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Short communication

PRELIMINARY RESULTS ON ESTIMATION OF THE DEGREE OF ACETYLATION OF CHITOSAN BY UV SPECTROPHOTOMETRY

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ABSTRACT

The degree of acetylation of chitosan is an important parameter to be taken into consideration when chitosan is used as starting material for realization of derived products with applications in pharmacy and medicine. Although considered a reliable method, the determination of the degree of acetylation of chitosan by UV spectrophotometry has as main drawback: the lack of accuracy. Starting from an already published method, based on the UV spectrophotometry of N-acetylglucosamine and glucosamine molecules and considering that UV absorbance of chitosan is due to the absorbance of the two monomers, some preliminary results of an improved method for estimation of the degree of acetylation of chitosan are proposed. The improvements are based on the use of phosphoric acid as solvent for chitosan (and of monomer molecules - N-acetylglucosamine and glucosamine) and the use of optical density at 197 nm.

Keywords: UV spectrophotometry, chitin, chitosan, degree of acetylation.

1. INTRODUCTION

Chitin is considered to be the second most abundant natural synthetized compound, after cellulose[1]. The most important source of natural chitin are invertebrates[2], of which crustaceans are by far the most available source[3]. In addition to its main importance as

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structural and building material in many types of organisms, chitin importance is rising due to unnumbered applications in almost all the fields of human activities. The success of chitin is, in fact, due to chitosan, the more soluble derivative of chitin. Considering the natural abundance that makes any application sustainable, chitin, together with chitosan, has been proposed for a broad range of industrial applications, including separation techniques[4], biotechnological processes[5], wastewater treatment[6], food, agriculture[7], cosmetics[8], pharmaceutical[9], medical, tissue engineering[10], biomaterials[11], and the list can continue almost endlessly. Through chemical modification, chitosan can be considered as a precursor to produce other materials, increasing its applications further.

Chitin and chitosan are poly[β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose], simply named N-Acetyl-D-glucosamine (NAG) and poly[β-(1→4)-2-amino-2-deoxy-D-glucopyranose], named also glucosamine (GN) respectively. Chitosans are the fully or partially N-deacetylated derivatives of the chitin polymers that are usually produced by treatment with alkali[12]. The molar fraction of the NAG units is defined as the degree of N-acetylation (DA)[13]. Chitin is the polymer with the highest DA, virtually equal to 1 (or 100%), and chitosan is the polymer with the lowest DA (ideally 0, or 0%). Solubility is greatly dependent on DA, as chitin is virtually insoluble and chitosan is quite soluble, at least in in dilute acidic solutions. When DA is around or below 50%, the polymer becomes soluble in weak acidic media as the amino group ($-NH_2$) will ionize as $-NH_3^+$. Beside solubility, many other chemical and biochemical properties of these polymers depend on DA[14]: mechanical properties, especially of the films of these polymers, cytotoxicity, cellular uptake, crystallinity, supramolecular aggregation, and so on.

Although at first glance considered simple, the methods used to determine DA of chitosan possess inherent drawbacks and limitations. Many methods have been published for determining the DA, including spectroscopy[15], Fourier-transform infrared[16], nuclear magnetic resonance spectroscopic methods[17], dye absorption[18], potentiometric and conductometric titrations[19], ninhydrin assay[19], elemental analysis[20], circular dichroism[21], chromatographic separations (GPC)[22], and many others. Some of the above-mentioned methods require high-priced instrumentation and high-skilled personnel and some of them have a high level of errors. Consequently, there is a need for an uncomplicated, trustworthy and accurate method for determining the DA of chitosan. Spectrometric methods generally enter in this category and many spectrometric techniques were proposed for calculation of the DA of chitosan. The group of Liu [23] have determined DA of chitin and chitosan by UV spectrophotometry using dual standards, proving that their method is a simple and accurate alternative. In this study, some preliminary results regarding the estimation of the degree of acetylation of chitosan based on direct UV spectrophotometry, similar with the method of Liu[23] with some minor modifications, are presented.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

The following chemicals were used in this study: glucosamine (Sigma, #G1514), N-Acetyl-D-glucosamine (Sigma, #A8625), chitin from shrimp shells (Sigma, #C7170), chitosan from shrimp shells, ≥75% (deacetylated)(Sigma, #C3646), partly deacetylated chitin (Roth #5375), orthophosphoric acid (Sigma, #438081). All other chemicals were of analytical reagent grade.

2.2. UV Spectra

UV spectra of standard and sample solutions were recorded on a double beam scanning spectrophotometer, model T90, from PG Instruments (Leicester, UK). The standards and samples were dissolved in 0.1 M orthophosphoric acid.

2.3. Methods

Stock solutions of glucosamine (GN) and N-Acetyl-D-glucosamine (NAG) with the concentration of 0.5 mM were realized in phosphoric acid 0.1 M. For standard curves, these solutions were diluted in the range 0.5 – 0.05 mM.

Stock solutions with concentrations between 0.1 – 0.15% of chitin and chitosan (2 batches from Sigma and 1 for Roth) were dissolved in phosphoric acid 0.1 M. Dilutions to have around 1 – 1.5 mg/100 mL chitin or chitosan in phosphoric acid 0.1 mM were performed and considered as unknown samples.

A chitin solution was subjected to deacetylation procedure, mixing it with 40 % NaOH, (final concentration of chitin 155 mg per 100 mL solution). The reaction mixture was boiled under reflux, and samples (0.5 mL) were taken at 0, 1, 2, 4, and 8 hours. The samples were dissolved in 4.5 mL of 0.1 M phosphoric acid and UV spectra was recorded. The final concentration of chitin, respectively chitosan, in the analyzed samples was considered to be 15.5 mg/100 mL solution.

All experiments were performed in duplicate, standard deviations and errors being calculated using MS Excel software.

3. RESULTS AND DISCUSSIONS

Usually, chitosan is obtained by deacetylation of chitin by treatment with hydrolytic enzymes, like chitinaze[13] or chitosanase[24], or with concentrated alkali at high temperature[12]. If the starting material, i.e. chitin, is highly purified, the chitosan obtained by these methods is quite pure, having very low levels of contaminations. Chitosan molecule is composed of two monomeric units N-acetylglucosamine (GNA) and glucosamine (GN). Both these units are far UV chromophoric groups. The results have shown that these two

groups, and especially amino and N-acetylamine functions, do not interact with the polymeric chain in a manner that will affect its adsorption in the UV region[23].

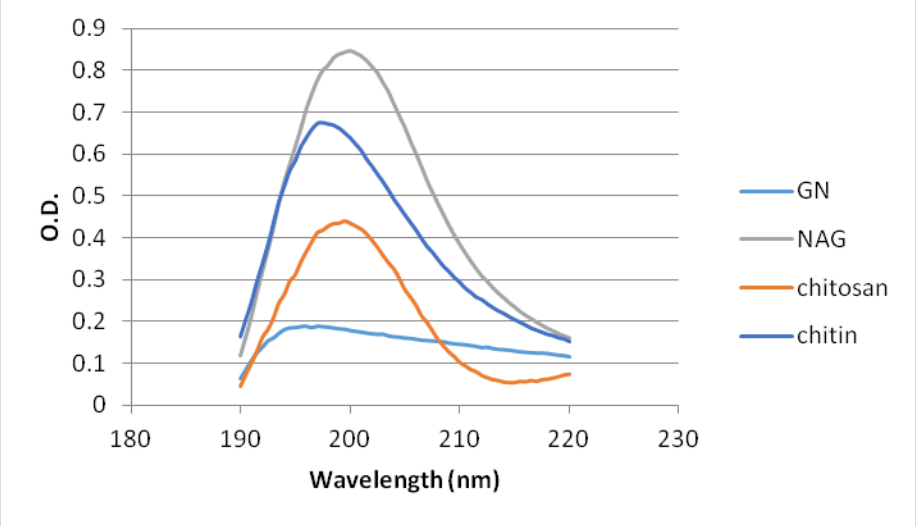

Figure 1. UV spectra of N-acetylglucosamine and glucosamine, chitosan and chitin

In this study, GNA and GN were used as standards to represent these units from chitosan. In Figure 1 there are presented the UV spectra of solutions of N-acetylglucosamine, glucosamine, chitin and chitosan. When comparing the spectra of GN and NAG at the same molar concentrations, one can observe that the molar absorptions (ϵ coeficients) have different values, i.e. $\epsilon_{NAG} > \epsilon_{GN}$. In fact, at low concentration, the contribution of NG can be neglected.

What is important to see from Figure 1 is that both chitosan and NAG have the same λ_{max}, their spectra being similar. These results are similar with those of Liu[23].

Based on the results presented above one may suppose that the monomer units, GNA and GN, contribute in a simple additive way to the total absorbance of the entire polymer. That means the optical density of a chitosan sample ($DO_{chitosan}$) will additively depend on the concentration (C_x) and absorptivities (ϵ_x) of GNA and GN, according with the following equation, made in accordance with Liu[23]:

$$DO_{chitosan} = C_{GNA} \cdot \epsilon_{GNA} + C_{GN} \cdot \epsilon_{GN} \quad \text{Eq. 1}$$

One may define de degree of acetylation (DA) of chitosan as the mole fraction of acetylated units in the polymer chain:

$$DA = \frac{C_{GNA}}{C_{GNA} + C_{GN}} \quad \text{Eq. 2}$$

Considering that the total concentrations of the two chromophoric groups is

$$C_T = C_{GNA} + C_{GN} \quad \text{Eq. 3}$$

one may transform these equations to obtain:

$$\frac{DO_{chitosan}}{C_T} = (\epsilon_{GNA} - \epsilon_{GN})DA + \epsilon_{GN} \quad \text{Eq. 4}$$

This can be considered as the equation of a straight line $y=ax+b$, where y is the absorbance of chitosan at a designated wavelength divided by the total concentration of polymer, DA is x, coefficient a is the term ($\epsilon_{GNA} - \epsilon_{GN}$) and the term ϵ_{GN} is the coefficient b. In fact, one may transform these equations to obtain a simplified one:

$$\frac{DO_{chitosan}}{C_T} = a \cdot DA + b \quad \text{Eq. 5}$$

Based on these, solutions with known concentrations of NAG and GN were prepared in phosphoric acid and their UV absorbance at λ_{max} =197 nm were recorded. In fact, the calculations have shown relatively high errors when the values at 197 nm were used, but lower errors when for calculations were used the averaged values recorded in the range 195 – 200 nm. These averaged values were labeled as the optical densities values at 197 nm.

The results presented in Table 1 and in Figure 2 show that the degree of acetylation is correlated with the concentrations of NAG and GN from the analyzed sample. In this case, DA is the concentration of NAG divided by the sum of concentrations of NAG and GN. One may assume that this will be true in the case of chitin and chitosan, as these polymers are from monomers of NAG and GN.

Table 1. Optical densities at 197 nm of standard mixtures of NAG with GN

No.	C_{GNA} (mM)	C_{GNA} (mM)	DA	$DO_{197\ nm}$	$DO_{197\ nm}/C_T$
1	0.05	0	1	0.795	1.590
2	0.045	0.005	0.9	0.755	1.510
3	0.04	0.01	0.8	0.701	1.402
4	0.03	0.02	0.6	0.547	1.094
5	0.02	0.03	0.4	0.358	0.716
6	0.01	0.04	0.2	0.147	0.294
7	0.005	0.045	0.1	0.185	0.370
8	0	0.05	0	0.078	0.156

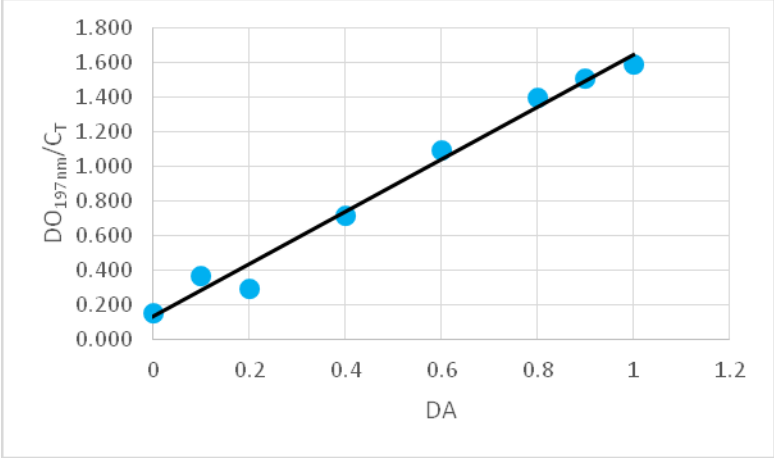

Figure 2. Dependence of the factor $DO_{207\ nm}/C_T$ on DA

The plot from Figure 2 proves that there is a linear correlation (R^2 = 0.992) between the optical density of NAG plus NG, divided to their total concentration and DA (slope = 1.512, intercept = 0.135).

To apply the standard curve (in fact the coefficients of the regression line) to chitin and chitosan samples, some calculations based on the equations presented above have to be performed. For this, one has to consider that the molecular weights of the monomer molecules are 203.2 for NAG monomer (instead of 221.2, which is the molecular weight of acetyl-D-glucosamine as single molecule, but in polymer, by condensation with other monomer molecules will lose a water molecule), and 161.1 for GN. For convenience, we have considered the concentration of chitin or chitosan in samples expressed as mg product per 100 mL solution (V factor in Eq. 6). Other factors from Eq. 6 are the slope *a* and the intercept *b* from curve presented in Figure 2. In these conditions, the equation used, very similar with that of Liu[23], to calculate the DA of chitin or chitosan samples is:

$$DA = \frac{161.1 \cdot DO_{chitosan} \cdot V - b \cdot m}{a \cdot m - 41.2 \cdot DO_{chitosan} \cdot V} \qquad \text{Eq. 6}$$

The Eq. 6 was used to calculate the degree of acetylation of chitin or chitosan samples. In Table 2 the DA was calculated for 3 chitosan samples (two different batches from Sigma and one from Roth) and a chitin sample. The samples were analyzed in duplicate and the error (as % from the average DA) was calculated. Considering that we are working with polymers with relatively low solubility, errors under 20% are considered as acceptable.

Table 2. DA of some samples of chitosan (2 batches from Sigma, 1 from Roth) and of chitin

No.	Sample	m (mg)	V (L)	$DO_{chitosan}$	DA	% Error
1	Chitosan (source 1)	12.5	0.1	0.715	0.61	7.85
2	Chitosan (source 2)	14.1	0.1	0.865	0.68	14.54
3	Chitosan (source 3)	17.7	0.1	0.842	0.48	8.74
4	Chitin	15.5	0.1	1.247	0.98	11.74

In Table 3 the DA of chitin subjected to deacetylation reaction in the presence of NaOH concentrated solutions are presented. The experiment was performed in duplicate and the errors (except for low DA value) are in the acceptable range. Taking into account that the values of DA calculated with Eq. 6 are in accordance with the time of deacetylation reaction, i.e. the DA decrease with the reaction time, one may consider that the results presented in Table 3 are accurate, even in the absence of results obtained with an alternative method.

Table 3. DA of some samples of chitosan subjected to deacetylation with 40% NaOH. The mass of chitin was 15.1 mg dissolved in 100 mL 0.1 M phosphoric acid

No.	Deacetylation time (h)	$DO_{chitosan}$	DA	STD	% Error
1	0	1.198	0.93	0.07	5.85
2	1	1.102	0.83	0.03	2.95
3	2	0.792	0.52	0.07	8.98
4	4	0.675	0.42	0.03	4.30
5	8	0.488	0.26	0.13	26.25

Following the method proposed by Liu[23], with some minor modifications, i.e. use of phosphoric acid as solvent for chitin and chitosan, use of other wavelengths for measurement of optical densities of samples (197 nm instead 207 nm, in fact the averaged values recorded in the range 195 – 200 nm), it was proved that the UV spectra of mixtures of NAG and GN are quite similar to the UV spectra of chitosan and the DA chitosan can be estimated with sufficient accuracy based on spectrophotometry measurements.

4. CONCLUSIONS

Some preliminary results of the attempt to improve the published method of determination of degree of acetylation of chitosan by UV spectrophotometry using dual standards were presented. The method is based on the fact that the UV absorption of chitosan depends on absorbencies of N-acetylglucosamine and glucosamine monomers that form the polymeric chain of chitosan. In order to improve the errors of this method, the UV absorbance values recorded on the range 195-200 nm, instead the absorbance value at a single wavelength was used. Another modification of the published method was the use of phosphoric acid as solvent for chitosan, N-acetylglucosamine and glucosamine, instead hydrochloric acid, modification that also seems to contribute to the stability of the optical densities of the samples and to decrease in a certain degree the magnitude of errors.

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