Zhou et al., 2005). However, FMOC-Su reacts with not only GlcN, but also with water
213 which was added to make the most solubility of FMOC-Su and to avoid formation of oily droplets during derivatization
214 procedure (Díaz et al., 1996). A further study to optimize the derivatization of GlcN with FMOC-Su should be conducted to
215 improve the recovery.

216 Even though the spectrophotometric detection of GlcN resulted in higher recovery than UPLC/FLR (Table 2), the
217 spectrophotometric method is not specific to GlcN. Other hexosamines and aldehydes based on the total carbohydrates present
218 in the reaction mixture, regardless of the source, could be deaminated and then reacting with MBTH to exhibit a blue color
219 under measuring condition (Sawicki, Hauser, Stanley & Elbert, 1961; Tsuji et al., 1969). The UPLC/FLR method is specific to

GlcN and a complete separation of the two GlcN-FMOC-Su isomers was achieved. Five spectra from each single GlcN-FMOC-Su peak had excellent match from 210 to 400 nm. Spectra from different peaks also showed good match either from standard or insect samples, indicating no interference or co-elution on both GlcN-FMOC-Su peaks. One example of the chromatograms is displayed in Fig. 3.

The hypothesis of this study was that insect chitin can be fully hydrolyzed into GlcN and thereafter determined by using either UPLC/FLR or spectrophotometry. It was further hypothesized that the amount of chitin as GlcNAc could be calculated from. However, the amount of GlcNAc would be a slight overestimation of chitin due to the unknown amount of N-acetylated units in insect chitin. It has been shown that the fraction of N-acetylation (Fa) is nearly 1 (between 0.9 and 1.0) in naturally existing chitin (Roberts, 1992). Moreover, it is postulated, although it is challenging to determine due to insolubility of chitin, that the length of chitin polymer, or degree of the polymerization, also have a slight effect on the conversation factor between GlcNAc and chitin. Should the difference between GlcNAc and chitin with regards to Fa and the degree of polymerization be small, the GlcNAc values could be applied to roughly estimate the insect chitin levels.

3.2. Chitin Amount in Selected Insects

The proteins were removed by alkaline treatment from the insect samples prior to chitin analysis in order not to interfere with the detection of GlcN-FMOC-Su. An example of chromatograms are shown to illustrate the effect of deproteinizing on the presence of interfering compounds (Fig. 4). Two GlcN-FMOC-Su peaks in cricket samples with and without deproteinizing were identified by comparing their retention times to those in the standard material. Without deproteinizing, the peaks of the interfering compounds were not separated from those of the target compounds. On the contrary, there were no interfering compound peaks appearing close to the target peaks in samples subject to deproteinizing prior to analysis of chitin as GlcN-FMOC-Su derivatives. In this study NaOH was successfully used for deproteinization although other methods e.g. enzymatic methods should be considered for environmental sustainability.

The amount of chitin was 4.6 ± 0.1%, DM in crickets (*A.domesticus)* and 4.5 ± 0.1 %, DM in mealworms (*T. molitor)* as measured by UPLC/FLR. The amount of chitin in crickets was significantly higher than that reported by Finke (2007) (0.007% DM) with using a calculation method based on determination of non-protein ADF. The chitin content in the mealworms was also much higher as compared to the value (0.01 %, DM) based on the non-protein ADF (Finke, 2007), but slightly lower as compared to the value (about 7.5%) based on acetyl group determination (Hahn et al., 2018). In addition to analytical methods,

246 the differences in chitin values may also be explained by differences in the insect feeding patterns as it is known that the rearing
247 conditions have a significant effect on composition even within the same species (Huis et al., 2013). The UPLC/FLR method
248 was also suitable for detecting very low amounts of chitin as the chitin content was less than 0.01% DM in the fractions (SPI)
249 isolated from *A.domesticus* and *T.molitor*.

250 At present, data on chitin contents in insects is most often indirectly calculated from acid detergent fibre (Finke, 2007) although
251 also other methods exist. A specific method of analysis of insect chitin is needed for accurate compositional (nutritional) data
252 and for enabling insect derived fractions to be allowed as safe novel food and feed ingredients. This study showed that a certain
253 amount of nitrogen from selected insects is contributed by chitin. Thus, it is suggested to use UPLC/FLR method for chitin
254 analysis in order to avoid overestimation of the crude protein content of insect materials with using the nitrogen-to-protein
255 conversion factor 6.25 unless applying suggested specific factors such as 4.76 for *T.molitor* (Janssen, Vincken, van den Broek,
256 Fogliano & Lakemond, 2017) and 5.09 for *A.domesticus* (Ritvanen, Pastell, Welling & Raatikainen, 2020). On the other
257 hand, the UPLC/FLR quantification of chitin also contributes to the more accurate estimation of fiber content since chitin is
258 the most common form of fiber in insects (Huis et al., 2013).

259 **4. CONCLUSION**

260 The UPLC/FLR method displayed a good performance resulting in sensitive and specific quantitation of insect chitin after
261 alkaline deproteinization and acid hydrolysis of chitin. This study shows that alkaline removal of protein prior to hydrolysis
262 of chitin is essential for UPLC/FLR as well as spectrophotometric analysis of GlcN. The chitin levels in both *A.domesticus*
263 and *T.molitor* were similar (~5% DM) with chitin expressed as the amount of GlcNAc. This expression may have resulted in
264 a slight overestimation of insect chitin considering the Fa units and the degree of polymerization of insect chitin. However,
265 chitin amount as measured by UPLC/FLR contributes to the more accurate analysis of both protein and fiber content of
266 edible insects.

267

268 **ACKNOWLEDGEMENT**

269 The authors would like to thank Miikka Olin, Dr. Minnamari Edelmann and Dr. Bhawani Chamlagain for their technical support.

REFERENCES

Aghazadeh-Habashi, A., Carran, J., Anastassiades, T., & Jamali, F. (2005). High performance liquid chromatographic determination of N-butyryl glucosamine in rat plasma. In *Journal of Chromatography B* (Vol. 819, Issue 1, pp. 91–96). https://doi.org/https://doi.org/10.1016/j.jchromb.2005.01.034.

Bierstedt, A., Stankiewicz, B. A., Briggs, D. E. G. & Evershed, R. P. (1998). Quantitative and qualitative analysis of chitin in fossil arthropods using a combination of colorimetric assay and pyrolysis–gas chromatography–mass spectrometry. Analyst, 123(1), 139–145. https://doi.org/10.1039/A704799A.

Chen, W., & Chiou, R. Y. (1999). A modified chemical procedure for rapid determination of glucosamine and its application for estimation of mold growth in peanut kernels and koji. *Journal of Agricultural and Food Chemistry*, *47*(5), 1999–2004. https://doi.org/jf981049f [pii].

Crespo, M. O. P., Martínez, M. V., Hernández, J. L., & Yusty, M. A. L. (2006). High-performance liquid chromatographic determination of chitin in the snow crab, Chionoecetes opilio. In *Journal of Chromatography A* (Vol. 1116, Issue 1, pp. 189–192). https://doi.org/https://doi.org/10.1016/j.chroma.2006.03.068.

Díaz, J., Lliberia, J. L., 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. In *Journal of Chromatography A* (Vol. 719, Issue 1, pp. 171–179). https://doi.org/https://doi.org/10.1016/0021-9673(95)00372-X.

EFSA, S. C. (2015). Risk profile related to production and consumption of insects as food and feed. *EFSA Journal*, *13*(10), 4257. https://doi.org/10.2903/j.efsa.2015.4257.

Eikenes, M., Fongen, M., Roed, L., & Stenstrøm, Y. (2005). Determination of chitosan in wood and water samples by acidic hydrolysis and liquid chromatography with online fluorescence derivatization. In *Carbohydrate Polymers* (Vol. 61, Issue 1, pp. 29–38). https://doi.org/https://doi.org/10.1016/j.carbpol.2005.02.006.

Einbu, A., & Varum, K. M. (2007). Depolymerization and de-N-acetylation of chitin oligomers in hydrochloric acid. *Biomacromolecules*, *8*(1), 309–314. https://doi.org/10.1021/bm0608535 [doi].

Ekblad, A., & Näsholm, T. (1996). Determination of chitin in fungi and mycorrhizal roots by an improved HPLC analysis of glucosamine. *Plant and Soil*, *178*(1), 29–35. https://doi.org/10.1007/BF00011160.

Finke, M. D. (2007). Estimate of chitin in raw whole insects. *Zoo Biology*, *26*(2), 105–115. https://doi.org/10.1002/zoo.20123 [doi].

Frey, B., Vilariño, A., SchÜepp, H., & Arines, J. (1994). Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular-arbuscular mycorrhizal fungus Glomus intraradices. In *Soil Biology and Biochemistry* (Vol. 26, Issue 6, pp. 711–717). https://doi.org/https://doi.org/10.1016/0038-0717(94)90263-1.

Funahashi, R., Ono, Y., Qi, Z.-D., Saito, T., & Isogai, A. (2017). Molar masses and molar mass distributions of chitin and acid-hydrolyzed chitin. In *Biomacromolecules, 18(12), 4357-4363.* https://doi.org/10.1021/acs.biomac.7b01413.

Janssen, R. H., Vincken, J. P., van den Broek, L. A., Fogliano, V., & Lakemond, C. M. (2017). Nitrogen-to-Protein Conversion Factors for Three Edible Insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens. Journal of agricultural and food chemistry, 65(11), 2275–2278. https://doi.org/10.1021/acs.jafc.7b00471

Hackman, R. H. (1962). Studies on chitin: action of mineral acids on chitin. *Aust J Biol Sci* 15(3):526-532.

Hagen, S. R. (1993). High-performance liquid chromatographic quantitation of phenylthiocarbamyl muramic acid and glucosamine from bacterial cell walls. In *Journal of Chromatography A* (Vol. 632, Issue 1, pp. 63–68). https://doi.org/https://doi.org/10.1016/0021-9673(93)80026-5.

Hahn, T., Roth, A., Febel, E., Fijalkowska, M., Schmitt, E., Arsiwalla, T., & Zibek, S. (2018). New methods for high-accuracy insect chitin measurement. *Journal of the Science of Food and Agriculture*, *98*(13), 5069–5073. https://doi.org/10.1002/jsfa.9044 [doi].

Huis, A., Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013). Edible insects: future prospects for food and feed security. In *FAO Forestry Paper* (p. xvi + 187 pp.).

Jongema, Y. (2017). List of edible insects of the world (April 1, 2017). https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm Accessed 21 October 2019.

Kardas, I., Struzczyk, M. H., Kucharska, M., Broek, L. A. M., Dam J. E. G. (2012). Chitin and chitosan as functional biopolymers for industrial applications. In: Navard P. Editor. The European polysaccharide network of excellence (EPNOE) research initiatives and results. Vienna: Springer. p 329-373.

Kramer, K. J., Hopkins, T. L., & Schaefer, J. (1995). Applications of solids NMR to the analysis of insect sclerotized structures. In *Insect Biochemistry and Molecular Biology* (Vol. 25, Issue 10, pp. 1067–1080). https://doi.org/https://doi.org/10.1016/0965-1748(95)00053-4.

Liang, Z., Leslie, J., Adebowale, A., Ashraf, M., & Eddington, N. D. (1999). Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC. In *Journal of Pharmaceutical and Biomedical Analysis* (Vol. 20, Issue 5, pp. 807–814). https://doi.org/https://doi.org/10.1016/S0731-7085(99)00085-0.

Liu, C., Damodaran, S., & Heinonen, M. (2019). Effects of microbial transglutaminase treatment on physiochemical properties and emulsifying functionality of faba bean protein isolate. In *LWT* (Vol. 99, pp. 396–403). https://doi.org/https://doi.org/10.1016/j.lwt.2018.10.003.

López-Cervantes, J., Sónchez-Machado, D. I., & Delgado-Rosas, K. E. (2007). Quantitation of Glucosamine From Shrimp Waste Using HPLC. *Journal of Chromatographic Science*, *45*(4), 195–199. https://doi.org/10.1093/chromsci/45.4.195.

Lukkari, T. (2018). Effect of processing on functional properties of proteins extracted from insects. Prosessoinnin vaikutus hyönteisistä eristetyn proteiinin toiminnallisiin ominaisuuksiin. [M.Sc. Thesis]. Available at: https://helda.helsinki.fi/handle/10138/236033.

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. In *Animal Feed Science and Technology* (Vol. 197, pp. 1–33). https://doi.org/https://doi.org/10.1016/j.anifeedsci.2014.07.008.

Matcham, S. E., Wood, D. A., & Jordan, B. R. (1984). The measurement of fungal growth in solid substrates. *Applied Biochemistry and Biotechnology*, *9*(4), 387–388. https://doi.org/10.1007/BF02798989.

Nation, J. L. (2016). *Insect physiology and biochemistry (2 ed.)*. Boca Raton USA, (Chapter 2).

Ride, J. P., & Drysdale, R. B. (1972). A rapid method for the chemical estimation of filamentous fungi in plant tissue. In *Physiological Plant Pathology* (Vol. 2, Issue 1, pp. 7–15). https://doi.org/https://doi.org/10.1016/0048-4059(72)90043-4.

Ritvanen, T., Pastell, H., Welling, A., & Raatikainen, M. (2020). The nitrogen-to-protein conversion factor of two cricket species - Acheta domesticus and Gryllus bimaculatus. Agricultural and Food Science, 29(1), 1–5. https://doi.org/10.23986/afsci.89101

Roberts, G. A. F. (1992). *Chitin chemistry* (Roberts GAF (ed.). Macmillan Education UK, (Chapter 1).

Rumpold, B. A., & Schluter, O. K. (2013). Nutritional composition and safety aspects of edible insects. *Molecular Nutrition & Food Research*, *57*(5), 802–823. https://doi.org/10.1002/mnfr.201200735 [doi].

Sawicki, E., Hauser, T. R., Stanley, T. W., & Elbert, W. (1961). The 3-Methyl-2-benzothiazolone Hydrazone Test. Sensitive New Methods for the Detection, Rapid Estimation, and Determination of Aliphatic Aldehydes. *Analytical Chemistry*, *33*(1), 93–96. https://doi.org/10.1021/ac60169a028.

Sipponen, M. H., Mäkinen, O. E., Rommi, K., Heiniö, R.-L., Holopainen-Mantila, U., Hokkanen, S., Hakala, T. K., & Nordlund, E. (2018). Biochemical and sensory characteristics of the cricket and mealworm fractions from supercritical carbon dioxide extraction and air classification. *European Food Research and Technology*, *244*(1), 19–29. https://doi.org/10.1007/s00217-017-2931-1.

Tsuji, A., Kinoshita, T., & Hoshino, M. (1969). Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-

methyl-2-benzothiazolone hydrazone hydrochloride. *Chemical & Pharmaceutical Bulletin*, *17*(7), 1505–1510. https://doi.org/10.1248/cpb.17.1505 [doi].

Wang, X., Chen, X., Chen, L., Wang, B., Peng, C., He, C., Tang, M., Zhang, F., Hu, J., Li, R., Zhao, X., & Wei, Y. (2008). Optimizing high-performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a pharmacokinetic study. *Biomedical Chromatography : BMC*, *22*(11), 1265–1271. https://doi.org/10.1002/bmc.1056 [doi].

Zhou, J. Z., Waszkuc, T., & Mohammed, F. (2005). Determination of glucosamine in raw materials and dietary supplements containing glucosamine sulfate and/or glucosamine hydrochloride by high-performance liquid chromatography with FMOC-Su derivatization: collaborative study. *Journal of AOAC International*, *88*(4), 1048–1058.

Zhu, X., Cai, J., Yang, J., & Su, Q. (2005). Determination of glucosamine in impure chitin samples by high-performance liquid chromatography. In *Carbohydrate Research* (Vol. 340, Issue 10, pp. 1732–1738). https://doi.org/https://doi.org/10.1016/j.carres.2005.01.045.

Table 1 Amount of GlcN-HCl of control sample, calculated using the external calibration curve in 7 days

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Weight of GlcN-HCl	(mg)	23.8	23.8	23.8	23.8	23.8	23.8	23.8
Calculated weight of GlcN-HCl	(mg)	24.1	23.0	23.9	25.7	26.5	22.9	24.7
Average	(mg)	24.4 （±5.5 %, RSD）						
Difference between the average value and true value		2.5 %						

363

364

Table 2 Recovery of GlcN-HCl by UPLC-FLR and spectrophotometric methods after acid hydrolysis of chitin with 6M HCl for 6 hours under 110°C and 100°C

Samples	Spiked (mg)	Recovery (%)		Spiked (mg)	Recovery (%)	
	UPLC	110°C	100°C	UV	110°C	100°C
De-proteined cricket	4.2	60.8 (± 6.8)	75.8 (± 9.3)	3.9	NA	92.2 (±4.0)
Hydroysis buffer	4.2	48.5 (± 9.3)	74.6 (± 9.1)	4.0	NA	96.5 (±0.8)
	No. of determination=3			No. of determination=2		NA: not analyzed

365

366

367

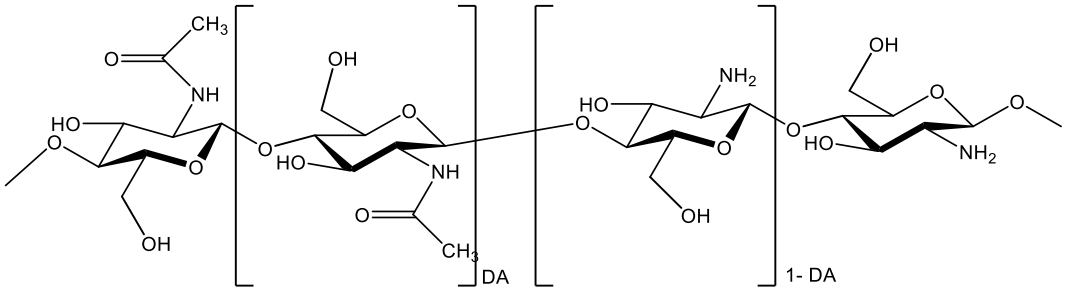
368

369

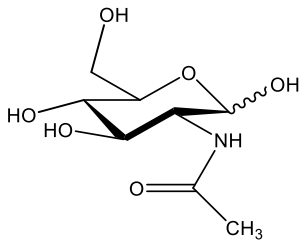
370

371

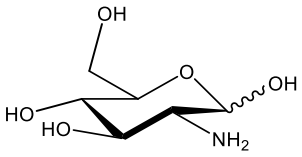
Figures



(a) Chitin / Chitosan



(b) N-acetyl-D-glucosamine (GlcNAc)



(c) Glucosamine (GlcN)

Fig. 1 Structures of (a) chitin poly (N-acetyl-β-D-glucosamine), when the average degree of acetylation (DA) is above 50 %; chitosan poly (D-glucosamine), when DA is less than 50 %. (b) N-acetyl-β-D-glucosamine (GlcNAc) and (c) D-glucosamine (GlcN). (Graphic program used: ChemDraw Professional)

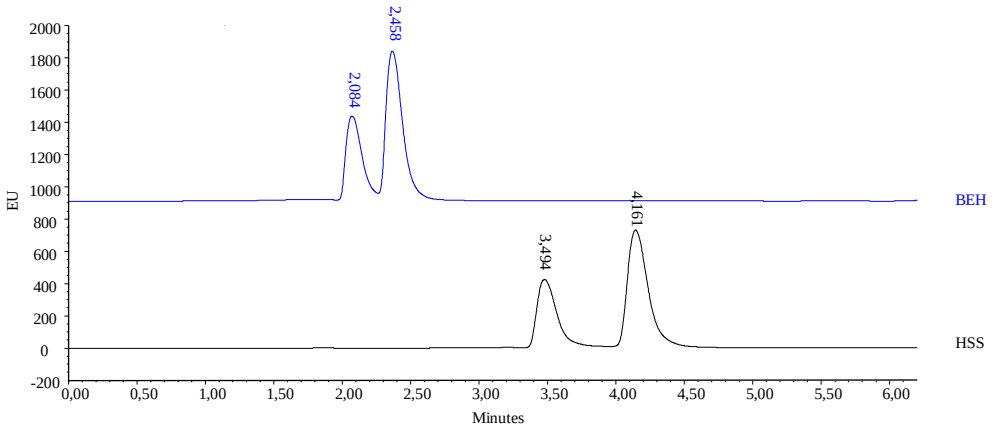
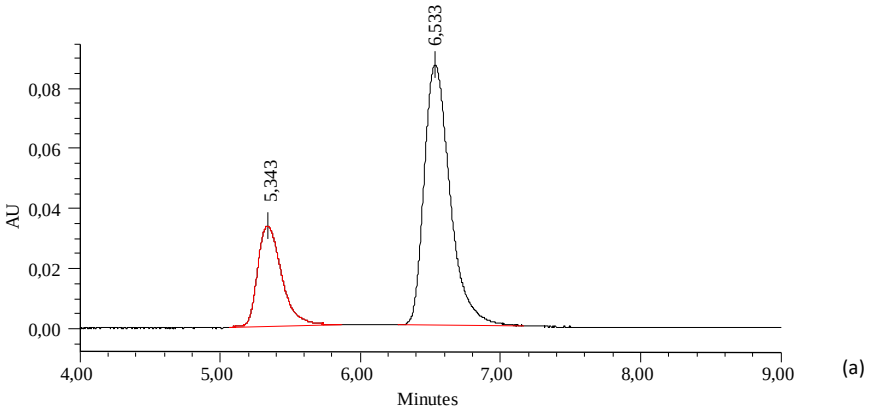
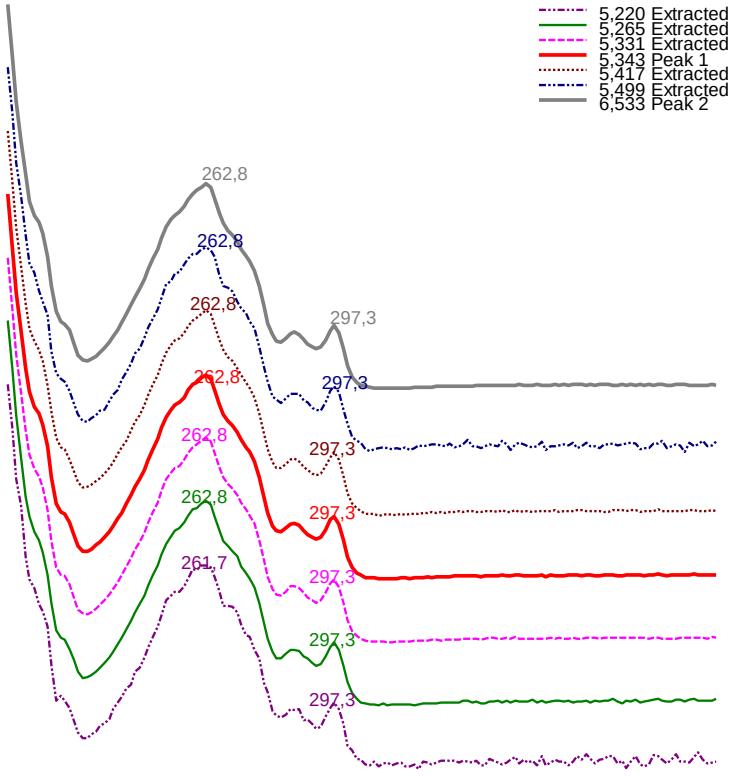


Fig. 2 Chromatograms showing the separation characteristics of two GlcN-FMOC-Su isomers from HSS and BEH columns from GlcN-HCl dietary supplement solution (1.2 ng/inj.), please see materials and methods for UPLC/FLR conditions



(a)



(b)

Fig. 3 UV spectrums of GlcN-FMOC-Su peaks of de-proteinized cricket flour from UPLC/PDA system: (a) chromatogram at wavelength 265 nm, extracted from PDA Spectrum (210-400 nm); (b) UV spectrums from two GlcN-FMOC-Su peaks (extracted at 5.34 and 6.53 min, separately) and one single GlcN-FMOC-Su peak (extracted at 5.22, 5.26, 5.33,5.41 and 5.49 min, separately).

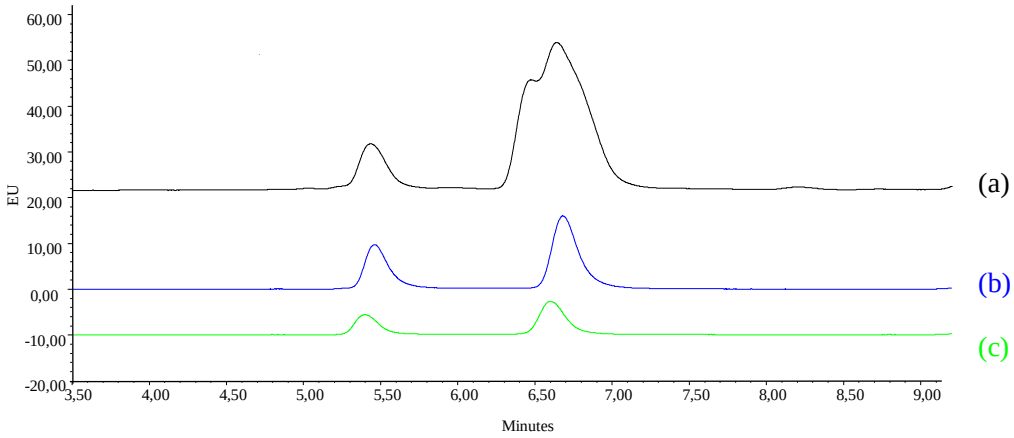


Fig. 4 Chromatograms of cricket flour treated with NaOH (0.5M) under different incubation time: (a) cricket flour without de-proteinization; (b) GlcN-HCl standard; (c) cricket treated with NaOH (0.5M), incubation time 4h.