Carbohydrate Polymers 79 (2010) 801–810

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Review

Determination of the degree of *N*-acetylation for chitin and chitosan by various NMR spectroscopy techniques: A review

Mohammad R. Kasaai *

Faculty of Agricultural Engineering, Sari Agricultural and Natural Resources University, Khazar Abad Road, Km. 9, P.O. Box 578, Sari, Mazandaran, Iran

ARTICLE INFO

Article history:
Received 18 October 2009
Accepted 21 October 2009
Available online 25 October 2009

Keywords:
Chitin
Chitosan
Degree of *N*-acetylation
^{1}H NMR
^{13}C NMR
^{15}N NMR

ABSTRACT

Up to know, several methods have been developed to determine degree of *N*-acetylation, DA, for chitin and chitosan. In this article, an effort has been made to review the available literature information on the DA for the two biopolymers by different techniques of nuclear magnetic resonance, NMR (^{1}H NMR, ^{13}C NMR, and ^{15}N NMR) spectroscopy. This article describes the effects of various parameters and different compounds as impurities of the original polymers (moisture, minerals, proteins, and pigments) on the accuracy of the DA values. This study allows one to choose an appropriate technique to determine the DA for the two polymers. These techniques have been compared for their performances and limitations. Among various NMR spectroscopy techniques, ^{1}H NMR is the most sensitive and precise technique and results in the most accurate data. ^{13}C NMR and ^{15}N NMR techniques have been employed for entire range of the DA.

1. Introduction

Both chitin and chitosan are copolymers of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl glucosamine, GlcNAc), and 2-amino-2-deoxy-D-glucose (glucosamine, $GlcNH_2$) with β-D-(1 → 4) glycoside linkages. The word "chitosan" generally is used for both partially deacetylated and fully deacetylated chitosan. The degree of *N*-acetylation, DA, has been employed to differentiate chitin from chitosan. The solubility of the polymers in organic acids and in the mixture of salt/organic solvent (LiCl/dimethyl acetamide) (Striegel & Timpa, 1995) have been also used to differ between chitin and chitosan.

Chitosan is a cationic polysaccharide and its cationic nature in acidic medium is unique among polysaccharides. It exhibits diverse biological activities such as antifungal activity (Allan & Hadwiger, 1979; El Ghaouth, Arul, Asselin, & Benhamou, 1992a); antibacterial activity (Sudarshan, Hoover, & Knorr, 1992; Tasi & Su, 1999); elicitation of plant defense (El Ghaouth, Arul, Grenier, & Asselin, 1992b; Mauch, Hadwiger, & Boller, 1984); cholesterol lowering effect (El Ghaouth, Arul, Wilson, & Benhamou, 1994); and wound-healing property (Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1998). Chitosan inhibited the growth of gram-positive and gram-negative bacteria isolated from fishery products (Biagini, Muzzarelli, Giardino, & Castaldini, 1992). Chitosan exhibits film and fiber forming ability (Cruz et al., 2006; Rathke & Hudson, 1994); and scavenging and antioxidant activities (Averbach, 1978; Yin, Lin, Zhang, & Yang, 2002). Chitin and chitosan with vast interesting properties can be used in agriculture, food, cosmetics, medicine, and chemical industries.

The expansion and stiffness of the chitin/chitosan macromolecular chain conformation, and tendency of the polymer to aggregation depend strongly on the DA (Ottøy, Värum, & Smidsrød, 1996). The DA of chitin/chitosan is the most important parameter influences in its various properties. The effectiveness of chitin/chitosan in various applications appears to be dependent on the DA (Olsen, Schwartzmiller, Weppner, & Winandy, 1989; Sandford & Hutchings, 1987). The determination of the DA for the two copolymers is essential to study their chemical structures, properties and structure–properties relationships. With knowledge on the DA of the polymers, their some properties and new applications can be predicted. Thus, to determine an appropriate technique giving acceptable and reasonable result for the DA is essential and desirable for researchers.

Several techniques other than NMR spectroscopy have been already developed or employed to determine the DA of chitin/chitosan. These techniques include: IR spectroscopy (Baxter, Dillon, Taylor, & Roberts, 1992; Brugnerotto et al., 2001; Duarte, Ferreira, Marvão, & Rocha, 2002; Domard & Rinaudo, 1983; Miya, Iwamoto, Yoshikawa, & Mima, 1980; Shigemasa, Matsuura, Sashiwa, & Saimato, 1996a, 1996b; Sannan, Kurita, Ogura, & Iwakura, 1978; Sabins & Block, 1997); near infrared spectroscopy (NIR) (Rathke & Hudson, 1994); UV-spectrophotometry (Muzzarelli & Rocchetti, 1985; Muraki, Yaku, Iyoda, & Kojima, 1993; Tan, Khor, Tan, & Wong, 1998); ninhydrin assay (Curotto & Aros, 1993; Prochazkova, Värum, & Østgaard, 1999); colloidal titration (Terayama, 1952);

* Tel.: +98 151 382 2574x834; fax: +98 151 382 2566.
E-mail address: reza_kasaai@hotmail.com

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.10.051

conductometric titration (Raymond, Morin, & Marchessault, 1993); potentiometric titration (Ke & Chen, 1990; Tolaimate et al., 2000); acidic (Niola, Basora, Chornet, & Vidal, 1993) or enzymatic hydrolysis-colorimetry or HPLC analysis (Nanjo, Katsumi, & Sakai, 1991); GPC-UV analysis (Aiba, 1986); elemental analysis (Kim, Kim, & Lee, 1996; Ottøy et al., 1996); and thermal analysis using Differential Scanning calorimetry, DSC (Guinesi & Cavalheiro, 2006).

NMR techniques have been also developed or employed to determine the DA of chitin/chitosan with different accuracies. These techniques include: proton nuclear magnetic resonance, ^{1}H NMR (Hirai, Odani, & Nakajima, 1991; Shigemasa et al., 1996a; Signini & Campana-Fitho, 1999; Värum, Anthonsen, Grasdalen, & Smidrød, 1991a); cross-polarization (CP)/magic-angle spinning (MAS) ^{13}C NMR (Duarte, Ferreira, Marvão, & Rocha, 2001a; Heux, Brugnerotto, Desbrie'res, Versali, & Rinaudo, 2000; Lamarque, Viton, & Domard, 2004; Peter, Grun, & Forster, 1984; Raymond et al., 1993; Tolaimate et al., 2000); and CP/MAS ^{15}N NMR (Heux et al., 2000; Yu, Morin, Nobes, & Marchessault, 1999) spectroscopy. Up to date, no review article on the DA determination by various NMR techniques has been published. There is no unique technique that can be applied for the entire range with a high precision. The objectives of this study are: to review various NMR techniques published on the DA determination with special attention on the information mostly given during ten recent years; to compare the three NMR techniques for their performances and limitations; and to present various factors affecting the experimental results. The validity of the DA values obtained from different NMR techniques will be also discussed.

2. Description of sample preparation and description of NMR spectra

2.1. Description of sample preparation

The most complicated problem, which is related to the sample preparation for determination of the DA, is the poor solubility of chitin/chitosan. The solubility of chitosan in aqueous acidic solution depends on the degree of crystallinity, degree of polymerization, degree of neutralization of amine groups, distribution of glucosamine and *N*-acetyl glucosamine residues in chitin/chitosan macromolecules, ionic strength of the solvent, pH and concentration of the polymer solutions. A solvent should have good solubility properties for chitin/chitosan. In addition, the resonance peaks of the chitin/chitosan sample should not be obscured by resonance peaks of the solvent. The determination of the DA is difficult when the sample is partially soluble (Yang & Montgomery, 2000). When chitosan samples are completely soluble, it results in homogenous solution, and the DA measurements is reproducible (Heux et al., 2000). The most commonly solvents for proton and carbon NMR are: D_2O/CD_3COOD; $D_2O/DCOOD$; and D_2O/DCl. A detailed description of several parameters influencing on the DA determination has been found elsewhere (Kasaai, 2008).

2.2. Description of NMR spectra

2.2.1. ^{1}H NMR spectroscopy

A typical ^{1}H NMR spectrum of chitosan was shown in Fig. 1. Each unit residue of the polymer has six carbon and seven hydrogen atoms and these atoms produce C–H linkages. In addition, each unit of the polymer residue has four hydrogen atoms those are bonded with four oxygen atoms and created O–H groups. Each of these bonds in the unit residue has its own characteristics environment and thus has its own chemical shift. The ^{1}H NMR spectrum of the polymer is a superposition of the spectra of the individual unit residues which are slightly modified because of their linkages to each other. The peak at 2.0–2.1 ppm represents three protons of *N*-acetyl glucosamine (GlcNAc) and the peak at 3.1–3.2 ppm represents H-2 proton of glucosamine (GlcN) residues. The non-anomeric protons which are connected to ring-skeleton in a glycosyl residue have similar electron densities and thus have similar chemical shifts. In the spectrum of the molecule, the signals of the non-anomeric protons partially overlap and produce a broad envelope of signals in the middle of the spectrum. All of the signals are observed between 3.5 and 4 ppm. Anomeric protons (H_1) are observed at higher chemical shifts. This is due to their neighboring glycosidic and ring oxygen. The protons of H-1 [GlcN (H-1D) and GlcNAc (H-1A)] resonate at 4.6 and 4.8 ppm, respectively. Among various bands of ^{1}H NMR spectra, the methyl protons ~2.0–2.1 ppm, possess the highest resolution. The protons of H-1 [$H_{1(GlcNAc)}$ ~4.8 and $H_{1(GlcN)}$ ~4.6 ppm] have the least resolution. The res-

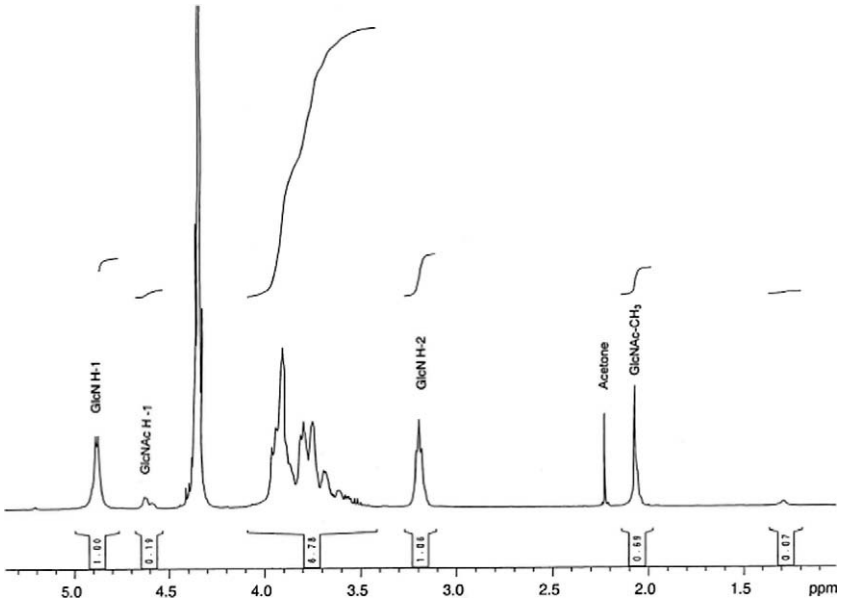

Fig. 1. ^{1}H NMR spectrum (600 MHz) of chitosan in D_2O at 65 °C. Acetone was used as a reference (2.2 ppm). Reproduced from Yang and Montgomery (2000).

Table 1
Chemical shifts for proton (in CD_3COOD/D_2O or DCl/D_2O) and carbon nuclei in chitins and chitosans determined by ^1H NMR or ^{13}C NMR spectroscopy.

Type of proton or carbon	Position[a] (δ, ppm)
H_1 ($H_{1\ of\ GluNAc}$)	4.62–4.85
H_1 ($H_{1\ of\ GluNH2}$)	4.85–4.97
H_2 ($H_{2\ of\ GluNH2}$)	3.18–3.24
H_2 ($H_{2\ of\ GluNAc}$)	3.38–3.65
H_3 ($H_{3\ of\ GluNH2}$)	3.52–3.87
H_3 ($H_{3\ of\ GluNAc}$)	3.52–3.65
H_3, H_4, H_5, H_6, H'_6	3.74–4.34
$H_{N-COCH3}$	1.95–2.09
CH_3COOH (AcOH)	2.09–2.11[b]
C_1	102.7–105.7
C_2	55.2–57.6
C_3	73.1–75.7
C_4	80.9–85.7
C_5	73.1–75.7
C_6	59.6–60.8
$N-CH_3$ (C_7)	22.8–23.3
N–C=O (C_8)	173.6–173.8

[a] The lowest and highest values correspond to fully *N*-deacetylated chitosan and fully acetylated chitin, respectively.
[b] Acetic acid is produced by hydrolysis of *N*-acetyl residue of chitosan when the sample is kept at high temperature for a long term.

olution of H_3, H_4, H_5, and H_6 protons are also low. The signals of the latter protons overlap with HOD signals of the solvent (D_2O/CD_3COOD) at 4.05 ppm. The average values for chemical shifts of various protons are listed in Table 1. The chemical shifts for residual protons of the solvents have been reported to be [D_20 (δ = 4.7 ppm) and CD_3COOD (δ = 2.05 and 8.5 ppm)]. D_2O enters into fast exchange reactions with protons and may eliminate resonance signals from –OH, $–NH_2$, and NH-CO-CH_3 species.

2.2.2. ^{13}C NMR spectroscopy

^{13}C NMR spectra of four chitin/chitosan samples having different DA [sample A (DA ≈ 1.0); sample B (DA ≈ 0.58); sample C (DA ≈ 0.21); and sample D (DA ≈ 0.0)] were illustrated in Fig. 2-1. There are essentially 8 signals in ^{13}C NMR spectrum of the chitin/chitosan sample. These signals are attributed to the 8 carbon atoms of *N*-acetyl glucosamine residues (see Table 1). Each signal occurs as singlet with the exception of carbon atoms for C_2 and $C_{C=O}$ which are doublets. This is due to the influence of the quadrupolar ^{14}N nucleus of the acetamide group (Gail et al., 1991; Tanner, Chanzy, Vincendon, Roux, & Gaill, 1990). In low acetylated chitosan, a doublet signal for C_4 was observed at around 86 ppm (Heux et al., 2000). The formation of *N*-acetyl group in the process of *N*-acetylation leads to a strong variation of the chemical shift of carbon atoms in ^{13}C NMR spectroscopy. The C-1 atom for cellulose, chitin and chitosan molecules resonate around 100 ppm and other carbon atoms resonate between 60 and 80 ppm. Carbon atom of –CH_2OH (C-6) group is clearly visible around 60 ppm. The large chemical shift dispersion of ^{13}C NMR spectra leads to well-resolved spectra. The anomeric carbon and methyl carbon atoms are observed between 95 and 110 ppm and within 15–25 ppm, respectively, when chitin/chitosan sample is dissolved in D_2O.

2.2.3. ^{15}N NMR spectroscopy

^{15}N NMR spectra of the four chitin/chitosan samples described in Section 2.2.2 are illustrated in Fig. 2-2. In the ^{15}N NMR spectrum of chitin/chitosan sample, there is only two major peaks corresponding to acetamide (NH–CO–CH_3) and amine (NH_2) groups. The two peaks are far from each other and well-separated (Brugnerotto et al., 2001; Heux et al., 2000; Yu et al., 1999). The peaks appeared around 110 and 10 ppm attributed to amide and amine groups, respectively.

3. Description of the DA determination by various types of NMR spectroscopy

The NMR spectra described in this manuscript obtained from a solid state or liquid state (in an appropriate solvent). Different models of pulse Fourier-Transform (FT) NMR spectrometer (fabricated by Bruker Company) have been employed to record the NMR spectra.

The DA of chitin/chitosan by various types of NMR (^1H NMR, ^{13}C NMR, and ^{15}N NMR) spectroscopy has been determined as described in the following: determination of the ratio of I_p/I_R: where I_p is integral of the probe signal (nucleus/nuclei); and I_R is the integral of reference signal (nucleus/nuclei). The integral of a signal is proportional to the number of nuclei contributing to the signal. The specific description for each technique is given in individual sections as follows.

3.1. ^1H NMR spectroscopy

A dilute solution (5–10 mg mL^{-1}) of the chitosan sample in a deuterated aqueous acid, CD_3COOD/D_2O or DCl/D_2O, at about pH 4 is prepared. The solute concentration depends on the molecular weight and the DA of the sample. The higher the molecular weight or the greater the DA the smaller amount of chitosan is required. CF_3COOD (99.9%) as a solvent has been used for highly crystalline chitin/chitosan sample (Hwang, Kim, Jung, Cho, & Park, 2003). The use of CF_3COOD enables one to determine the DA in a wider range. However, chain scission and *N*-deacetylation in the latter solvent may be occurred. To minimize the effect of HOD signal on chitosan spectrum, the samples were freeze-dried using D_2O (99.9%) to exchange labile protons by deuterium atoms (Heux et al., 2000; Hwang et al., 2003).

The chitosan samples were prepared and the spectra between 0 and 10 ppm were recorded using a proton NMR spectrometer as described in several reports (Hirai et al., 1991; Rinaudo, Le Dung, Gey, & Milas, 1992; Värum et al., 1991a). The methyl protons have been selected as probe nucleus/nuclei (Hirai et al., 1991; Rinaudo et al., 1992; Värum et al., 1991a). The DA was determined using Eqs. (1) (Hirai et al., 1991; Tan et al., 1998), (2) (Värum et al., 1991a), and (3) (Rinaudo, Milas, & Dung, 1993):

$$DA = \left[(1/3 \times I_{CH_3})/(1/6 \times I_{(H2-H6)})\right] \times 100 \quad (1)$$

$$DA = \left\{7\left[I_{H1\ (GcNAc)} + I_{CH_3\ (GlcNAc)}\right]/4\left[I_{H1\ (GlcN)} + I_{H2\ (GlcNAc)}\right] + I_{CH_3\ (GlcNAc)}\right\} \times 100 \quad (2)$$

$$DA = \left[I_{CH_3}/(3 \times I_{H\text{-}1\ proton})\right] \times 100 \quad (3)$$

where $I_{(H2-H6)}$ is the summation of integrals of H_2, H_3, H_4, H_5, and H_6. Eq. (3) has been used to determine the DA% in the range of 2-21 (Rinaudo et al., 1993). The DA value was determined using the integral of $–CH_3$ signal (at about 2.0 ppm) compared with the integral of H_1 proton considered as an internal standard (Tolaimate et al., 2000). Kasaai, Arul, Chin, and Charlet (1999) have used the experimental procedure proposed by Hirai et al. (1991), but they determined the DA according to:

$$DA = \left[(1/3\, I_{N-COCH_3}/I_{H\text{-}2}(GlcNAc)\right] \times 100 \quad (4)$$

where $I_{N-COCH3}$ and $I_{H\text{-}2\ proton}$ are integrals of methyl protons and H-2 proton of GlcNAc, respectively. A validated method has been established for the DA smaller than 52% (Lavertu et al., 2003). These authors performed all of validation steps (the precision, ruggedness, robustness, specificity, and accuracy) and proposed the following equation for DA < 10:

$$DA = 100 \times (I_{H1\text{-}D}/I_{H1\text{-}D} + I_{H1\text{-}A}) \quad (5)$$

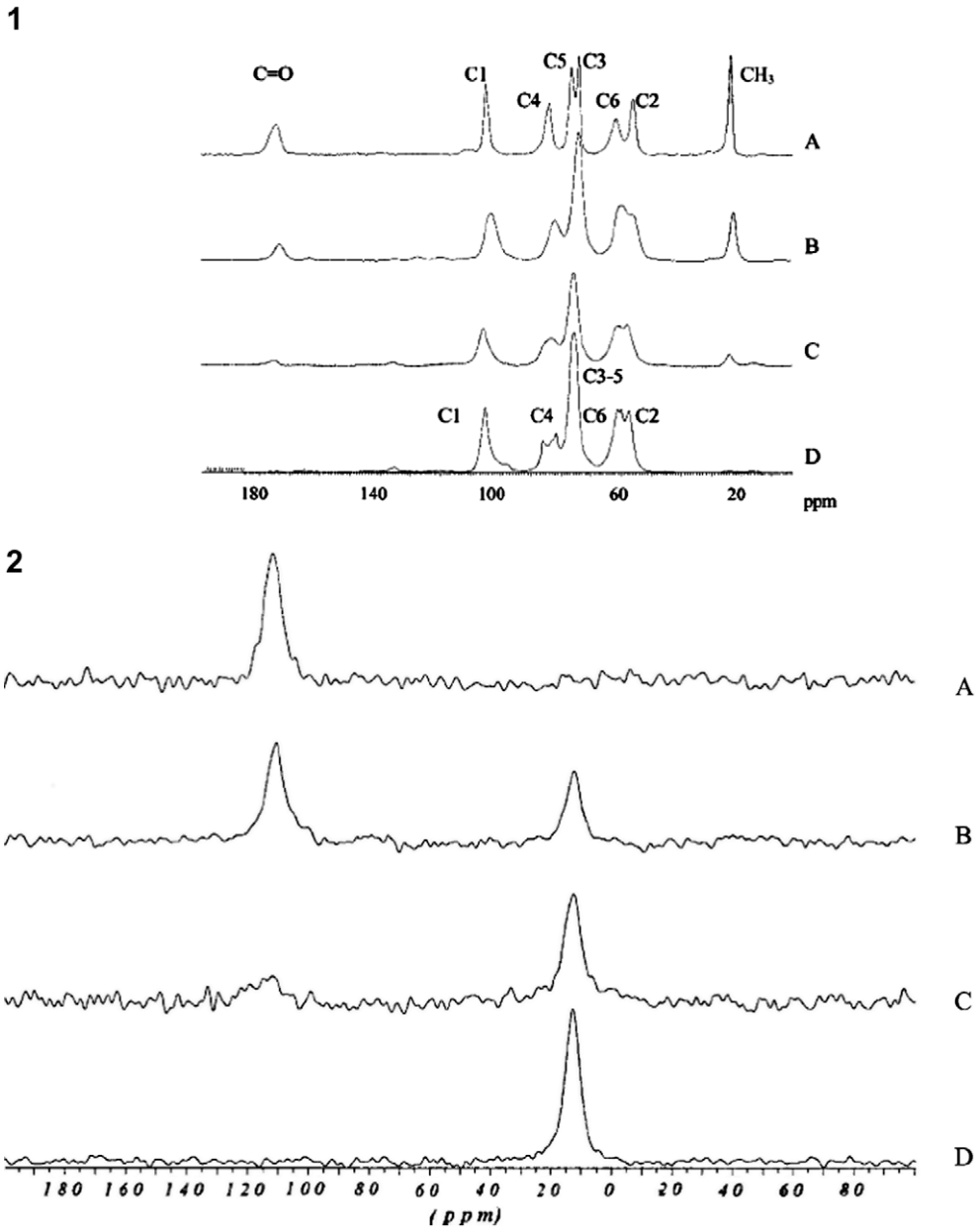

Fig. 2. (1) ^{13}C CP-MAS NMR spectra of chitosans samples (with decreasing DA from A to D); and (2) ^{15}N CP-MAS NMR spectra of chitosan samples (with decreasing DA from A to D). Reproduced from Heux et al. (2000).

where $I_{H1\text{-}D}$ is the integrals of H1 atom for the deacetylated monomer unit and I_{H1}-A is the integral of H_1 for the acetylated monomer unit. Sato et al., (1998) determined the DA by excluding H_2 peak of GlcNAc unit and H_3–H_6 peaks of pyranose ring. This is due to their low resolution and overlap with HOD signal (4.05 ppm) of the solvent peak. They have determined the DA using the following equation:

$$DA = (I_{H1} + I_{Ac}/3)/(I_{H1} + I_{H2} + I_{H1'} + I_{Ac}) \times 100 \quad (6)$$

where I_{H1}, $I_{H1'}$, I_{H2} and I_{Ac} are the integrals for: $H_{1\ (GlcN)}$; $H_{1'\ (GlcNAc)}$; $H_{2\ (GlcN)}$; and $H_{Ac\ (GlcNAc)}$, respectively. Shigemasa et al. (1996a, 1996b) have determined the DA using ^{1}H NMR spectroscopy through the following ratios:

$$A/B = \left(I_{H2(GlcNAc)} + I_{H3,\ H4,\ H5\ and\ H6\ protons\ of\ hexosamine\ residue}\right)/I_{H2(GlcN)} \quad (7)$$

$$A/C = I_{H2(GlcNAc),\ H3,\ H4,\ H5\ and\ H6\ protons\ of\ hexosamine\ residue}/I_{methyl\ protons\ of\ \textit{N}\text{-acetyl group}} \quad (8)$$

$$B/C = I_{H2(GlcN)}/I_{N-CO-CH_3} \quad (9)$$

where A is the integral of H-2 proton of GlcNAc residue plus the integrals of summation of H-3, H-4, H-5, H-6 protons of the hexamine residue; B is the integral of H-2 proton of GlcN residue; and C is the integral of methyl proton of *N*-acetyl group. They have employed ^{1}H NMR spectroscopy to compare and verify the DA determined by IR spectroscopy.

3.2. ^{13}C NMR spectroscopy

^{13}C NMR spectra of chitin/chitosan in solid state were recorded from 0 to 250 ppm (Raymond et al., 1993). In more modern instrument, cross-polarization (CP), magic-angle spinning (MAS), and high power ^{1}H decoupling (HPDEC) have been employed (Saito, Tabeta, & Hirano, 1982; Värum, Anthonsen, Grasdalen, & Smidrød, 1991b; Raymond et al., 1993; Ottøy et al., 1996; Heux et al., 2000; Duarte et al., 2001a). The procedure of Ottøy et al. (1996) has been

modified with changes in relaxation delay and contact times (Duarte, Ferreira, Marvão, & Rocha, 2001b; Tolaimate et al., 2000). The contact and delay times are the most variable parameters to record the CP/MAS ^{13}C NMR spectrum (Duarte et al., 2002; Guinesi & Cavalheiro, 2006; Tolaimate et al., 2000). The carbon atom of carbonyl or methyl group has been used as measure of nucleus/nuclei. The DA was calculated from the integral of methyl carbon divided by the summation integrals of carbon atoms of the D-glucopyranosyl ring [C_1–C_6 atoms (δ 50–105 ppm)] (Duarte et al., 2002; Guinesi & Cavalheiro, 2006; Ottøy et al., 1996; Raymond et al., 1993; Tolaimate et al., 2000; Värum et al., 1991a):

$$DA = 100 \times I_{N-CH3}/[1/6\ (I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})]$$
$$= 100 \times (I_{N-CH_3}) \Big/ \left(1/6 \sum I_{\text{main chain carbons}}\right) \quad (10)$$

Guinesi and Cavalheiro (2006) have employed a ^{13}C NMR spectrometer resonate at frequency of 75.43 MHz with a magic angle of 4.5 kHz. The contact and delay times were set at 1.8 ms and 13 s, respectively.

3.3. ^{15}N NMR spectroscopy

^{15}N NMR spectra of chitin/chitosan in solid state were recorded using NMR spectrometer operating at 30 or 200 MHz from 0 to 200 ppm (Heux et al., 2000; Yu et al., 1999). Two major peaks have been recognized in the spectra of chitin/chitosan, and the DA was calculated according to:

$$DA = (I_{N\text{-acetyl group}}) / (I_{N\text{-acetyl group}} + I_{\text{amine group}}) \quad (11)$$

Heux et al. (2000) have used a contact time of 2 ms to record ^{15}N NMR spectrum, whereas Yu et al. (1999) have employed different contact times. The latter conditions have been employed to minimize errors for quantitative evaluation of the DA. This is due to differing cross-polarization rates involving amine (NH_2) group versus *N*-acetyl (NH–CH_3) group.

4. Description of various parameters affecting the DA determination

4.1. General aspects

Chitin naturally occurs associated with proteins, organic pigments and minerals (Muzzarelli, 1977; No, 1995). Among them, proteins possess functional (amine and NH–C=O) groups similar to chitin/chitosan. NMR techniques are given signals for any chemical compounds present in chitin/chitosan samples. Small differences in the compositions result in significant difference in NMR spectra. The impurities and moisture create interference peaks and change the positions and intensities of some peaks. Chitin/chitosan being a hygroscopic material produces highly intense signal for OH group in the ^1H NMR spectrum and induces a limitation for the DA determination. The sample can be dried to eliminate the interference peak of moisture. The determination of the DA is difficult when the sample solutions contain impurities (Yang & Montgomery, 2000). Eq. (5) can be used for the DA calculation when the chitosan sample contaminated by acetic acid (Lavertu et al., 2003).

Solid-state spectroscopy (^{13}C NMR, ^{15}N NMR) has the possibility of determining the DA in entire range of the DA. ^1H NMR needs solution preparations and can not be used for highly acetylated chitin and block copolymers of chitin/chitosan.

Approximately 5, 300 and 200 mg of chitin/chitosan samples have been used to record ^1H NMR, ^{13}C NMR, and ^{15}N NMR spectra, respectively (Hirai et al., 1991; Ottøy et al., 1996; Yu et al., 1999). A larger amount of the polymer sample and/or a longer acquisition time is required for a solid-state sample compare to a liquid-state sample. The amount of the polymer sample required for analysis depends also on the sensitivity and limit of detection of the instruments. Generally, the instruments made with high and advanced technologies required smaller amounts of samples for analysis.

4.2. Sensitivity, resolution, and other parameters affecting the quantitative evaluation

The sensitivity of NMR depends on the nucleus, its abundance, and its chemical environment (Neiss, 2000). Low sensitivity of ^{13}C NMR and ^{15}N NMR are associated with low natural abundance of ^{13}C and ^{15}N, particularly when NMR spectrometer having a low magnetic field strength is employed (Bush, 1996; Colquhoun & Goodfellow, 1994). In order to increase the sensitivity, it is necessary to use chitosan enriched with ^{13}C and ^{15}N. The low sensitivity of ^{13}C NMR and ^{15}N NMR spectroscopy leads to a long time accumulation for low concentration of the polymer. The properties of different nuclei present in chitin/chitosan (spin, natural abundance, relative sensitivity, absolute sensitivity, range for resonance, gyromagnetic ratio, resonance frequency) (Bush, 1996; Colquhoun & Goodfellow, 1994; Csuk & Glänzer, 1988; De Nooy, Capitani, Masci, & Crescenzi, 2000; Freeman, 1988; Webb, 2000) for a comparative evaluation are given in Table 2. A highly abundant nucleus with a large gyromagnetic ratio such as ^1H is very sensitive and therefore is the first choice for detection in NMR experiments. The higher magnetic fields, the art of sample preparation and fast MAS capabilities dramatically increase the sensitivity and resolution of spectra (Saalwächter & Ramamoorthy, 2006).

Another important aspect of the NMR technique, with a negative effect on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, particularly in pulsed repetitive experiments. All characteristics of a signal (chemical shift, multiplicity, line width, coupling

Table 2
Properties of the nuclei present in chitin and chitosan.

Nucleus	Spin quantum number (*I*)	Natural abundance (%)	Relative sensitivity[a]	Absolute sensitivity[b]	Range (ppm)	Gyromagnetic ratio	Resonance frequency (MHz)		
							$1.409 \times \tau$[c]	$2.349 \times \tau$	$4.698 \times \tau$
^1H	1/2	99.98	1.000	0.9998	20	2.793	60.000	100.000	200.000
^2D (deuterium)	1	1.5×10^{-2}	1.45×10^{-6}	2.17×10^{-8}	–	–	–	15.35	–
^{12}C	0	98.89	–	–	–	–	–	–	–
^{13}C	1/2	1.108	1.59×10^{-2}	1.8×10^{-3}	250	0.702	15.097	25.144	50.288
^{14}N	1	99.63	1.04×10^{-3}	4×10^{-3}	–	0.283	–	7.22	–
^{15}N	1/2	0.365[c]	3.85×10^{-6}	4×10^{-3}	1000	0.283	–	10.13	–
^{16}O	0	99.96	–	–	–	–	–	–	–
^{17}O	5/2	3.7×10^{-2}	1.08×10^{-5}	–	–	–	–	13.56	–

[a] Relative sensitivity at constant magnetic field for equal number of nuclei.
[b] Absolute sensitivity equals to the product of relative sensitivity and natural abundance.
[c] τ = testla (in kilogauss).

constants, and relative intensity) contribute analytical information. Inadequate specimen preparation or incorrect instrumental and parameters adjustment may lead to poor resolution, decrease sensitivity, spectral artifacts, and erroneous data (Braun, Kalinowski, & Berger, 1996; Colquhoun & Goodfellow, 1994).

Solid-state NMR spectroscopy (^{13}C NMR and ^{15}N NMR) often give broad NMR spectra. In solid state, there are up to three additional interactions compared to liquid-phase which cause dramatic increase in line width (Chippendale, 1997; Neiss, 2000). The broadness, low signal-to-noise ratio and asymmetries make difficult the quantitative analysis. Peak asymmetries cause problems in measuring of accurate chemical shifts values. Reliable information can be obtained by lines fitting using Gaussian functions and take into consideration of full width at half-height (fwhh). These considerations reduce the error of the DA particularly arising from low value of the DA (Heux et al., 2000).

4.3. Reference materials

The reference compound should have the following properties: (a) stability in a variety of solvents; and (b) an unchanged chemical shift value over a wide range of temperature and pH values (Teng, 2005). The reference compound can be added into the samples as an internal reference or can be used alone as an external reference. An internal standard is used, when unknown samples do not contain this compound, and the reference should not have similar physical and chemical properties to unknown samples (Teng, 2005).

Tetrametylsilane (TMS) is the most widely used material as a reference for proton and carbon (^{1}H and ^{13}C) NMR spectroscopy. The insolubility of TMS in aqueous solutions makes it a poor reference sample for proteins and polysaccharides (Teng, 2005). Two following chemical compounds have been commonly used as reference materials for ^{1}H NMR in aqueous solutions: (i) 2,2-dimetyl-2-silapentane-5-sufonic acid sodium salt (DSS); and (ii) 3-(trimethyl silyl) propane sulfonic acid sodium salt (TPSS). The two latter references adopted by IUPAC, just after TMS (Harris, Becker, De Menezes, Goodfellow, & Granger, 2001; Harris, Becker, de Menezes, Goodfellow, & Granger, 2002; Hirai et al., 1991; Teng, 2005; Värum et al., 1991a). For aqueous solutions, DSS is a better reference, because of its high water solubility, and insensitivity to a wide range of pH (2–11) and temperature (25–90 °C) (Teng, 2005). DSS was used as an external reference for ^{1}H and ^{13}C nuclei for biological samples. The resonance frequency of TPSS is close to the TMS signal. Proton of acetone has been also employed as a reference (2.25 ppm) for evaluation of the DA for chitin and chitosan (Yang & Montgomery, 2000). The signal of methyl proton was used as an internal reference (Rinaudo et al., 1993; Rinuado, Milas, & Desbrières, 1997; Shigemasa et al., 1996a). This band is the best internal reference, because: (I) it is well-resolved peak with a flat baseline in both sides of the peak; and (ii) its intensity is three times higher than other protons. Ideally an internal standard is desirable since this removes the necessity of correcting for any magnetic bulk susceptibility difference between the sample and the standard (Webb, 2000).

In addition to TMS, methanol, benzene and glycine have been used as external references for ^{13}C NMR spectroscopy. Their corresponding chemical shifts have been reported to be 50.05, 128.05, and 176.03 ppm from the value for TMS (0 ppm), respectively (Heux et al., 2000; Muzzarelli, Louch, & Emanuelli, 1987; Saitô, Tabeta, & Ogawa, 1987).

The selection of reference for ^{15}N is complicated, because there is no any compound similar to TMS, DSS or TPSS available for ^{15}N as a reference. A variety of reference systems have been used to define 0.00 ppm for ^{15}N (Teng, 2005). Ammonium ion (NH_4^+ of enriched ammonium nitrate) has been used as external standard for DA determination of chitin/chitosan by ^{15}N NMR spectroscopy (Heux et al., 2000).

Standard chitin/chitosan samples with known DA can be used as external references. Since chitin/chitosan with known DA values as standard references are not commercially available, a validated method can be used to verify the accuracy of the DA for chitin/chitosan samples having known DA, and then, the latter samples can be used as standard references. The mixtures of chitin and chitosan, having random distribution of the co-units can be used as a reference for chitin/chitosan having random distribution, but they can not be representative of the block ones. Ideally, individual monomer of *N*-acetyl glucosamine and glucosamine or mixtures of these two monomers can not be used as references for NMR spectroscopy. The mixtures of monomers are not real representatives of chitin or chitosan samples. There are inter-and intra-molecular interactions between macromolecules chains in the polymers, whereas such interactions are not present in the mixtures of monomers.

5. Evaluation of reported results

5.1. ^{1}H NMR spectroscopy

Chemical shifts vary by changing in the distribution of co-units of the macromolecules. Depending on the nature of the neighboring units, the chemical shifts of a particular resonance signal change (Schanzenbach & Peter, 1997). Knowledge on the effects

Table 3
Relaxation times for different protons of chitosans having different DA in two solvents at 70 or 27 °C.

Protons	Relaxation time (s)					
	[a]DA = 3%, solvent = CH_3COOD/D_2O, T = 70 °C	[a]DA = 3%, solvent = DCl/D_2O, T = 70 °C	[b]DA = 52%, solvent = DCl/D_2O, T = 70 °C	[b]DA = 0.0, solvent = DCl/D_2O, T = 70 °C	[c]DA = 15.5%, solvent = DCl/D_2O, T = 70 °C	[c]DA = 15.5% solvent = DCl/D_2O, T = 27 °C
H_1 ($H_{1\ of\ GluNAc}$)	0.96	0.92	1.12	–	–	–
H_1 ($H_{1\ of\ GluNH2}$)	0.97	0.99	1.12	1.01	–	–
H_2 ($H_{2\ of\ GluNH2}$)	1.23	1.69	1.42	1.37	1.73	1.46
H_2 ($H_{2\ of\ GluNAc}$)	1.23	1.71	<1.10	–	1.14–1.48	–
H_3 ($H_{3\ of\ GluNH2}$)	–	–	–	–	–	–
H_3 ($H_{3\ of\ GluNAc}$)	–	–	–	–	–	–
H_3, H_4, H_5, H_6, H'_6	1.00, 0.77, 0.82, 0.92	1.07, 1.06, 0.92, 0.69, 0.76	1.42	1.37	1.14–1.48	1.23–1.36
$H_{N-COCH3}$	1.70	1.0	1.56	1.07	–	1.35
CH_3COOH	–	12	–	–	10.35	2.60

[a] Hirai et al. (1991).
[b] Lavertu et al. (2003).
[c] Fernandez-Megia1 et al. (2005).

of solvent, temperature and the distribution of the co-units are necessary to achieve a precise data of ^{1}H NMR spectrum. Due to the association of the molecules by hydrogen bonds, the resonance signals have been shifted to a lower field, because of diminution of the electric shielding of the protons.

Among various conditions proposed in the literature for determining the DA of chitosan by ^{1}H NMR (Hirai et al., 1991; Shigemasa et al., 1996a, 1996b), the procedures proposed by Hirai et al. (1991) and Värum et al. (1991a) have been widely accepted. However, Värum et al. (1991a) have taken into account different types of a proton, i.e. $H_{1\ (GlcN)}$ and $H_{1\ (GlcNAc)}$. Several Eqs. (1)–(9) have been already given to determine the DA (see Section 3.1). In addition, the DA can be also determined using different combinations of peaks, i.e. this technique is internally consistent. The use of methyl proton peak as an internal peak has advantages over other resonance peaks. This is because this peak is three times more intense than that of the other peaks and it is well-resolved. In the case of DA < 10, the summation of two peaks [$H_{1\ (GlcN)} + H_{1\ (GlcNAc)} = H_{1\text{-}D} + H_{1\text{-}A}$] (Lavertu et al., 2003) have been used as an internal reference. The H_2 peak of deacetylated monomer as an internal reference has been proposed by Shigemasa et al. (1996b). The latter is not an appropriate peak for the evaluation of the DA, because of its overlapping with the peaks appeared between 3.8 and 4.2 ppm (see Table 1).

The relaxation time, T_1 [as a measure of the time taken to reestablish equilibrium of the nuclear spin following a radio frequency (r.f.) pulse of different protons] of chitosan samples having different DA in two solvents (CH_3COOD/D_2O; DCl/D_2O) at 27 and 70 °C are given in Table 3 (Hirai et al., 1991; Lavertu et al., 2003; Fernandez-Megia1, Novoa-Carballal, Quiñoá, & Riguera, 2005). The T_1 was measured by means of inversion recovery pulse sequence [a 180° pulse inverts the magnetization, which is then measured by a 90° pulse after a delay time, τ (180–τ–90)] (Colquhoun & Goodfellow, 1994). The value of T_1 was found to be lower than 1.6 s (Hirai et al., 1991; Lavertu et al., 2003). The pulse repetition delay times were reported to be 6 and 40 s for CH_3COOD/D_2O and DCl/D_2O, respectively (Hirai et al., 1991). The pulse repetition delay time reported by Hirai et al. (1991) was significantly high value (40 s); this is because the relaxation time for CH_3 residue of acetic acid was 12 s (Hirai et al., 1991). Lavertu et al. (2003) noted that the hydrolytic cleavage of the acetyl groups of chitosan by dilute acid as proposed by Hirai et al. (1991) was found to be quite slow and therefore it was not necessary to use a large relaxation delay time in order to quantify the amount of acetic acid resulting from the deacetylation. The T_1 is closely related to molecular mobility. As the mobility decreases the relaxation time, T_1, reaches to the minimum value. The relaxation time increases with an increase the solution temperature because of rising in the molecular mobility. A significant reduction of T_1 for acetic acid from 10.38 s at 70 °C to 2.60 s at 27 °C was achieved. The value of T_1 is slightly affected by DA, since acetic acid presents a T_1 value much higher than the rest of protons at 27 °C (see Table 3). An accurate DA value can be attained at this temperature (Fernandez-Megia1 et al., 2005).

The precision of ^{1}H NMR technique was estimated by performing the experiment in triplicate (Lavertu et al. (2003). The coefficient of variation was found to be less than 0.8% by these authors. The accuracy of ^{1}H NMR spectroscopy has been shown by mixing solutions of chitosans having DA of 0.52 and 0.0 with different volumetric ratios [the ratios: (80: 20); (50:50); and (20: 80)]. The expected the DA values were 40.6%, 24.5%, and 9.6%, respectively. The DA was found to be 40.1%, 24.2%, and 10.4%, respectively (Lavertu et al. (2003). The deviation of the experimental results from the actual value for the DA has been reported to be less than 5% (Rinaudo et al., 1992, 1993; Värum et al., 1991a).

5.2. The effect of temperature on 1H NMR spectrum of chitosan

Chitosan as a polyelectrolyte with high molecular weight generates high solution viscosity. It is necessary to reduce the viscosity as well as to increase the mobility of the molecule in order to obtain clear and resolved a spectrum. In order to diminish the viscosity of solution and line width of ^{1}HNMR signals, ^{1}H NMR spectrum has been taken using a dilute solution at temperatures between 70 and 90 °C (Heux et al., 2000; Hirai et al., 1991; Tan et al., 1998; Värum et al., 1991a). However, the experimental time at high temperature is several times longer than that of room temperature. Fernandez-Megia1 et al. (2005) have demonstrated that the time of experiment significantly reduced (more than six times) by recording the spectrum at room temperatures compared to high temperature as high as 70 °C. This is due to the longer T_1 at 70 °C (Fernandez-Megia1 et al., 2005). Molecular mobility as well as relaxation time decreases with a decrease in temperature of the polymer solution. The solvent peak as well as the residue of HOD signal moved away from the integration areas of different chitosan signals and did not interfere with any of chitosan's peaks when the experiment was performed at 27 °C (Lavertu et al., 2003). At this temperature, the solvent proton resonates within 4.6 and 4.7 ppm. Chemical shifts depend on temperature (Koenig, 1992; Vincendon, 1985). The effect of temperature on the chemical shift is small (Koenig, 1992) and reported to be approximately 10^{-3} ppm K^{-1}The sensitivity of various protons such as (CH_3, OH, and NH_2) and the protons involved in intra-molecular and intermolecular hydrogen bonds to temperature are different.

Table 4
The DA ranges, performances/advantages and limitations/disadvantages for different NMR spectroscopy methods.

Method	DA (%)	Performances and advantages	Limitations and disadvantages
^{1}H NMR	0–60	Highly sensitive; gives accurate data; gives information on the distribution of the co-units	Require sample preparation, applicable for a limited range of the DA, where the sample is soluble in the solvent
^{13}C NMR	0–100	No need to prepare the solution; applicable for entire range of the DA; gives information on sequential distribution; no need to dry the sample; the higher sensitive instruments generally results in higher precision	Low resolution and low sensitivity, availability of the instrument is a limitation due to the cost, specialist consideration and sophistication, especially for more modern instruments having higher stronger magnetic fields; the limit of detection is more than 5%
^{15}N NMR	0–100	It gives clear, simple and well-resolved spectra; no need to prepare the solution; no need to dry the sample; impurities do not induce any problem; applicable for entire range of the DA; an appropriate technique for a composite or a blend of chitin/chitosan with other polysaccharides; the higher sensitive instruments generally results in higher precision; using cross-polarization and strong magnetic fields yield in higher accuracy of the results and improve limit of detection	A possible error if the samples contain proteins as impurities; low sensitivity due to low natural abundance of N (0.3%); all limitation items as disadvantage points given in ^{13}C NMR are valid here

5.3. ^{13}C NMR spectroscopy

Two types of carbon atoms (CH_3 and C=O) were used to determine the DA. CH_3 signal is more appropriate than C=O signal, because a longer relaxation time for carbon nucleus of carbonyl group results in underestimated value for the DA (Ottøy et al., 1996; Raymond et al., 1993; Saito et al., 1982). Duarte et al. (2001b) determined the DA of chitin/chitosan samples using the procedure proposed by Raymond et al. (1993). The spectra of accurately weighed mixtures of chitin/chitosan and glycine were recorded at different contact times, relaxation times, and proton spin–lattice relaxation times. The intensities of carbonyl group for glycine molecule and carbon atoms for chitin/chitosan molecule in the spectrum of their mixture allow one to estimate the DA for the chitin/chitosan sample. Ottøy et al. (1996) have determined the DA using both ^{13}C NMR and ^{1}H NMR spectroscopy. The DA of chitosan samples determined by ^{13}C NMR in the range of 20–55 was consistent with the DA values obtained from ^{1}H NMR.

5.4. ^{15}N NMR spectroscopy

The chemical shift for nitrogen of *N*-acetyl group reported to be between 101 and 110 depending on its neighbors (Heux et al., 2000; Yu et al., 1999) and smaller than 10 ppm for the amine groups (Heux et al., 2000; Yu et al., 1999). The difference in the chemical shifts reported by the two research groups could be due to the chitins having different DA values. Yu et al. (1999) determined the DA% in the range (60–95), whereas Heux et al. (2000) determined the DA% in the range (0–100). The lowest value of the chemical shift is attributed to fully *N*-deacetylated chitosan and the highest value is corresponded to fully acetylated chitin.

^{15}N NMR is less sensitive than ^{13}C NMR spectroscopy. One can not expect to detect the DA level lower than 10% with ^{15}N NMR technique, when line broadening effects are observed. Line width or full width at half-height (fwhh) in ^{15}N NMR technique gives a good indication of crystallinity of the sample. ^{15}N NMR technique has been employed to evaluate the DA for chitin in association with (1 → 3)-β-D-glucans or structural polysaccharides of fungi (Heux et al., 2000). The results of ^{15}N NMR spectroscopy were consistent with the results of ^{13}C NMR in the range of 60–95% (Yu et al., 1999).

6. Comparison of different types of NMR spectroscopy

The DA range, advantages and disadvantages of various types of NMR spectroscopy for the DA determination are given in Table 4. Among various NMR techniques, ^{1}H NMR technique results in more reliable data than that of ^{13}C NMR and ^{15}N NMR techniques. ^{1}H NMR spectroscopy was used to verify the validity of several techniques and it has been usually employed as a standard method to calibrate other methods (Brugnerotto et al., 2001; Shigemasa et al., 1996a, 1996b) for the DA determination. ^{1}H NMR spectroscopy has been chosen as a standard method by the American Standard Test Method organization to determine the DA for chitosan (ASTM, 2003). The limitation of liquid phase of ^{1}H NMR technique for the DA determination of chitin/chitosan is its poor solubility in acidic aqueous solution. The solubility depends on the DA, the distribution of *N*-acetyl groups in partially *N*-deacetylated chitin as well as partially *N*-acetylated chitosan, and their molecular weights. The higher the DA as well as the larger the macromolecules, the poorer the solubility. The combination of the three techniques enables one to employ NMR technology for the DA determination in entire range for chitin/chitosan, their some derivatives and composites. The results of solid-state ^{13}C CP/MAS were in good agreement with the results of ^{15}N CP/MAS in entire range of the DA (Heux et al., 2000). These authors have compared the results of the three NMR techniques and they concluded that the three methods were in good agreement.

The ^{13}C NMR and ^{15}N NMR spectra of chitin/chitosan generally show much better chemical shift dispersion than do the ^{1}H NMR spectra. The interpretation of spectrum and evaluation of the DA by ^{15}N NMR is simple, since it gives only two signals. Among polysaccharides, the N nucleus is only present in chitin and chitosan, thus the evaluation of the DA in the two polymers associated with other polysaccharides using ^{15}N NMR spectroscopy is possible without any purification process. Interpretation of a ^{13}C NMR spectrum is not a difficulty because ^{13}C nucleus has a wide range of chemical shifts (0–250 ppm), and different carbon atoms signals having different chemical shifts and are well-resolved.

^{13}C NMR and ^{15}N NMR can be used for entire range of the DA. Solid-state NMR (^{13}C and ^{15}N) spectroscopy has been used to determine the DA of insoluble samples such as highly acetylated chitin, highly crystalline, cross-linked, and block copolymer of chitin/chitosan samples. CP/MAS ^{13}C NMR, and CP/MAS ^{15}N NMR are appropriate methods for insoluble chitin/chitosan samples even the samples are associated with impurities and humidity (Ottøy et al., 1996). One could not expect to detect the DA less than 10% in ^{15}N NMR and ^{13}C NMR techniques due to line broadening effects, their high coefficient of variation (≈10%) and low sensitivity. In this sense, solid-state ^{15}N NMR is less sensitive than ^{13}C NMR spectroscopy (Heux et al., 2000).

Various types of NMR spectroscopy provide information on the chemical structure of chitin/chitosan, sequential distribution of amine and *N*-acetyl groups (De Nooy et al., 2000; Värum et al., 1991a, 1991b). NMR spectroscopy also measures short-range or molecular order (Colquhoun & Goodfellow, 1994). Chemical modifications such as deacetylation, acetylation, decomposition, depolymerization and fragmentation should change various types of NMR spectra by increasing/decreasing the intensity or shifting the position of some peaks. The objective of this study was not to examine the effects of chemical modifications on specific absorption bands used to determine the DA, nor their influence on the accuracy of the DA measurement.

It is desirable to take into consideration the following major parameters for quantitative analysis: precision of the different techniques; and accuracy of the experimental results and calculations. In this way, ^{1}H NMR spectrometry is an appropriate method.

7. Conclusions

The following conclusions are made from this review: (i) NMR spectroscopy is non-destructive and noninvasive technique. It is applicable to determine the DA for chitin/chitosan in different physical states and heterogeneous systems; (ii) among various NMR spectroscopy techniques, ^{1}H NMR is the most sensitive and precise technique and results in the most accurate data. ^{1}H NMR technique can be chosen as a reference method to verify the results obtained from other techniques or to verify the validity of other techniques. This is due to its highest sensitivity compared to the other techniques. ^{1}H NMR technique is applicable for a limited range of the DA, where the polymer samples are soluble in a solvent; (iii) ^{13}C NMR and ^{15}N NMR can be used for both soluble and non-soluble samples in entire range of the DA. The combination of the three techniques enables one to employ NMR technology for the DA determination in entire range for chitin/chitosan, their some derivatives and composites; (iv) the use of solid-state (^{13}C or ^{15}N) NMR technique provides a number of advantages such as it does not need solvent and sample preparation. The NMR spectra of highly crystalline chitin/chitosan samples can be used as references to interpret complicated spectra acquired from less crystalline or amorphous samples; (v) these techniques can be

used for determination of acetyl content not only in chitin and chitosan but also in amino sugars, hetero-polysaccharides and glycoproteins, which are carrying acetyl, *N*-acetyl and *O*-acetyl groups; (vi) ^{15}N NMR spectroscopy results in clear, simple and well-resolved spectra; (vii) impurities (minerals, proteins and pigments) and moisture may induce interferences to obtain accurate results. To achieve accurate results, care must be taken to identify the peaks corresponding the impurities and moisture; and (viii) NMR spectroscopy yields information on sequential distribution of the glucosamine and *N*-acetyl glucosamine residues.

8. Future work and perspectives

The nuclei of predominant interest in NMR studies for chitin and chitosan are ^{1}H, ^{13}C, ^{17}O and ^{15}N. No information is available on ^{17}O NMR of chitin/chitosan. ^{17}O NMR spectroscopy, two-dimensional NMR (proton and carbon) and three dimensional (proton, carbon and nitrogen) NMR spectroscopy are useful techniques to determine or estimate chemical structure (sequence, linkage positions, and size) of chitin/chitosan and evaluate the DA. The use of modern and more powerful NMR instrument increases the sensitivity and limit of detection.

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.

Allan, C. R., & Hadwiger, L. A. (1979). The fungicidal effect of chitosan on fungi of varying cell wall composition. *Experimental Mycology, 3*, 285–287.

American Standard Test Method, ASTM International. (2003). 13.01, F2260-03. Available from http://www.astm.org.

Averbach, B. L. (1978). Film-forming capabilities of chitosan. In R. A. A. Muzzarelli & E. R. Pariser (Eds.), *Proceedings of the first international conference on chitin/chitosan* (pp. 199–209). Cambridge, MA, USA: MIT Press.

Baxter, A., Dillon, M., Taylor, K. D., & Roberts, G. A. F. (1992). Improved method for IR determination of the degree of N-acetylation of chitosan. *International Journal of Biological Macromolecules, 14*, 166–169.

Biagini, G., Muzzarelli, R. A. A., Giardino, R., & Castaldini, C. (1992). Biological materials for wound healing. In C. J. Bruine, P. A. Sandord, & J. P. Zikakis (Eds.), *Advances in chitin and chitosin* (pp. 16–24). London: Elsevier Applied Science.

Braun, S., Kalinowski, H.-O., & Berger, S. (1996). *100 and more basic NMR experiments: A practical course*. New York: VCH.

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

Bush, A. (1996). Polysaccharides and complex Oligosaccharides. In D. M. Grant & R. K. Harris (Eds.), *Encyclopedia of nuclear magnetic resonance* (pp. 3746–3750). New York: John Wiley & Sons.

Chippendale, A. M. (1997). Structural determination by nuclear magnetic resonance spectroscopy. In G. L. Trigg (Ed.). *Encyclopedia of Applied Physics* (Vol. 20, pp. 119–158). New York: Wiley-VCH, Inc.

Colquhoun, I. J., & Goodfellow, B. J. (1994). Nuclear magnetic resonance spectroscopy. In R. H. Wilson (Ed.), *Spectroscopic technique for food analysis* (pp. 87–145). New York: VCH Publisher, Inc.

Cruz, Z., Lauzon, H. L., Olabarrieta Arboleya, J. C., Nuin, M., Amaria, F. & Martinez de Marañón, I. (2006). Impact of chitosan on growth inhibition of micro-organisms isolated from fishery products. In *Presented at 10th international conference on chitin/chitosan* (10 ICCC). Montpollier, France.

Csuk, R., & Glänzer, B. I. (1988). *NMR spectroscopy of fluorinated monosaccharides* (Vol. 46, pp. 73–79). *Advances in carbohydrate chemistry and biochemistry*. London: Academic Press.

Curotto, E., & Aros, F. (1993). Quantitative determination of chitosan and the percentage of free amino groups. *Analytical Biochemistry, 211*, 240–241.

De Nooy, A. E. J., Capitani, D., Masci, G., & Crescenzi, V. (2000). Ionic polysaccharides hydrogels via the passerini and Ugi multicomponent condensations: Synthesis, behavior and solid-state NMR characterization. *Bio-macromolecules, 1*, 259–267.

Domard, A., & Rinaudo, M. (1983). Preparation and characterization of fully deacetylated chitosan. *International Journal of Biological Macromolecules, 5*, 49–52.

Duarte, M. L., Ferreira, M. C., Marvão, M. R., & Rocha, J. (2001a). Chitin and chitosan: An optimized methodology by FTIR spectroscopy. In T. Uragami, K. Kurita, & T. Fukamizo (Eds.), *Chitin and chitosan in life science* (pp. 86–89). Tokyo: Kodansha Scientific Ltd.

Duarte, M. L., Ferreira, M. C., Marvão, M. R., & Rocha, J. (2001b). Determination of the degree of acetylation of chitin materials by ^{13}C CP/MAS NMR spectroscopy. *International Journal of Biological Macromolecules, 28*, 359–363.

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

El Ghaouth, A., Arul, J., Asselin, A., & Benhamou, N. (1992a). Antifungal activity of chitosan on post-harvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. *Mycological Research, 96*, 769–779.

El Ghaouth, A., Arul, J., Grenier, J., & Asselin, A. (1992b). Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology, 82*, 398–402.

El Ghaouth, A., Arul, J., Wilson, C., & Benhamou, N. (1994). Ultrastructural and biochemical aspects of the effect of chitosan on decay of bell pepper fruit. *Physiological and Molecular Plant Pathology, 44*, 417–432.

Fernandez-Megia1, E., Novoa-Carballal, R., Quiñoá, 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.

Freeman, F. (1988). *A handbook of nuclear magnetic resonance*. UK: Longman scientific & Technical.

Gail, F., Persson, J., Sugiyama, J., Vuong, R., Tanner, S., & Chanzy, H. (1991). Ɓ-chitin from deep sea hydrothermal vent worms. In C. J. Brine, P. A. Sanford, & J. P. Zikaris (Eds.), *Advances in chitin and chitosan* (pp. 216–224). London: Elsevier Applied Science.

Guinesi, L. S., & Cavalheiro, E. T. G. (2006). The use of DSC curves to determine the acetylation degree of chitin/chitosan samples. *Thermochim Acta, 444*, 128–133.

Harris, R. K., Becker, E. D., De Menezes, S. M. C., Goodfellow, R., & Granger, P. (2001). NMR nomenclature, nuclear spin properties and conventions for chemical shifts (IUPAC recommendations). *Pure and Applied Chemistry, 73*, 1795–1818.

Harris, R. K., Becker, E. D., de Menezes, S. M. C., Goodfellow, R., & Granger, P. (2002). NMR nomenclature: Nuclear spin properties and conventions for chemical shifts (IUPAC recommendations). *Magnetic Resonance in Chemistry, 40*, 489–505.

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

Hirai, A., Odani, H., & Nakajima, A. (1991). Determination of degree of deacetylation of chitosan by ^{1}H NMR spectroscopy. *Polymer Bulletin, 26*, 87–94.

Hwang, K. T., Kim, J. T., Jung, S. T., Cho, G. S., & Park, H. J. (2003). Properties of chitosan-based biopolymer films with various degrees of deacetylation and molecular weights. *Journal of Applied Polymer Science, 89*, 3476–3484.

Kasaai, M. R. (2008). A review of several reported procedures to determine the degree of *N*-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydrate Polymers, 71*, 497–508.

Kasaai, M. R., Arul, J., Chin, S. L., & Charlet, G. (1999). The use of intense femtosecond laser pulses for the fragmentation of chitosan. *Journal of Photochemistry Photobiology A, 120*, 201–205.

Ke, H., & Chen, Q. (1990). Potentiometric titration of chitosan by linear method. *Huaxue Tongbao, 10*, 44–46.

Kim, S. S., Kim, S. H., & Lee, Y. M. (1996). Preparation, characterization and properties of β-chitin and N-acetylated β-chitin. *Journal of Polymer Science, Part B. Polymer Physics, 34*, 2367–2374.

Koenig, J. L. (1992). *Applications of IR spectroscopy to polymers* (pp. 77–112). *Spectroscopy of polymers*. Washington, DC: American Chemical Society.

Lamarque, G., Viton, C., & Domard, A. (2004). Comparative study of the first heterogeneous deacetylation of α- and β-chitins in a multi-step process. *Biomacromolecules, 5*(3), 992–1001.

Lavertu, M., Xia, Z., Serreqi, A. N., Berrada, M., Rodrrigues, A., Wang, D., et al. (2003). A validated H NMR method for the determination of the degree of deacetylation of chitosan. *Journal of Pharmaceutical and Biomedical Analysis, 32*, 1149–1158.

Mauch, F., Hadwiger, L. A., & Boller, T. (1984). Ethylene: Symptom, not signal for the induction of chitinase and b-1-3-glucanase in pea pods by pathogens and elicitors. *Plant Physiology, 76*, 607–611.

Miya, M., Iwamoto, R., Yoshikawa, S., & Mima, S. (1980). I.R spectroscopic determination of CONH content in highly deacetylated chitosan. *International Journal of Biological Macromolecules, 2*(5), 323–324.

Muraki, E., Yaku, F., Iyoda, J., & Kojima, H. (1993). Measurement of degree of deacetylation in D-glucosamine oligosaccharides by UV absorption. *Bioscience, Biotechnology, and Biochemistry, 57*, 1929–1930.

Muzzarelli, R. A. A. (1977). *Chitin*. Oxford: Pergamon Press Ltd.

Muzzarelli, R. A. A., & Rocchetti, R. (1985). Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectrophotometry. *Carbohydrate Polymers, 5*, 461–472.

Muzzarelli, R. A. A., Louch, C., & Emanuelli, M. (1987). The molecular weight of chitosans by laser light-scattering. *Carbohydrate Research, 164*, 433–442.

Nanjo, F., Katsumi, R., & Sakai, K. (1991). Enzymatic method for determination of the degree of deacetylation of chiosan. *Analytical Biochemistry, 193*, 164–167.

Neiss, T. G. (2000). Nuclear magnetic resonance instrumentation. In R. A. Meyers John (Ed.). *Encyclopedia of analytical chemistry: Application, theory and instrumentation* (Vol. 14, pp. 12089–12113). New York: John Wiley & Sons.

Niola, F., Basora, N., Chornet, E., & Vidal, P. F. (1993). A rapid method for the determination of the degree of N-acetylation of chitin–chitosan samples by acid hydrolysis and HPLC. *Carbohydrate Research, 238*, 1–9.

No, H. K. (1995). Preparation and characterization of chitosan and chitosan. A Review. *Journal of Aquatic Food Product Technology, 4*(2), 27–52.

Olsen, R., Schwartzmiller, D., Weppner, W., & Winandy, R. (1989). Biomedical applications of chitin and its derivatives. In G. Skjak-Braek, T. Anthonsen, & P. Sandford (Eds.), *Chitin and chitosan* (pp. 813–828). London: Elsevier Applied Science.

Ottøy, M. H., Värum, K. M., & Smidsrød, O. (1996). Compositional heterogeneity of heterogeneously deacetylated chitosans. *Carbohydrate Polymers, 29*(1), 17–24.

Peter, M. G., Grun, L., & Forster, H. (1984). CP/MAS-^{13}C-NMR spectra of sclerotized insect cuticle and of chitin. *Angewande Chemie International Edition, 23*, 638–639.

Prochazkova, S., Värum, K. M., & Østgaard, K. (1999). Quantitative determination of chitosans by ninhydrin. *Carbohydrate Polymers, 38*, 115–122.

Rathke, T. D., & Hudson, S. M. (1994). Review of chitin and chitosan as fiber and film formers. *Journal of macromolecule Science Review Macromolmolecule Chemical Physics, C34*(3), 375–437.

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., Le Dung, P., Gey, C., & Milas, M. (1992). Substituent distribution on *O*,*N*-carboxymethyl chitosans by H and C NMR. *International Journal of Biological Macromolecules, 14*, 122–128.

Rinaudo, M., Milas, M., & Dung, P. L. (1993). Characterization of chitosan. Influence of ionic strength and degree of acetylation on chain expansion. *International Journal of Biological Macromolecules, 15*, 281–285.

Rinuado, M., Milas, M., & Desbrières, J. (1997). Characterization and solution properties of chitosan and chitosan derivatives. In M. F. A. Goosen (Ed.), *Application of chitin and chitosan* (pp. 89–101). Lancaster, PA: Technomic Publishing Co. Inc.

Saalwächter, K., & Ramamoorthy, A. (2006). Sensitivity enhancement by inverse detection in solids. In A. Ramamoorthy (Ed.), *NMR of Biological Solids* (pp. 151–175). Boca Raton: Taylor and Francis.

Sabins, S., & Block, L. H. (1997). Improved infrared spectroscopic method for the analysis of degree of N-deacetylation of chitosan. *Polymer Bulletin, 39*, 67–71.

Saito, H., Tabeta, R., & Hirano, S. (1982). A high resolution ^{13}C NMR study of chitin, chitosan and *N*-acylchitosans by cross polarization/magic angle spinning (CP/MAS) NMR spectroscopy. In S. Hirano & S. Tokura (Eds.), *Proceedings of the second international conference on chitin and chitosan* (pp. 77–81). Tokyo: The Japanese Society of Chitin and Chitosan.

Saitô, H., Tabeta, R., & Ogawa, K. (1987). High resolution solid state ^{13}C NMR study of chitosan and its salts with acids: Conformational characterization of polymorphs and helical structures as viewed from the conformation-dependent C Chemical shifts. *Macromolecules, 20*, 2424–2430.

Sandford, P. A., & Hutchings, G. P. (1987). Chitosan – A natural, cationic biopolymer: Commercial applications. In M. Yalpani (Ed.), *Industrial polysaccharides: Genetic engineering, structure/property relations and applications* (pp. 363–375). Amsterdam: Elesvier Science Publishers.

Sannan, T., Kurita, K., Ogura, K., & Iwakura, Y. (1978). Studies on chitin: (7) IR spectroscopic determination of degree of deacetylation. *Polymer, 19*, 458–459.

Sato, H., Mizutani, S.-I., Tsuge, S., Ohtani, H., Aoi, K., Takasu, A., et al. (1998). Determination of the degree of acetylation of chitin/chitosan by pyrolysis-gas chromatography in the presence of oxalic acid. *Analytical Chemistry, 70*, 7–12.

Schanzenbach, D., & Peter, M. G. (1997). NMR spectroscopy of chito-oligosaccharides. In R. A. A. Muzzarelli & M. G. Peter (Eds.), *Chitin handbook* (pp. 199–204). Atec, Grottammate: European Chin Society.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimato, H. (1996a). An improved IR spectroscopic determination of degree of deacetylation of chitin. In A. Domard, C. Jeuniaux, R. A. A. Muzzarelli, & G. A. F. Roberts (Eds.). *Advances in chitin science* (Vol. 1, pp. 204–209). Lyon: André Publisher.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimato, H. (1996b). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules, 18*, 237–242.

Signini, R., & Campana-Fitho, S. P. (1999). On the preparation and characterization of chitosan hydrochloride. *Polymer Bulletin, 42*(2), 159–166.

Striegel, A. M., & Timpa, J. D. (1995). Molecular characterization of polysaccharides dissolved in Me_2NAc–LiCl by gel permeation chromatography. *Carbohydrate Research, 267*, 271–290.

Sudarshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology, 6*, 257–272.

Sugano, M., Watanabe, S., Kishi, A., Izume, M., & Ohtakara, A. (1998). Hypocholestrolemic action of chitosans with different viscosity in rats. *Lipids, 23*, 187–191.

Tan, S. C., Khor, E., Tan, T. K., & Wong, S. M. (1998). The degree of deacetylation of chitosan: Advocating the first derivative UV spectrophotometry method of determination. *Talanta, 45*, 713–719.

Tanner, S. F., Chanzy, H., Vincendon, M., Roux, J. C., & Gaill, F. (1990). High-resolution solid-state carbon-13 nuclear magnetic resonance study of chin. *Macromolecules, 23*, 3576–3583.

Tasi, G. J., & Su, W. H. (1999). Antibacterial property of shrimp chitosan against *Escherichia coli*. *Journal of Food Protection, 62*, 239–243.

Teng, Q. (2005). *Structural biology: Practical NMR applications* ((pp. 1–54)). New York: Springer.

Terayama, H. (1952). Method of colloid titration (a new titration between polymer ions). *Journal of Polymer Science, 8*, 243–253.

Tolaimate, A., Desbrières, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). 0n the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer, 41*, 2463–2469.

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

Värum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidrød, O. (1991b). ^{13}C NMR studies of the acetylation sequences in partially *N*-acetylated chitins (chitosans). *Carbohydrate Research, 217*, 19–27.

Vincendon, M. (1985). NMR conformational analysis of chitin in lithium chloride solution. In R. A. A. Muzzarelli, C. Jeuniaux, & G. M. Gooday (Eds.), *Chitin in nature and technology* (pp. 343–345). New York: Plenum Press.

Webb, G. A. (2000). Nitrogen NMR. In J. C. Lindon, G. E. Tranter, & J. L. Holmes (Eds.), *Encyclopedia of spectroscopy and spectrometry* (pp. 1504–1514). New York: Academic Press.

Yang, B. Y., & Montgomery, R. (2000). Degree of acetylation of heteropolysaccharides. *Carbohydrate Research, 323*, 156–162.

Yin, X., Lin, Q., Zhang, Q., & Yang, L. C. (2002). O_2-sacvenging activity of chitosan and its metal complexes. *Chinese Journal of Applied Chemistry, 19*, 325–328.

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.