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Research Article

A New Rapid and Sensitive Spectrophotometric Method for Determination of a Biopolymer Chitosan

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A novel approach of spectrophotometric quantification of chitosan based on one-step depolymerization with sodium nitrite followed by reaction of the end product with thiobarbituric acid has been proposed, optimized, and validated. In this process, chitosan is converted into 2,5-anhydro-D-mannose that reacts with thiobarbituric acid to form pink color. The color that resulted from the reaction was stabilized and measured at 555 nm. The method optimization was essential as many procedural parameters influenced the accuracy of the determination including hydrolysis conditions, thiobarbituric acid concentration, reaction time, pH, reaction temperature, and color stability period. Under given optimized conditions that appeared to be critical, chitosan was quantitatively analyzed and the calibration graph was linear over the range of 10–50 μg/mL ($r^2 = 0.999$). This approach was applied for determination of chitosan in pharmaceutical formulation (chitocal) and had a recovery rate of higher than 96%. The developed method is easy to use and highly accurate.

1. Introduction

Recently, much attention has been paid to chitosan as a potential polysaccharide resource. Chitosan is a linear amino polysaccharide of glucosamine and *N*-acetylglucosamine units and is obtained by alkaline deacetylation of chitin [1]. The following major characteristics of chitosan make this polymer advantageous for numerous applications: (1) it has a defined chemical structure; (2) it can be chemically and enzymatically modified; (3) it is physically and biologically functional; (4) it is biodegradable and biocompatible with many organs, tissues, and cells; (5) it can be processed into several products including flakes, fine powders, beads, membranes, sponges, cottons, fibers, and gels. Therefore, chitosan becomes of great interest as a new functional material of high potential in various fields and the discovery or development of methods for chitosan determination is imperative [2–5].

Although research activities dealing with chitosan are numerous, a generally accepted simple method for direct quantitative analysis is lacking. Chitosan can be degraded to glucosamine monomer by hydrolysis, and there are several reports on the determination of glucosamine by chromatographic, colorimetric, and fluorimetric techniques, or a combination of these [6–8]. Eikenes et al. [6] developed a method for determination of chitosan in wood and water samples based on acidic hydrolysis of chitosan to glucosamine followed by online derivatization by *o*-phthalaldehyde, chromatographic separation, and fluorescent detection. Roseman and Daffner [9] determined the concentration of glucosamine by acetylating followed by photometric detection of *N*-acetyl glucosamine.

A novel approach of colorimetric quantification of chitosan based on the derivatization reaction of amino groups in chitosan with *o*-phthalaldehyde and a thiol-*N*-acetyl-L-cysteine has been developed by Larionova co-workers [10]. The procedure conditions have been optimized for chitosan assay in the presence of polyanionic electrolyte dextran sulphate. The method has proven to be convenient and reliable for quantitative determination of either the concentrations of chitosan of various molecular weights or their degree of deacetylation.

Based on the fact that, in dilute solutions, the amino group is at its most accessible form for optimum quantification, several spectrophotometric approaches have been reported. The determination of aminoglucose by the indole reaction [11] and the formation of a complex of chitosan with ninhydrin are the most used protocols [12, 13]. These methods did not involve any pretreatment (i.e., hydrolysis) of the solution, but the method was highly dependent on the degree of deacetylation of chitosan. More reliable analytical methods are colorimetric assay of chitosan with various anionic dyes, for instance, C.I. Acid Orange 7, C.I. Acid Red 13, C.I. Acid Red 27, Orange II, Alizarin S, Alizarin GG, Congo Red [14], Cibacron Brilliant Red 3B-A [15, 16]. However, these techniques based on the reactions between free amino groups of chitosan and acting agents are ineligible for determination of chitosan in presence of substances bearing primary amino groups, including peptides [17]. Recently, an electrochemical method based on the oxidation-reduction peaks produced by chitosan solubilisation in an acidic buffer has been reported as a sensitive measurement for this polymer [18]. However, the application of this analysis to complex mixtures would be difficult.

Most quantitative analysis of polysaccharides such as chitosan entails total hydrolysis to monosaccharides followed by subsequent characterization of the monomers [6, 19, 20]. However, the glycosidic linkages of chitosan are resistant to acid hydrolysis such as the anthrone or phenol-sulphuric acid methods [21]. In addition, such hydrolysis methods are difficult to apply. Chitosanases may be used for depolymerization of chitosan, but availability and costs limit the use of such enzymes [22].

In the present work, a selective and sensitive method for determination of chitosan was developed using sodium nitrite hydrolysis and online derivatization with thiobarbituric acid in basic medium to form pink color. Sodium nitrite causes depolymerization and deamination of chitosan and converts it to 2,5-anhydro-D-mannose [19, 20]. The procedure conditions have been optimized and evaluated for chitosan assay in chitocal as a pharmaceutical formulation.

2. Materials and Methods

2.1. Principle of the New Assay. The method is based on determination of chitosan after deaminative cleavage by sodium nitrite ($NaNO_2$) and coupling of the 2,5-anhydro-D-mannose thus produced with thiobarbituric acid to give pink product. 2,5-Anhydro-D-mannose represents the sum of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc). Depolymerization of chitosan by using of $NaNO_2$ is selective, rapid, easily controlled, no side reactions have been observed with chitosan, and, as a consequence, the stoichiometry and reaction products are well established [23, 24]. Furthermore, because chitosan is soluble in dilute acids, the reaction with $NaNO_2$ is homogeneous. The depolymerization mechanism involves a deamination of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose unit forming 2,5-anhydro-D-mannose at the new reducing end as shown in Figure 1(a) [24–27]. The end product contains an aldehyde which is determined colorimetrically with thiobarbituric acid. Thiobarbituric acid undergoes a Knoevenagel condensation reaction with aldehydes to give the color-condensed product according to Tietze and Beifuss [28] (Figure 1(b)). The observed level of pink product was related to the chitosan present in the sample.

2.2. Assay Reagents. All experiments were performed with analytical reagent grade chemicals. Low molecular weight of acid-soluble chitosan (made from coarse ground crab with 89% degree of deacetylation) was purchased from Sigma-Aldrich Co. (USA). Chitosan stock solution was made by dissolving 100 mg of chitosan in 100 mL of 1% (v/v) aqueous acetic acid; the chitosan dissolves readily on shaking. From this solution, suitable dilutions can be made when required. An aqueous solution of 0.5 M $NaNO_2$ was prepared, and the solution should be kept between 0 and 4°C. 2-Thiobarbituric acid standard solution (0.04 M) was prepared by dissolving 0.576 g of reagent (≥98%, Sigma-Aldrich Co., USA) in 100 mL of distilled water with warming for 15 min.

2.3. Procedure. Chitosan solution was pipetted into the test tube, and the $NaNO_2$ reagent was added from a micropipette (0.1 mL of 0.5 M). The mixture was shaken briefly. The tubes were kept at 80°C for 30 min in a water bath to complete the depolymerization-deamination reaction. After depolymerization, the pH was raised to 8 by adding 0.2 mL NaOH (0.1 M) and the solution was then shaken, and 0.5 mL of the thiobarbituric acid solution (0.04 M) was finally added. The tubes were incubated a second time at 80°C for 10 min in a water bath. The solution was briefly cooled, and the absorbance was measured at 555 nm by Spectrophotometer (Unico 1200-Spectrophotometer, USA) against blank.

2.4. Assay of Chitosan in Pharmaceuitical Formulation. In Chitocal capsules (500 mg + 100 mg ascorbic acid + 80 mg gymnema sylvestre, manufactured by Aldebeiky Group-DBK Pharma, Cairo, Egypt), the well-mixed powdered content of 10 capsules was used in the assay. A weight of 100 mg of the powder was transferred into volumetric flask and dissolved in 50 mL aqueous acetic acid solution (1%, v/v) then adjusted to volume. Different aliquots (20, 40, 60, 80, and 100 μL) of this solution were analyzed according to the proposed procedure as previously mentioned. For the recovery study, the standard addition method has been used. Different amounts of the corresponding pure chitosan were added to a fixed amount of dosage form (capsule) where chitosan had been determined. Results are the average of triplicate readings of all determinations in the solutions.

2.5. Statistical Methods. Linear regression analysis of the absorbance at 555 nm versus chitosan concentration, determination of confidence intervals, and other statistical tests were carried out with SPSS 12.0 software program (Statistical Package for Social Sciences, USA).

3. Results and Discussion

3.1. Spectral Analysis. Firstly, we checked the use of acetic acid to dissolve chitosan because this is the usual solvent for

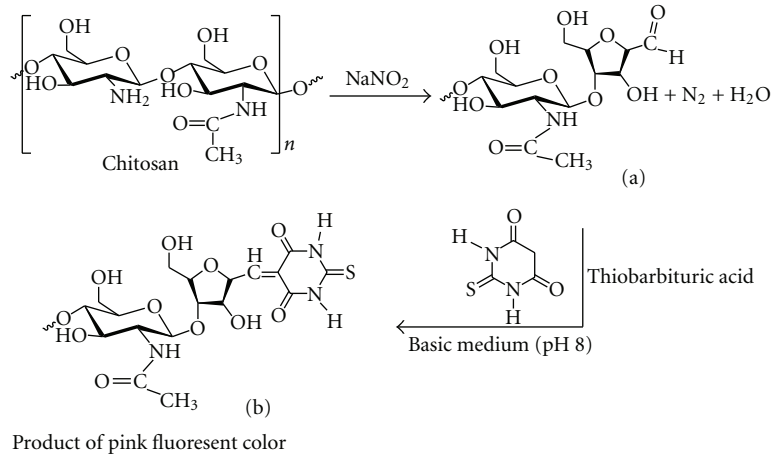

Figure 1: The mechanism by which the nitrous acid reaction leads to chain cleavage resulting in a 2,5-anhydro-D-mannose-reducing end (**a**) and proposed reaction of the reducing end in basic medium with thiobarbituric acid to generate a pink fluorescent dye (**b**).

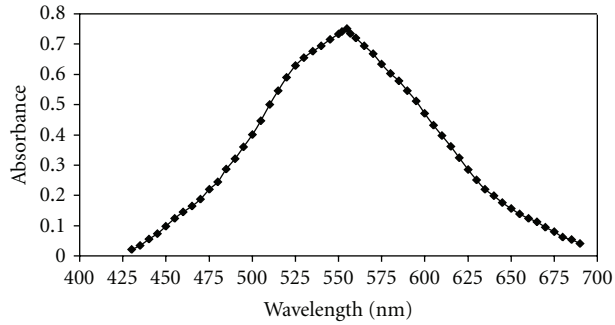

Figure 2: Absorbance spectrum of the pink-fluorescent product from the reaction between reducing end of chitosan and thiobarbituric acid. 400 to 700 nm spectrum is plotted and clearly shows the maximum wavelength is 555 nm for the pink color formed.

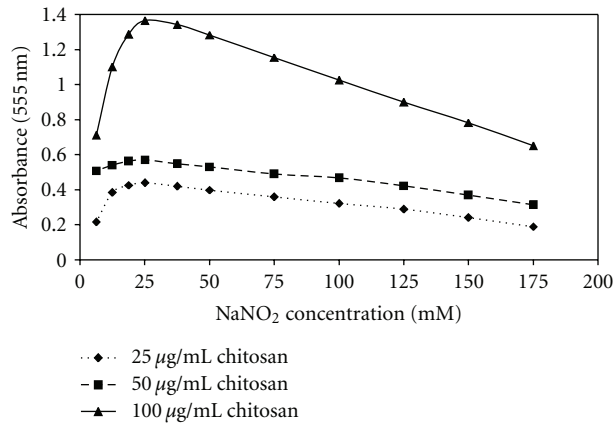

Figure 3: Effect of sodium nitrite concentration that used as a depolymerized reagent of chitosan for 30 min on the rate and intensity of the color development.

chitosan solubility. Absorption scans in the range of 400 to 700 nm were performed on 25 μg/mL chitosan solution in 1% (v/v) aqueous acetic acid solution. The same solution without chitosan was used as blank. The color developed within seconds of the mixing of thiobarbituric acid solution with the reagents and reached stability after 10 min. The spectral absorbance of this color is shown in Figure 2. The product reacts with thiobarbituric acid giving rise to a pink-colored derivative with excitation maximum located at 555 nm. Therefore, all absorbance measurements were performed at this wavelength. To obtain high sensitivity, it is necessary to investigate the effect of all parameters that could influence the chemical reactions and the performance of the colored complex.

3.2. Optimization of the Reaction Conditions. For survey of the optimal reaction conditions, seven factors were considered including the sample size, the amount of $NaNO_2$, the pH, the temperature, the depolymerization time, the amount of the thiobarbituric acid reagent, and the stability of the color formed. Three chitosan concentrations (10, 25 and 50 μg/mL) were selected to allow study the effect of these factors. Therefore, the sequence was adopted as follows: chitosan, $NaNO_2$, pH increase to 8, thiobarbituric acid, and then the absorbance measured. Using this sequence, when the 25 mM $NaNO_2$ was added to chitosan solution, incubation at 80°C for 30 min, and 10.8 mM thiobarbituric acid as added immediately after the pH adjusted to 8, the maximal absorbance was obtained.

3.2.1. Effect of the Sodium Nitrite Concentration. The effect of $NaNO_2$ concentration was investigated within the range of 6.25 to 175 mM. The results revealed that the absorbance increased by increasing the reagent concentration up to 25 mM and decreased at higher concentrations (Figure 3). Therefore, 25 mM $NaNO_2$ was the optimum concentration and was applied in the proposed method. It could be deduced from the curves that 25 ug/mL chitosan was more sensitive to $NaNO_2$ degradation compared with that of 50 and 100 ug/mL chitosan. It seems that the depolymerization of chitosan was influenced not only by the concentration of

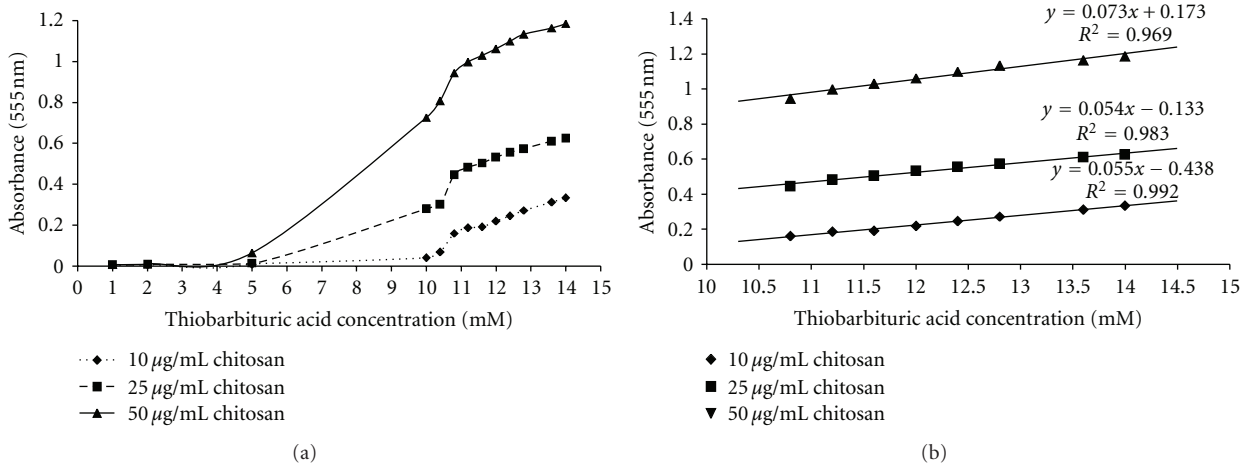

Figure 4: Influence of the concentration of thiobarbituric acid on the intensity of the color development (a) and the linear correlation between thiobarbituric acid and color development (b).

$NaNO_2$ but also by the initial concentration of chitosan. This phenomenon is related to the structure of chitosan in the solution. Normally chitosan molecule takes the shape of an extended random coil in the solution. When the concentration is high, due to the larger viscosity of the solution and strong intermolecular interactions, accessible chain segments can only stretch in a limited area, decreasing the contact probability with $NaNO_2$, thus resulting in a lower degradation rate. In this case, low chitosan concentration would be preferred to yield end product in a short time.

3.2.2. Effect of the TBA Concentration on Development of the Color. In order to study the effect of thiobarbituric acid, three chitosan concentrations (10, 25, and 50 ug/mL) were tested then the absorbencies at 555 nm were then plotted against serial concentrations of the thiobarbituric acid (Figure 4(a)). The influence of the thiobarbituric acid concentration was studied in the range between 1 to 14 mM. The rate of the reaction was found to increase continuously (presumably as reducing ends are produced) with a concentration of the thiobarbituric acid reagent up to 10 mM and then remain constant between 11.2 to 14 mM. A concentration of 10.8 mM of thiobarbituric acid was selected as the optimum concentration for the three chitosan concentrations. Moreover, regression analysis of the thiobarbituric acid concentrations ranged from 10.8 to 14 mM yielded a linear correlation with correlation coefficients of 0.992, 0.983, and 0.969 for 10, 25, and 50 μg/mL chitosan, respectively (Figure 4(b)).

3.2.3. Effect of the Reaction Time on the Color Development. In order to investigate the effect of the reaction time, keeping $NaNO_2$ and thiobarbituric acid at 25 and 10.8 mM, respectively, chitosan concentrations from 5 to 100 μg/mL were degraded by $NaNO_2$ in 1% acetic acid solution (v/v) and the reaction was examined after 15, 30, and 45 min, and the corresponding samples were processed in the same way as that described before. The absorbance values of the samples were determined at 555 nm, and the results are illustrated in Figure 5(a). It will be observed from the readings given in this figure that the maximum color intensity developed after the solutions have been heated for 30 min. This means that complete depolymerization or total acid hydrolysis of all chitosan concentrations occurred mainly in the first 30 min. It has been found that the colors obtained after heating of the chitosan solutions with $NaNO_2$ reagent for 45 min or longer are of a slightly different tint from those obtained with solutions which have been heated for 30 min. Therefore, this time was selected as the optimum for the determination reaction.

3.2.4. Effect of the pH. Another possible reason for the low color yields could be differences in the pH optimum for the reaction. The thiobarbituric acid reaction was carried out in the pH that ranged from 5 to 12 (Figure 5(b)). The pink complex gave its maximal absorbance only within a narrow pH interval, 7-8. The three chitosan concentrations gave highest color yield at pH $\approx$ 8. Therefore, this pH value was selected as the optimum for the determination reaction.

3.2.5. Calibration Curve and Final Optimized Assay. Different concentrations of chitosan ranged from 5 to 60 μg/mL were mixed in test tubes with 100 μL of $NaNO_2$ (0.5 M). After incubating for 30 min at 80°C in a static water bath, the pH was increased to 8 by adding 200 μL NaOH (0.1 M). 540 μL of thiobarbituric acid (0.04 M) was added, and the tubes were incubated a second time at 80°C for 10 min in a water bath and then cooled to room temperature. The absorbance was measured at 555 nm by spectrophotometer. The plot of absorbencies against serial concentrations of chitosan gave straight line as shown in Figure 6.

3.2.6. Stability of the Color Complex. The color of complex formed (Table 1) remained stable between 5 and 180 min after color development. Fading of the color with time was found to be very slow, and no fading occurred during the

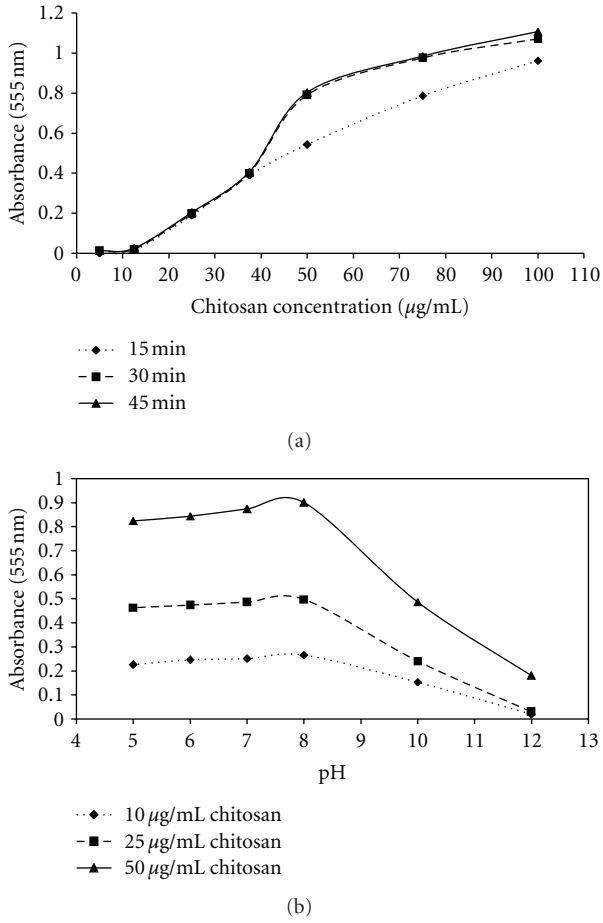

Figure 5: The influence of the time of heating with the sodium nitrite reagent on the subsequent color development (a) and the function of pH for reaction of the reducing end of chitosan with thiobarbituric acid after 30 min reaction time (b).

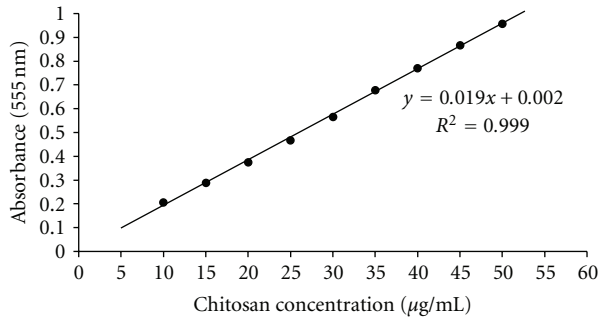

Figure 6: Standard analytical curve for chitosan at optimum conditions in the proposed method at 555 nm. Each value is the mean of four determinations.

first 180 min, even when exposed to indoors illumination as long as direct sunlight was avoided. After 12 h, decrease in absorbance was recorded, not exceeding 1% per hour.

3.3. Analytical Sensitivity. As for the slope of the calibration line, analytical sensitivity for chitosan determination was found to be 0.0019 absorbance/amount (0.1 μg/mL chitosan).

Table 1: Stability of color complex by the proposed method.

Time after color development (min)	Absorbance (555 nm) with the following amount of chitosan (μg/mL)		
	5	10	25
5	0.086	0.151	0.410
10	0.086	0.152	0.412
15	0.091	0.153	0.415
20	0.095	0.154	0.411
25	0.098	0.152	0.410
30	0.098	0.152	0.411
35	0.101	0.154	0.412
40	0.100	0.152	0.411
45	0.100	0.154	0.409
50	0.102	0.157	0.409
55	0.097	0.155	0.404
60	0.100	0.157	0.405
90	0.092	0.153	0.402
120	0.092	0.153	0.409
150	0.092	0.150	0.407
180	0.092	0.150	0.414
1080 (18 h)	0.046	0.080	0.231

The values are means of four replicates.

3.4. Linearity of the Color Responses. A linear relationship between the absorbance and the chitosan quantity was observed within the range of 10–50 μg/mL. The upper limit of the linearity in the assay was 50 μg/mL chitosan. In the regression analysis, the r value was 0.999, $P < 0.001$, the slope was 0.0191, and the intercept was 0.0028.

3.5. Lower Detection Limit. The detection limit of the method was determined by evaluating the zero calibrator 10 times. The limit of detection (LoD), defined as the mean chitosan value of the zero calibrator (limit of blank, LoB) + 1.645 (SD low concentration sample) according to the method of Armbruster and Pry [29], was found to be 0.185, where LoB = mean blank + 1.645 (SD blank).

3.6. Applications. To evaluate the performance of the method for analysis of real samples, determination of chitosan in pharmaceutical formulation of Chitocal capsules was investigated. The results presented in Table 2 revealed that the capsules product presented contents of chitosan in the range of 73.82 to 80.81% of that reported on the label with average of 76.11%. In addition, the recoveries for the addition of different concentrations of chitosan to Chitocal samples are in the range of 96.67 to 98.46% with average of 97.68% and indicate that the proposed method is suitable for determination of trace amounts of chitosan in the real samples.

Table 2: Determination of chitosan in pharmaceutical formulation by the proposed method.

Sample	Chitosan content declared in label (mg)	Sample taken (μL)	Chitosan calculated (mg)	Chitosan found (mg)	% of chitosan in formulation ± SE	Recovery (%)[a] ± SE
Chitocal capsule	500 mg	20	0.029	0.022	73.82 ± 1.54	96.67 ± 1.75
		40	0.059	0.044	74.86 ± 1.87	97.28 ± 0.88
		60	0.088	0.067	75.52 ± 1.84	97.89 ± 0.56
		80	0.118	0.089	75.59 ± 2.19	98.11 ± 1.15
		100	0.147	0.119	80.81 ± 2.51	98.46 ± 0.82
Mean ± SE					76.11 ± 1.55	97.68 ± 0.32

[a]Recovery (%) values were calculated from the amount of chitosan that had been determined after addition of its different amounts to a fixed amount of dosage form (capsule). The data are average of three determintaions.

In spite of the availability of several methods for quantitative determination of chitosan, only isolated examples have been found on the estimation of the content of this polymer in different samples. In the present study, a method for quantifying the chitosan in pure and commercial products was developed. This method is based on the previous reports on its unique degradability in nitrous acid solution. In previous reports, hydrochloric acid was used for the hydrolysis of chitin and chitosan prior to glucosamine analysis [19, 30–33]. However, hydrolysis by HCl is usually a time-consuming process to achieve complete depolymerization and high yield. Bosworth and Scott [19] reported that the maximum yield of hydrolysis of chitosan with 5.5 N HCl at 102°C was not reached in less than 23 h. Chen and Chiou [30] recovered only 63.7% of chitin as glucosamine after 60 min of hydrolysis with 2.5 M HCl at 140°C. Wu et al. [34] used 6 M HCl at 110°C and recovered 93.0% of chitosan after 3 h and only 68.7% of chitin after 12 h as glucosamine. Zhu et al. [33] got 85.3% recovery of chitin at 110°C after 4 h. In all of these works, the sum of free and acetylated glucosamine was reported. The recoveries of chitosan from the commercial preparation with the present method were >98% (Table 2), indicating high performance of the method for the analysis of materials containing this biopolymer.

In earlier methods, glucosamine has been quantified by hydrolysis in HCl solutions. However, part of the sample that was not depolymerized by HCl and stayed in the solid form was not included in the measurement [19, 30, 33, 34]. However, in the present method, chitosan samples dissolved in aqueous acetic acid solution and became completely soluble and the chitosan is not precipitated from the solution. In this case, the whole initial sample is considered for the estimation of chitosan content.

4. Conclusions

A colorimetric method is described for the determination of chitosan in pure and pharmaceutical formulation. This method depends on the acid hydrolysis of chitosan molecule by one step with sodium nitrite treatment to convert it into 2,5-anhydro-D-mannose at the new reducing end which reacts with thiobarbituric acid in basic medium to give rise very stable pink-colored solution. The procedure has been applied for estimation of the chitosan in Chitocal capsules as a pharmaceutical formulation with high recoveries of 96.67 to 98.46%, indicating that the proposed method is suitable for determination of trace amounts of chitosan in the real samples.

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