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An approach to understanding the deacetylation degree of chitosan

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ABSTRACT

Chitin, a polysaccharide isolated from crustaceans, is a homopolymer composed of 2-acetamide-2-deoxy-D-glucopyranose. The proportion of D-glucosamine and *N*-acetyl-D-glucosamine determines the classification of the biopolymer as chitin or chitosan. Chitosan a straight-chain copolymer is obtained by partial deacetylation of chitin.

This study sought to compare ^{1}H NMR and conductometry evaluating mixtures of D-glucosamine hydrochloride (DG-HCl) and *N*-acetyl-D-glucosamine (NADG) because these carbohydrates are monomers of chitosan. The degree of deacetylation of three different chitosan samples was evaluated also by these two techniques. Mixtures of DG-HCl/NADG of known concentration were prepared and determined by ^{1}H NMR and conductimetry. The results obtained with these two techniques were in agreement with the known content of the solutions.

A novel approach to understanding the deacetylation degree of chitosan and comparative studies of conductimetry and ^{1}H NMR employing the monomers of chitosan is described.

1. Introduction

Chitin and chitosan, typical marine polysaccharides, have distinctive biological and physicochemical characteristics. The biopolymer is characterized as either chitin or chitosan according to the degree of deacetylation (DD) which is determined by the proportion of D-glucosamine and *N*-acetyl-D-glucosamine. Structurally, chitosan is a straight-chain copolymer composed of glucosamine and *N*-acetylglucosamine being obtained by the partial deacetylation of chitin (Guibal, 2004; Klug, Sanches, Laranjeira, Favere, & Rodrigues, 1998; Kubota, Tastumoto, Sano, & Toya, 2000; Kurita, 2006).

Chitosan solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups in the polymeric chain, therefore on the proportion of acetylated and non-acetylated glucosamine units. The amino groups (pK_a from 6.2 to 7.0) are completely protonated in acids with pK_a smaller than 6.2 making chitosan soluble. Chitosan is insoluble in water, organic solvents and aqueous bases and it is soluble after stirring in acids such as acetic, nitric, hydrochloric, perchloric and phosphoric (Anthonsen & Smidsroed, 1995; Rinaudo, 2006; Sankararamakrishnan & Sanghi, 2006).

Chitosan has been largely employed in many areas, such as photography, biotechnology, cosmetics, food processing, biomedical products (artificial skin, wound dressing, contact lens, etc.), system of controlled liberation of medicines (capsules and microcapsules), treatment of industrial effluents for removal of metallic and colouring ions (Kawamura, Mitsuhashi, & Tanibe, 1993; Kumar, 2000; Longhinotti et al., 1998). Therefore, the characterization of the polymer in either chitin or chitosan is extremely important according to the structure-properties relationship, defining a possible industrial application.

The chitosan amino groups are responsible for adsorption of metallic ions and many techniques are available to determine the degree of deacetylation, such as conductometric titration (Raymond, Morin, & Marchessault, 1993), chromatography (Brugnerotto, Desbrieres, Roberts, & Rinaudo, 2001), spectrometric methods as ^{1}H nuclear magnetic resonance (^{1}H NMR) (Fernandez-Megia, Novoa-Carballal, Quinoa, & Riguera, 2005; Varum, Anthonsen, Grasdalen, & Smidsrod, 1991), ^{13}C-CP-MAS-NMR (Heux, Brugnerotto, Desbrieres, Versali, & Rinaudo, 2000), ^{15}N-CP-MAS-NMR (Yu, Morin, Nobes, & Marchessault, 1999), infrared (Brugnerotto et al., 2001; Duarte, Ferreira, Marvao, & Rocha, 2002), and ultra-violet (Aiba, 1986).

Chitin and chitosan samples contain broadly different amounts of *N*-acetyl groups, depending on their origin and isolation procedure. The conductometric titration is commonly employed to quantify acidic groups with good precision and simplicity. Solubility is one limiting factor in the choice of the technique as samples with a high degree of acetylation are insoluble in most solvents. Therefore CP-MAS NMR (solid state nuclear magnetic resonance) is the technique most indicated for samples highly acetylated where conductometric titration could not be used (Raymond et al., 1993). We report studies on the mixtures of D-glucosamine hydrochloride (DG-HCl) and *N*-acetyl-D-glucosamine (NADG)

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employing ^{1}H NMR and conductimetry aiming to understand the deacetylation degree of chitosan polymers. In the course of these studies we were able to compare these two techniques for determining the deacetylation degree of chitosan samples.

2. Experimental

2.1. General methods

Commercial chitosan (CC) was supplied by Purifarma Distribuidora Química e Farmacêutica Ltda (Brazil). Low molar mass chitosan (CA) was from Sigma–Aldrich (St. Louis, MO). D-(+)-Glucosamine hydrochloride (DG-HCl, more than 99% purity) was from Fluka (China). *N*-Acetyl-D-glucosamine (NADG, more than 99% purity) was from Sigma–Aldrich (St. Louis, MO). Deuterium oxide (D_2O, 99.9%) was from Sigma–Aldrich (Milwaukee, WI). Other reagents were of analytical grade and were used without further purification.

All chitosan samples were dried in an oven at 60 °C for 24 h and kept in desiccator over silica gel desiccant under vacuum for 2 h as chitosan is hygroscopic. All samples were prepared in triplicate.

The NMR spectra were acquired using a Varian Mercury 300 MHz spectrometer. Mestre-C software for PC was used for processing the spectra. A high pass convolution filter (4.967 ppm) was applied to suppress the HOD signal followed by Fourier transform and phase correction of the spectrum. A smooth exponential apodization and polynomial baseline correction was performed.

Conductances during titrations were measured with Schott Geräte HANDYLAB LF 1 conductivity meter equipped with LF 613 conductometric cell. Titrations were conducted under agitation and temperature control (25 °C) using a thermostatic bath.

2.2. General conditions for ^{13}C NMR acquisition

The ^{13}C NMR spectra of all samples were obtained with acquisition time and delay of 0.8680 and 1.132 s respectively, except for chitosan where the delay was 3.0 s. The spectra of chitosan were obtained with temperature control of 30 °C and all the other samples were maintained at 25 °C during acquisition.

2.2.1. Chitosan

Chitosan (25.00×10^{-3} g) was suspended in 1.500 g of D_2O containing 10.00×10^{-3} g of concentrated HCl. The mixture was stirred for 24 h at room temperature affording a viscous solution. The spectra of 0.7000 g of this solution were acquired with 14,000 transients.

2.2.2. NADG in D_2O

The ^{13}C NMR spectra of a solution of NADG (20.00×10^{-3} g) in D_2O (0.7000 g) were acquired with 496 transients.

2.2.3. NADG in aqueous HCl

NADG (0.2000 g) in aqueous hydrochloric acid (40.00 mL, 50.00×10^{-3} mol L^{-1}) was stirred at room temperature for 24 h. We have inserted a capillary tube (2.000 mm outside diameter) with D_2O into the 5.000 mm tube containing this sample (0.6000 g) to obtain the deuterium lock. The spectra were acquired with 5392 transients.

2.3. General conditions for ^{1}H NMR acquisition

The acquisition time was 3.642 s and the delay was 1.500 s. All spectra were acquired with 16 transients. We have used the same chitosan samples for both ^{1}H and ^{13}C NMR.

2.3.1. Chitosan

The ^{1}H NMR spectra were acquired at 70 °C to improve resolution, as chitosan is not very soluble at this concentration even at low pH.

2.3.2. DG-HCl/acetic acid in D_2O

The ^{1}H NMR spectra of DG-HCl (20.00×10^{-3} g; 1.000 M eq.) and acetic acid (5.570×10^{-3} g; 1.000 M eq.) in D_2O were acquired at 25 °C.

2.3.3. Mixtures of DG-HCl and NADG

A mixture of 0.9000 equimolar DG-HCl (35.90×10^{-3} g, 216 g mol^{-1}, 166.0×10^{-6} mol) and 0.1000 equimolar NADG (4.100×10^{-3} g, 221 g mol^{-1}, 18.50×10^{-6} mol) were dissolved in 0.7000 g of D_2O.

A mixture of 0.8000 equimolar DG-HCl (31.80×10^{-3} g, 216 g mol^{-1}, 147.0×10^{-6} mol) and 0.2000 equimolar NADG (8.200×10^{-3} g, 221 g mol^{-1}, 37.10×10^{-6} mol) were dissolved in 0.7000 g of D_2O.

The ^{1}H NMR spectra of both samples were acquired at 25 °C.

2.4. Conductometric titration

The samples (0.2000 g) in HCl (54.00×10^{-3} mol L^{-1}, 40.00 mL) were stirred at room temperature for 18 h and titrated with NaOH (165.0×10^{-3} mol L^{-1}) at 25 °C.

A mixture of 0.9000 equimolar DG-HCl (0.1796 g, 216 g mol^{-1}, 833.0×10^{-6} mol) and 0.1000 equimolar NADG (20.40 mg, 221 g mol^{-1}, 92.20×10^{-6} mol) in HCl (54.00×10^{-3} mol L^{-1}, 40.00 mL) were titrated with NaOH (165.0×10^{-3} mol L^{-1}) at 25 °C.

A mixture of 0.8000 equimolar DG-HCl (0.1592 g, 216 g mol^{-1}, 738.0×10^{-6} mol) and 0.2000 equimolar NADG (40.80×10^{-3} g, 221 g mol^{-1}, 184.0×10^{-3} mol) in HCl (54.00×10^{-3} mol L^{-1}, 40.00 mL) were titrated with NaOH (165.0×10^{-3} mol L^{-1}) at 25 °C.

Sodium hydroxide solution was added in portions of 0.5000 mL in 20 s interval. The values of conductance (mS cm^{-1}) with the corresponding titrant volumes were plotted in a graphic to find the linear variation before and after the equivalence point. The interception of the slopes with an acute angle with projection in abscise affords the corresponding volume of the equivalence point (Cienfuegos, 2000).

2.5. Purification of commercial chitosan

Chitosan (1.000 g) was suspended in aqueous acetic acid (300.0 mL, 0.5000 mol L^{-1}) and magnetically stirred for approximately 24 h. The viscous solution was filtered through quantitative filter paper followed by filtration through glass fibre micro filter of 12.00×10^{-3} mm and cellulose membrane of 0.4500×10^{-3} mm (Millipore). Concentrated ammonium hydroxide (60.00 mL) was added to the filtrate for precipitation of chitosan. Chitosan was filtered, and rinsed with distilled water until pH 7.0 (approximately 1.000 L) followed by ethanol (60.00 mL). Chitosan was dried in an oven at 60 °C for 24 h and kept in desiccator over silica gel desiccant under vacuum for 2 h. Chitosan was crushed in mortar with pestle and sieved (105.0×10^{-3} mm opening) to afford a fine powder.

3. Results and discussion

In order to make an approach to understanding the deacetylation degree of chitosan we have carried out conductometric titration of D-glucosaminehydrochloride (DG-HCl),

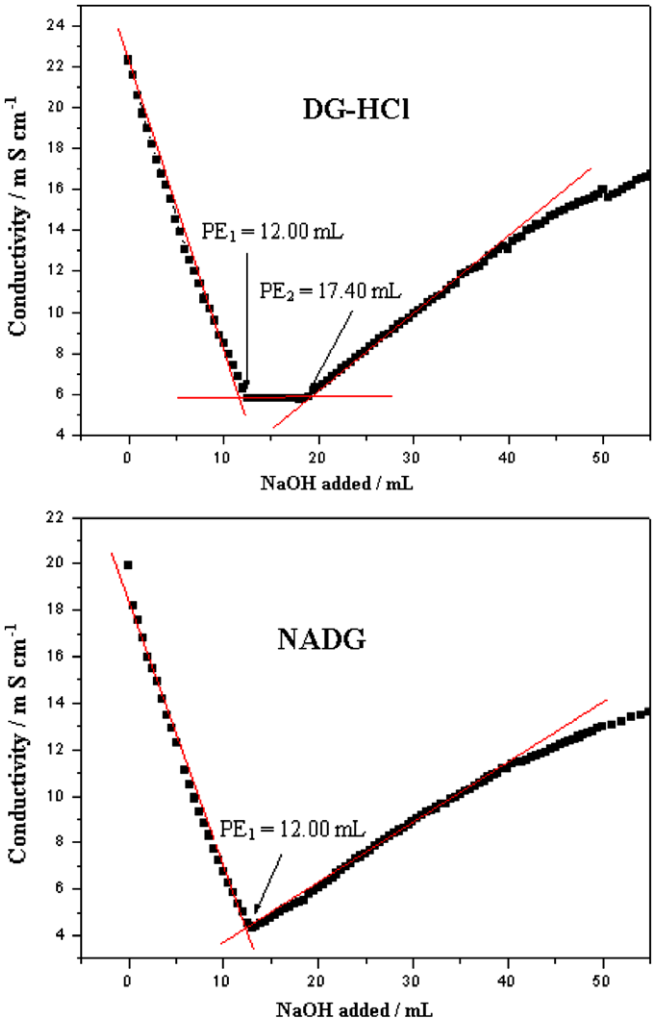


Fig. 1. Conductometric titration of: DG-HCl (0.2000 g) and NADG (0.2000 g) in HCl 54.00×10^{-3} mol L^{-1} (40.00 mL) with NaOH 0.1650 mol L^{-1} at 25 °C.

N-acetyl-D-glucosamine (NADG) and mixtures of DG-HCl and NADG of known concentrations. We have used DG-HCl and NADG because they are the monomers of chitosan.

Conductimetry is the measurement of a solution conductance (electrical conductivity) (Cienfuegos, 2000). The variation of solution electrical conductivity is measured during the chemical reaction in conductometric titrations. These titrations are based on the substitution of ions with different conductivity and the equivalent point shows when the change is complete.

Conductimetry is a function of the sum of conductivity of H^+ and OH^- in solution as these are the most conductive ions. Conductance depends on temperature and concentration of electrolytes in solution. The kinetic energy of the ions and consequently the conductance is increased when temperature is raised, thus titration was carried out under temperature control (25 °C, in a thermostatic bath) (Ewing, 1969). Two inflection points are observed in the conductivity graph of DG-HCl (Fig. 1). The first equivalence point corresponds to neutralization of the acid (in excess) and the second point corresponds to neutralization of the DG-HCl ammonium group.

The undergoing reactions for the titration of a solution of DG-HCl in hydrochloric acid by sodium hydroxide are represented in Scheme 1.

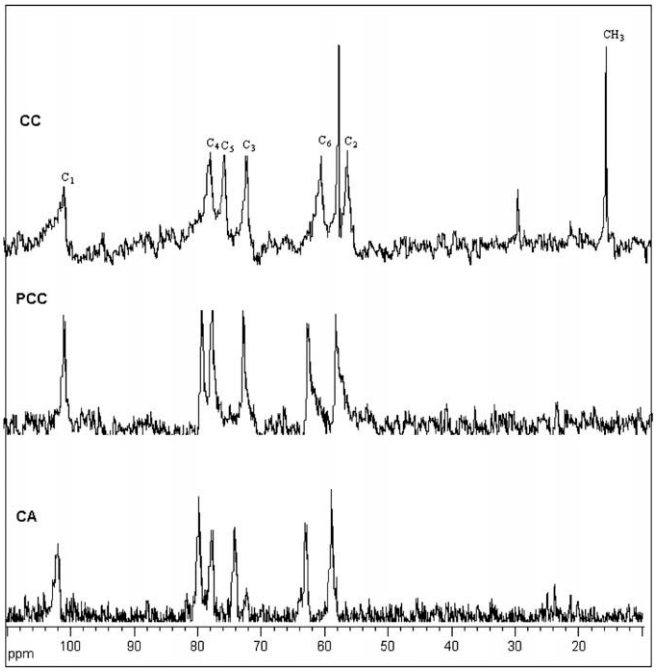


Fig. 2. ^{13}C NMR spectra of the chitosan samples (CA, CC and PCC) in 1% DCl at 30 °C.

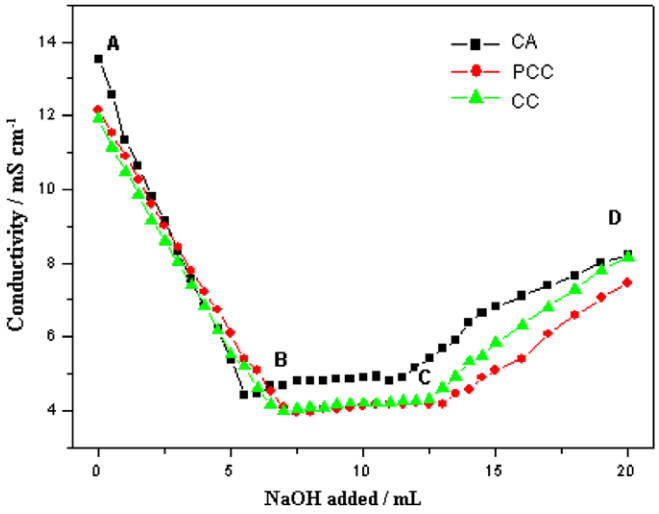
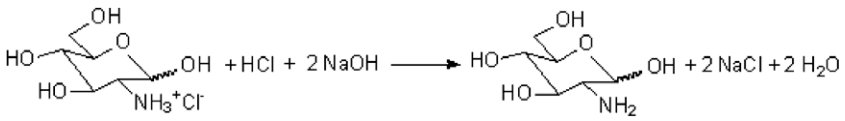


Fig. 3. Conductometric titration of the chitosan samples (0.2000 g) in HCl 54.00×10^{-3} mol L^{-1} (40.00 mL) with NaOH 165.0×10^{-3} mol L^{-1} at 25 °C.



Scheme 1. Neutralization of HCl and the amonium group of DG-HCl by NaOH during titration.

Table 1
Percentage of deacetylation by conductometric titration and ^{1}H NMR.

Samples[a]	Conductimetry[b]	^{1}H NMR[b]
CA	82.00 ± 0.95	87.30 ± 0.75
CC	77.00 ± 0.95	84.90 ± 0.75
PCC	83.50 ± 0.95	83.50 ± 0.75
M_1	90.32 ± 0.93	89.90 ± 0.65
M_2	80.93 ± 0.93	80.00 ± 0.65

[a] CA, chitosan from Aldrich; CC, commercial chitosan; PCC, purified commercial chitosan; M_1, mixture of 179.6×10^{-3} g of DG-HCl and 20.40×10^{-3} g of NADG; M_2, 159.2×10^{-3} g of DG-HCl and 40.80×10^{-3} g of NADG.
[b] All values are the means of three determinations ± standard deviation.

The neutralization of the hydrochloric acid in excess affords only one inflection point in the conductivity graph of NADG (Fig. 1). The acetamide group is not protonated by hydrochloric acid thus the second inflection point could not be seen due to titration of the ammonium group. The amide group has not been hydrolysed either, otherwise there would be two inflection points.

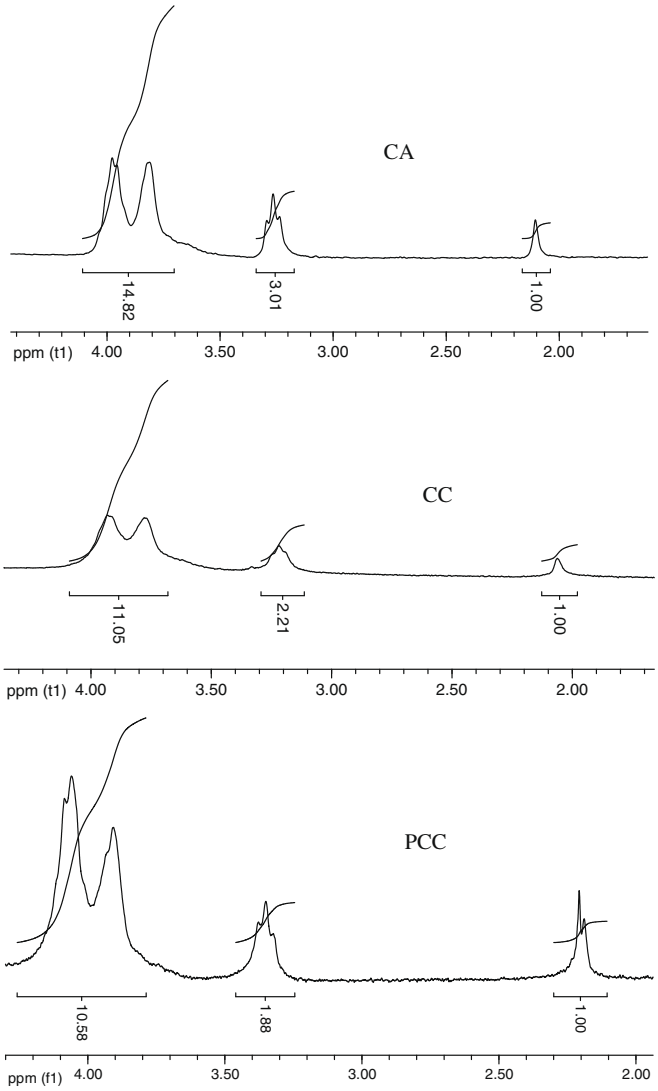


Fig. 4. ^{1}H NMR of CA, CC, and PCC in 1% DCl at 70 °C.

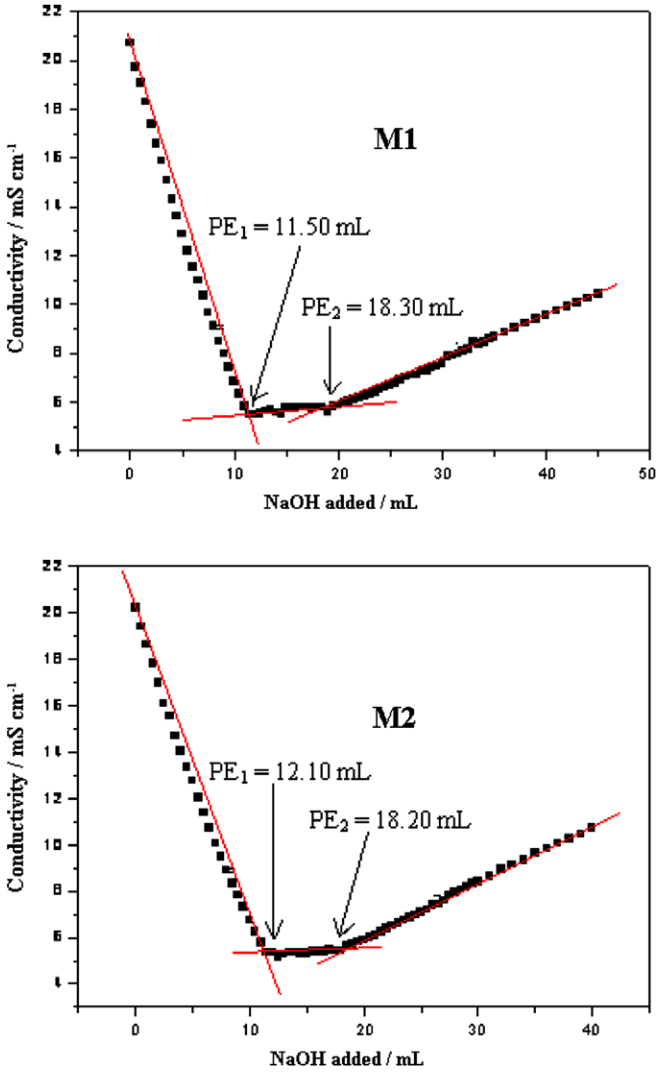


Fig. 5. Conductometric titrations of: DG-HCl (0.1796 g) and NADG (20.40 mg) in HCl 54.00×10^{-3} mol L^{-1} (40.00 mL; mixture M_1) with NaOH 0.1650 mol L^{-1} at 25 °C; DG-HCl (0.1592 g) and NADG (40.80 mg) in HCl 54.00×10^{-3} mol L^{-1} (40.00 mL; mixture M_2) with NaOH 165.0×10^{-3} mol L^{-1} at 25 °C.

^{13}C NMR spectra of NADG in either D_2O or HCl have been obtained to corroborate the results of the conductometric titration. The solution of NADG in HCl was stirred for 24 h at room temperature to find out if the amide group would be either hydrolysed or protonated. The spectra of NADG in D_2O and HCl were identical showing that neither protonation nor hydrolysis of the amide group has occurred in acid solution even after 24 h.

This reasoning could be extended for the chitosan conductometric titration. Therefore the amino groups in the polymeric chain are protonated by hydrochloric acid and neutralized by sodium hydroxide during titration. The amide group from the polymeric chitosan should not be either hydrolysed or protonated in HCl like NADG.

3.1. Purification of commercial chitosan (CC)

The purification procedure was reproducible allowing recovery of 90% of the initial mass of chitosan. The purified commercial chitosan (PCC) was ground and sieved to afford a white powder. The PCC samples were insoluble in distilled water and soluble in aqueous acetic acid and in aqueous hydrochloric acid.

3.2. ^{1}H and ^{13}C NMR and conductometric titration of three samples of chitosan, DG-HCl, NADG and mixtures of DG-HCl and NADG

The chitosan samples were characterized by conductometry, ^{1}H and ^{13}C NMR (Fernandez-Megia et al., 2005; Varum et al., 1991). The ^{13}C NMR spectra of CC, PCC, and CA are displayed in Fig. 2. Three extra signals were observed in the ^{13}C NMR of CC. The signal in $\delta = 17$ is probably from the acetyl group. The achievement of the purification procedure is shown in Fig. 2 where ^{13}C NMR of PCC did not display these three impurity signals.

Conductometric titrations of chitosan CA, CC, PCC are shown in Fig. 3. Three line segments are observed. The first rapid descending branch corresponds to neutralization of HCl in excess (A–B), the second segment refers to neutralization of the ammonium group (B–C) and the third to the excess of base (D).

The two stoichiometry points are found by intersection of the three lines and the difference between the two points corresponds to the volume of base required to neutralize the ammonium groups.

The percentage of amino groups represented by the degree of deacetylation (DD) was calculated using the following equation:

$$\%DD = \frac{[base](V_2 - V_1)161}{m} \quad (1)$$

where [base] is the concentration of the NaOH solution (in mol L^{-1}), V_1 and V_2 are the volume of NaOH (in mL) used in the titration, 161 is the molar mass of the monomer ($C_6H_{11}O_4N$) and m is the mass of chitosan (in mg). The percentages of deacetylation calculated by conductometric titration are shown in Table 1.

The results obtained by conductometric titration were compared to those values calculated using ^{1}H nuclear magnetic resonance. The ^{1}H NMR spectra were obtained at 70 °C to increase chitosan solubility (Fernandez-Megia et al., 2005). The signals and their respective integrals for determining the deacetylation degree are shown in Fig. 4.

The degrees of acetylation and deacetylation of chitosan were calculated from the areas of the signals in 2.1 ppm (methyl) and the sum of the areas from 3.2 to 4.2 ppm (H2, H3, H4, H5, H6, and H6') in the ^{1}H NMR according to Eqs. (2) and (3), respectively. The percentages of deacetylation of chitosan samples employing ^{1}H NMR are shown in Table 1.

$$\%DA = \left(\frac{2xA_{CH_3}}{A_{H2-H6}}\right) \times 100 \quad (2)$$

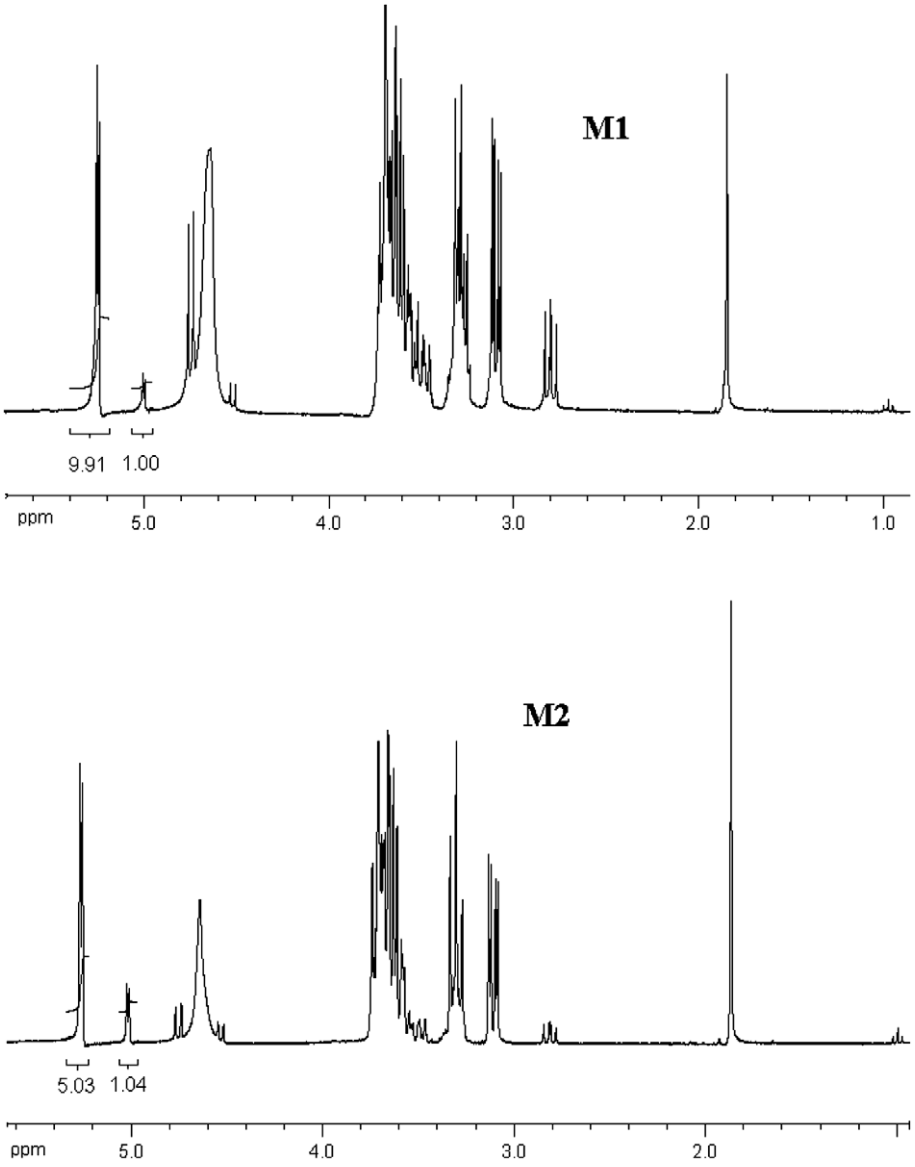


Fig. 6. ^{1}H NMR of M_1 and M_2 in 1% DCl at 25 °C.

$$\%DD = 100 - \%DA \quad (3)$$

Conductometric titrations were carried out also for mixtures of DG-HCl and NADG in two different proportions and are shown in Fig. 5. Two equivalent points are observed corresponding to neutralization of HCl and the ammonium group (from DG-HCl). The degree of deacetylation of these mixtures by conductometric titration and ^{1}H NMR are presented in Table 1 and the results are statistically in agreement.

The degree of deacetylation of the mixtures was calculated dividing the area of the peak in 5.0 ppm (H2 of NADG) by the area in 5.2 ppm (H2 of DG-HCl). These choices were made by comparing the ^{1}H NMR of NADG in D_2O and DG-HCl/acetic acid in D_2O. These peaks did not superimpose over with any other signals in the spectrum.

^{1}H NMR of M_1 and M_2 in HCl/D_2O (1% v/v) at 25 °C are shown in Fig. 6.

The degree of acetylation (%DA) and deacetylation of the mixtures were calculated using Eqs. (4) and (5) and the results are presented in Table 1.

$$\%DA = \left(\frac{A_{NADG}}{A_{DG\text{-}HCl}}\right) \times 100 \quad (4)$$

$$\%DD = 100 - \%DA \quad (5)$$

4. Conclusions

Reliable and quick techniques that measure acetyl contents of chitosan are needed. Conductometric titration and ^{1}H NMR are two examples of such techniques. Solutions of DG-HCl/NADG of known concentration were prepared and studied by conductimetry and ^{1}H NMR. The content of the mixtures determined by these two techniques were not significantly different to their known concentration.

We have shown that conductometric titration could work even with mixtures with high NADG content. Titration of neat NADG displayed only one inflexion point due to neutralization of HCl in excess. Titration of neat DG-HCl displayed two inflexion points due to neutralization of the acid in excess and the ammonium group. Therefore the difference of deacetylation degrees obtained by conductometric titration and ^{1}H NMR of CA and CC (chitosan samples which were not previously purified) should not be attributed to the high acetyl content but to the presence of some kind of impurity which affects the results of one technique but not the other. The matching results obtained by these two techniques for PCC (the purified chitosan) corroborate this conclusion.

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References

Aiba, S. (1986). Studies on chitosan: 1. Determination of the degree of *N*-acetylation of chitosan by ultraviolet spectrophotometry and gel permeation chromatography. *International Journal of Biological Macromolecules, 8*, 173–176.

Anthonsen, M. W., & Smidsroed, O. (1995). Hydrogen ion titration of chitosans with varying degrees of N-acetylation by monitoring induced ^{1}H NMR chemical shifts. *Carbohydrate Polymers, 26*, 303–305.

Brugnerotto, J., Desbrieres, J., Roberts, G., & Rinaudo, M. (2001). Characterization of chitosan by steric exclusion chromatography. *Polymer, 42*, 9921–9927.

Brugnerotto, J., Lizardi, J., Goycoolea, F. M., Argüelles-Monal, W., Desbrières, J., & Rinaudo, M. (2001). An infrared investigation in relation with chitin and chitosan characterization. *Polymer, 42*, 3569–3580.

Cienfuegos, F. (2000). *Análise instrumental*. Rio de Janeiro: Interciência. p. 606.

Duarte, M. L., Ferreira, M. C., Marvao, M. R., & Rocha, J. (2002). An optimised method to determine the degree of acetylation of chitin and chitosan by FTIR spectroscopy. *International Journal of Biological Macromolecules, 31*, 1–8.

Ewing, G. W. (1969). *Instrumental methods of chemical analysis*. New York: McGraw-Hill. p. 627.

Fernandez-Megia, E., Novoa-Carballal, R., Quinoa, E., & Riguera, R. (2005). Optimal routine conditions for the determination of the degree of acetylation of chitosan by ^{1}H NMR. *Carbohydrate Polymers, 61*, 155–161.

Guibal, E. (2004). Interactions of metal ions with chitosan-based sorbents: A review. *Separation and Purification Technology, 38*, 43–74.

Heux, L., Brugnerotto, J., Desbrieres, J., Versali, M.-F., & Rinaudo, M. (2000). Solid state NMR for determination of degree of acetylation of chitin and chitosan. *Biomacromolecules, 1*, 746–751.

Kawamura, Y., Mitsuhashi, M., & Tanibe, H. (1993). Adsorption of metal ions on polyaminated highly porous chitosan chelating resin. *Industrial & Engineering Chemistry Research, 32*, 386–391.

Klug, M., Sanches, M. N. M., Laranjeira, M. C. M., Favere, V. T., & Rodrigues, C. A. (1998). Análise das isotermas de adsorção de Cu(II), Cd(II), Ni(II) e Zn(II) pela N-(3, 4-dihidroxibenzil) quitosana empregando o método da regressão não linear. *Quimica Nova, 21*, 410–413.

Kubota, N., Tastumoto, N., Sano, T., & Toya, K. (2000). A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents. *Carbohydrate Research, 324*, 268–274.

Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive & Functional Polymers, 46*, 1–27.

Kurita, K. (2006). Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology, 8*, 203–226.

Longhinotti, E., Pozza, F., Furlan, L., Sanches, M. N. M., Klug, M., Laranjeira, M. C. M., et al. (1998). Adsorption of anionic dyes on the biopolymer chitin. *Journal of the Brazilian Chemical Society, 9*, 435–440.

Raymond, L., Morin, F. G., & Marchessault, R. H. (1993). Degree of deacetylation of chitosan using conductometric titration and solid-state NMR. *Carbohydrate Research, 246*, 331–336.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*, 603–632.

Sankararamakrishnan, N., & Sanghi, R. (2006). Preparation and characterization of a novel xanthated chitosan. *Carbohydrate Polymers, 66*, 160–167.

Varum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrod, O. (1991). Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r spectroscopy. *Carbohydrate Research, 211*, 17–23.

Yu, G., Morin, F. G., Nobes, G. A. R., & Marchessault, R. H. (1999). Degree of acetylation of chitin and extent of grafting PHB on chitosan determined by solid state ^{15}N NMR. *Macromolecules, 32*, 518–520.