Quantitation of Glucosamine From Shrimp Waste Using HPLC

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Abstract

This work presents a high-performance liquid chromatography (HPLC) method for the quantitation of glucosamine in chitin. The method includes an acid hydrolysis of chitin. The chromatographic separation is achieved using a Hypersil ODS 5-µm column (250 × 4.6 mm) at 38°C, with precolumn derivatization with 9-fluorenylmethyl-chloroformate and UV detection (λ = 264 nm). The mobile phase is a mixture of mobile phase A [30mM ammonium phosphate (pH 6.5) in 15:85 methanol–water (v/v)], mobile phase B [15:85 methanol–water (v/v)], and mobile phase C [90:10 acetonitrile–water (v/v)], with a flow rate of 1.2 mL/min. The HPLC method proposed showed adequate repeatability (relative standard deviation, 5.8%), accuracy (92.7% recovery), and sensitivity, with a detection limit of 2 µg/mL. The method is successfully applied to the quantitation of glucosamine for the determination of the purity of chitin from shrimp waste.

Introduction

Chitin is a natural polysaccharide found particularly in the exoskeleton of crustaceans, the cuticles of insects, and the cell walls of fungi. Because chitin is one of the most abundant biopolymers next to cellulose, much interest has been paid to its biomedical, biotechnological, and industrial applications (1,2). Chitin is substantially composed of 2-acetamide-2-deoxy-D-glucopyranose (*N*-acetyl-D-glucosamine) units linked by β-(1→4) linkage. Chitosan obtained from chitin, mainly by *N*-deacetylation with an alkaline hydrolysis, is chiefly composed of 2-amino-2-deoxy-D-glucopyranose (D-glucosamine) units (3–6). Glucosamine is an amino monosaccharide, which participates in the constitution of glycosaminoglycans, a major class of extracellular complex polysaccharides. Glucosamine sulphate, glucosamine hydrochloride, and *N*-acetyl-glucosamine are commonly used alone or as part of the mixture produced by the pharmaceutical industry, as medicine, to help rebuild damaged joints, tendons, cartilage, and soft tissue (7,8).

The main industrial source of chitin is shellfish waste from processing of crab and shrimp. Shrimp production in Sonora, México, was around 49,000 metric tons in 2004 (9). Only 55% of the animal is edible, the rest is composed of inedible cephalothorax and exoskeleton (10–12). This waste is rich in chitin, protein, and calcium with small amounts of pigments. Lactic acid fermentation of shrimp waste has been reported as an efficient and economical technique to protect this biomass from bacterial decomposition. The fermented waste forms a silage containing a protein rich liquor and the insoluble chitin (13–18). The purification of chitin from the solid fraction consists mainly of depigmentation, deproteinization, demineralization, and blanching steps (19–21).

A variety of different methods are available for determining glucosamine after hydrolysis of chitin with acid, alkali, or enzymes (22–24). The traditional method for analysis of glucosamine is spectrophotometry (25–27), but in most cases such techniques are laborious, time consuming, and unstable. Gas chromatographic methods are very sensitive and have a high specificity, but the derivatization procedure of the hydrolysis products into volatile components is time-consuming, and the laboratory work requires a significant amount of experience. With the exception of electrochemical detection (28), high-performance liquid chromatography (HPLC) also requires derivatization steps to determine glucosamine. Typical derivatization reagents include 9-fluorenylmethyl-chloroformate (FMOC-Cl), *o*-phthalaldehyde (OPA), and phenylisothiocyanate (PITC). Each of these reagents has specific advantages and limitations; for example, FMOC-Cl and PITC react with primary and secondary amino sugars, but those produced chromatograms with various peaks, whereas the method using OPA produced chromatograms with only peaks (29). Some HPLC methods have been reported to quantitate glucosamine in humic substances, wood, and water samples with OPA and fluorescent detection (30,31), in bacterial cell walls with PITC and UV detection (32), and in fungi and mycorrhizal roots with FMOC-Cl and fluorescent detection (33). Recently, a method has been published for the quantitation of glucosamine in chitin from biological materials (29), and another method has been published for glucosamine HPLC with FMOC-succinimide (Su) for derivatization and was selected by AOAC as the most appropriate method for further laboratory validation (34). To our knowledge, there are no studies relating to

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the glucosamine content in chitin samples obtained from fermented shrimp waste.

In the present work, an HPLC method, after derivatization with 9-fluorenylmethyl chloroformate, was used for the analysis of glucosamine. Additionally, the data validating the method and the results of its application for the determination of glucosamine in chitin samples from fermented shrimp waste is reported.

Experiment

Standards and reagents

HPLC-grade methanol and acetonitrile were obtained from EMD Chemicals (Darmstadt, Germany). Glucosamine standard was purchased from Fluka (Steinheim, Switzerland). Glacial acetic acid, boric acid, anhydrous ammonium monohydrogen phosphate, anhydrous dihydrogen phosphate, sodium hydroxide, EDTA, and HCl were purchased from Products Monterrey (Monterrey, Nuevo Leon, México). Chitin, hydroxylamine hydrochloride, FMOC, and 2-(methylthio)-ethanol were purchased from Sigma (St. Louis, MO). Takabate 380 was purchased from ENMEX (Tlanepantla, Estado México, México). All reagents were of analytical grade, unless otherwise noted. All aqueous solutions were prepared with ultra-pure water purified with NANO pure Diamond UV system (Barnstead International, Dubuque, Iowa). A 2M stock solution of monohydrogen and $(NH_4)_2$ HPO_4 was used to prepare HPLC eluents, adjusting the pH to 6.5 with ammonium dihydrogen phosphate, $NH_4H_2PO_4$. The glucosamine standard (105.8 mg/mL) was dissolved in ultra-pure water and diluted to obtain different concentrations and calculated from calibration plots. The purity of the reference standard was ≥ 99.0%. For the determination of glucosamine in shrimp chitin, the stock solution was, in all the cases, analyzed together with the samples. The standard and other solutions were stored at approximately 4°C in the dark. Analyte concentrations in samples were estimated on the basis of peak area units. All samples were analyzed in duplicate.

The solution used for the derivatization of glucosamine, FMOC-Cl, was dissolved in acetonitrile (4 mg/mL). A borate buffer was prepared from a 250mM boric acid solution adjusted to pH 8.5 with 1M sodium hydroxide solution prepared from sodium pellets. The alkaline cleavage reagent was prepared daily in 1000 µL batches by mixing 680 µL of 850mM sodium hydroxide solution with 300 µL of 500mM hydroxylamine hydrochoride solution and 20 µL of 2-(methylthio)-ethanol. The quench reagent was acetonitrile–acetic acid (8:2).

Samples

Slightly thawed minced shrimp waste samples were fermented at 30°C for 36 h. The silage was centrifuged to obtain the chitin-rich fraction (sediment or raw chitin), the protein rich liquor, and the lipid fraction (35). The raw chitin was depigmentated with an organic mixture consisting of petroleum ether–acetone–water, deproteinized with commercial proteolytic enzymes (0.15% w/w, Takabate 380), demineralized by dilution with hydrochloric acid 1 N, and blanched with sodium hypochlorite solution. The chitin was oven-dryed at 105°C. After that, the samples were stored in a desiccator and in darkness until their analysis. Also, the samples were analyzed from pure and commercial chitin.

Sample hydrolysis

The conditions used for hydrolysis were modified from those proposed by Ekblad and Näsholm (33). Each sample (100 mg) was placed in screw-cap tube and hydrochloric acid (6 N, 5 mL) was then added; the tubes were closed under nitrogen, placed in an electric oven at 110°C for 24 h, cooled, and their contents were vacuum-filtered through a Whatman no. 41. The filtrate was diluted to 100 mL with ultra-pure water in a volumetric flask, readying the solution for the derivatization process.

Derivatization

A method to derivatize glucosamine (36) was slightly modified by Lopez-Cervantes et al. (37) and used for this research. Before derivatization, samples of hydrolysate (300 µL) were placed in a tube and dried in a vacuum oven for 6 h at 110°C, the residues were then dissolved in a borate buffer (300 µL), preparing the solution for the derivatization process. To derivatize, 300 µL of the glucosamine standard solution, the prepared sample, was deposited in a 1.5-mL vial, and then 300 µL of FMOC reagent was added and vortex mixed for 90 s. Then the cleavage reagent was added (180 µL), and the tubes were vortexed for 15 s. After allowing time for the reaction, 5 min at room temperature, 420 µL of quench reagent was added; the resulting solution was vortexed for 15 s and filtered with a 0.45-µm membrane. A 20-µL sample of this solution was injected onto the column of the HPLC system.

Equipment

The HPLC system (GBC, Dandenong, Australia) was equipped with an auto injector LC 1650, an online solvent degasser LC1460, a system controller WinChrom, a pump LC1150, a column oven LC1150, a 20-µL injection loop (Rheodyne, Cotati, CA), and a photodiode array detector LC5100. Chromatographic analysis was performed using an analytical scale (4.6 × 250 mm) SGE Hypersil ODS C18 column with a particle size of 5 µm (SGE, Dandenong, Australia). The mobile phase was a gradient prepared from three solutions, A [30mM ammonium phosphate (pH 6.5) in 15:85 methanol–water (v/v)], B [15:85 methanol–water (v/v)], and C [90:10 acetonitrile–water (v/v)]. The elution gradient used is shown in Table I. The flow rate was constant at 1.2 mL/min, and the column was maintained at 38°C. Detection was performed using a UV wavelength of 264 nm.

Statistical analysis

For the descriptive and regression statistical analyses, the computer program used was SPSS 11.0 for Windows (SPSS, Chicago, IL).

Results and Discussion

Sample preparation

Several studies have been published about the conditions for

the release of glucosamine from chitin that have indicated that acid hydrolysis is the preferred method (33,38). As noted in the Experimental section, 100 mg of sample with 5 mL of 6N hydrochloric acid was hydrolyzed. These conditions were selected on the basis of preliminary trials to establish optimal conditions for our samples (100, 200, or 300 mg of sample; 5 or 10 mL hydrochloric acid), which were prepared with a slightly modified version of the Ekblad and Näsholm method (33). Optimal conditions were identified on the basis of peak areas in chromatography.

Glucosamine identification

Glucosamine does not contain a chromophore with an absorption range useful for LC with UV detection. FMOC-Cl as a derivatization reagent reacts with primary and secondary amino sugars or amino acids. Additionally, the precolumn derivatization with FMOC-Cl requires a relatively short time for both derivatization and for passage of FMOC derivatives through the column. The total time required for derivatization was 8 min, and the total time between injections was 43 min.

Glucosamine has two natural steroisomers (α and β), and the interconversion of these two in an aqueous solution is not preventable, resulting in two peaks in the chromatogram (29,30). However, other research reports that glucosamine gives three peaks (32,39). Conceivably, peak 3, might be due to the open form of glucosamine (32). The chromatograms of glucosamine standard also showed three peaks (15.86 ± 0.03 min, 17.29 ± 0.03 min, and 17.96 ± 0.03 min, for n = 6). This pattern was also shown for hydrolyzed chitin. The ratio between the three peaks did not vary between different samples or different concentrations. Peak 2 is bigger than the other two and tends to coelute with either peak 1 or 3. The sum of the areas of these three peaks is used for the quantitation of the glucosamine. Typical HPLC chromatograms of FMOC derivatizates of glucosamine is shown in Figure 1. No peak interfered with glucosamine because the chitin shrimp waste samples were deproteinized.

Peak identification from glucosamine was performed by comparing the retention times with pure standards, and it was confirmed with characteristic spectra obtained from the photodiode array detector, which also permitted the confirmation of the purity of the peaks. The absorption spectrum for the glucosamine is shown in Figure 2. Similarity indexes between the spectra from standard and sample were greater than 0.9992.

The elution conditions used were based on a method developed, by our group, for free amino acid determination in shrimp waste, with minor modifications (37). Finally, it was found that better reproducibility was obtained at 38°C, 1.20 mL/min, and using the gradient program shown in Table I.

Table I. Gradient Program Employed for the Separation of FMOC Derivatives of Glucosamine

Time (min)	% Eluent A	% Eluent B	% Eluent C
0	17	68	15
32	10.8	43.2	46
34.05	0	0	100
36.50	0	0	100
36.55	17	68	15
43	17	68	15

Analytical characteristics

The linearity of standard curves (Table II) was expressed in terms of the determination coefficient (r^2) from plots of the integrated peak area versus the concentration of the standard (µg/mL). This equation was obtained over a wide concentration range in concordance with the level of the glucosamine found in the samples analyzed. The curve is based on the analysis of at least 4 dilutions of the corresponding standard. The relationships between the concentration and peak area was linear, with coefficients of determination greater than 0.999.

The precision study was comprised of repeatability and reproducibility studies. A total of 10 replicate determinations of a sample were performed under optimum conditions to determine repeatability. Five replicate analyses of the same sample were made on different days to determine reproducibility. Table II shows results obtained from these assays. The relative standard deviations (RSDs) of the repeatability and the reproducibility are 5.8 and 4.3, respectively. These results indicate that the present method can be used for quantitative analysis of glucosamine in

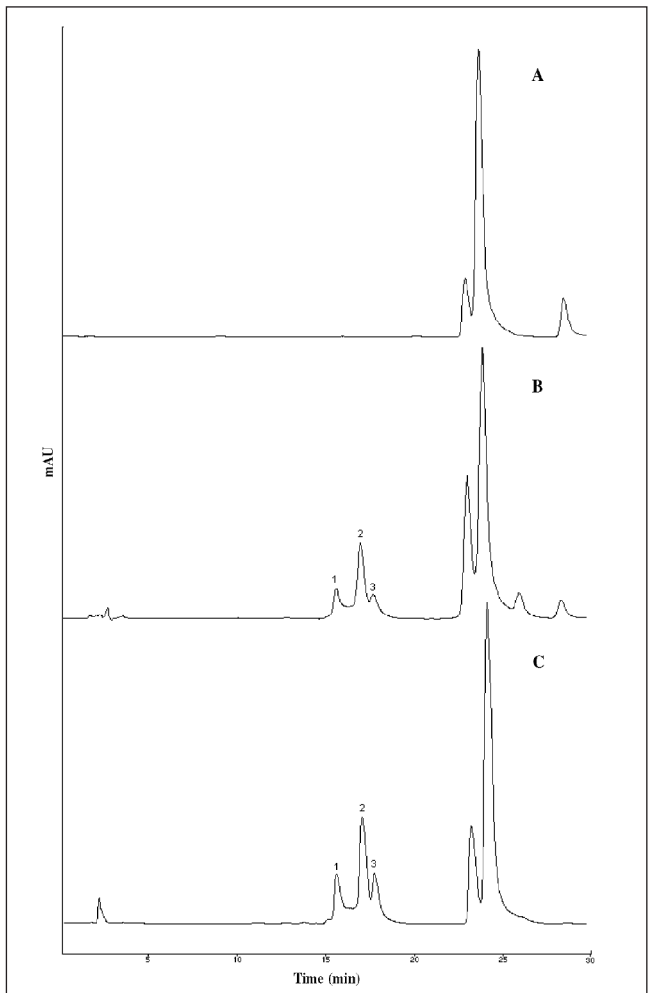

Figure 1. HPLC chromatograms (at 264 nm) of FMOC derivatives of glucosamine: blank (A); glucosamine reference standard (B); shrimp chitin (C).

chitin of shrimp samples and is in agreement with values reported in related previous studies (28,29).

The detection limit was determined on the basis of a signal-to-noise ratio (3:1) as per American Chemical Society guidelines (40). It was not possible to compare this result because the references did not give a detection limit for glucosamine; however, the detection limit obtained (2 µg/mL) was smaller than those presented for the determination of glucosamine in pharmaceutical formulations (41).

Accuracy was estimated by means of recovery assays. For evaluation of recovery, six samples of powdered chitin shrimp waste were spiked with a known concentration of glucosamine prior to hydrolysis, extraction, derivatization, and quantitation. Recovery was good, in line with a previous evaluation of a similar method for the analysis of glucosamine in chitin samples in biological materials (29). Table II shows the recovery of glucosamine.

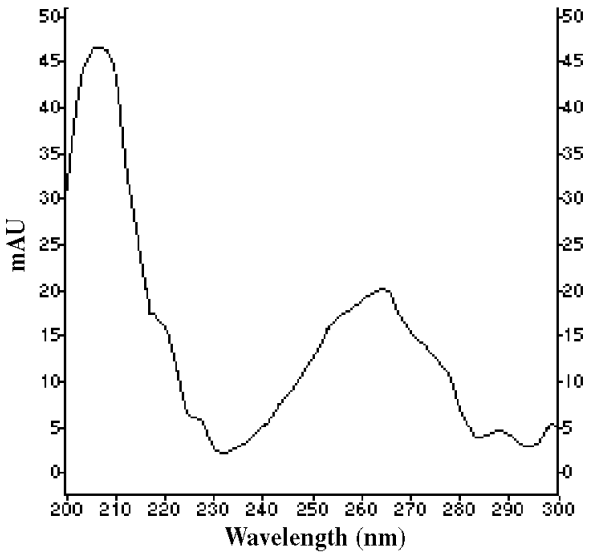

Figure 2. Absorption spectrum of the glucosamine.

Table II. Analytical Quality Parameters of the Proposed Method

Parameter	Glucosamine
Linearity	
Range (ng/mL)	338–1354
Equation	y = 2739x – 6883.5
r^2*	0.9995
Repeatability (*n* = 10)	
Mean ± SD (mg/g dry mass)	7.55 ± 43.5
RSD (%)†	5.8
Reproducibility (*n* = 5)	
Mean ± SD (mg/g dry mass)	737 ± 31.7
RSD (%)	4.3
Recovery	
Mean value (mg/g dry mass)	672.0
Spiked concentration (mg/g dry mass)	302.6
Amount found (mg/g dry mass)	952.4
Mean (%)	92.7
RDS (%)	2.56
Detection limit (µg/mL)	2

* r^2 is the determination coefficient.
† RSD is the relative standard deviation-average value in percentage.

Sample characteristics

The practical applicability of the method was assessed by analysis of different samples: 10 from shrimp chitin, three from commercial chitin, and three from pure chitin. For shrimp chitin, glucosamine contents ranged from 890.7 to 988.2 mg/g of dry mass. This variability can be attributed to the sample's source, purification process, and hydrolysis conditions. The mean glucosamine levels determined in the present study for shrimp chitin (958.6 ± 35.7 mg/g of dry mass) and for pure chitin (968.4 ± 34.8 mg/g of dry mass) were similar, but the mean content in commercial chitin was lower (918.7 ± 49.1 mg/g of dry mass). This method can be applied to the determination of the purity of chitin and the quality of chitin products, these results could suggest that the purity from shrimp waste is satisfactory and with higher values than reported in related previous studies (29).

Conclusion

The proposed HPLC–UV method may be useful for the quantitation of glucosamine in chitin samples from fermented shrimp waste and possibly other types of samples, such as several pharmaceutical formulations. The method developed is specific, sensitive, precise, and accurate. This method may be suitable for routine analysis and for studying the purity of chitin.

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