DETERMINATION OF DEGREE OF DEACETYLATION OF CHITOSAN - COMPARISION OF METHODS

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Abstract

Degree of deacetylation (DD) is one of the main parameters characterizing chitosan. The most precise measurements of DD require sophisticated equipment (NMR spectrometer), not available at many laboratories worldwide working on chitosan. There is a need for low-cost, simple, yet sturdy and reliable methods and procedures for DD determination. The aim of this work was to test and compare – on the same set of chitosan samples - a few of the existing analytical techniques and provide recommendation for selecting DD determination methods. Tests were performed on four chitosans of nominal DD in the range 70 - 95%. Three different methods of titration, two different methods of spectroscopy UV / Vis and infrared spectroscopy using various calculation approaches were used. The results are summarized and compared with values obtained by ^{1}H NMR, considered as the reference method. Moreover, evaluation of the ease of performance and availability of reagents in the given methods was performed. On that basis, recommendations for selection of DD determination methods were formulated.

Key words: *chitosan, degree deacetylation, methods.*

1. Introduction

Chitosan is typically obtained by partial deacetylation of chitin. The product is a copolymer of *N-acetylglucosamine* units and D-*glucosamine units*. Molar fraction of *N-acetylglucosamine* units in the chain, defined as:

$$DD = 100 \frac{n_{GlcN}}{n_{GlcN} + n_{GlcNAc}} \quad (1)$$

is called the degree of deacetylation (DD), where, n_{GlcN} – average number of D-*glucosamine units*, n_{GlcNAc} - average number of N-*acetylglucosamine* units. In some works, degree of acetylation is used, DA = 100 – DD. Several methods for determining the degree of deacetylation have been elaborated, from simple, such as pH-metric titration, UV-Vis spectroscopy [1], infrared spectroscopy [1, 2], elemental analysis [3], to complex ones, which require complicated and expensive equipment, such as ^{1}H NMR spectroscopy [4 - 6] and ^{13}C NMR spectroscopy [7, 8]. Within the same method, there are usually many analytical procedures for performing measurements and calculations and many ways of interpretation of results. It is obvious that not every research laboratory has an NMR spectrometer. Moreover, this method, although probably the most precise, is not suitable for routine measurements of DD due to high costs (deuterated solvents, depreciation of equipment, specialized staff time) and time-consuming sample preparation procedure. Most laboratories must work on less advanced, simpler and cheaper techniques. Therefore it is important to determine which of these methods lead to reliable, reproducible results in line with 'standard' by NMR. Indication of such methods is also important in the case of parallel research conducted by many laboratories (e.g., international projects, collaboration with industrial research laboratories, etc.) to provide the ability to compare the results. The selected technique must be precise, accurate, reproducible and inexpensive.

Authors' interest in this topic has been stimulated by participation in a large international project led by the International Atomic Energy Agency (IAEA) - Co-ordinated Research Programme "Development of Radiation-Processed Products of Natural Polymers for Application in Agriculture, Healthcare, Industry and Environment", where interlaboratory comparison of results emerged as an important problem.

The aim of this work was to test and compare – on the same set of chitosan samples - a few of the existing analytical techniques and procedures, and to provide recommendation for selecting DD determination methods. Tests were performed on four chitosans of nominal DD in the range 70 - 95 %.

2. Materials and methods

2.1 Materials

In this study four chitosan samples were used, of similar nominal viscosity (ca. 1000 mPas at 1% in 1% acetic acid, 20 °C) but different degrees of deacetylation. Samples have been manufactured and donated by Heppe Medical Chitosan GmbH. Chitosans were of high purity (Chitoscience® product line) and had individual certificates of analysis. These samples are denoted here as S1, S2, S3 and S4. DD values of these samples determined by

^{1}H NMR technique were taken as the reference values for all other determination methods. Chitosan samples were dried at 40 °C in vacuum until constant mass. All reagents were of analytical purity.

2.2 Methods

Some of the most commonly applied methods of DD determination have been used in our tests. They are described below.

2.2.1. ^{1}H NMR Spectroscopy [6]

Dilute solutions (5–7 mg cm^{-3}) of chitosan samples in a deuterated aqueous acid DCl/D_2O, at about pH 4 were prepared. 0.05 g - 0.07 g chitosan was weighted in plastic tube. Next, 6 cm^3 D_2O and 1 cm^3 deuterium chloride was added to every sample to obtain a solution. D_2O and DCl were further added to the final volume of 10 cm^3 and pH of ca. 4. The samples were twice freeze-dried using D_2O (99.9%) to exchange labile protons by deuterium atoms. Measurements were made for three samples of every kind of chitosan. Spectra between 0 - 6 ppm were recorded using a NMR spectrometer Bruker Avance II (700 MHz) at the temperature 27 °C and 60 °C. Integrals under characteristic signal are used to calculation degree of deacetylation.

2.2.2. Titration methods

2.2.2.1. Titration I [9 - 11]

Dried chitosan (0.2 g) was dissolved in 20 cm^3 0.1 M hydrochloric acid and 25 cm^3 deionized water. After 30 minutes continuous stirring, next portion of deionized water (25 cm^3) was added and stirring continued for 30 minutes. When chitosan was completely dissolved, solution was titrated with a 0.1 mol·dm^{-3} sodium hydroxide solution using automatic burette (0.01 cm^3 accuracy). Degree of deacetylation (DA) of chitosan was calculated using formula:

$$DA[\%] = 2.03 \cdot \frac{V_2 - V_1}{m + 0{,}0042 \cdot (V_2 - V_1)} \tag{2}$$

where: m – weight of sample, V_1, V_2 – volumes of 0.1 mol·dm^{-3} sodium hydroxide solution corresponding to the deflection points, 2.03 – coefficient resulting from the molecular weight of chitin monomer unit, 0.0042 – coefficient resulting from the difference between molecular weights of chitin and chitosan monomer units.

2.2.2.2. Titration II [12]

Titration II was based on modified procedure described by Tan et al. [12]. Chitosan (0.20–0.25 g) was dissolved in 20 cm^3 of 0.1 mol·dm^{-3} HCl and diluted with 10 cm^3 of deionized water. Under continuous stirring, 0.5 cm^3 of 0.1 mol·dm^{-3} sodium hydroxide was added, allowed to equilibrate and the pH recorded. This sequence was repeated until the pH reached a value of 3.

A value of f(x) corresponding to the volume of NaOH added, was calculated using the following formula:

$$f(x) = \left(\frac{V_0 + V}{N_B}\right) \times ([H^+] - [OH^-]) \tag{3}$$

where: V_0 is the volume of chitosan solution in cm^3 (30 cm^3), V is the volume of NaOH added in cm^3, N_B is the concentration of NaOH (0.1 mol·dm^{-3}), [H$^+$] is the concentration of [H$^+$] in mol·dm^{-3}, [OH$^-$] is the concentration of [OH$^-$] in mol·dm^{-3}.

The dependence of f(x) on the hydroxide sodium volume is linear. By extrapolating the linear titration curve to the x-axis, the volume of NaOH at the end point can be determined.

The DD of the chitosan sample was calculated using the following formula [12]:

$$DD\ (\%) = \frac{\phi}{\frac{(W-161\times\phi)}{204}+\phi} \times 100 \tag{4}$$

where:

$$\phi = \frac{(N_A \times V_A - N_B \times V_B)}{1000} \tag{5}$$

N_A is the concentration of HCl in mol·dm^{-3}, V_A is the volume of HCl in cm^3, N_B is the concentration of NaOH in mol·dm^{-3}, V_B is the volume of NaOH at the end point in cm^3, W is the sample mass in g.

2.2.2.3. Titration III [13]

Chitosan of 0.125 g was dissolved in 25 cm^3 aqueous solution of 0.1 mol·dm^{-3} hydrochloric acid and stirring by 30 minutes until totally dissolved. The solution was titrated with 0.1 mol·dm^{-3} NaOH. The degree of deacetylation was calculated as follow:

$$NH_2\% = \frac{(C_1V_1 - C_2V_2)\cdot 0{,}016}{G\cdot(100-W)} \times 100 \tag{6}$$

where: C_1 is HCl concentration in mol·dm^{-3}, C_2 is NaOH concentration in mol·dm^{-3}, V_1 is volume of HCl solution in cm^3, V_2 is volume of NaOH solution in cm^3, 0.016 is molecular weight of NH_2 in 1 cm^3 0.1 mol·dm^{-3} HCl in g, G is the sample weight in g, W is the water percentage of sample in %.

$$DD\ (\%) = NH_2\%/9{,}94\% \times 100\% \tag{7}$$

where 9.94 % is the theoretical NH_2 percentage.

2.2.3. UV-VIS Spectroscopy methods

2.2.3.1. Method I [14]

Establishing the calibration curve:

The calibration curve was made by plotting the first derivative UV values at 203 nm as a function of *N*-acetyl-D-glucosamine (GlcNAc) and D-(+)-glucosamine hydrochloride. Standard solutions of GlcNAc and GlcN were prepared in 0.85 % phosphoric acid at concentrations of 0, 12.8; 25.6; 38.4; 51.2 and 64.0 μg·cm^{-3}. 0.85 % H_3PO_4 was used as the reference liquid.

Sample preparation and determination of DA:

Aliquots of 100 ± 10 mg chitin or chitosan were heated in 20 cm^3 85% phosphoric acid for 40 min at 60 °C with constant stirring. After 40 min, when chitin/chitosan was completely dissolved, 1 cm^3 clear solution was taken and diluted to 100 cm^3 with deionized water. The dilution was necessary to get the chitin/chitosan concentration to the range detectable by a spectrophotometer. The diluted solutions were incubated at 60 °C for 2 h prior the UV measurement. Next the UV/Vis spectra were carried out in range 190 – 400 nm. 0.85% H_3PO_4 was used as the reference liquid.

The degree of acetylation of chitin and chitosan samples was calculated as:

$$DA\ (\%) = \frac{\frac{m1}{203.21}\times 100}{\frac{m1}{203.21}+\frac{m2}{161.17}} \tag{8}$$

where: m1 is the mass of N-acetylglucosamine in 1 cm^3 chitin/chitosan solution, calculated from the calibration curve by the corresponding λ = 203 nm, m2 is the mass of glucosamine in 1 cm^3 chitin/chitosan solution, m2 = M - m1, M is the mass of chitin/chitosan in the 1 cm^3 solution.

$$M = (M1 \times M3)/(M1 + M2),$$

where M1 is mass of solid chitin/chitosan sample taken for analysis (100 ± 10 mg), M2 is mass of 20 cm^3 85% phosphoric acid (34 g), M3 is mass of 1 cm^3 chitosan solution in concentrated phosphoric acid (1.7 g).

2.2.3.2. Method II [15]

Establishing the calibration curve:

Aqueous solutions containing various proportions of of D-glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) in 0.1 mol·dm^{-3} HCl are prepared to simulate chitosan samples of various DD. UV spectra of these solutions are recorded. Calibration curve is generated by relating the ratio absorbance / total concentration (C_S, see below) to the degree of acetylation (DA = 1 - DD). Absorbance is measured at λ = 201 nm. The DA of the solution is defined as the concentration of *N*-acetylglucosamine divided by the total concentration of *N*-acetylglucosamine and D-glucosamine hydrochloride.

Preparation of chitosan solution and determination degree of deacetylation

7 - 9 mg chitosan is dissolved in 25 cm^3 of 0.1 mol dm^{-3} HCl. Next UV spectrum is collected in the range w of wavelength 190 – 230 nm. On the basis of the absorbance of chitosan solution at λ = 201 nm, the DD is calculated by equation:

$$DD\ (\%) = \left(1-\frac{161.1\cdot A\cdot V\cdot C\cdot m}{k\cdot m-42.1\cdot A\cdot V}\right)\times 100 \tag{9}$$

where: 161.1 is the molecular weight of GlcN residue is g·mol^{-1}, 42.1 is difference between the molecular weight of N-acetylglucosamine and the molecular weight of GlcN residue in g·mol^{-1}, A is absorbance at the λ = 201 nm, V is the volume of solution in dm^3, m is mass of chitosan sample in mg, C is intercept with x-axis in the standard curve, k is slope in the standard curve.

2.2.4. Infrared spectroscopy

Measurements have been performed in the transmission mode, with chitosan contained in potassium bromide (KBr) tablets. Potassium bromide was mixed with chitosan in mass ratio 100:1 (200 mg KBr and 2 mg chitosan) [16]. KBr was placed in an oven at 300 °C for 24 h before mixing. Substances were mixed in agate mortar and pressed to tablet form. Tablets were dried for 24 hours at 50 °C in order to remove moisture. For every kind of chitosan 3 tablets were produced. The spectra of chitosan samples were obtained within a frequency range of $\lambda = 400 - 4000$ cm^{-1}, each spectrum is an average of 64 scans with a resolution of 2 cm^{-1}.

3. Results and discussion

3.1. ^{1}H NMR Spectroscopy

NMR spectroscopy is one of the most accurate methods for determining the degree of deacetylation. It has been chosen as a standard method by the American Standard Test Method [17]. Interpretation of results is clear, and the values obtained are reproducible. ***Figure 1*** shows ^{1}H NMR spectra of chitosan with different degrees of deacetylation. It can be seen that with increasing DD the signal from the methyl group and hydrogen H-1GlcNAc decreases because the molar content of N-acetylglucosamine in chitosan molecule goes down.

Various expressions have been worked out to calculate degree of deacetylation. In our calculations we have chosen five of them, the most often used. Formulas (10)

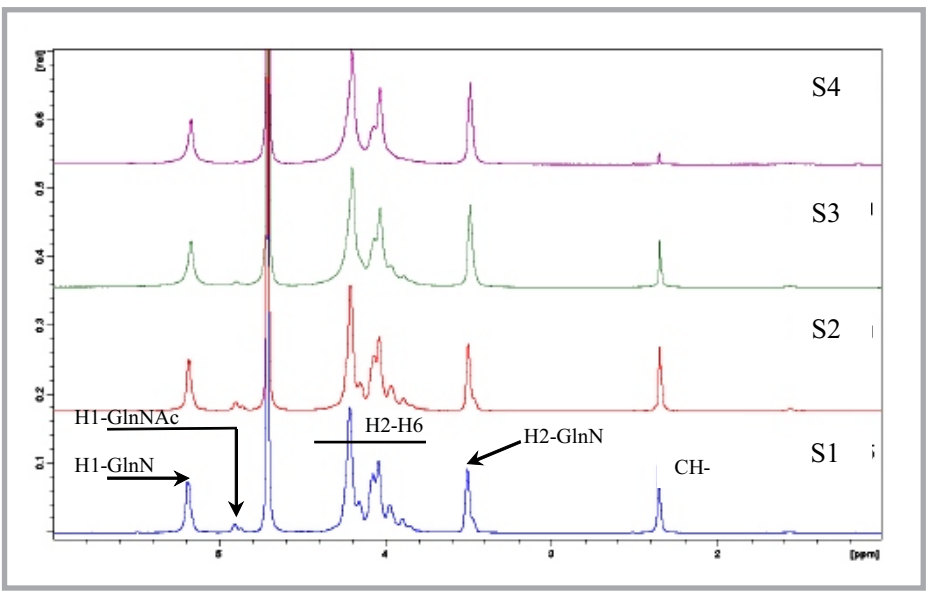

Figure 1. *^{1}H NMR spectra of chitosan with different degrees of deacetylation in DCl/D_2O at 60 °C (700MHz).*

[5, 12] and (14) [18] are used in all range of DD, (11) is recommended for chitosans in DD range 79-98% [19], the (12) for chitosan with DD > 48% [20]; and the (13) for DD < 90% [18].

$$DD\ [\%] = [1-\frac{\frac{1}{3}\times I_{CH_3}}{\frac{1}{6}\times I_{(H2-H6)}}]\times 100 \quad (10)$$

$$DD\ [\%] = [1-\frac{I_{CH_3}}{3\times I_{H1-GlnNAc}}]\times 100 \quad (11)$$

$$DD\ [\%] = [1-\frac{\frac{1}{3}I_{CH_3}}{I_{H2-GlnN}}]\times 100 \quad (12)$$

$$DD\ [\%] = \frac{I_{H1-GlnN}}{I_{H1-GlnN}+I_{H1-GlnNAc}}\times 100 \quad (13)$$

$$DD\ [\%] = \frac{I_{H1-GlnN}}{I_{H1-GlnN}+\frac{1}{3}I_{CH_3}}\times 100 \quad (14)$$

were: $I_{H1\text{-}GlnN}$ is integrals for H1 (GlcN), $I_{H1\text{-}GlnNAc}$ is integrals for H1 (GlcNAc); I_{CH3} is integral of – CH_3 signal, $I_{(H2\text{-}H6)}$ – the summation of integrals of H2, H3, H4, H5, and H6.

DD values calculated on the basis these formulas are shown in collective ***Table 1***. Results from formula (10) were taken as reference point to compare with data from other methods.

3.2. Titration methods

To determine the degree of deacetylation by titration methods, three different methods were selected. The first one (Titration I) seems to be the simplest in terms of performance and calculation. By using this method, on the basis of titration of chitosan solution, a curve with two inflexion points is obtained [***Figure 2***]. The difference of the volumes of these two points (V_1 i V_2) corresponds to the acid consumed by the amine groups and allows to calculate degree deacetylation. Determination of the first derivative helps in precise reading of V_1 and V_2. Measurements were carried out for minimum five samples. Average values were shown in ***Table 1***.

In method Titration II, the linear relation between function f(x) and corresponding volume of NaOH was shown in ***Figure 3***. Calculation procedure is described in the Experimental section above. Measurements were performed for five samples of every kind of chitosan. Average values are shown in ***Table 1***.

Third method of titration, in terms of the measurement procedure, is similar to the first method (Titration I). However, the calculations are made based on the position of the first point on the titration curve, and not based on the difference between the position of the first and second inflection point as in method "Titration I". ***Figure 4*** shows a titration curve for chitosan solution using method of Titration III. The first maximum corresponds to neutralization of HCl, the second to the amount of NaOH needed for reaction with H^+ on

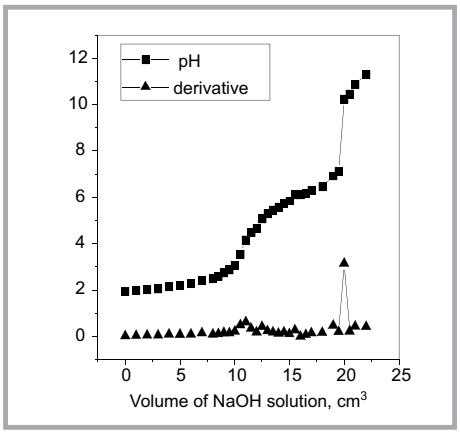

Figure 2. *Determination of the degree of deacetylation DA by pH-metric titration of chitosan solution (10-1mol dm-3 hydrochloric acid by 10-1 mol dm-3 sodium hydroxide).*

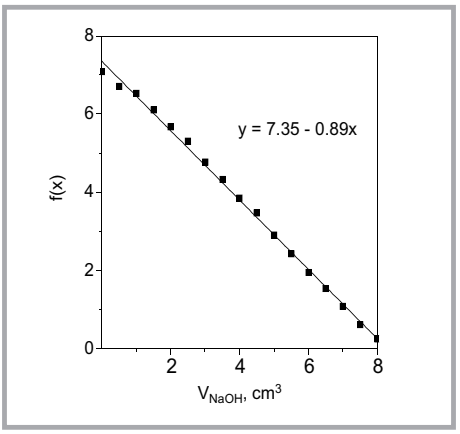

Figure 3. *Determination of the final point of titration chitosan solution (sample S3) (7.22·mg cm-3 0.1 mol dm-3HCl).*

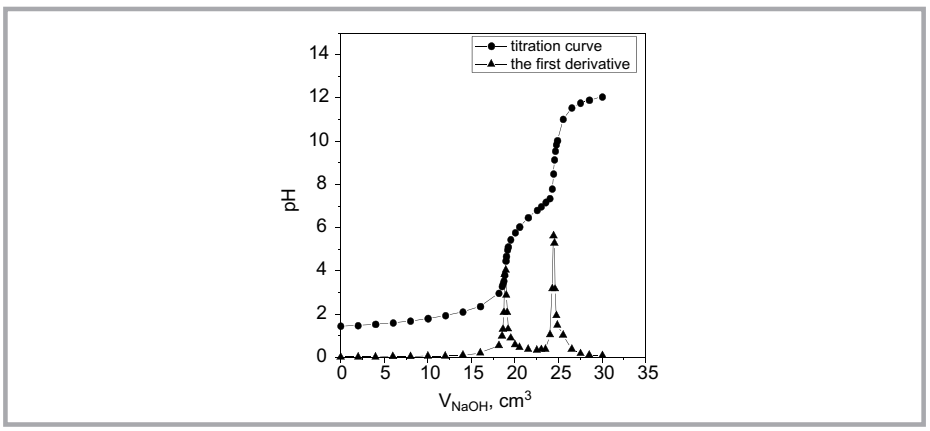

Figure 4. *Determination of degree of deacetylation DA by pH-metric titration of chitosan solution (4.99·mg cm-3 in 10-1 mol dm-3 hydrochloric acid by 10-1 mol dm-3 sodium hydroxide).*

amine groups. Because chitosan was earlier dried to the constant mass, expression (100-W) was omitted. Degrees of deacetylation were collected in ***Table 1***.

Methods of determination of degree deacetylation by titration are cheap and based on readily accessible reagents and apparatus, while a disadvantage is the relatively long time used for sample preparation and for the titration itself. The difficulty of mixing for higher values of pH, due to precipitation of chitosan, can be the cause of error in Titration I. The advantages of Titration II is lack of potential sources of error due to precipitation of chitosan (titration is limited to low pH range) and the relatively short measurement time, compared to the Titration I where the full range of pH is used. Additionally, the values are closer to the

reference values obtained by ^{1}H NMR, standard deviations are small. This does not mean that the agreement of results with the results of the reference is very good throughout in all the range. It seems that this method gives more relevant results for samples with a low degree of deacetylation. However, disadvantage of this method is, as before, quite long time for sample preparation and complex procedure of calculation. The advantages of method Titration III is, as for other methods of this group: low cost, readily available reagents and apparatus.

Potentiometric titration seems to be the simplest and most robust method for determination of DD, when proper protocol is chosen and care is taken to use acid and base solutions of precisely known concentrations (ready-to-use volumetric standards; base solutions either used at once or stored in a way which prevents reaction with atmospheric carbon dioxide). If we consider Titration I, II and III, an interesting observation is that the obtained results were (in all cases except one) lower than those obtained by 1H NMR (on average, by 3.53%), which seems to indicate some systematic difference, independent of the titration protocol. Our preference is protocol I and protocol III, since laborious calculations and extrapolations based on a relatively narrow range of data such as in method II are not necessary. Protocol I yields results that are closest to those obtained by ^{1}H NMR; on the other hand measurements according to protocol III are not disturbed by precipitation at pH > 7.

3.3. UV-VIS Spectroscopy methods

Figure 5 shows the first derivative UV spectra of standard solutions containing N-acetylglucosamine and D-glucosamine at different concentrations, in the wavelength range 190 – 220 nm, obtained as proposed by Wu et al. (UV method) [14]. The linear regression was made at 203 nm.

The presence of D-glucosamine at the tested concentrations did not impose noticeable interference to the calibration curve, whereas N-acetylglucosamine resulted in a good linear regression in the study range of concentration (***Figure 6***).

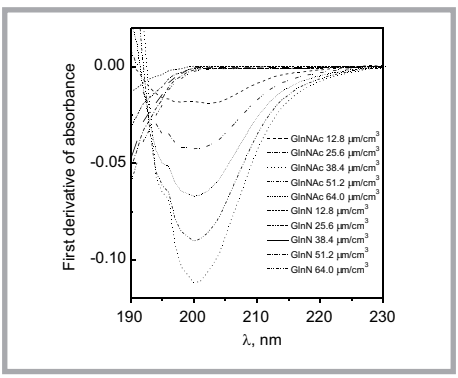

Figure 5. *First derivative UV spectra of N-acetylglucosamine (GlcNAc) and D-glucosamine (GlcN) standards at concentrations ranged from 12.8 to 64 μg·cm^{-3}.*

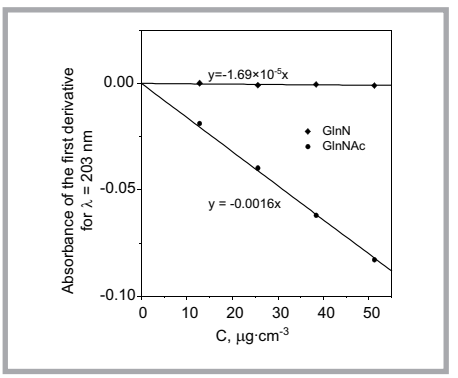

Figure 6. *First derivative value at 203 nm against concentrations of D-glucosamine (GlcN) and N-acetylglucosamine (GlcNAc).*

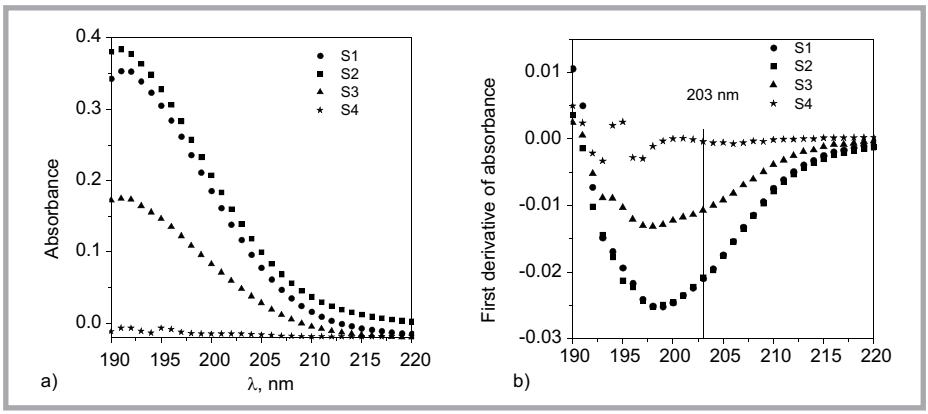

Figure 7. *UV spectra of the examined chitosan samples a) absorbance b) the first derivative of absorbance. Chitosans were dissolved in 0.85 % aqueous solution phosphoric acid.*

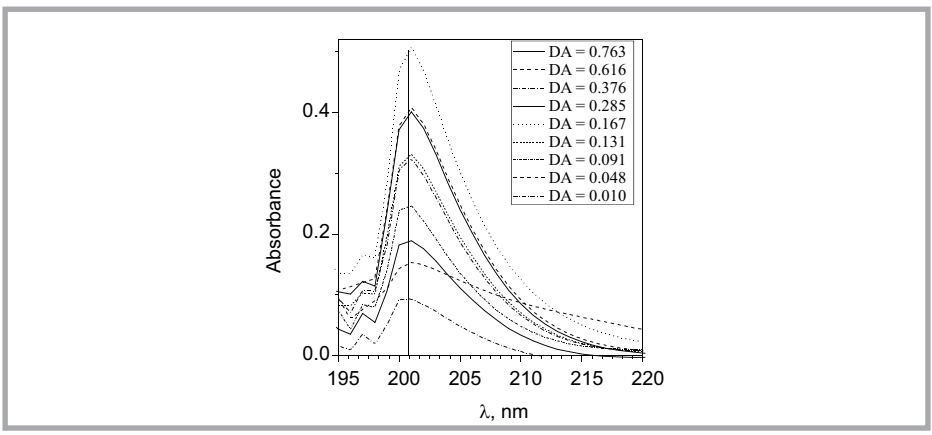

Figure 8. *UV spectra N-acetylglucosamine and D-glucosamine hydrochloride admixture in 0.1 mol dm^{-3} hydrochloric acid solution, DA of chitosan is defined as the mole fraction of acetylated units in the mixture.*

Figure 7 illustrates the absorbance and the first derivative of absorbance for tested chitosans in the range of 190-220 nm. Derivative values were read at 203 nm. Using standard curve concentrations of N-acetylglucosamine, degree of deacetylation of chitosan samples were determined and calculated. Results obtained by this method were shown in ***Table 1***.

Method II of UV/ViS spectroscopy is similar to the method I. Both of them require preparation of calibrations curves using D-glucosamine and *N*-acetylglucosamine at various molar ratios. ***Figure 8*** shows the UV spectra of the standards solution. It shows that the *N*-acetylglucosamine and glucosamine hydrochloride in common solution have a maximum at 201 nm in a 0.1 mol dm^{-3} hydrochloric acid solution, and this maximum is mainly due to *N*-acetylglucosamine.

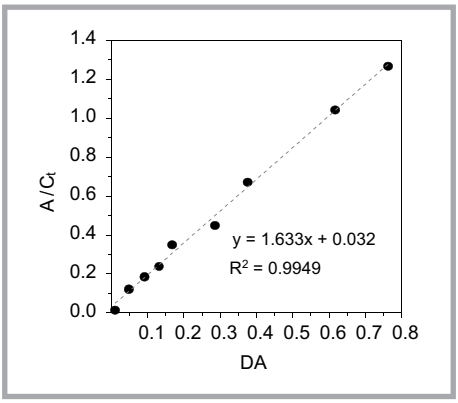

Figure 9. *A/C_t versus DA of standard solutions.*

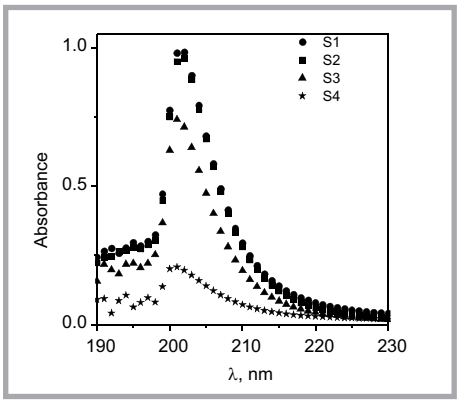

Figure 10. *UV spectra of chitosans in 0.1 mol dm^{-3} hydrochloric acid solution.*

Standard curve (***Figure 9***) indicates a linear dependence of the ratio of absorbance to the total molar concentration of repeating units (A/C_S) on degree of acetylation at 201 nm.

Degrees of deacetylation determined by this method for all chitosan samples are shown in ***Table 1***. Corresponding UV/Vis spectra for these chitosan are presented in ***Figure 10.***

Method II UV/Vis is similar to methods I UV/Vis. Both of them require preparation of calibrations curves using D-glucosamine and N-acetylglucosamine in a different molar relationships. The advantages of these methods are: generally available equipment (UV-Vis spectrometer) and easy measurements. Disadvantages include: using expensive chemicals, laborious and time-consuming procedure (calibration curve, etc.), complex procedures of calculation and the fact that the presence of contaminants (absorbing below 210 nm) can influence spectra and hence the final results.

Unfortunately, Method II UV/Vis, being somewhat easier to perform than Method I (no need for re-calculating the spectra into first derivatives), failed to produce results comparable with the standard data obtained by ^{1}H NMR. The obtained DD data were systematically on the low side. Therefore, based on our experience, we cannot recommend Method II as reliable.

3.4. Infrared spectroscopy

Like in the NMR spectroscopy, also in the FTIR spectroscopy, several procedures and equations are described in literature for calculation of degree of deacetylation. These equations were derived on the basis of calibration curves, where the calibration values of DD were determined by absolute methods like NMR. Calculation procedures are based on absorbance ratios of various spectral bands [21, 22]. In this project four procedures were chosen. The equations used in these procedures are listed below.

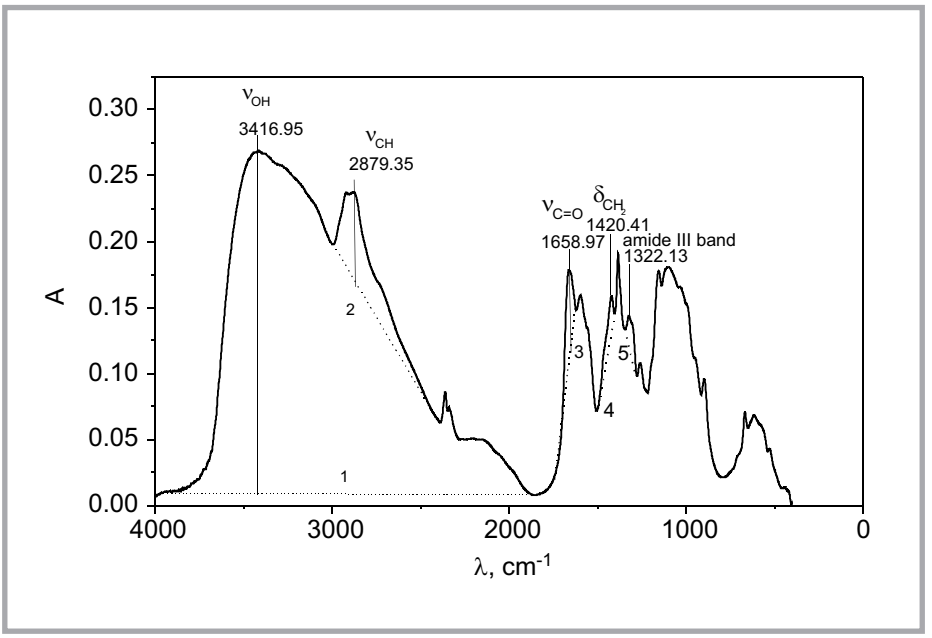

Figure 11. *Rereferences bands and corresponding baselines, based on Duarte et al. [21] (1 - 3) and Brugaretto et al. [22] (4 - 5) for FTIR spectrum of chitosan sample S2.*

$$DA\ [\%] = \frac{A_{1655}}{A_{3450}} \times 100/1{,}33 \text{ [17, 23]} \quad (15)$$

$$DA\ [\%] = \frac{A_{1655}}{A_{2870}} \times 100/1{,}33 \text{ [23]} \quad (16)$$

$$DA\ [\%] = \frac{A_{1655}}{A_{3450}} \times 115 \text{ [17]} \quad (17)$$

$$DA\ [\%] = (\frac{A_{1320}}{A_{1420}} - 0{,}03822)/0{,}03133 \text{ [22]} \quad (18)$$

where: A_{3450}, A_{2870}, A_{1655}, A_{1420}, A_{1320}, are values of absorbance from baseline 1, 2, 3, 4, 5 to maximum, respectively. In ***Figure 11***, on the basis IR spectrum of chitosan S2, baseline settings and individual bands ascribed for characteristic groups in chitosan are presented.

Our choice of expressions used for calculation of DD was based on literature recommendations. Expressions 15 and 17 were chosen because some authors maintain that calculations based on equations proposed by Baxter et al. [24] are - in a sense - absolute methods. These equations were set up without comparing the DA values to a range of samples with known DA and were with good agreement with values from NMR. Next two formulas (16, 18) were taken for DD calculations because: in (16), the stretching band at 2870 cm^{-1}, as a reference band, is convenient – its position and intensity reportedly do not change with water content; in (18): DD obtained from the ratio A_{1320}/A_{1420} was in agreement with DD determined from ^{1}H NMR and ^{13}C NMR [22] and positions at 1320 cm^{-1} and 1420 cm^{-1} do not change with humidity.

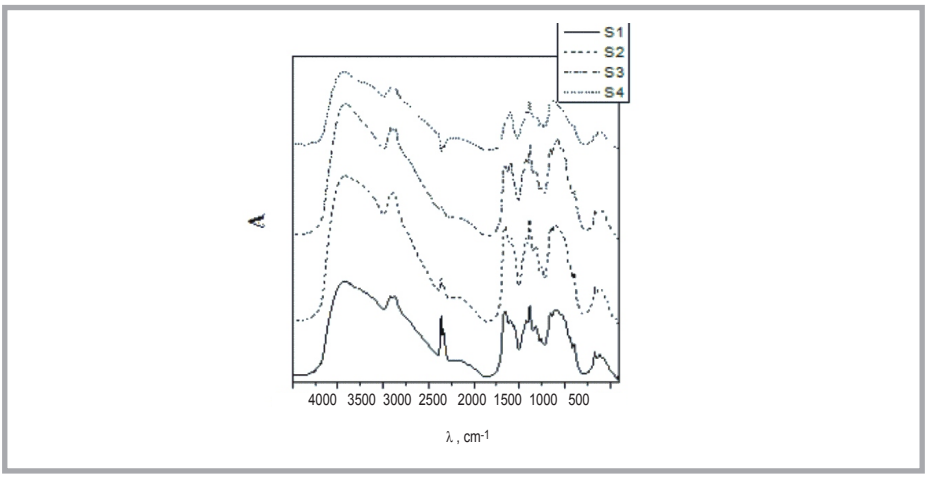

Figure 12. *Exemplary FTIR spectrum of the chitosans: S1, S2, S3 and S4.*

Exemplary FTIR spectra for all chitosan samples are presented in ***Figure 12***. It can be seen that with an increase of degree of deacetylation the band 1655 cm^{-1} (>C=O) undergoes changes and finally decays, the shape of bands between 1500 - 1750 cm^{-1} is changed too. Significant differences can also observed in shape of 3000 - 3500 cm^{-1} band. Using the four above formulas degrees of deacetylation were calculated, the results are shown in ***Table 1***.

Our general impression is that the FT-IR method, although undoubtedly having some advantages, can be recommended only for rough estimation of the DD of chitosan, and even when used for this purpose it must be performed with care. This is mainly due to problems with reproducibility. Absorbance of some of the peaks may be influenced by humidity. Baseline setting is troublesome and operator-dependent (in our opinion this is one of the major sources of error). Signals resulting from impurities may overlap with the signals from chitosan. Probably also instrument settings (like spectral resolution) may influence on the results. Out of the four tested calculation procedures, we found No. (16) as producing totally unreliable results (which may be a result of some systematic error either in derivation of this procedure itself or in our understanding). The best results (*i.e.*, being systematically closest to the ^1H NMR data) were obtained by applying procedure (15).

3.5. Comparison of results

The mean values of degree of deacetylation obtained by all tested methods are presented in ***Table 1.***

4. Conclusions

The aim of this study was to test and select the method of determining the degree of deacetylation of chitosan, which could be easily performed in most laboratories deal-

Table 1. *Comparison degree of deacetylation obtained by different methods; 1) n = 3, t = 60°C, 2 n ≥ 5, 3) n = 3, 4) for DD 79 - 98%, 5) for DD < 90%, 6) n = 6, 7) n =3, n – number of measurements.*

Chitosan sample	^{1}H NMR[1]				Titration[2]			UV/Vis		FTIR[3]			
	Formula									Formula			
	10	11[4]	12	13[5]	14	I	II	III	I[6]	II[7]	15	16	17	18
S1	79.72 ± 0.05	77.92 ± 0.38	78.99 ± 0.20	81.93 ± 1.15	81.92 ± 0.26	76. 65 ±0.48	75.27 ± 0.87	74.67 ± 1.80	80.14 ± 0.27	67.83 ± 1.19	76.61 ± 1.92	64.22 ± 2.94	-8.50 ± 13.03	64.49 ± 7.14
S2	81.23 ± 0.19	80.23 ± 0.47	80.84 ± 0.29	82.42 ± 0.79	83.49 ± 0.33	81. 47 ±0.91	76.29 ± 1.15	77.28 ± 1.51	82.55 ± 1.58	72.47 ± 0.28	77.84 ± 2.36	66.11 ± 2.36	-12.50 ± 23.30	74.60 ± 3.60
S3	89.44 ± 0.32	90.42 ± 0.69	91.27 ± 0.25	84.87 ± 1.27	91.26 ± 0.57	87. 67 ±1.41	85.29 ± 0.63	85.59 ± 0.35	90.55 ± 0.92	81.09 ± 0.19	85.61 ± 2.04	78.00 ± 2.04	43.86 ± 9.19	89.50 ± 5.85
S4	97.47 ± 0.31	98.00 ± 0.38	98.20 ± 0.19	100.00 ± 0.00	98.04 ± 0.36	94. 69 ±1.25	91.47 ± 0.67	94.65 ± 1.11	100.29 ± 0.73	96.79 ± 0.34	96.20 ± 0.89	94.19 ± 1.35	83.16 ± 0.80	76.48 ± 10.77

ing with this polysaccharide. There seem to be a general agreement in the literature that ^{1}H NMR is the best and most accurate method currently available for determination of DD of chitosan. While this technique itself cannot be treated as ideal or absolute, it definitely deserves to be the reference method, to which other techniques, better suited for general lab use and not requiring access to very expensive equipment, can be compared. In our study we have tested, on the same set of chitosan samples, determination of DD by potentiometric titration, FT-IR spectroscopy and UV-Vis spectroscopy. For each of these techniques, numerous detailed procedures exist in literature. By direct comparison of these procedures on the same set of samples which have been also analyzed by the benchmark method of ^{1}H NMR we were able to indicate which of those procedures yield reliable results, and also to assess their advantages and disadvantages (see final parts of the corresponding chapters). Similarly, we tried to compare the techniques themselves, in terms of their reliability, weak and strong sides. As expected, there are no ideal solutions, but some general recommendations can be formulated. In our opinion, potentiometric titration, when based on selected procedures and standard analytical solutions of well-known concentrations, is the most reliable and robust of the non-NMR methods. Here we indicate procedures I and III to yield best results in our hands. The titration technique is followed by UV-Vis (procedure I) and FT-IR spectroscopy (procedure (15)). UV-Vis when carefully performed seems to produce good results, but the procedure is quite time-consuming (the need of calibration) and potentially sensitive to impurities. FT-IR offers faster measurements, but quantitative reproducibility of the spectra and the somewhat operator-dependent procedures for setting the baselines for reading absorbances are the weak sides of this technique.

5. Acknowledgments

The authors would like to thank the Heppe Medical Chitosan GmbH for donating chitosan samples used in this study, as well as Dr. Torsten Richter (HMC GmbH, Halle, Germany), Prof. Murat Sen

(Hacettepe University, Ankara, Turkey) and Prof. Saphwan Al-Assaf (Glyndwr University, Wrexham, U.K.) for valuable discussions.

- *This work has been financed in part by International Atomic Energy Agency ((UN) IAEA CRP F22046) and Ministry of Science and Higher Education, Poland (2379/IAEA/2012/0).*

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