



Influence of Stirring Rate and Reaction Time on the Chitosan Trimethylation Process

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Abstract Chitosan is a derivative of chitin, a natural polymer present in the exoskeleton of crustaceans such as crabs and lobsters. This polymer is characterized by its high biodegradability, biocompatibility and mucoadhesivity, which is a raw material of interest for the pharmaceutical and food industry. However, its applications are limited by the insolubility at pH greater than 6. One of the most important derivatives of chitosan is N, N, N-trimethyl chitosan hydrochloride, a polymer obtained by a quaternization process of the amino groups present in chitosan. The objective of this work was to evaluate the influence of stirring rate and reaction time on the chitosan trimethylation process. A methylation process of the chitosan was performed, using as alkylating agent dimethyl sulfate and absolute ethanol as the precipitating agent. The influence of the speed of agitation and reaction time on this process was studied. It was found that the stirring rate influences the process. In conclusion, the results obtained in this work showed that with the use of dimethyl sulfate as an alkylating agent, applying a stirring speed of 300 rpm and a time of 2 h, it is possible to transform chitosan in N, N, N trimethylchitosan chloride.

Keywords Chitosan, N,N,N-trimethylchitosan hydrochloride, Degree of quaternization, Stirring rate, Reaction time

1. Introduction

The carapace of lobsters and other crustaceans constitute a solid residue of the fishing industry. This waste degrades rapidly causing emanation of unpleasant odors and toxic gases that affect the health of the population. On the other hand, these crustacean exoskeletons are rich in chitin and its functional derivative is chitosan, compounds that are widely used in the pharmaceutical, food, biotechnology and cosmetics industries [1-4]. The use of these wastes as a source of raw material solves an economic problem and an environmental problem.

Chitin is a natural biopolymer that has a high potential for application in the fields of chemistry, biochemistry, pharmacology, food science and macromolecular science, which justifies its great commercial value. One of the fundamental derivatives of chitin is chitosan, a natural polysaccharide that is obtained by basic hydrolysis of chitin through a process of deacetylation. Both compounds (chitin and chitosan) are not presented as a single molecule; their molecular masses can vary, as well as the degrees of deacetylation or free amino groups, which determine the quality and use of these polymers. Cuba produces chitin from the carapace of the lobster (*Panulirus Argus*). In recent years, it has been developed a technological process for obtaining chitosan from this chitin for the use in the pharmaceutical, cosmetic and food industries [2, 5, 6]. However, despite the advantages presented by these two biopolymers, their applications are limited by the insolubility having both components at higher pH 6

The polysaccharides are polymers, constituted by monosaccharides, repeatedly linked by glycosidic bonds. Their chemical properties are determined fundamentally by the functional groups present in the molecule. By modifying the structure and length of the chain, it is possible to vary its properties [7, 8]. Alternatively modify or derivatize the



functional groups present in the macromolecule in which the main chain is not altered, but its properties vary significantly [8].

Chitosan has three types of reactive functional groups. An amino or acetamido group and two hydroxyl groups at C-2, C-3 and C-6, respectively. Most reactions of chitosan involve specific reactions of the primary amino group, a powerful nucleophile that can undergo N-acylation, quaternization, reductive amination and Schiff reactions among others. This allows to include different functional groups in the molecule [9, 10].

Chitosan has an approximate pKa value of 5.6, being soluble only in acid solutions with pH values less than 6. This has motivated that in order to extend the pH range in which the biopolymer can be used, different routes for the quaternization of the molecule have been developed [11].

One of the routes used has been the alkylation of the amino groups with formation of the N,N,N-trimethylchitosan hydrochloride. Different alkylating agents have been used and different reaction conditions have been studied [12]. The quaternization of the amino group present in the structure of this polysaccharide allows introducing a permanent positive charge in the molecule, which gives the chitosan a permanent cationic polyelectrolyte character, regardless of the pH of the medium. A material with a higher charge density would surely generate a higher solubility; therefore, one of the proposed routes to achieve this goal is to achieve high efficiency in the quaternization of the amines groups in the polymer chains [13].

The pH, the degree of crystallinity of the polymer and the penetration of the alkylating agent into the polymer are factors that influence the alkylation reaction of a polymer. This last factor is preceded by a process of swelling of the polymer allowing the penetration of the solvent and the alkylating agent inside it. This favors the alkylation reaction [8, 14]. The objective of this work was to evaluate the influence of stirring rate and reaction time on the chitosan trimethylation process.

2. Experimental

Chitosan supplied by the Centro de Investigación y Desarrollo de Medicamentos (CIDEM, Habana, Cuba) was used. Batch quality parameters (10001) were Deacetylation degree of 77.7%, Total ash of 0.7%, Moisture content of 9.1%, Insoluble matter of 1.19% and Molecular mass of 310 kDa [5].

2.1. Synthesis Method

Chitosan trimethylation process was performed at laboratory scale (0.2 L) according to the methodology established by Britto and Assis [15], with the modification that was used as precipitating agent absolute ethanol (pa) (Merck, Germany). Glass reactor (stirred tank type) 1L capacity, marine propeller stirrer and heating system and gas extraction were used.

10 g of chitosan were placed in the reactor, 150 ml of dimethyl sulfate were added and the mixture was stirred. Subsequently, 40 ml of a solution containing 12 g of sodium hydroxide and 8.8 g of sodium chloride was added. After the reaction, the solution was filtered. 40 ml of absolute ethanol were added, stirred for 10 minutes and left to stand for 24 h. The precipitate formed was separated by vacuum filtration using as filter medium cotton (XX, 2 mm, Filtronic, Brazil). Dried at 40 °C for 6 h. Stored in a dry place and protected from light.

2.2. Study of the influence of stirring rate and reaction time in the trimethylation process

To evaluate the degree of influence of the stirring rate and the reaction time on the process of trimethylation, a statistical analysis was carried out using the multiple linear regression method, defining these two parameters as independent variables. The studies were carried out applying the experimental methodology proposed in Table 1.

Table 1 Applied experimental methodology

Experiment	Stirring rate (rpm)	Reaction time (h)
E1	150	6
E2		2
E3	300	6
E4		2



Yield, viscosity, and solubility were determined for each experiment. The viscosity determinations were carried out according to the methodology described by de la Paz *et al.* [16]. To measure the viscosity a rotary viscometer (Visco STAR PLUS, Spain) was used using the L1 spindle, at a speed of 200 rpm at 25 °C. A 1% w/v N, N, N-trimethyl chitosan hydrochloride solution was prepared in sodium chloride solution (0.2 mol/L). The determinations were made five times. Meanwhile, the solubility determinations were carried out according to the methodology described by Jia *et al.* [17] consisting of weighing 0.4 g of N, N, N-trimethylchitosan hydrochloride sample and transferring to a test tube. 2 mL of deionized water were added slowly and it was observed if the solution becomes clear. If not, 2 mL more are added slowly until total dissolution. The solubility in water was expressed in g/mL.

2.3. Determination of the degree of quaternization, dimethylation and acetylation

DQ (degree of quaternization), DM (degree of dimethylation) and DA (degree of acetylation) were determined by Nuclear Magnetic Resonance (¹H NMR) to the experiments that presented yields above 90%. The determinations were made by two methods [13]. The first method (M-1) uses the methodology developed by Kotze *et al.* [18]. It is based on the integration of the signals of all the methyl hydrogens of the polymer, taking as reference the protonic signal corresponding to the anomeric carbon of the glycosidic ring. The second method (M-2) uses the methodology established by Curti *et al.* [19]. It is based on the measurement of the intensity of the signals of all the methyl hydrogens present in the spectrum, not requiring the use of the H signal in carbon C1 as a reference.

The ¹H NMR spectra was acquired using a Bruker/Avance DPX-250F (Germany) device configured with a 5 mm QNP-250 probe. For this analysis, samples were dissolved in deuterated chloroform (CDCl₃) at a concentration of 10 g/L at a temperature of 80 °C to ensure maximum resolution during the analysis. The parameters for the acquisition of the NMR spectra were obtained at a frequency of 250.13 MHz, with 32 sweeps, 8k of data acquisition, a spectral width of 1500 Hz, and a pulse of 7 μs (90°). The spectra were processed with the software MestReNova version 6.0.2-5475 with a Fourier transform modulated by an exponential function (LB 0.3Hz). Chemical shifts are recorded on the δ scale (ppm). The signal of trimethylsilane (TMS) was used as reference.

2.4. Statistical analysis

The statistical evaluations were carried out with the statistical program Statgraphic plus 5.1 (USA). The adjustment of the models was performed by multiple regression applying the least squares method. The significant coefficients were determined by applying the procedure of sequential elimination of the non-significant coefficients (t Student). The adjustment of the model was carried out applying the Fisher criterion. Results were considered significant when $p < 0.10$.

3. Results

Table 2 shows the results of the evaluation of the three variants studied. The best yields were obtained in the E-3 and E-4 experiments, which correspond to the highest stirring rate studied (300 rpm) at times of 6 and 2 h, respectively. Statistical analysis using the method of multiple linear regression to evaluate the influence of the stirring rate (X1) and reaction time (X2) on the synthesis showed that both variables did not influence significantly on the yield ($p = 0.1782$ and $p = 0.5997$, for stirring rate and reaction time, respectively). The analysis of variance of the model applying ANOVA the p-value was higher than 0.1 ($p = 0.2672$), so there was no statistically significant relationship between the yield and the variables studied for a 90% confidence. According to these results, the equation of the adjusted model was: $\text{Yield} = 71,3 + 0,10 (X1) - 0,81 (X2)$.

Therefore, when evaluating the equation of the system it is observed that the value of the intercept of the curve is high. This is indicative that one of the variables influences the system or are other factors that influence. Based on this hypothesis the reaction time variable was eliminated because it was the variable that showed the highest p value. In this way the system was simplified and the statistical analysis using the method of simple regression analysis was evaluated.



Table 2: Results of the analysis of the four variants studied

Experiment	Yield (%)	Viscosity (cP)	Solubility (g/mL)
E-1	79.6	5.05 ± 0.084	0.1365
E-2	87.2	6.22 ± 0.041	0.1506
E-3	99.3	6.18 ± 0.041	0.1397
E-4	98.9	8.27 ± 0.234	0.0594

A logarithmic-X model was the most adequate to describe the relationship between performance and stirring rate. The variance analysis of the p-value of the model ($p = 0.0578$) is lower than 0.1, so there is a statistically significant relationship between the performance and the stirring rate for a 90% confidence. According to these results, the equation of the adjusted model was: $\text{Yield} = -2.2 + 22.1 \ln(X1)$. This result confirms the influence of the stirring rate on the reaction yield.

A viscosity behavior between 5 and 7 cP was observed. In all cases, the values decrease with respect to the viscosity value of the chitosan without derivatizing (49.3 ± 2.4 cP) [5]. This means that there was cleavage of the polymer chain due to the reaction conditions.

Statistical analysis to evaluate the influence of the stirring rate and reaction time on the synthesis showed that both variables did not influence significantly on the viscosity ($p = 0.5213$ and $p = 0.5049$, for stirring rate and reaction time, respectively). The analysis of variance of the model applying ANOVA the p-value was higher than 0.1 ($p = 0.5929$), so there was no statistically significant relationship between the performance and the variables studied for a 90% confidence. According to these results, the equation of the adjusted model was: $\text{Viscosity} = 6.3 + 0.01 (X1) - 0.67 (X2)$.

The solubility test showed that the four experiment variants studied allow obtaining soluble products. Only the solubility of sample E-4 was different from the rest of the experiments. Statistical analysis to evaluate the influence of the stirring rate and reaction time on the synthesis showed that both variables did not influence significantly on the viscosity ($p = 0.3910$ and $p = 0.4108$, for stirring rate and reaction time, respectively). The analysis of variance of the model applying ANOVA the p-value was higher than 0.1 ($p = 0.4507$), so there was no statistically significant relationship between the performance and the variables studied for a 90% confidence. According to these results, the equation of the adjusted model was: $\text{Viscosity} = 0.14 - 0.0003 (X1) - 0.01 (X2)$.

Nuclear Magnetic Resonance (NMR) is a strong alternative for the evaluation of chitosan and its derivatives. It allows to characterize the structure of the basic skeleton of these polysaccharides and also to determine the degree of quaternization (DQ%), the degree of methylation (DM%) and the degree of acetylation (DA%) of the amino group. These parameters are fundamental in the characterization of these compounds [23]. Considering that, the E3 and E4 experiments were that higher yields achieved, it was decided to continue the study with the samples obtained under the conditions of these experiments.

The ^1H NMR spectrum of the experiments E-3 and E-4 (Figure 1) showed a singlet at 3.31 ppm corresponding to the protons of the methyl groups of the quaternary nitrogen ($-\text{N}(\text{CH}_3)_3$) which shows that the quaternization process of the amino group. A signal at 3.03 ppm is observed which corresponds to protons of dimethyl groups bonded to nitrogen ($-\text{N}(\text{CH}_3)_2$), which indicates that the quaternization did not occur at 100%. Similar results have been reported in the literature by authors who have applied this alkylation variant [15, 23]. A group of signals $\delta = 3,4 < \delta < 4,4$ that correspond to the rest of the protons linked to the carbons C3, C4, C5 and C6 were observed. The DQ (%), DM (%) and DA (%) were determined from the ^1H -NMR spectra of samples E-3 and E-4. The results are shown in Table 3.



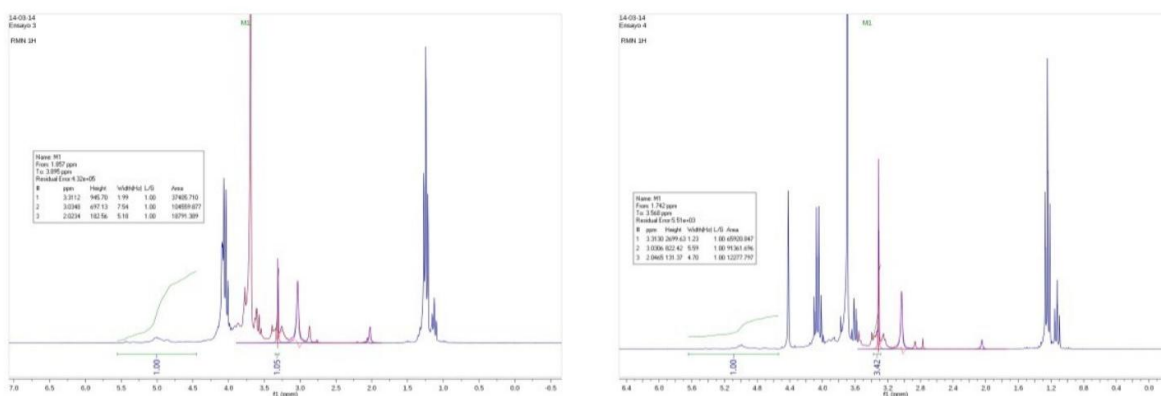


Figure 1: NMR spectra of samples E-3 and E-4

Table 3: Values of DQ (%), DM (%) and DA (%)

Experiment	DQ (%)		DM (%)		DA (%)	
	M-1	M-2	M-1	M-2	M-1	M-2
E-3	35.4	34.3	62.0	43.2	25.5	22.5
E-4	50.3	43.2	58.3	37.7	13.7	11.9

4. Discussion

Of all the methods described in the literature to obtain N, N, N-trimethylchitosan chloride, the process reported by Britto *et al.* [15] was selected for this work. In this process, dimethyl sulfate and non-methyl iodide (product with high toxicity) is used as an alkylating agent. On the other hand, the proposed process achieves the modification of the polysaccharide in a single step of synthesis, in 6 hours at room temperature and without the need to use other solvents.

This reaction occurs through a bimolecular nucleophilic substitution mechanism (S_N2) where the amine acts as a nucleophile (Lewis base) since the non-shared (non-bonding) electron pair of the nitrogen can form a bond with an electrophile such as sulfate dimethyl. The dimethyl sulfate is added in excess, in an amount sufficient to completely alkylate the amine, avoiding the formation of mixtures of different alkylated products. This process is known as exhaustive alkylation with formation of tetraalkylammonium salt. It is necessary in this process to add a not very strong base (diluted solution of sodium hydroxide) to avoid the protonation of the intermediate alkylated amines that can form and neutralize the large amount of sulfuric acid that is formed [7].

Table 2 shows that the best yields were achieved by applying a stirring rate of 300 rpm. It is known that the crystalline zone controls the hydration of the polymer determining the accessibility to the internal sites [14]. The high crystallinity in the polymers, causes a high rigidity and resistance. This aspect delays the swelling process and the penetration of the solvent into the polymer mass. The agitation helps the swelling and rupture of the polymer chains favoring the synthesis process. The stirring speed significantly influences the quality and performance of the final product [8]. At higher stirring rate, the mass transfer effect decreases, favoring the process of wetting and swelling of the polymer, improving its dispersion in the medium and therefore favoring the reaction [20]. Statistical analysis confirmed that the stirring rate had a marked significant influence on the yield of the synthesis

On the other hand, a statistically significant influence of the stirring rate on viscosity and solubility was not observed. However, the viscosity values decrease with respect to the viscosity value of the chitosan before being treated and the product obtained from the synthesis improves its solubility.



The viscosity of a solution is influenced by the size of the molecule and the nature of the solvent. The size of the molecule determines the flow properties and their modification, produces changes in the solvent-polymer interaction and a decomposition in smaller molecules, which leads to changes in the viscosity of the solution [21]. Although the stirring rate did not influence statistically the viscosity, the results obtained in this study showed that under the established reaction conditions the polymer chains are broken, obtaining shorter chain products that favor a decrease in the viscosity of the system. These results were similar to those reported by Britto *et al.* [15].

With regard to solubility, it has been reported in the literature that the solubility of chitosan can be improved by modifications that allow the introduction of hydrophilic groups in the polymer chain, which transform the polymer into water-soluble polyelectrolyte with a high charge density [17, 22]. The quaternization method applied in this work transformed the chitosan obtained from the chitin of the lobster shell into a trimethylated product, which improves its water solubility properties.

The determination of the degree of quaternization by the ^1H NMR method showed that sample E-4 has a degree of quaternization higher than sample E-3. This result indicates that under the conditions in which the experiment was carried out, quaternization of the amino group is more efficient. It is verified that the increase of the stirring rate guarantees adequate trimethylation in a shorter time, obtaining products with a low content of ortho methylation. The quaternization value obtained is similar to the values reported in the literature [15, 19, 23].

The results of the calculation of the degree of dimethylation show that in both samples there are dimethyl groups, being higher in the sample corresponding to the E-3 experiment. This indicates that the quaternization process does not occur stoichiometrically, with part of the amino groups partially methylated. These results coincide with the results reported in the literature [13, 15, 19, 23].

This aspect is very discussed in literature, suggesting several alternatives such as making successive alkylations or increase the reaction time in order to achieve a higher degree of alkylation. On this last aspect, Torres-Vergara [23], suggested that it should be well studied because an increase in the reaction time, applying the synthesis methodology proposed by Britto and Assis [15], could increase the degree of trimethylation but at the cost of a parallel increase in methoxylation of the product what is undesirable. The strategy would be to achieve a trimethylation process with a high degree of trimethylation in a shorter time. On the other hand, Torres-Vergara [23], in his work suggests that the control of methoxylation is an aspect that should be more studied, because it will not always be an undesired phenomenon.

The degrees of acetylation DA (%) of both samples are adequate because although acetamide groups are present in the final product, from the starting chitin, their values are low which indicates that the degree of conversion of chitin into chitosan by deacetylation was satisfactory [5].

5. Conclusions

In this study, the influence of stirring rate and reaction time on the alkylation process of chitosan was evaluated. It was found that the stirring rate influences the process. The yields of the synthesis process were high with a substantial decrease in time. This aspect is important, since one of the difficulties presented by the other variants of synthesis is the high time in which the final product is obtained. In these working conditions, the modification of the chitosan is guaranteed, which significantly improves its solubility properties, with a decrease in viscosity with respect to the starting chitosan. The increase in solubility favors the use of this polymer in the development of finished forms in the pharmaceutical industry, the viscosity values obtained being suitable for pharmaceutical excipients. In conclusion, the results obtained in this work showed that with the use of dimethyl sulfate as an alkylating agent, applying a stirring speed of 300 rpm and a time of 2 h, it is possible to transform chitosan, derived from chitin from carapace of lobster, in N, N, N trimethylchitosan chloride.

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