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Determination of glucosamine in impure chitin samples by high-performance liquid chromatography

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Abstract—A simple, rapid, selective, and specific high-performance liquid chromatography (HPLC) method was developed to quantitate glucosamine, and its application for estimating purity of chitin was investigated. The chromatographic separation was achieved using a reversed-phase C_8 column, pre-column derivatization with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) and ultraviolet detection ($\lambda = 254$ nm). The mobile phase consisted of CH_3CN and H_2O. The optimum conditions of acid hydrolysis of chitin (concentration of HCl, temperature, and heating time) was obtained by performing the orthogonal array design (OAD) procedure and the released glucosamine was determined by the above HPLC method. The accuracy of the method was checked by the standard addition technique. The method was found to be specific with good linearity, accuracy, precision, and well suited for quantitation of glucosamine and determination of the purity of chitin in biological materials and food products.

Keywords: Glucosamine; Chitin; Derivatization; High-performance liquid chromatography

1. Introduction

Chitin, the most abundant natural amino polysaccharide, occurs mainly in the exoskeleton of crustaceans, insects, and in the cell walls of some microorganisms, and is estimated to be produced annually almost as abundantly as cellulose. Chitosan is (1→4)-2-amino-2-deoxy-β-D-glucan produced by the *N*-deacetylation of chitin. Due to their biodegradability and low toxicities, chitin, chitosan, and their ramifications have found a variety of applications in various fields, including biomedicine, the food, textile, and cosmetic industries and wastewater treatment.[1] Glucosamine, a natural component of glycoproteins found in connective tissues and gastrointestinal mucosal membranes, has therapeutic potential for the treatment of a variety of diseases, including arthritis, inflammatory bowel disease, and general inflammatory damage.[2,3]

The traditional source of chitin is shellfish waste from the processing of shrimp, Antarctic krill, crab, and lobster.[4,5] The production of chitin and chitosan from crustacean shells as a food industry waste is economically feasible. However, chitin, and chitosan are often found in association with proteins and such minerals as calcium carbonate. Various industrial sources, production process, and preparation conditions of these polymers cause variability of the product properties and afford different grades of product purity. Today's markets for chitin and its ramification in North America and Europe are moving most of the available products into dietary-supplement and cosmetics applications. Both of these applications require such properties as high purity, high binding capacity, and high viscosity, and, it is thus of great importance to determinate the purity of chitin and chitosan.

Since chitin is not soluble in water and most solvents, it is necessary to hydrolyze it with acid, alkali, or enzymes to yield glucosamine for determination. The characteristics of release of glucosamine from chitin

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Table 1. Results of hydrolysis of chitin ($L_9(3)^4$ orthodoxy table)

No.	Concentration of HCl (mol L^{-1})	Temperature (°C)	Heating time (h)	Average ratio[a] (%)	RSD[b] (%)
1	6	100	2	22.1	3.1
2	6	110	4	46.1	1.9
3	6	120	6	41.9	3.9
4	8	100	4	80.8	2.8
5	8	110	6	85.3	3.6
6	8	120	2	70.3	3.1
7	10	100	6	49.8	2.6
8	10	110	2	58.9	3.4
9	10	120	4	53.1	2.4

[a] Average ratio = $m/G \times 100\%$, m—the amount of glucosamine hydrochloride in the hydrolysate of chitin sample, G—the weight of chitin sample.
[b] RSD = Relative standard deviation, $n = 3$.

hydrolyzed under various conditions have been extensively investigated,[6–8] and have indicated that acid hydrolysis is the preferred method.[9] In general, chitin can be hydrolyzed with acid to cleave the β-(1→4) glucosidic bonds and removing the acetyl group, to produce glucosamine for subsequent quantitation.

The traditional method for analysis of glucosamine is spectrophotometry,[6,7,10–13] but in most cases such techniques are laborious, time-consuming, and unstable. Other analytical methods, including gas–liquid chromatographic analysis of amino sugars as volatile derivatives, and liquid-chromatographic procedures, are in common use, involving separation of either unmodified or chemically derivatized amino sugars. Methods involving gas chromatography are fast and sensitive, nevertheless they require prior derivatization of the amino sugar.[14,15] Methods for liquid-chromatographic analysis of amino sugars using silica-based column packing materials with refractive index (RI) detection showed insufficient sensibility.[16,17] Methods involving pre-column derivatization are time-consuming and need several stages of drying under vacuum.[18] Methods for ion chromatography with integrated pulsed ampero-metric detection (IC-IPAD) analysis of industrial sugar samples often exhibit extra, interfering small peaks.[19]

The purpose of this work was to develop a specific, and accurate HPLC method for glucosamine that could be routinely applied to the quantitation of this compound, and determine the purity of chitin in biological materials and evaluate the quality of chitin products.

2. Results and discussion

2.1. Optimum conditions of acid hydrolysis of chitin

During acid hydrolysis of chitin, the concentration of HCl, temperature, and heating time are prime attributes affecting glucosamine recovery.[16,20] Excessive acid treatment results in the breakdown of glucosamine and decrease of recovery. To optimize these conditions, a three-level orthogonal array design (OAD) was employed. The concentration of HCl, the temperature, and time were the optimized variables with the constant sample amount (40 mg) and volume of hydrochloric acid solution (20 mL). The results of hydrolysis of chitin are presented in Table 1. After the OAD procedure has been conducted, a graph with the sum of the average ratio of glucosamine and chitin with the same solvent volume or extraction temperature level was drawn (Fig. 1) to examine the key variable.

From the graph, it may be seen that the concentration of HCl as well as temperature are the important variables influencing the average ratio. The sum of the average ratio obtained with 8 mol L^{-1} HCl (236.4) was much higher than those obtained with 6 mol L^{-1} (110.1) or 10 mol L^{-1} (161.8). Similarly, the sum of the average ratio obtained with 110 °C (190.3) was much higher than those obtained with 100 °C (152.7) or 120 °C (165.3). The heating time in the range studied played a less important role. Therefore, the optimum concentration of HCl, temperature, and heating time for hydrolysis of chitin samples were 8 mol L^{-1}, 110 °C and 4 h, respectively.

2.2. Separation of Fmoc-amino sugars and Fmoc alcohol

Glucosamine hydrochloride does not contain a chromophore absorbing in the wavelength range useful for liquid chromatography with ultraviolet detection.

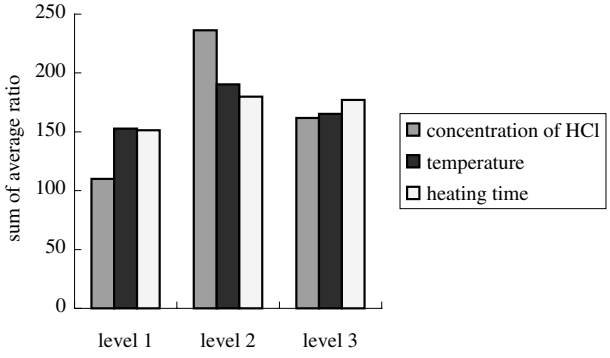

Figure 1. Effects of concentration of HCl, temperature, and heating time on the average ratio of glucosamine and chitin.

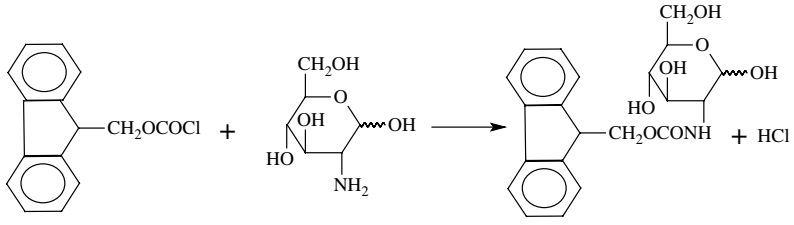

Figure 2. Derivatization reaction of Fmoc-Cl with glucosamine.

Fmoc-Cl as a derivatization reagent reacts with primary and secondary amino sugars.[21,22] The reaction of Fmoc-Cl with glucosamine is shown in Figure 2.

Fmoc-Cl also reacted spontaneously with water to yield the corresponding alcohol as a hydrolysis product.[23] As shown in Figure 3, Fmoc-alcohol increased sharply with a rise of time and finally approximately constant while Fmoc-Cl was inversely proportional to Fmoc-alcohol. At the same time, glucosamine gave two peaks on the column used, presumably representing the anomers[24] in equilibrium and, in consequence, two peaks (t_R = 3.94 min, 4.19 min) appeared in the chromatogram. The remaining of Fmoc-Cl (t_R = 10.45 min) and Fmoc-alcohol (t_R = 6.94 min) eluted after glucosamine.

2.3. Optimum conditions of derivatization

As mentioned in Ref. 23, the derivatization reaction was accompanied by hydrolysis, and therefore, the reaction conditions, namely temperature, time, and pH, were very important for both the derivatization and the hydrolysis.

2.3.1. Temperature and time of derivatization. The effects of temperature and time on the derivatization of glucosamine are shown in Figure 4(a)–(e). These results showed first that, the rate of derivatization increased with the temperature from 10 to 20 °C, but then decreased sharply with temperature from 20 to 60 °C. The rise of temperature increased the rate of hydrolysis of Fmoc-Cl, so that the rate of derivatization decreased. When the temperature was raised from 60 to 80 °C, it appeared that a divergent reaction had occurred. At 20 °C, 30 min was enough to complete the derivatization reaction. Therefore, the optimum temperature and time were 20 °C and 30 min, respectively.

2.3.2. pH of the reaction medium. The pH of the medium was important in controlling both the derivatization and the hydrolysis. The rate of derivatization increased with increasing pH, but was paralleled by an increased rate of hydrolysis of Fmoc-Cl. The optimum conditions were those under which the reactive amino groups more effectively compete for the limited amount of reagent available. The effect of pH on the derivatization of glucosamine is shown in Figure 5. These results suggest that the optimum pH value for derivatization of glucosamine is 7. Under these conditions, the rate of derivatization was the highest while the rate of hydrolysis was the lowest.

2.4. Precision and accuracy of the method

The concentrations of the standards were calculated by using the observed peak areas and the straight-line parameters, and all were within 5% of the nominal value. These observations showed a satisfactory fit to the linear model. The standard curves for glucosamine

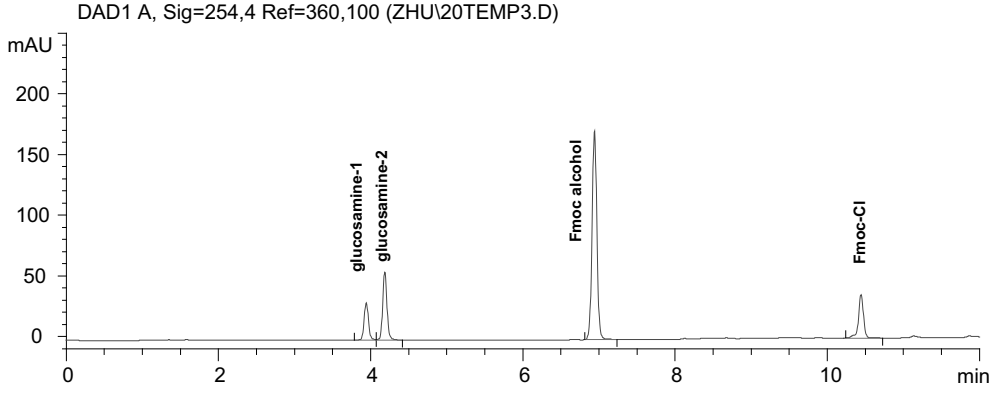

Figure 3. HPLC chromatograms of Fmoc derivatizatives of glucosamine.

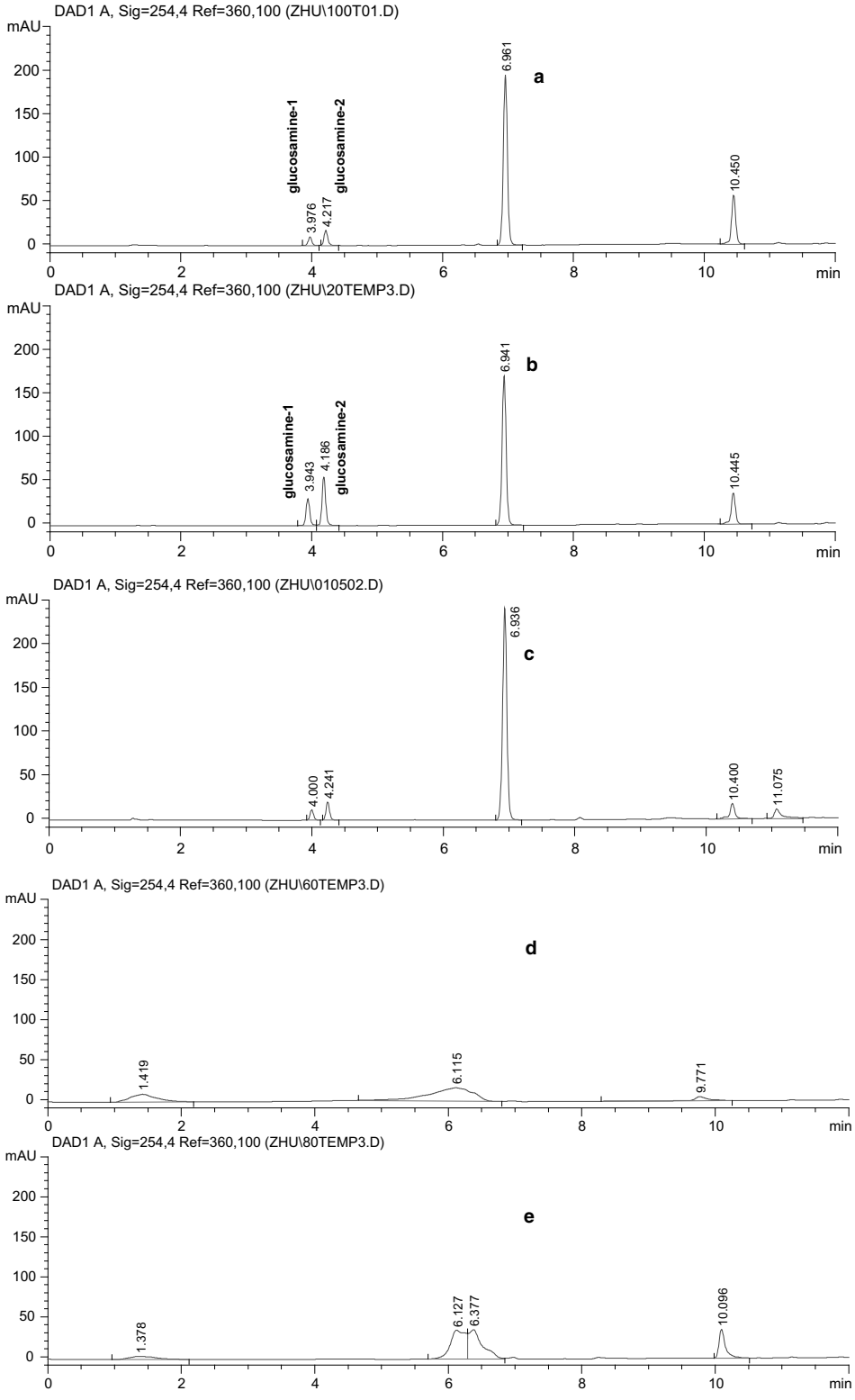

Figure 4. HPLC chromatograms of Fmoc derivatizatives of glucosamine under different temperatures: (a) 10 °C, (b) 20 °C, (c) 40 °C, (d) 60 °C, (e) 80 °C).

hydrochloride showed linearity over the selected concentration range from 100 to 500 $\mu g\,mL^{-1}$ for raw materials with consistent slopes, and excellent correlation coefficients ($r \geqslant 0.99$) throughout the validation runs. In addition, the ruggedness of the HPLC method as a function of time, at room temperature, was carried out for different concentrations of the glucosamine. The results are shown in Table 2. The assay

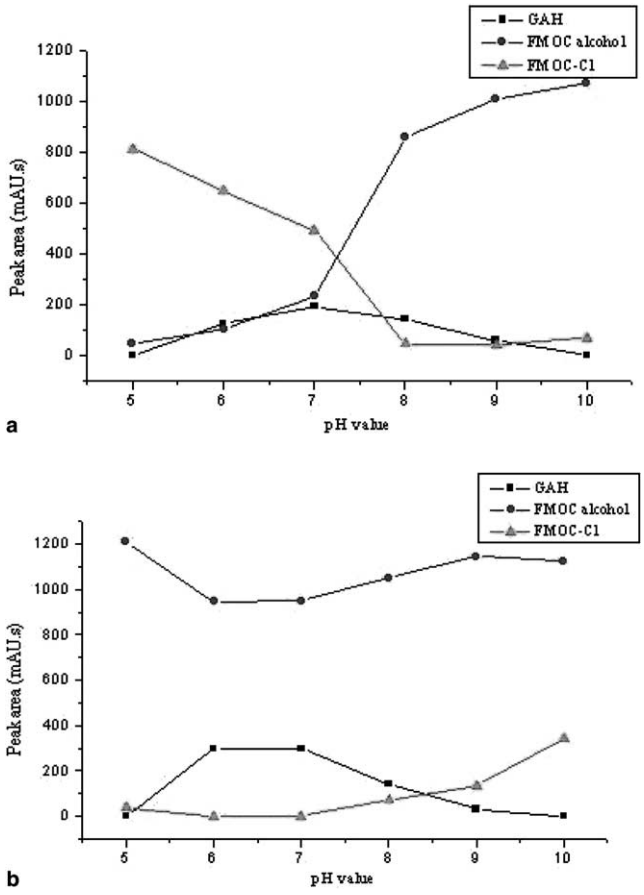

Figure 5. The effect of pH on the derivatization of glucosamine: (a) reaction time: 10 min, (b) reaction time: 30 min.

was precise, as the standard deviation was less than 4.0%.

The assay accuracy of the method was checked by using the standard addition technique, namely, to assess glucosamine hydrochloride three times from the chitin sample spiked with two concentration levels of glucosamine hydrochloride. The results are shown in Table 3. The recovery ranged from 90.1 to 98.7% for glucosamine hydrochloride.

2.5. Determination of the purity of chitin sample and comparison with the spectrophotometric method

The suggested HPLC method was found effective for determining the purity of five chitin samples in biological materials. Marked differences in purity of chitin could be observed from different source samples. This may result from various industrial sources, production process, and preparation conditions of these polymers.[5,25] The quality and properties of the chitin sample was different. These data can screen the quality of chitin products and supervise chitin processing.

Further comparison with the compendial colorimetric method[26] was performed. As shown in Table 4, glucosamine appeared to be degraded at higher concentrations (>50 μg mL^{-1}) by the spectrophotometry (SP) method, resulting in deviations from results by the HPLC method. Therefore, the spectrophotometric method was less accurate the suggested HPLC method. Moreover, the relative standard deviation by the HPLC method was lower than by the spectrophotometric method. In addition, the spectrophotometric method was more laborious and unstable.

In conclusion, a simple, rapid and accurate HPLC method for analyzing purity of chitin in raw biological materials and foods has been developed. The optimum conditions of acid hydrolysis of chitin were found at 110 °C with 8 mol L^{-1} hydrochloric acid for 4 h. Compared with the spectrophotometric method, the method required less time and provided better recovery and reproducibility. Therefore, the method should be

Table 2. Precision for assay of glucosamine hydrochloride

Experiment No.	Taken (μg mL^{-1})	Found (μg mL^{-1})	Accuracy (%)	RSD (%)	
				Intra-day	Inter-day
1	50.0	50.3	101.0	3.2	1.9
2	100.0	100.4	100.0	2.1	1.0
3	200.0	199.6	99.8	0.8	3.6
4	400.0	401.8	100.5	3.2	1.9
5	500.0	499.4	99.9	1.6	4.2

Table 3. Accuracy for assay of glucosamine hydrochloride

No.	Initial (μg mL^{-1})	Pure added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)
1	59.9 ± 0.5	100.0	158.6	98.7
		200.0	248.5	94.3
2	186.2 ± 0.4	100.0	278.7	92.5
		200.0	366.4	90.1

Table 4. Determination of purity of chitin from different samples

No.	Source	Ash (%)	Moisture (%)	Purity of SP method (%) (%RSD)	Purity of HPLC method (%) (%RSD)
1	Alaska crab leg	1.00	6.4	86.9 (4.6)	88.1 (3.3)
2	Self-made (from shrimp)	0.16	8.3	89.5 (3.9)	91.3 (1.5)
3	Wuxi 0105	1.74	1.3	88.2 (4.8)	90.6 (1.8)
4	Wuxi 0202	0.12	3.1	75.7 (5.4)	76.3 (3.1)
5	Pure chitin	—	—	99.5 (4.9)	99.5 (2.5)

applicable for routine determination of glucosamine and the purity of chitin in raw biological materials and foods.

3. Experimental

3.1. Materials and reagents

Standard chitin and D-glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride were purchased from Sigma Chemical Co. The purity of the chitin was found to be 99.5 ± 0.6 by spectrophotometry. Analytical grade-Fmoc-Cl was purchased from Shanghai Supelco Co.

All chemicals and solvents were analytical grade or HPLC grade. Deionized water was purchased from the Wahaha purified water Co.

Raw chitin materials were purchased from Alaska (USA), Zhejiang, and Wuxi (China).

3.2. Instrumentation and chromatographic conditions

An Agilent 1100 series high-performance liquid chromatograph equipped with a 1312A binary gradient pump, 1329A thermostatted autosampler, G1315A diode-array detector, and a G1319A Chemstation was used. The analytical column, a *ZORBAX* Eclipse XDB- C_8 (150 mm × 4.6 mm i.d., 4 μm, Agilent, USA) was used for separation. The system was operated at ambient temperature.

The HPLC mobile phase A (water) and B (acetonitrite) lines were, respectively, equipped with aqueous and organic solvent in-line filters. A binary gradient elution was performed from 30% to 100% of mobile phase B over 12 min, the column was then regenerated with 100% mobile phase B during 2 min before returning to initial conditions.

The mobile phase was delivered at a flow rate of 1.0 mL min^{-1} and the eluent was evaluated at a wavelength of 254 nm.

3.3. Preparation of borate acid buffering solution

Boric acid (1.24 g) was dissolved in 50 mL of deionized water and adjusted to pH 5, 6, 7, 8, 9 and 10, respectively, with 0.2 mol L^{-1} sodium hydroxide, and then completed to 100 mL with deionized water. This buffer could be stored at room temperature for two weeks.

3.4. Derivatization of glucosamine with Fmoc-Cl

Derivatization was performed in a 2.0-mL microvial equipped with a screw-cap. 100 μL of 0.2 mol L^{-1} borate buffer (pH 7.0), 100 μL of 129.35 mg L^{-1} Fmoc-Cl in acetonitrile and 10 μL of aqueous solution containing 1–10 nmol of glucosamine hydrochloride standard or sample (after neutralization with 2.0 mol L^{-1} sodium hydroxide) were added to the vial successively. The mixture was allowed to react at 20 °C for 30 min. After derivatization was complete, 5 μL of the mixture was injected onto the column.

3.5. Construction of calibration curve

Glucosamine standards were prepared in concentrations ranging from 100 to 500 μg mL^{-1} from a stock solution of glucosamine hydrochloride (1000 μg mL^{-1}). The quantitative determination of glucosamine hydrochloride under the suggested derivatization and HPLC procedure, is valid in concentration range from 100 to 500 μg mL^{-1}. The linear regression equation was found to be

$$y = 8.8 + 1.0288x,$$

where y is the sum of area of double peak under the curve and x is the concentration of glucosamine hydrochloride in μg mL^{-1} with a coefficient of determination 0.9997.

3.6. Acid hydrolysis and calculation of purity of chitin

Each chitin sample (including pure chitin) was weighed precisely (between 10 and 50 mg of dry chitin material) and put in a PTFE container, and 20 mL of 8 mol L^{-1} hydrochloric acid solution was added to each container. The containers were then firmly closed and kept for the selected time at the selected temperature. The containers were then cooled to room temperature. From each solution, 1.0 mL was withdrawn and deposited in a glass tube. The pH of the solution was adjusted to 7.0 using 2.0 mol L^{-1} sodium hydroxide. After pH adjustment, the solution was adjusted to 10 mL with deionized water. The concentration of glucosamine in the hydrolyzate was measured by the preceding HPLC method. Calculation of the purity of chitin was as follows:

$$X = \frac{mG_0(203 - 42\mathrm{DD})}{203m_0G} \times 100\%,$$

where X—the purity of chitin sample (%); m—the amount of glucosamine hydrochloride in the hydrolyzate of chitin sample; m_0—the amount of glucosamine hydrochloride in the hydrolyzate of the pure chitin sample; G—the weight of chitin sample; G_0—the weight of pure chitin sample; DD—degree of deacetylation of chitin sample.

3.7. Recovery test

The chitin sample (40 mg) and 1 or 2 mL of glucosamine standard solution (100 μg/mL) were put in a PTFE container and 20 mL of 8 mol L^{-1} hydrochloric acid solution was added. The container was then firmly closed and treated under the optimum conditions for 4 h. The container was then cooled to room temperature. After

pH adjustment with 2.0 mol L^{-1} sodium hydroxide, the concentration of glucosamine in the hydrolyzate was measured by the HPLC method, and the global recovery rate calculated.

Acknowledgements

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