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Chitosan analysis using acid hydrolysis and HPLC/UV

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ABSTRACT

In this study, a method for the characterization and quantification of chitosan was designed using acid hydrolysis, glucosamine derivatization, and high performance liquid chromatography. Based on a kinetic study of acid hydrolysis, we have demonstrated that chitosan can be quantitatively hydrolyzed into glucosamine in 6 h with either 10 M hydrochloric acid (HCl) at 105 °C, or 12 M HCl at 90 °C. Following N-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) derivatization, the glucosamine content can be separated from the rest of the hydrolysates and quantified using reverse-phase HPLC with UV detection. This method was validated for linearity, precision (repeatability and reproducibility), and accuracy using both chitosan and a dietary supplement formulation containing chitosan.

1. Introduction

Chitosan is a copolymer of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine, which can be derived from chitin (β-(1–4)-2-acetamido-2-deoxy-D-glucan), a natural amino polysaccharide (Toan, Ng, Aye, Trang, & Stevens, 2006). Chitosan has been shown to be non-toxic, biodegradable, biocompatible, and highly soluble in aqueous acidic solutions and it has found widespread application in food, dietary supplement, agricultural, pharmaceutical, and biotechnological industries (Li, Dunn, Grandmaison, & Goosen, 1996; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Yi et al., 2005).

To characterize and quantify chitosan, various direct analytical methods can be used. These include capillary electrophoresis, size exclusion chromatography, and colorimetric detection. Capillary zone electrophoresis can separate chitosan and its acidic derivatives to provide quantitative information (Fu, Huang, Zhai, Li, & Liu, 2007). Average molecular weights of chitosan can be determined using size exclusion chromatography (Nguyen, Winnik, & Buschmann, 2009; Terbojevich, Cosani, Focher, & Marsano, 1993). These methods are useful in characterizing chitosan products but they are not specific enough and too labor intensive for routine analysis of chitosan in complex matrices.

Utilizing the reactivity of the primary amines present in chitosan, ninhydrin, o-phthalaldehyde (OPA), and anionic dyes are used for colorimetric determination of chitosan purity and content (Larionova, Zubaerova, Guranda, Pechyonkin, & Balabushevich, 2009; Leane, Nankervis, Smith, & Illum, 2004; Muzzarelli, 1998). However, due to the wide presence of amine groups, these colorimetric methods lack satisfactory selectivity for analyzing chitosan content in complex formulations due to interference with other ingredients.

In indirect characterization methods, chitosan is hydrolyzed into glucosamine whose concentration is determined by colorimetry, ion exchange, or HPLC methods (Jang et al., 2005; Yu Ip, Veda, Robert, & Hennessey, 1992; Zhu, Cai, Yang, & Su, 2005). Hydrochloric, sulfuric, phosphoric, or trifluoroacetic acids have been used for acid hydrolysis of chitosan (Novikov, 2004; Nud'ga, Petrova, Kever, & Makarova, 2002; Zamani, Jeihanipour, Edebo, Niklasson, & Taherzadeh, 2008). Chitinase or chitosanase, derived from bacteria, have been used for enzymatic hydrolysis (Il'ina, Zueva, Lopatin, & Varlamov, 2004; Ramirez-Coutino, Marin-Cervantes, Huerta, Revah, & Shirai, 2006).

The analytical methods for quantifying glucosamine are well documented. Glucosamine levels can be determined by colorimetric methods using the reactive primary amine or the reducing aldehyde groups (Cheng, Labavitch, & VanderGheynst, 2011; Rondle & Morgan, 1955). It can be derivatized to aldononitrile acetate and analyzed with gas chromatography (Whiton, Lau, Morgan, Gilbart, & Fox, 1985). Capillary electrophoresis and ionic chromatography, equipped with electrochemical detectors, have also been used for separating and determining glucosamine (Lee, 1996; O'Shea, Lunte, & LaCourse, 1993).

HPLC, coupled with pre-column derivatization, is another method for glucosamine analysis. AOAC International has published an official method for the analysis of glucosamine in raw

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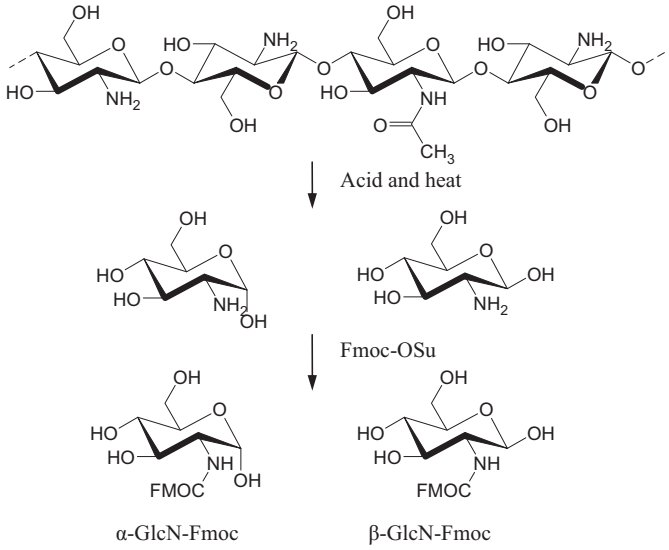

Fig. 1. Schematic view of chitosan acid hydrolysis and glucosamine Fmoc-OSu derivatization.

materials and finished products (OMA, 2008; Zhou, Waszkuc, & Mohammed, 2005). In this method, glucosamine is derivatized with Fmoc-OSu (N-(9-fluorenylmethoxycarbonyloxy) succinimide), and the derivatives are separated and quantified by HPLC with UV detection.

In this study, we demonstrated an indirect chitosan analytical method in which we hydrolyzed chitosan under acidic conditions into glucosamine, derivatized the liberated glucosamine with Fmoc-OSu, and quantified the level of glucosamine derivative in the resultant mixture with HPLC (Fig. 1). The method was validated for routine laboratory analysis.

2. Experimental

2.1. Materials

All the reagents were used as purchased without further purification. Glucosamine hydrochloride ($C_6H_{13}NO_5HCl$), N-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu), Acetonitrile (ACN), and sodium borate were purchased from Sigma–Aldrich (St. Louis, MO). Chitosan (95% purity) was obtained from Wilke Resources (Lenexa, KS). Trifluoroacetic acid (TFA), acetic acid, and hydrochloric acid (HCl, trace metal grade) were purchased from Fisher Chemical (Fairlawn, NJ). Different concentrations of hydrochloric acid were prepared by diluting concentrated HCl with deionized (DI) water.

Chitosan was blended with excipients (silicon dioxide, maltodextrin, stearic acid, plant extracts, and silicified microcrystalline cellulose) to a target content of 33% by weight and the dietary formulation was used to analyze method precision and accuracy.

2.2. Chromatographic conditions and analysis

HPLC analysis was conducted using an Agilent 1100 system equipped with degasser, quaternary pump, autosampler, and photodiode array detector (PDA). Agilent Zorbax SB-C18, 5 μm, 150 mm × 4.6 mm column was used for the chromatography. To analyze glucosamine Fmoc derivatives, the HPLC conditions were as described in the AOAC method (OMA, 2008; Zhou et al., 2005). In brief, the elution gradient begins at 70% of 0.05% TFA in water (A) and 30% ACN (B). After 6 min, B is elevated to 100% ACN in 5 min. The elution rate was held at 0.8 mL/min and the injection volume was 10 μL for both samples and standards. The chromatogram was recorded at 265 nm UV, integrated, and analyzed with Agilent Chemstation, a chromatography data system.

2.3. Sample hydrolysis and derivatization

Samples containing either 50 mg chitosan or glucosamine were weighed into thick-walled glass digestion tubes, 2 mL of 1% acetic acid solution was added, and the mixture vortexed until it formed a consistent gel. Chitosan was hydrolyzed following the addition of 10 mL concentrated HCl (8, 10, or 12 M) and heat (90 or 105 °C). After a designated period of hydrolysis (for kinetic studies, the samples were taken at 30 min intervals), the digestion mixture was cooled to room temperature and subsampled (1 mL) into a mixture of sodium borate (3.8 g) and DI water (30 mL). The pH was adjusted to 7.00 with 10 M HCl and the solution volume adjusted to 50 mL using 0.2 M borate buffer, pH 7.0.

Glucosamine in the hydrolysate was derivatized by mixing 1 mL of the dilute neutralized solution with 1 mL of 10 mg/mL Fmoc-OSu in acetonitrile and the reaction was allowed to progress to completion at ambient temperature for at least 4 h without agitation. After derivatization, the sample was diluted with 3 mL HPLC mobile phase (0.05% TFA/ACN, 1:1 (v/v)) for analysis.

Chitosan content was calculated as follows:

$$C(\%) = \frac{m_g(\text{mg})}{215.7 \times m_o(\text{mg})}[161.2 + DA(\%) \times 42 + (1 - DA(\%)) \times M_A] \times 100\%$$

where C is the chitosan content (%); m_g is the glucosamine content as determined by HPLC; 215.7 is the glucosamine (HCl) molecular weight; m_o is the sample weight for analysis; 161.2 is the mole weight of glucosamine repeating unit in chitosan; DA is the degree of acetylation in chitosan; 42 is the mole weight of one acetyl group and M_A is the molecular weight of counter acid in chitosan.

2.4. Method validation

For validation of the method, we analyzed the response linearity of the glucosamine standard, and method precision (repeatability and reproducibility) and accuracy. Five different weights of glucosamine standard (30–90 mg) were dissolved in 1% acetic acid solution. The standard solutions were processed and derivatized as described for the derivatization of sample hydrolysates. The derivatized standards were analyzed for linearity of detection. To test method repeatability, five replicate samples were prepared and analyzed following the procedure outlined in the previous sections. For method reproducibility, a second set of replicate samples ($n = 5$) were prepared and analyzed by a second analyst using a different instrument. The results were then compared to the results of the first analyst. Method accuracy was determined using a blank matrix spiked with chitosan at 50, 100, or 150% of the formulation target content (33% chitosan) and analyzed.

3. Results and discussion

3.1. Glucosamine separation and quantitative determination

Chitosan and a formulation containing chitosan were hydrolyzed, derivatized, and analyzed with HPLC. The chromatograms for the hydrolysates were the same as those for glucosamine standards. As shown in the chromatogram (Fig. 2), glucosamine derivatives were detected in two peaks at 6 and 7.5 min. This is due to the presence of two possible conformations of free glucosamine (α- and β-glucosamine) in solution (Zhou

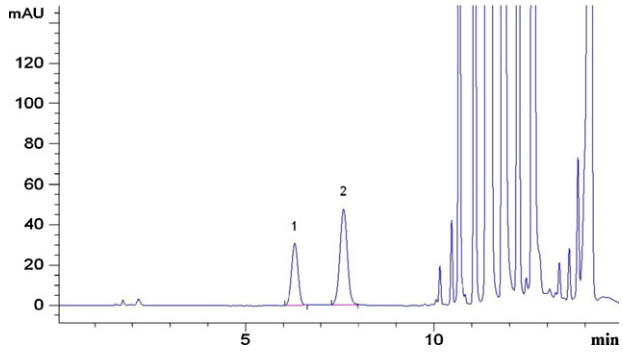

Fig. 2. The HPLC chromatogram of Fmoc-OSu glucosamine derivatives. (1) α-Glucosamine Fmoc derivative and (2) β-glucosamine Fmoc derivative.

et al., 2005). The sum of the two peak areas was used to calculate total glucosamine content.

3.2. Chitosan hydrolysis

The mechanism of chitosan hydrolysis was studied by Shabrukova, Shestekova, Zainetdinova, and Gamayurova (2002) and Einbu, Grasdalen, and Varum (2007). In strong acid, chitosan could be fully hydrolyzed to acetic acid and glucosamine. When the hydrolysis is incomplete, acetyl glucosamine is observed. When weak acid is used at low temperature, oligosaccharides are dominant in the hydrolysate products.

3.2.1. Effect of acid concentration and temperature on glucosamine yield

Chitosan was first dissolved in acetic acid to aid its dispersion in the concentrated HCl solutions. The dispersed chitosan was then hydrolyzed at two different temperatures. The glucosamine content in the hydrolysate solution was analyzed and plotted against hydrolysis time. Fig. 3 shows the glucosamine yield during the hydrolysis process with respect to hydrolysis time at various acid concentrations and temperatures.

As shown in Fig. 3, using 8 M HCl, after 8 h of hydrolysis, chitosan is not fully hydrolyzed into glucosamine, and less than 40% glucosamine yield is detected at 90 °C. The glucosamine yield reaches 98% using a digestion condition of 10 M HCl and 105 °C in 6 h. We also observed similar recovery using 12 M HCl and 90 °C in 6 h. Based on these results, hydrolysis conditions of 10 M HCl, 105 °C, and six hour duration were selected as the optimum conditions for method validation. The hydrolysis conditions of 12 M HCl and 105 °C result in complete glucosamine conversion in two hours, but the endpoint is difficult to control because glucosamine decomposes rapidly under these conditions (Fig. 3).

3.2.2. Activation energy of chitosan hydrolysis

The initial stage of the hydrolysis (up to 5 h) was fitted with a first order reaction model, $x = 1 - e^{kt}$, where x is glucosamine yield, k the reaction constant and t the reaction time. The correlation coefficient is above 0.9. The reaction constant is correlated with temperature (T) using Arrhenius equation, $k = Ae^{-Ea/RT}$, where A is constant, R the gas constant and Ea is the activation energy. The activation energy is determined to be 78 kJ/mol and is independent of acid concentration.

3.3. Effects of acid concentration and temperature on glucosamine decomposition

Since glucosamine is known to be deaminated by acid hydrolysis (Shabrukova et al., 2002), it is necessary to minimize the decomposition to achieve an accurate result for chitosan quantitation. To determine the degree of decomposition, glucosamine samples were weighed into digestion tubes and treated with the selected hydrolysis condition (105 °C, 10 M HCl). At the end of each hour interval, a sample was taken and analyzed for glucosamine. The results were recorded and are shown in Fig. 4. The degradation of glucosamine fits a first-order decomposition model. After 6 h of hydrolysis, we observed approximately a 20% loss of glucosamine. However, the decomposition kinetics during the chitosan hydrolysis process are complicated by the processes of chitosan depolymerization and deacetylation, and glucosamine generation. The glucosamine decomposition during chitosan hydrolysis does not appear to have a significant impact on chitosan recovery. The glucosamine content of the chitosan plain material is 93.6% (w/w, average results from two analysts, Table 1) which is experimentally close to the nominal chitosan content of the plain material (95%). The recovery of glucosamine from the formulation was greater than 99%, demonstrating that the decomposition of glucosamine during chitosan hydrolysis at the specified conditions has minimal impact on assay accuracy.

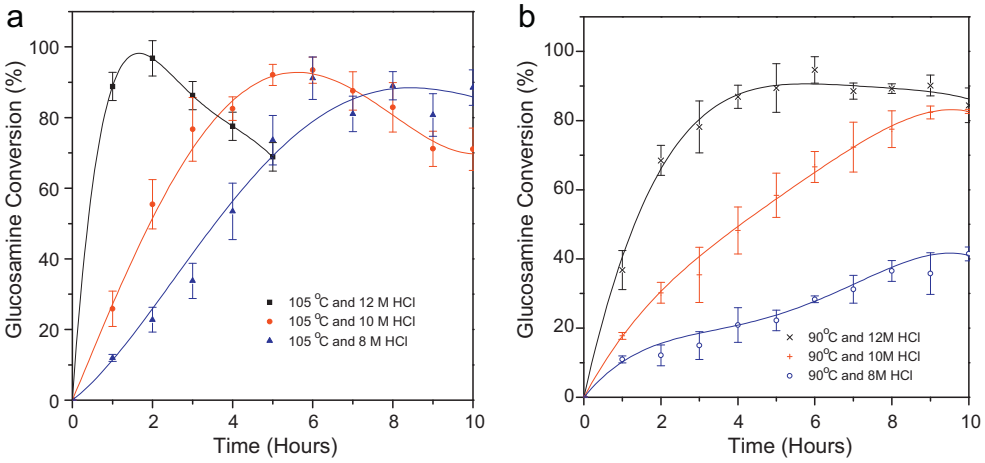

Fig. 3. Effects of acid concentration and temperature on glucosamine yield in chitosan hydrolysis. (a) Percent glucosamine conversion following chitosan hydrolysis with 8 M, 10 M, and 12 M HCl at 105 °C. The glucosamine conversion is calculated based on HPLC measurement and theoretical content. Data is presented as best fit curves. (b) Similar to (a), except that the hydrolysis temperature is 90 °C.

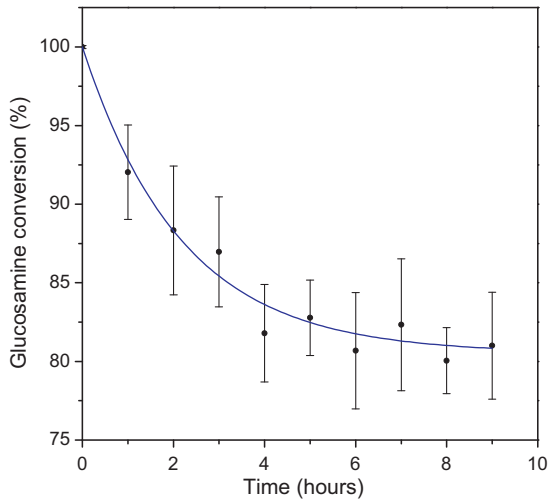

Fig. 4. Glucosamine decomposition at selected digestion conditions (10 M, 105 °C) with first-order decomposition model fitting.

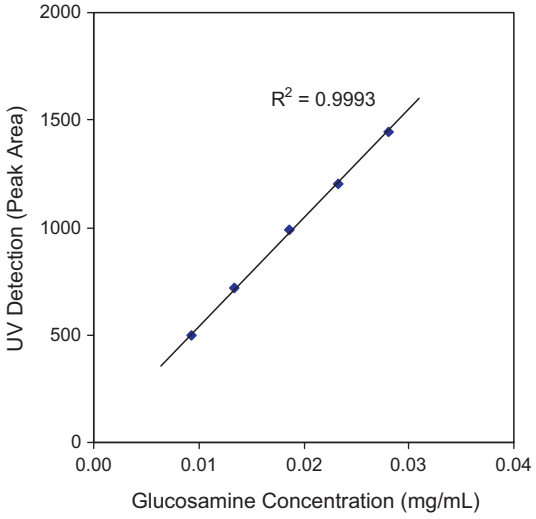

Fig. 5. Linear correlation between UV detection of glucosamine derivatives to their initial glucosamine concentrations.

3.4. Method validation

Five concentrations of glucosamine standards were analyzed and the results plotted as peak area counts against glucosamine concentration (Fig. 5). The correlation between the two parameters is linear with a correlation coefficient $R^2 > 0.999$.

The assay repeatability and intermediate precision were calculated by determining the relative standard deviation of the results, as recorded in Table 1. The relative standard deviations (RSD) of five replicate analyses (repeatability) for the chitosan and formulations are 0.93% and 1.67%, respectively, for analyst 1, and 5.25% and 5.49%, respectively, for analyst 2. The overall RSD (reproducibility) were 4.52% for the analysis of chitosan and 3.84% for the analysis of the formulation. The assay reproducibility for measuring glucosamine content was within 5% of the nominal content for both chitosan and the formulations.

The recovery of chitosan from the matrix samples was calculated as percentage of the glucosamine spike. Chitosan recovery for the three formulations (50, 100, and 150% of target chitosan) was between 99 and 103%, demonstrating the accuracy of the method.

Table 1
Method precision (reproducibility and repeatability)[a] for the analysis of chitosan and a formulation containing chitosan.

Sample type	Analyst	Chitosan as glucosamine (%)	SD_r (%)	RSD_r (%)	SD_R (%)	RSD_R (%)
Chitosan	1	96.23	0.89	0.93		
	2	91.05	4.78	5.25	4.24	4.52
Formulation	1	34.82	0.58	1.67		
	2	34.98	1.92	5.49	1.34	3.84

[a] SD_r, repeatability standard deviation; RSD_r, repeatability relative standard deviation; SD_R, reproducibility standard deviation; RSD_R, reproducibility relative standard deviation.

4. Conclusions

Based on a kinetic study of acid hydrolysis, we demonstrated that chitosan can be quantitatively hydrolyzed into glucosamine in 6 h with either 10 M HCl at 105 °C, or 12 M HCl at 90 °C. Chitosan content is calculated based on the glucosamine content in the hydrolysate. Using derivatization and HPLC, the current method is more selective than colorimetric methods. The simplicity of the method makes it suitable for routine quantitative analysis of chitosan in both plain materials and dietary supplement formulations.

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