Enhancing the Specificity of Chitin Determinations through Glucosamine Analysis via Ultra-Performance LC-MS

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Abstract

As chitin is gaining an increased attention as feedstock for industry, quantification thereof is becoming increasingly important. While gravimetric procedures are long, not specific, and highly labour-intensive; acidic hydrolysis of chitin into glucosamine followed by quantification of the latter is more performant. Even though several quantification procedures for the determination of chitin can be found in the literature, they give inconsistent results and their accuracy was not assessed due to the lack of certified analytical standards. Therefore, in the present study commercially available chitin from practical grade was characterised in detail, allowing the assessment of method accuracy. The procedure for the hydrolysis of chitin into glucosamine and subsequent quantification via UPLC-MS was investigated in detail as well. Using 9-fluorenylmethyl chloroformate (FMOC-Cl) as derivatisation reagent, glucosamine was quantified using reversed-phase chromatography. For the chitin hydrolysis, the highest glucosamine recovery was obtained with 8.0 M HCl for 2 hours at 100 °C. The entire procedure for chitin quantification, including the hydrolysis, was characterised by a high inter- and intraday precision and accuracy. The specificity of the procedure was assessed as well by analysing different mixtures of cellulose and chitin. Chitin recoveries from these analyses ranged from 98.8 to 105.8 % while no signal was observed for 100 % cellulose, indicating the high specificity of the procedure. It was also concluded that the procedure is much faster and less labour-intensive compared to the gravimetric procedure.

Key words

Chitin, Glucosamine, Derivatisation, FMOC-Cl, Ultra-Performance Liquid Chromatography

45 Graphical abstract

47 1. Introduction

48 With the awareness raising that chitin has a massive potential in all types of sectors;
 49 quantification thereof is becoming increasingly important. Chitin, the second most abundant
 50 natural polymer after cellulose, is a linear polysaccharide composed of β -1,4-linked subunits of
 51 mainly N-acetyl-D-glucosamine and to a lesser extent D-glucosamine (Fig. 1). Depending on
 52 the natural resource from which it is isolated, chitin occurs in three allomorphs with different
 53 physicochemical properties (1). While α -chitin (e.g., fungi and arthropods) is characterised by
 54 an antiparallel configuration of multiple polymer chains, all chains in β -chitin (e.g., squid pens)
 55 are arranged parallel. In γ -chitin (e.g., stomach of the *Loligo* squid), the polymer chains are
 56 predominantly arranged in an alternating pattern in such a way that when two chains run in a
 57 certain direction, a third chain runs in the opposite direction (1,2). Because of said antiparallel
 58 configuration in α -chitin and the resulting strong inter- and intramolecular bonds, this form is
 59 highly crystalline and characterised by a poor solubility in aqueous solutions and organic
 60 solvents. Partial deacetylation of chitin, however, yields its derivative chitosan which is soluble
 61 in diluted acidic solutions. Both biodegradable polymers are non-toxic and their applications
 62 include food production, seed treatment, cosmetics, pharmaceuticals, and medical applications,
 63 among others (3). Moreover, the presence of a nitrogen atom in the N-acetylglucosamine

backbone can allow for the production of platform chemicals, making the use of the energy-intensive Haber-Bosch process redundant (4–6).

The chitin content is more than often determined via gravimetric procedures originally developed for other fibre determinations (7–14). In general, these procedures consist of several solubilisation steps with acids, bases, and/or detergents to remove minerals, proteins, lipids, etc. while leaving only fibre behind. Consequently, these results are often displayed as crude fibre as these procedures do not differentiate between different types of fibres and other insoluble compounds. Thus, specifically measuring the chitin content in complex mixtures containing other types of fibres and/or other insoluble compounds is not possible, making them not specific enough for accurate chitin analyses. Examples of such complex mixtures include but are not limited to: whole insects including their gut contents, insect rearing residues (i.e., non-consumed substrate, frass, and exuviae), mycorrhizal roots (15), certain fungi (e.g. *Pythium ultimum*) (16), and certain algae (e.g., *Cladophora glomerata*, *Oedogonium irregulare*) (17). In addition, despite their wide-spread use, these procedures are long, labour-intensive, and they produce large quantities of chemical waste.

An alternative approach for determining the chitin content is via spectrophotometry or chromatography after acidic hydrolysis of the polymer into its building blocks: (N-acetyl)glucosamine (Fig. 1) (18). While this has been studied a number of times in the past for fungi, crabs, crustaceans, and chitin derived thereof (19–22), only but one very recent study has been published regarding insects despite the fact that the latter are becoming increasingly important as a raw material for human food and animal feed (23). Though, due to the lack of commercially available, certified analytical standards of chitin, none of their proposed procedures could be validated in terms of their accuracy. This lack of certified standards is likely due to the complex interactions and crosslinks in the exoskeleton of crustaceans, insects, and fungi; making pure chitin from these sources challenging (3,24–26). For this reason,

commercially available chitin does not have a purity listed in its specifications and is often labelled practical grade.

When trying to implement the hydrolysis and analysis procedures listed in the literature in our own lab, inconsistent results were observed. Consequently, it was decided to further investigate the hydrolysis of chitin into glucosamine followed by a chromatographic analysis with ultra-performance liquid chromatography (UPLC) and mass spectrometry (MS). Due to the lack of a certified chitin standard, a commercially available chitin from shrimp shells (practical grade) was subjected to a detailed analysis. Based on the presumption that this chitin did not contain any other type of fibres, a gravimetric procedure was used for the determination of its chitin content. In addition, it was analysed accurately for possible contaminants (moisture, minerals, amino acids), for its carbon and nitrogen content, and by its degree of acetylation. With its composition characterised, this chitin was used as a reference standard to determine intra- and inter-day precision as well as the accuracy of the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis). To the authors' knowledge, this is the first time that a commercially available chitin was characterised in detail in order to validate the accuracy of a chitin quantification procedure.

2. Materials and methods

2.1. Chemicals and reagents

Hydrochloric acid (37 %, ACS Reagent), chitin from shrimp shells (practical grade), cellulose fibres (medium), 9-fluorenylmethyl chloroformate (FMOC-Cl, > 99.0 %), *N*-acetyl-D-glucosamine (GlcNAc, > 98 %), and D-glucosamine hydrochloride (GlcN, analytical standard) were all supplied by Sigma-Aldrich. Lithium hydroxide (98+ %) was purchased from Merck Millipore. Sodium hydroxide (AnalaR NORMAPUR) and sodium tetraborate (GPR Rectapur) were supplied by VWR Chemicals. Diatomaceous earth (Celite® 545) was purchased from Honeywell. All reagents for the elemental analysis, including the tin pressed capsules (8 mm ×

5 mm, standard clean) were bought from OEA Laboratories Ltd. The analytical standard of acetanilide was purchased from Thermo Fisher. Helium gas was supplied by Nippon Gases. Acetonitrile and formic acid were both ULC-MS grade and supplied by Biosolve. Ultrapure water was obtained by means of the Arium Pro® UV from Sartorius.

2.2. Characterisation of the chitin standard

Because of the lack of a certified analytical standard for chitin, the chitin (practical grade) was subjected to a gravimetric method to determine the chitin content as described previously with minor modifications (7). To this end, around 1.0000 g of chitin (m_{chitin}) was accurately weighed and first treated with 1.0 M HCl (30 minutes at 100 °C) to remove minerals and catechols (9). For the sake of solubilising all proteins, three consecutive steps with 1.0 M NaOH were applied (4, 17, and 5 hours at 80 °C). To speed up the analysis, the prolonged filtrations in between the solubilisation steps have been replaced by a centrifugation step at 4000 g (Heraeus Multifuge 3S) for 15 minutes. The supernatant was decanted and replaced with fresh solution for the next solubilisation step. After the third and final deproteinisation step, the mixture was filtrated using a glass filter crucible (pore size 10-16 µm, VWR International) which contained 1.0 g of diatomaceous earth as filter aid. The diatomaceous earth had previously been placed in a muffle furnace (LT 5/12, Nabertherm) at 500 °C until constant weight to ensure that it contained no organic matter. After filtration, the crucibles were placed in a forced-air oven at 105 °C for one hour to remove moisture. Subsequently, they were allowed to cool down to room temperature in a desiccator before weighing them (m_1) up to the nearest 0.1 mg. The crucibles were then placed in the muffle furnace at 500 °C until constant weight was achieved (m_2). Considering that only chitin remained after the demineralisation and deproteinisation steps, the weight loss in the muffle furnace was only attributed to chitin (27). As such, the mass fraction of chitin in the sample on dry matter basis ($w_{\text{chitin on dry matter}}$) could be calculated using Eq. 1.

$$w_{\text{chitin on dry matter}} [\%] = \frac{m_2 [\text{g}] - m_1 [\text{g}]}{m_{\text{sample}} [\text{g}] \times w_{\text{dry matter}} [\%]} \times 100 \% \quad (\text{Eq. 1})$$

The chitin standard was also analysed for impurities such as moisture, ash, and amino acids. The nitrogen and carbon content as well as its degree of acetylation were also determined. The dry matter content of the chitin ($w_{\text{dry matter}}$) was determined by oven drying (Mettler UF 500) at 55 °C for 2.5 hours (11). The ash content was determined according to a previously published study (10), with minor modifications. Briefly, about 3.0000 g of chitin was accurately weighed and first charred in a porcelain crucible (VWR International) before the sample was incinerated in a muffle furnace (LT 5/12, Nabertherm) until constant weight was achieved. The amino acid content was determined via UPLC-MS after acidic and basic hydrolysis of the chitin sample using a previously published procedure (10) with minor modifications. Since low quantities of amino acids were expected, the sample amount was increased from 25.00 to 50.00 mg. As asparagine and glutamine convert into aspartic acid and glutamic acid respectively, the results are shown as their sums Asx and Glx (28). The mass fraction of nitrogen (w_N) and carbon (w_C) in the chitin standard were determined with the Flash 2000 Organic Elemental Analyzer (Interscience). Briefly, around 4.00 mg of the sample was accurately weighed in tin pressed capsules using an analytical balance (R160P, Sartorius). After closing, the capsules were combusted in the equipment with the temperature of the combustion and reduction ovens kept at 950 °C and 840 °C, respectively. A water scrubbing tube containing magnesium perchlorate was included as well. The carrier gas (250 mL/min) was helium and the total runtime of the analysis was 450 seconds. The response of the thermal conductivity detector was calibrated with different weights of an analytical standard of acetanilide. The degree of acetylation (DA) was calculated from the results of the elemental analysis using Eq. 2 (8).

$$\text{DA} [\%] = \frac{w_C [\%] / w_N [\%] - 5.14}{1.72} \times 100\% \quad (\text{Eq. 2})$$

With the composition characterised, the commercially available chitin from practical grade will be further referred to as the chitin standard. Using the data from the characterisation, the actual chitin content of the chitin standard was calculated so it could be used for assessing the accuracy of the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis) for chitin quantification.

2.3. Optimisation of the glucosamine derivatisation, UPLC-MS method, and chitin hydrolysis

2.3.1. Derivatisation conditions

The derivatisation of glucosamine with FMOC-Cl was adapted from a previous published study (19). Briefly, 10 μ L of syringe filtered sample (Chromafil PET 0.20 μ m, Macherey-Nagel) was added to 100 μ L borate buffer (100 mM, pH 7.0) after which 100 μ L of a FMOC-Cl solution in acetonitrile (650 ppm) was added. After thorough mixing, the vials were placed in the Sample Manager of the UPLC-MS which was kept at 7.0 ± 0.5 °C. To optimise the derivatisation reaction, 10 μ L of an analytical standard of glucosamine (100 ng/ μ L) was derivatised and subsequently analysed via UPLC-MS. Preliminary tests showed that a reaction time of 30 minutes at room temperature was not sufficient to yield complete derivatisation of all glucosamine as the peak area continuously increased with repetitive injections of the same standard. Consequently, injections were made at regular intervals in order to monitor the derivatisation yield in function of time and to determine the end-point of the derivatisation reaction. The derivatisation yield was calculated as the ratio of the total peak area at a certain time to the maximum area obtained at the plateau.

To speed up the lengthy derivatisation reaction, a sonication step at 45 kHz (USC 300 THD, VWR International) at 40 °C was included as ultrasound shows great potential to accelerate or improve analytical processes (29). The temperature was kept at 40 °C since previous research has shown that divergent reactions occur at higher temperatures (21). The pH of the borate buffer and molar excess of FMOC-Cl were not altered for the optimisation as previous research

has already shown that the values described in the previous paragraph are optimal values for the derivatisation of glucosamine (21,30). After sonication, the mixture was diluted with 500 μ L of the initial mobile phase. The derivatisation yield in function of time was monitored in the same manner as described in the previous paragraph.

2.3.2. *Chromatographic conditions*

To quantify the derivatised glucosamine, a reversed-phase chromatographic procedure with MS detection was developed. The chromatographic analysis was performed on the ACQUITY H-Class System (Waters Co.) consisting of a Quaternary Solvent Manager, a Sample Manager-Flow Through Needle, and a Column Manager. The MS detector was the ACQUITY QDa Detector (Waters Co.). The ACQUITY UPLC BEH Amide column (1.7 μ m, 2.1 mm $\times$ 150 mm, Waters Co.) and the ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 mm $\times$ 50 mm, Waters Co.) were both kept at 45.0 ± 0.5 °C throughout the analysis. An injection volume of 1.0 μ L was used for all standards and samples. The QDa detector was operated in electrospray positive mode with a capillary voltage and temperature of 0.8 kV and 600 °C, respectively. After performing a full scan, the most intense ions of glucosamine (m/z 180.1, $[M + H]^+$), *N*-acetylglucosamine (m/z 204.1, $[M + H - H_2O]^+$), and glucosamine derivatised with FMOC-Cl (m/z 424.2, $[M + Na]^+$) were selected for further experiments. An overview of the mass spectra of (*N*-acetyl)glucosamine is provided in the Supplementary Information. The mass spectra of the FMOC-derivative is shown in Fig. 2. It was observed that the most intense ion of *N*-acetylglucosamine did not correspond to its $[M+H]^+$ mass (i.e., 222.2). Instead, the base peak corresponded to the mass of its oxonium ion (i.e., $[M + H - H_2O]^+$) obtained through the loss of one water molecule (also denoted as $[GlcNAc]^+$ in literature) (31,32). All subsequent data points were acquired in selected ion monitoring (SIM) mode at an acquisition rate of 10 Hz. To optimise the chromatographic separation, different gradients of ultrapure water and acetonitrile, both containing 0.1 % formic acid as modifier, were tested.

2.3.3. Chitin hydrolysis

Resulting from a literature survey, it was opted to use 8.0 M HCl for the hydrolysis of the chitin standard into glucosamine (19,21). To investigate the dependence of time and temperature on the hydrolysis, a face-centered central composite design with one centerpoint was used. During the optimisation, this design was augmented with three additional runs. An overview of the augmented design is shown in Table 1.

Table 1 Face-centered central composite design for the optimisation of the hydrolysis of chitin into glucosamine with 8.0 M HCl. Three additional runs (10 through 12) were included to augment the design as the optimum was on the edge of the first design.

Run	Heating time (h)	Hydrolysis temperature (°C)
1	2.0	100.0
9	6.0	120.0
8	6.0	110.0
2	2.0	110.0
5	4.0	110.0
3	2.0	120.0
7	6.0	100.0
6	4.0	120.0
4	4.0	100.0
10	0.5	90.0
11	0.5	110.0
12	4.0	90.0

The hydrolysis procedure itself consisted of weighing 20 mg of the chitin standard in a glass tube to the nearest 0.01 mg (R160P, Sartorius), followed by the addition of 10.0 mL of 8.0 M HCl. The tubes were then capped and placed in a forced-air oven (Mettmert UF 500) at the desired temperature for the appropriate amount of time according to Table 1. After the required heating time has passed, the tubes were cooled rapidly to room temperature under running tap water. An aliquot of 1000 µL of the hydrolysate was then diluted up to 25.0 mL in a volumetric flask using ultrapure water. Subsequently, a portion was filtered through a syringe filter prior to derivatisation with FMOC-Cl and analysis with UPLC-MS using to the optimised conditions described in section 2.4. All hydrolyses and subsequent analyses were performed in triplicate.

2.4. Optimised procedure for chitin analysis

Around 20.00 mg of sample was accurately weighed into a glass tube with screw cap. A positive control (i.e., the chitin standard) was analysed simultaneously with the samples. After addition of 10.0 mL of 8.0 M HCl, the tubes were capped and placed in a forced-air oven at 100 °C for 120 minutes. Subsequently, the hydrolysis tubes were cooled rapidly to room temperature under running tap water. An aliquot of 1000 µL was first diluted with ultrapure water to 25.0 mL in a volumetric flask after which a portion was filtered through a 0.20 µm syringe filter. The diluted and filtered sample was then derivatised by mixing 10 µL with 100 µL of 100 mM borate buffer (pH 7.0) and 100 µL of FMOC-Cl (650 ppm in acetonitrile). The mixture was sonicated for 30 minutes at 40 °C and subsequently diluted with 500 µL of initial mobile phase.

The UPLC-MS analysis was carried out on an ACQUITY H-Class System (Waters Co.) with an ACQUITY QDa Detector (Waters Co.). The ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 mm × 50 mm, Waters Co.) which was kept at 45.0 ± 0.5 °C. The mobile phase consisted of (A) ultrapure water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. The mobile phase was delivered at a flowrate of 0.500 mL/min and the following gradient was used: 26 % B at 0 – 2.2 min, 26 – 90 % B at 2.2 – 2.3 min, 90 % B at 2.3 – 3.8 min, 90 – 26% B at 3.8 – 3.9 min, followed by equilibration at 26 % B for a total run time of 6.0 minutes. An injection volume of 1.0 µL was used for all standards and samples. The QDa Detector was operated in electrospray positive mode with a probe temperature and voltage of 600 °C and 0.8 kV, respectively. The derivatised glucosamine was detected in SIM mode at m/z 424.2 ($[M + Na]^+$) with a cone voltage of 4 V and acquisition rate of 10 Hz. It should, however, be noted that although MS detection was used; the derivatised glucosamine can also be detected by UV or fluorometric detection as well. For preparing calibration curves, both peaks of the derivatised β-glucosamine and α-glucosamine as well as the plateau between both peaks were integrated and the total peak area was used.

Quantification of glucosamine in the hydrolysates was achieved through linear calibration curves which were obtained by making appropriate dilutions of an analytical standard with ultrapure water, also followed by derivatisation with FMOCCl. The chitin content of the chitin standard, expressed as the mass fraction of *N*-acetylglucosamine (w_{GlcNAc}), was calculated with Eq. 3. In this equation, V_{sample} is 0.0100 L, the dilution factor (DF) is 25, M_{GlcNAc} and M_{GlcN} are the molecular masses of *N*-acetylglucosamine and glucosamine, and a and b denote the intercept and slope of the linear calibration curve of glucosamine, respectively.

$$w_{\text{GlcNAc}} = \frac{(\text{Total peak area} - a) \times V_{\text{sample}} [\text{L}] \times \text{DF} \times M_{\text{GlcNAc}} \left[\frac{\text{g}}{\text{mol}} \right]}{b \times m_{\text{sample}} [\text{mg}] \times M_{\text{GlcN}} \left[\frac{\text{g}}{\text{mol}} \right]} \times 100 \% \quad (\text{Eq. 3})$$

2.5. Validation of the procedure

Calibration curves were prepared by injecting six analytical standards of GlcN (1.00 – 50.0 ng/ μ L) in triplicate. The total area (peak 1, plateau, and peak 2) was plotted against the standard concentration. Linearity of the calibration curve was assessed by regression analysis using JMP Pro 15.1.0 (SAS Institute). The limit of detection (LOD) and limit of quantification (LOQ) were evaluated based on a signal-to-noise (S/N) ratio of 3 and 10, according to the American Chemical Society (ACS) (33).

Both the intra- and inter-day precision of the entire optimised procedure were assessed by means of the relative standard deviation (RSD) of replicate analyses. To this end, 20.00 mg of the chitin standard was hydrolysed, derivatised, and analysed via UPLC-MS in triplicate. This was repeated for a total of four days over a timespan of two months. Fresh derivatisation reagent and mobile phases were prepared daily. Moreover, the characterised chitin standard was used for the purpose of assessing method accuracy. The latter was calculated as the ratio of the chitin content determined via UPLC-MS (Eq. 3) to the chitin content determined according to the gravimetric procedure.

In order to assess the specificity of the optimised procedure, cellulose fibres were mixed with the chitin standard in four different ratios and the resulting mixture was subjected to the optimised procedure in triplicate. Alongside these samples, the chitin standard and cellulose fibres were also analysed as such as positive and negative control, respectively. The chitin recovery of each mixture was calculated as the ratio of the experimentally determined chitin content (Eq. 3) to the expected mass fraction of chitin in the fibre mixture, taking into account the actual chitin content of the standard (Table 2).

3. Results and discussion

3.1. Composition of the chitin standard

The results of the characterisation of the chitin standard can be found in Table 2. It could be concluded that the dry matter content of the standard was around 95 %. Resulting from the gravimetric chitin analysis, it was found that the chitin standard contained only 87.30 ± 1.77 % of chitin on dry matter basis. As such, it was hypothesised that the chitin standard still contained impurities, which was confirmed by the results of the elemental analysis. Even though it was expected that the amounts of nitrogen and carbon in the chitin standard were somewhere in between the composition of 100 % acetylated chitin (6.89 % N and 47.29 % C) and 100 % deacetylated chitosan (8.69 % N and 44.72 % C), the elemental analysis showed that the chitin standard contained only 6.72 ± 0.12 % N on dry matter. While minerals and proteins were initially suspected as being the major contaminants; the results showed very low amounts of ash and amino acids with the individual amino acid contents ranging from 0.01 to 0.09 g/100 g on dry matter. Moreover, no cystine, methionine, and tryptophan were detected in the chitin standard.

As a result, the remaining 12 % must therefore have originated from other contaminants which were removed during the acidic and basic solubilisation steps of the gravimetric procedure to determine the chitin content. It should be noted that the exoskeleton of crustaceans and insects

is heavily cross-linked, with several amino acid residues (e.g. lysine, alanine) being involved together with dopamine and other catecholamines, resulting in a highly complex network of chitin, proteins, and phenolic derivatives among others (3,24–26). It is plausible that a fraction of these compounds was still present in the chitin. Though, according to the literature these catechols, catecholamines, and modified amino acids (e.g., *N*- β -alanyldopamine) are removed during an acidic solubilisation step (13,26). Since the used UPLC-MS procedure could not detect such heavily modified amino acids or proteins, the presence of these components could not be confirmed nor denied.

Table 2 Composition of the chitin from shrimp shells (practical grade). Results are shown as the mean of three replicates $\pm$ standard deviation.

Quantity		Individual amino acids ²			
Dry matter ^a	94.66 $\pm$ 0.60	His	0.02 $\pm$ 0.00	Lys	0.01 $\pm$ 0.01
Chitin ^b	87.30 $\pm$ 1.77	Arg	0.01 $\pm$ 0.00	Cyss + cys	n.s.
Ash ^b	0.18 $\pm$ 0.01	Ser	0.01 $\pm$ 0.00	Tyr	0.01 $\pm$ 0.00
Nitrogen ^b	6.72 $\pm$ 0.12	Gly	0.05 $\pm$ 0.01	Met	n.s.
Carbon ^b	45.76 $\pm$ 0.46	Asx ^c	0.03 $\pm$ 0.01	Val	0.03 $\pm$ 0.01
		Glx ^c	0.05 $\pm$ 0.01	Ile	0.01 $\pm$ 0.00
DA (%)	96.91 $\pm$ 2.11	Thr	0.01 $\pm$ 0.00	Leu	0.02 $\pm$ 0.00
		Ala	0.03 $\pm$ 0.01	Phe	0.02 $\pm$ 0.00
Sum amino acids ^b	0.38 $\pm$ 0.07	Pro	0.07 $\pm$ 0.01	Trp	n.s.

^a g/100 g *as is*; ^b g/100 g dry matter; ^c sum of resp. aspartic acid with asparagine, and glutamic acid with glutamine; DA, degree of acetylation; n.s., no signal was observed during analysis.

3.2. Optimisation of the glucosamine derivatisation, UPLC-MS method, and chitin hydrolysis

3.2.1. Derivatisation conditions

Preliminary tests showed that when derivatisation conditions described in the literature were applied in our lab, the derivatisation reaction (Fig. 3a) was not yet complete. Therefore, in order to determine the end-point of the derivatisation reaction, a glucosamine standard was derivatised as described previously (19) and samples were taken at regular intervals. These samples were then injected onto the UPLC-MS and the derivatisation yield at (1) room temperature and (2) 40 °C with sonication was monitored in function of time. As can be observed from Fig. 3b, the reaction at room temperature took very long to complete as it only levelled off after 6 hours. Looking at the right of Fig. 3c, it was concluded that the derivatisation

reaction with FMOC-Cl proceeded much faster when the mixture was sonicated at elevated temperature as the reaction was already completed after $\pm$ 20 minutes. Consequently, it was opted to include a sonication step of 30 minutes at 40 °C in the derivatisation step instead of performing it at room temperature.

3.2.2. *Chromatographic conditions*

In order to quantify the glucosamine content of the chitin hydrolysates, the separation was first optimised with the ACQUITY UPLC BEH Amide (1.7 μ m, 2.1 mm $\times$ 150 mm) column using hydrophilic interaction chromatography (HILIC) as this column type is highly promising for neutral, polar molecules. After method development using standards, chromatographic conditions were found that produced stable and repeatable chromatograms with almost symmetrical peaks. The retention time of *N*-acetylglucosamine was determined as 1.36 min while the retention time of glucosamine was 1.76 min. Injection of the hydrolysed chitin standard, however, resulted in a fluctuating baseline and a broad (> 2 min) distorted glucosamine peak which eluted much later (t_R = 4.50 min). Furthermore, standard injections after a sample was injected also had fluctuating baselines and shifting retention times, presumably because the high ionic strength of the sample altered the equilibrated conditions in the column which were required for the several different separation mechanisms of HILIC (34). Despite best efforts, no useable and repeatable chromatograms were obtained with the amide column.

Instead, it was opted to optimise the separation on the ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 mm $\times$ 50 mm) as this column has shown to be more robust when using high ionic strength samples such as neutralised protein hydrolysates (35). It was, however, observed that both glucosamine and *N*-acetylglucosamine were not retained sufficiently on the column as they eluted in the void volume of the system. Although 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is suggested as derivatisation reagent for glucosamine in a previous study

(36), the AQC derivative was also not retained sufficiently on the current system. Consequently, FMOCl was used as derivatisation reagent as it is less polar compared to AQC and is also used in several studies on the chromatographic separation of glucosamine (19,21,37). Since FMOCl reacts both with primary and secondary amines but not with amides, only the separation of glucosamine was optimised.

Analysis of a glucosamine standard using the chromatographic conditions described in section 2.4, resulted in a chromatogram which showed two distinct peaks which were not entirely baseline separated (Fig. 4). The MS spectra of both peaks (Fig. 2) were, however, highly similar as they exhibited both a base peak at m/z 424.2, which belonged to the sodium adduct of glucosamine derivatised with FMOCl ($[M + Na]^+$). Moreover, both spectra showed a high resemblance to those reported in the literature with two other abundant ions at m/z values 384.2 and 440.1 which corresponded presumably to $[M + H - H_2O]^+$ and $[M + K]^+$, respectively (19). The formation of an oxonium ion of the derivatised glucosamine through the loss of one water molecule was plausible as the MS spectrum of underivatised glucosamine also showed a high abundant peak at $[M + H - H_2O]^+$ (i.e., m/z 162.1) as can be seen in Fig. S1 of the Supplementary Material. As such, it was concluded that the two peaks corresponded to two anomeric forms of glucosamine. The ratio of the peak areas was calculated as 25.8/74.2 which was in good agreement with previously published results (38,39), meaning that the obtained peaks corresponded to the β -anomer ($t_R = 0.87$ min) and α -anomer ($t_R = 1.03$ min) of glucosamine, respectively. The incomplete baseline separation can be attributed to the interconversion between the two anomers through mutarotation, as reported previously (15,38–40). For calibration and quantification, both peaks as well as the plateau were integrated and the total area was used. It was shown that this did not influence the calibration as long as the integration was consistent, similarly to previously published results (19,21,39). While MS detection was used for the present study, it should be noted that UV or fluorescence detection are also suitable

for detection of the glucosamine derivatised with FMOC-Cl. Using MS detection, however, has the advantage of a considerably enhanced specificity. Hence, a better confirmation of the peak identity and less matrix interference can be obtained (41,42).

3.2.3. Chitin hydrolysis

As mentioned in the materials and methods section, a face-centered central composite design was used for the optimisation of the chitin hydrolysis. After analysing all data, however, it was found that the optimal conditions were located on the edge of the design. As such, the design was augmented with three more runs to enlarge the design space (Table 1). Using the optimised derivatisation and chromatographic conditions, the glucosamine content in the hydrolysate was determined via UPLC-MS. This glucosamine content was then used to calculate the chitin content, expressed as the mass fraction of N-acetylglucosamine. Subsequently, a contour plot was made with this mass fraction as the response and the hydrolysis time and temperature on the x- and y-axes, respectively (Fig. 5). As can be observed from the graph, a prolonged heating time at higher temperature resulted in a decreased recovery, which was caused by the breakdown of glucosamine (18). On the other hand, a very short hydrolysis time was not preferential either because not all chitin could be hydrolysed, resulting in lower recoveries. This hypothesis was confirmed visually as chitin particles were observed in the respective hydrolysis tubes.

Resulting from this optimisation, it was shown that hydrolysis with 8.0 M HCl for 2 hours at 100 °C resulted in the highest chitin content. It was, however, hypothesised that not all N-acetylglucosamine might have been deacetylated into glucosamine under these conditions, which would have resulted in an underestimation of the chitin content. In order to test this hypothesis, the hydrolysed chitin standard (2 hours at 100 °C) was also injected onto the ACQUITY UPLC BEH Amide column (see section 3.2.2). Despite the same aforementioned

issues reoccurring during the analysis, no peak was detected on the SIM channel of *N*-acetylglucosamine which confirmed the complete deacetylation.

Considering that the optimised hydrolysis took 2 hours, the derivatisation reaction 30 minutes, and the UPLC-analysis only 6 minutes; the entire procedure was considered much faster compared to the gravimetric procedure (± 2 days) even when additional time was included for sample handling, pipetting, etc. The proposed procedure was also perceived as less labour-intensive compared to the gravimetric procedure and in addition, higher sample throughput could be achieved.

3.3. Validation

After the optimal conditions were identified, the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis) was validated in terms of its linearity, precision and accuracy. In addition, the specificity was also assessed by analysing different fibre mixtures with known chitin content. Resulting from the regression analysis of the calibration curve of glucosamine, a highly significant ($p < 0.0001$) linear correlation was found. Although a plateau was observed in between the peaks of β - and α -glucosamine, integrating this plateau as well and using the total peak area has shown no adverse effects on the calibration as a correlation coefficient of 0.998 was obtained. The values of the LOD and LOQ were determined as 0.25 and 0.85 ng/ μ L, respectively. These values were lower compared to previously reported values (19,43), which were obtained with UV detection.

The precision and accuracy of the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis) were assessed as well. The intraday precision was determined by performing the procedure on the chitin standard three times on the same day. This was performed for a total of four times over the timespan of two months to also assess the interday precision. As can be observed from Table 3, the intra-day precision of the GlcNAc content ranged from 1.73 to 2.77 % while the inter-day precision was found to be 3.49 %. Since all values were lower than

5 %, it could be concluded that the entire procedure had a high precision. Since an analytical standard for chitin is lacking, the commercially available chitin from practical grade was characterised in detail and was used for assessing method accuracy. The accuracy of the entire procedure was considered high with values ranging from 95 to 112 %. However, because of this rather broad variation over the course of the study period, it was suggested to also include a positive control (i.e., the chitin standard) when analysing real samples and correcting for its recovery. Correcting the analytical result for recovery values can be applied to estimate the true concentration and make results more universally comparable (44); a procedure which is often found in the literature (23,45–47).

Table 3 Overview of the statistical evaluation of the calibration curve of glucosamine derivatised with 9-fluorenylmethyl chloroformate; the intra- and inter-day precision and accuracy of the entire procedure applied on the chitin standard over the timespan of two months; and the mean results ($n = 3$) of the recovery for assessing the specificity of the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis) for chitin analysis ($n = 3$)

Validation parameters		Fibre mixture	Recovery (%)
Calibration range	1.00 – 50.0 ng/ μ L	100 % cellulose	n.s.
Regression equation	$Y = 2.59 \times 10^3 X - 843$	10 % chitin – 90 % cellulose	101.6 ± 4.7^c
R-squared	0.998	25 % chitin – 75 % cellulose	99.0 ± 0.4^c
S ^a	910	50 % chitin – 50 % cellulose	98.8 ± 2.0^c
F-test (p-value)	< 0.0001	75 % chitin – 25 % cellulose	105.8 ± 0.6^c
LOD (ng/ μ L)	0.25	100 % chitin	111.6 ± 2.3
LOQ (ng/ μ L)	0.85		
Intraday precision ^b (%)	1.73 to 2.77		
Interday precision ^b (%)	3.49		
Intraday accuracy (%)	95.2 to 111.6		
Interday accuracy (%)	99.8		

^a standard error of the regression; ^b calculated as relative standard deviation (RSD); ^c corrected for recovery; n.s., no signal was observed during analysis

A drawback of the gravimetric procedure for chitin determinations is that it cannot differentiate different types of fibres and other insoluble compounds. Hence, the specificity of the proposed procedure was assessed as well by analysing different mixtures of cellulose and chitin. Cellulose was chosen for this purpose based on its similarity to chitin and its high abundance in nature. Recoveries close to 100 % were found for all fibre mixture (corrected for recovery) while no signal was observed for the samples containing 100 % cellulose. Both indicated that

the proposed procedure can indeed differentiate chitin from cellulose, which can allow for much more accurate chitin determinations. As such, the results of this validation clearly indicated that the proposed procedure is not only characterised by a high precision but also by a high accuracy and a high specificity.

4. Conclusions

In the present study, a procedure for determining chitin after acidic hydrolysis into glucosamine followed by quantification using UPLC with MS detection was investigated and validated. To this end, commercially available chitin from practical grade was first characterised for its chitin content via a gravimetric procedure and for impurities since a commercially available certified standard is currently lacking. Following the optimisation of the derivatisation reaction of glucosamine with FMOC-Cl, it was shown that a sonication step at 40 °C decreased the required reaction time substantially. The derivatised glucosamine was then quantified via a simple reversed-phase chromatographic procedure using the ACQUITY UPLC BEH C18 column. Although MS detection was used in the present study, detection of the FMOC-derivative of glucosamine can be performed with a UV or fluorometric detector as well. Nevertheless, MS detection has the advantage of a higher specificity which results in less matrix interference. After examining the influence of time and temperature on the acidic hydrolysis with 8.0 M HCl, the highest chitin content was obtained after 2 hours at 100 °C. Moreover, no *N*-acetylglucosamine was detected by using these conditions, confirming the complete deacetylation. The results of the validation showed that a highly significant linear correlation ($p < 0.0001$; $R^2 = 0.998$) was obtained for the calibration range 1.00 – 50.0 ng/μL. The limit of detection and quantification were calculated as 0.25 and 0.85 ng/μL, respectively. In addition, the entire procedure (hydrolysis, derivatisation, and UPLC-MS analysis) was characterised by both a high intra- and interday precision as these values were always below 3.50 % over the timespan of two months. Furthermore, the entire procedure was also characterised by a high

specificity as chitin recoveries close to 100 % were obtained when mixtures of chitin and cellulose in different ratios were analysed, with inclusion of a positive control. Finally, this procedure for determining the chitin content by hydrolysing it into glucosamine followed by quantification with UPLC-MS was also much faster and less labour-intensive compared to the gravimetric procedure.

Declarations

Conflicts of interest

The authors declare that they have no conflict of interest.

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611 **Figures**

612

613 Fig. 1 Chemical structures of chitin and its deacetylated derivative chitosan. During the acid hydrolysis of
 614 chitin, depolymerisation and deacetylation take place simultaneously, yielding both N-acetyl-D-
 615 glucosamine and D-glucosamine. Depending upon the reaction circumstances, a complete conversion into
 616 glucosamine is achieved

617

618 Fig. 2 Mass spectra of the two peaks in the chromatogram of a glucosamine standard derivatised with 9-
619 fluorenylmethyl chloroformate, injected on the ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 mm $\times$
620 50 mm). Probe temperature and voltage were 600 $^{\circ}$ C and 0.8 kV, respectively. The lower signal intensity
621 of the first peak compared to the second peak could also be observed on the chromatogram obtained in
622 selected ion monitoring mode (shown in Fig. 4)

Fig. 3 **a** Derivatisation reaction of D-glucosamine with 9-fluorenylmethyl chloroformate. **b** The influence of the reaction time on the derivatisation yield at room temperature ($n = 1$) and **c** with sonication at 40 °C ($n = 3$)

Fig. 4 Chromatogram of a glucosamine standard (20 ng/ μ L) derivatised with 9-fluorenylmethyl chloroformate, injected on the ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 mm $\times$ 50 mm). Two distinct peaks of the β -anomer (0.87 min) and α -anomer (1.03 min) could be identified. A plateau was observed between the two peaks, originating from the interconversion between the anomers through mutarotation

Fig. 5 Contour plot of the chitin content (expressed as mass fraction N-acetylglucosamine, w_{GlcNAc}) of the chitin standard in function of hydrolysis time and temperature. The black dots represent the experiments of the original design while the black squares represent the three additional runs