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# Optimal routine conditions for the determination of the degree of acetylation of chitosan by $^{1}$H-NMR

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**Abstract**

Chitosan applications and properties mostly depend on its degree of acetylation, so methods that allow its accurate determination are needed. $^{1}$H-NMR taken in 2% DCl solutions at 70 °C is usually employed for that purpose but suffers from long experimental times. Here, it is demonstrated that this time can be dramatically reduced more than six-fold by simply performing the $^{1}$H-NMR experiment at 27 °C (the standard NMR probe temperature) with 90° pulses. Routine conditions allowing DA determinations in less than 3.5 min of NMR time per sample are described.


*Keywords:* Chitosan; Degree of acetylation; $^{1}$H-NMR

## 1. Introduction

Chitosan (Fig. 1) is a linear polysaccharide comprising variable proportions of glucosamine (GluN) and *N*-acetylglucosamine (GluNAc) through β(1–4) linkages. Although naturally present in some microorganisms and fungi, commercial chitosan is industrially produced by partial deacetylation of chitin, the second most abundant natural polysaccharide. Recently, chitosan has emerged as an interesting biopolymer with applications ranging from the production of fibers (Agboh & Qin, 1997) to catalyst support (Quignard, Choplin, & Domard, 2000; Vincent & Guibal, 2002). However, due to the low toxicity, and high biodegradability and biocompatibility associated to chitosan, biological and pharmaceutical issues have recently attracted most of the attention on the polymer (Singla & Chawla, 2001). Thus, antimicrobial (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003) and cholesterol-lowering (Ylitalo, Lehtinen, Wuolijoki, Ylitalo, & Lehtimäki, 2002) properties of chitosan have been described, as well as applications to the delivery of drugs, proteins, and DNA (Hejazi & Amiji, 2003; Janes, Calvo, & Alonso, 2001; Liu & Yao, 2002). Furthermore, it has been demonstrated the ability of chitosan to perturb cell membranes, a mechanism claimed as crucial in chitosan based drug delivery systems (Fang & Chan, 2003).

However, most of the above applications, as well as chitosan solubility (Cho, Jang, Park, & Ko, 2000; Kubota & Eguchi, 1997), biodegradability (Kristiansen, Vårum & Grasdalen, 1998), $pK_a$ (Sorlier, Denuzière, Viton, & Domard, 2001), and self-aggregation (Liu et al., 2003; Schatz, Pichot, Delair, Viton, & Domard, 2003) depend on the proportion between acetylated and non-acetylated glucosamine units, the so called degree of acetylation (DA). Therefore, methods that allow an accurate DA determination are needed. Several procedures have been described in the literature based on different techniques such as $^{1}$H-NMR (Hirai, Odani, & Nakajima, 1991; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996; Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991), solid state $^{13}$C-CP-MAS-NMR (Duarte, Ferreira, Marvão, & Rocha, 2001; Heux, Brugnerotto, Desbrières, Versali, & Rinaudo, 2000; Raymond, Morin, & Marchessault, 1993) and $^{15}$N-CP-MAS-NMR (Heux et al., 2000; Yu, Morin, Nobes, & Marchessault, 1999), IR (Baxter, Dillon, Taylor, & Roberts, 1992; Brugnerotto et al., 2001; Duarte, Ferreira, Marvão,

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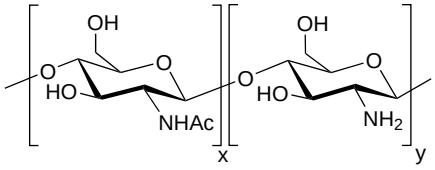


Fig. 1. Chitosan.

& Rocha, 2002; Miya, Iwamoto, Yoshikawa, & Mima, 1980; Sabnis & Block, 1997; Shigemasa et al., 1996) and UV (Aiba, 1986; Muraki, Yaku, Iyoda, & Kojima, 1993; Muzzarelli & Rocchetti, 1985; Tan, Khor, Tan, & Wong, 1998) spectroscopies, circular dichroism (Domard, 1987), ninhydrin assay (Prochazkova, Vårum, & Østgaard, 1999), acidic (Niola, Basora, Chornet, & Vidal, 1993) or enzymatic (Nanjo, Katsumi, & Sakai, 1991) hydrolysis-HPLC, conductometric titration (Raymond et al., 1993), and pyrolysis-gas chromatography (Sato et al., 1998). However, most of the above methods suffer from serious drawbacks. While solid state NMR, titrations, and enzymatic or pyrolysis based methods are time consuming; others such as IR, UV, circular dichroism, the ninhydrin assay, and acidic hydrolysis-HPLC relay on the use of standards and calibrations. Also, IR spectroscopy requires a difficult deconvolution of the amide band, and, along with $^{15}$N-CP-MAS-NMR, generally underestimates the degree of acetylation of low DA samples. Therefore, it is not surprising that $^{1}$H-NMR spectroscopy has been recognized as the method of choice for the determination of DA of chitosan (Desbrières, Martinez, & Rinaudo, 1996; Heux et al., 2000; Rinaudo, Le Dung, Gey, & Milas, 1992). Thus, $^{1}$H-NMR does not relay on previous calibrations and allows accurate determination of even low DA values, typical of commercial chitosans. Furthermore, $^{1}$H-NMR is run under routine basis in many laboratories and industries and allows the quick identification of impurities sometimes present in commercial chitosans. The importance of $^{1}$H-NMR in the determination of DA of chitosan is also demonstrated by the fact that $^{1}$H-NMR data are usually employed as standards to calibrate alternative methods (Brugnerotto et al., 2001; Shigemasa et al., 1996).

Among the various conditions proposed in the literature for determining the DA of chitosan by $^{1}$H-NMR (Hirai et al., 1991; Shigemasa et al., 1996; Vårum et al., 1991), those by Hirai and coworkers using 2% DCl solutions at 70 °C have been widely accepted. However, the long NMR times associated to the FID acquisition and the high temperature employed to reduce sample viscosity hamper, in our opinion, their use on a routine basis.

In this paper, we will show that more user-friendly routine conditions including short NMR times, for determination of chitosan DA, can be achieved by performing the NMR experiment at ambient temperature, if the acquisition parameters are judiciously selected.

## 2. Experimental section

### 2.1. Chitosan samples

Overall, nine samples of chitosan with different molecular weights and DA, were used in this study. Samples FL, FM and FH were obtained from Fluka, and correspond to Chitosan low $M_W$ (catalog number 22741, lot 407568/1, DA = 15.5%, $M_W \sim 150{,}000$, FL), Chitosan medium $M_W$ (catalog number 22742, lot 398416/1, DA = 15.3%, $M_W \sim 400{,}000$, FM) and Chitosan high $M_W$ (catalog number 22743, lot 371936/1, DA not provided by producer, $M_W \sim 600{,}000$, FH). Two other commercial samples of chitosan were obtained from Pronova Biocare as hydrochloride salts: Protasan Cl 110 (product code 28071110, batch number 310-490-01, DA = 13%, P110) and Protasan UP Cl 113 (product number 28073113, batch number FP-110-02, DA = 14%, P113). Four more samples (FL-1, FL-2, P113-1 and P113-2) were prepared by controlled acetylation of FL and P113 (Vachoud, Zydowicz, & Domard, 1997).

To remove any uncertainty from sample unhomogeneity (Domard, 1987), prior to NMR studies, FL, FM and FH chitosans were dissolved in 0.5% AcOH and then sequentially dialyzed against $10^{-3}$M HCl, $5.5\times10^{-3}$M $NH_4OH$, and $H_2O$, and lyophilized.

### 2.2. SEC-MALLS

Weight-average molecular weight ($M_W$) of commercial chitosans was determined by size exclusion chromatography-multiangle laser-light scattering (SEC-MALLS) (Schatz et al., 2003). An Iso Pump G1310A (Hewlett Packard) was connected to a PSS Novema GPC column 10 μ (8×50 mm) and a PSS Novema 3000 column (8×300 mm). A PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation) operating at 660 nm and a G1362A refractive index detector (Agilent) were connected on line. A 0.15M $NH_4OAc$/0.2M AcOH buffer (pH = 4.5) was used as eluent. Polymer solutions (0.1%, w/v) were filtered on 0.45 μm pore size membranes (Millipore) before injection (Schatz et al., 2003). Refractive index increments d$n$/d$C$ for each DA were taken from literature (Schatz et al., 2003). The resulting $M_W$ determined for the above commercial chitosans were: 152,000 for FL, 407,000 for FM, 661,000 for FH, 87,000 for P110 and 113,000 for P113. Acetylated products FL-1, FL-2, P113-1 and P113-2 exhibited $M_W$ values as expected.

### 2.3. NMR sample preparation

Five milligram of commercial or acetylated chitosan were added to a 5 mm NMR tube containing 0.5 mL of 2% DCl solution in $D_2O$ and heated at 70 °C for 1 h in order to speed up the dissolution. Most of the samples were completely dissolved after this time, except sample FL-2 that required 3 h. The resulting solutions remained clear at

27 °C (the standard NMR working temperature) for at least 50 min, enough time to acquire $^1$H-NMR spectra under the conditions proposed here.

### 2.4. NMR acquisition and processing.

$^1$H-NMR spectra were recorded on Bruker DRX 500 and AMX 500 spectrometers (500 MHz) at 27 °C. Acquisition time (AQ) and relaxation delay (D1) were set at 1 and 12 s, respectively. When 90° pulses were used, 128 scans were acquired (NMR time = 28 min). The spectral width and data points were 8000 Hz and 32 K points, respectively. $^1$H chemical shifts were expressed in ppm and referred to internal sodium 3-trimethylsilylpropane sulfonate (TSP).

For comparison, $^1$H-NMR spectra of chitosan were also recorded under the conditions proposed by Hirai.[13] In this case, the NMR probe temperature was set at 70 °C, the pulse angle was calibrated at 45°, AQ and D1 were set at 1 and 39 s, respectively, and 256 scans were acquired (NMR time = 2 h 51 min).

MestRe-C software for PC was used for processing the spectra (Cobas & Sardina, 2003). A 32 K zero filling was applied to the FID in order to increase the spectral resolution. Also, a linear prediction with MestRe-C default parameters [basis points 7499, coefficients (sinusoids) 16] was applied with the aim of removing baseline distortions and truncation of the HOD signal. For the spectra run with non-calibrated pulses, a smooth exponential apodization was performed. After Fourier transform and phase correction of the spectrum, a polynomial baseline correction was carried out.

$^1$H-NMR signal assignment was based on previous work (Shigemasa et al., 1996; Vårum et al., 1991). $^1$H-NMR $\delta$ (2% DCl, 27 °C) 2.08 (s, NAc), 2.09 (s, AcOH), 3.21 (s, H2 of GluNH2) 3.38–4.34 (m, H2 of GluNAc, H3-H6), 4.62 (s, H1 of GluNAc), 4.92 (s, H1 of GluNH2).

### 2.5. Determination of DA

DA values were determined from the relative integrals of acetyl (*N*-acetyl and AcOH) and combined H2-H6 protons. Usually, intervals 1.7–2.4 and 2.7–4.4 ppm were selected for integration.

### 2.6. Inversion recovery experiments

A solution of chitosan sample FL in 2% DCl was used for the determination of $T_1$ at 27 °C on a Bruker DRX 500 (inversion recovery). Variable delays of 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 11.5, and 12.0 s were selected for this experiment. A similar experiment run at 70 °C, showed that *N*-acetyl hydrolysis occurs at this temperature, increasing the amount of AcOH and therefore disturbing $T_1$ measurements.

Accurate $T_1$ values at 70 °C could be obtained using a 2% DCl solution of a completely deacetylated chitosan (prepared from sample FL) (Mima, Miya, Iwamoto, & Yoshikawa, 1983), to which AcOH (30 μL, 0.07M in $D_2O$) was added. Variable delays of 0.01, 2.5, 5.0, 7.5, 10.0, 12.5, 14.0, 15.0, 16.0, 17.5, 19.0, 20.0, 22.5, 25.0, 27.5, and 30.0 s were employed in this experiment.

$T_1$ values obtained at both temperatures are summarized in Table 1.

Table 1
$T_1$ (s) in 2% DCI at 27 and 70 °C

| Protons | $T_1$ at 27 °C | $T_1$ at 70 °C |
|---|---|---|
| NAc | 1.35 | – |
| AcOH | 2.60 | 10.38 |
| H2 (GluN) | 1.46 | 1.73 |
| H2 (GluNAc), H3-H6 | 1.23–1.36[a] | 1.14–1.48[a] |

[a] Maximum and minimum $T_1$ values found within the multiplet.

## 3. Results and discussion

When acquiring an NMR spectrum with quantitative purposes, it should be taken into account that immediately after a pulse of angle $\theta$, the nuclear magnetization ($M$) begins to return to equilibrium by spin–spin and spin–lattice relaxation. Since multiple and successive pulses are used in NMR experiments, a repetition time ($\tau$) [consisting of acquisition time (AQ) and relaxation delay (D1)] long enough to allow complete relaxation of all the nuclei should be introduced after each pulse and before the next one. Otherwise, and due to differential saturation, the relative intensities of the resonances, especially those with long relaxation times, will no longer correlate with the exact number of nuclei originating that signal (Rabenstein, 1984; Rabenstein & Keire, 1991). In order to avoid this, when using 90° pulses, repetition times $\tau$ at least five times the longest $T_1$ in the sample should be employed. This figure allows to recover 99.33% of the magnetization along the $z$-axis (Rabenstein, 1984; Rabenstein & Keire, 1991).

In general, aqueous 2% DCl is considered to be the best solvent for NMR of chitosan. Naturally, this acid media produces the hydrolysis of some of the *N*-acetyl groups and production of some free AcOH. Therefore, for DA determination, both the *N*-Acetyl and the AcOH signals should be considered for integration.

Since chitosan solutions are generally viscous, their NMR spectra have been usually taken at temperatures as high as 70 °C. Unfortunately, at this temperature the acetyl protons of AcOH present a very long $T_1$ value of 12 s (Hirai et al., 1991), more than six times greater than that of the remainder protons in the sample ($T_1$ lower than 2 s). Therefore, reliable integration can only be achieved if sufficiently long delay times between pulses ($\tau = 40$ s for 45° pulses) are introduced (Hirai et al., 1991). As a result, very long time-consuming NMR experiments are needed for DA determination under these conditions.

As complete dissolution of chitosan in more diluted DCl solutions impeding NAc hydrolysis is a very lengthy process (more than 24 h), we reasoned that shorter NMR times could be attained by lowering the $T_1$ of AcOH.

The known dependence of $T_1$ on molecular weight and temperature, anticipates that a reduction of the temperature should drastically diminish the $T_1$ of low molecular weight species as AcOH, leaving unchanged the $T_1$ of polymeric and oligomeric materials (Becker, 1980; Bovey & Mirau, 1996).

Indeed, when a commercial chitosan sample dissolved in 2% DCl, was submitted to inversion recovery experiments at 27 (the standard NMR probe temperature) and 70 °C, a very significant reduction of $T_1$ for AcOH from 10.38 s at 70 °C to 2.60 s at 27 °C (see Table 1 and Experimental section) was achieved. Consequently, and taking into account the correlation between $T_1$ and $\tau$, a parallel drastic reduction in the NMR time can be expected by simply lowering the experimental probe temperature from 70 to 27 °C.

On the other hand, although chitosan $T_1$ values are slightly affected by DA (Fernandez-Megia, Novoa-Carballal, Quiñoá, & Riguera), since AcOH presents a $T_1$ much higher than the rest of the protons at 27 °C, accurate

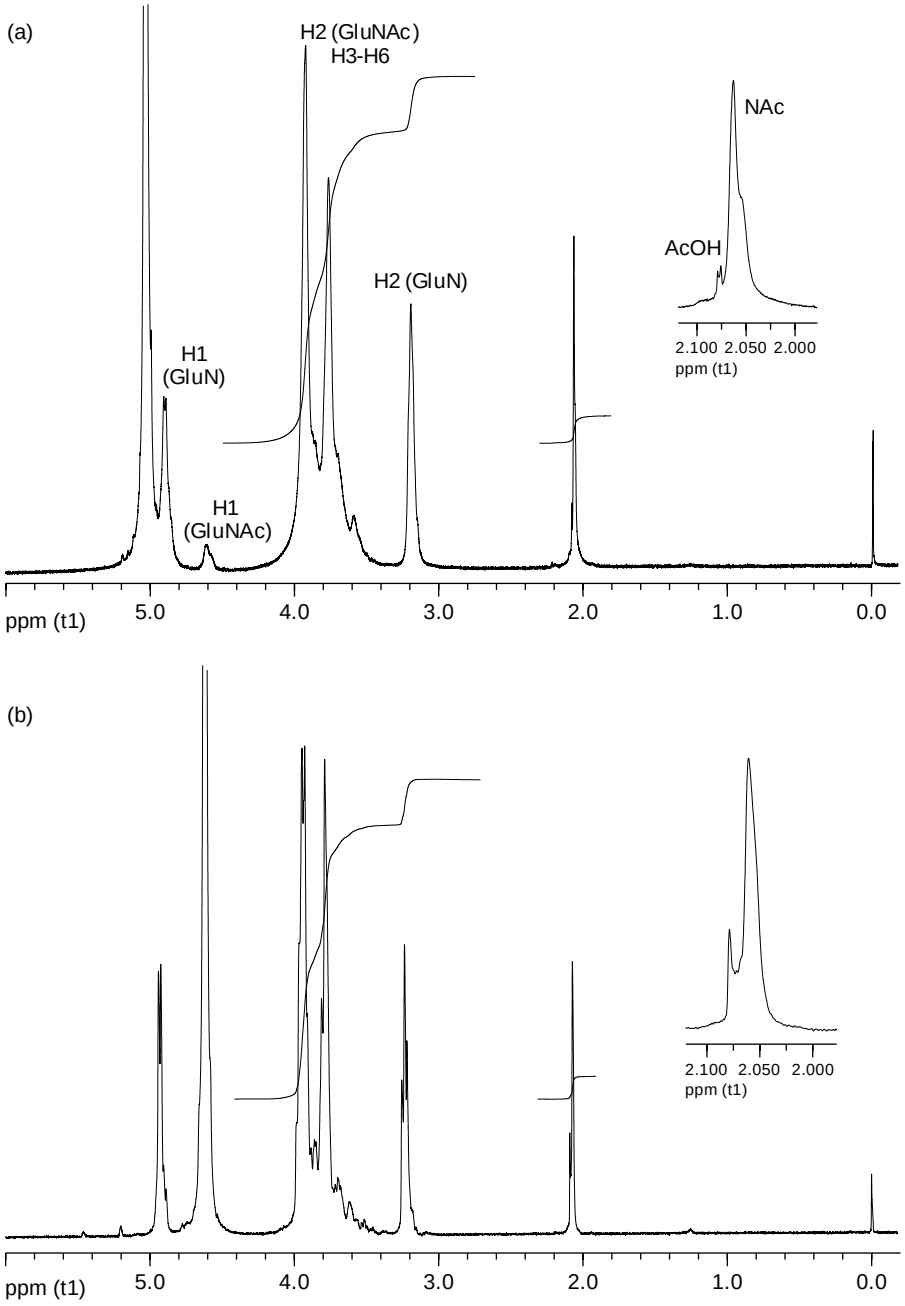


Fig. 2. $^1$H-NMR spectra of chitosan FM: (a) 27 °C, $\theta = 90°$; (b) 70 °C, $\theta = 45°$.

Table 2
DA of chitosan at 27 at 70 °C

| Entry | Chitosan | DA (70 °C)[a] | DA (27 °C)[b] |
|---|---|---|---|
| 1 | FL | 17 | 17 |
| 2 | FL-1 | 27 | 27 |
| 3 | FL-2 | 39 | 40 |
| 4 | FM | 14 | 14 |
| 5 | FH | 19 | 19 |
| 6 | P110 | 7 | 7 |
| 7 | P113 | 14 | 14 |
| 8 | P113-1 | 22 | 22 |
| 9 | P113-2 | 24 | 23 |

[a] AQ = 1 s, DI = 39 s, $\theta$ = 45°, 70 °C, 256 scans, 2 h 51 min.
[b] AQ = 1 s, DI = 12 s, $\theta$ = 90°, 27 °C, 128 scans, 28 min.

determinations in the whole range of DA can be attained at this temperature.

The benefits of determining the DA of chitosan by $^1$H-NMR at 27 °C, were demonstrated with several samples of chitosans with different molecular weights and acetylation degrees (see Experimental section). For comparison, the spectra were run both at 27 and 70 °C. The NMR experiments at 27 °C were carried out with a $\tau$ of 13 s (equivalent to five times the $T_1$ of AcOH) and 90° pulses. In this way, not only complete magnetization recovery, but also higher signal-to-noise ratio than with lower pulses (45°) are assured (Rabenstein, 1984; Rabenstein & Keire, 1991). The NMR spectra at 70 °C were performed in accordance

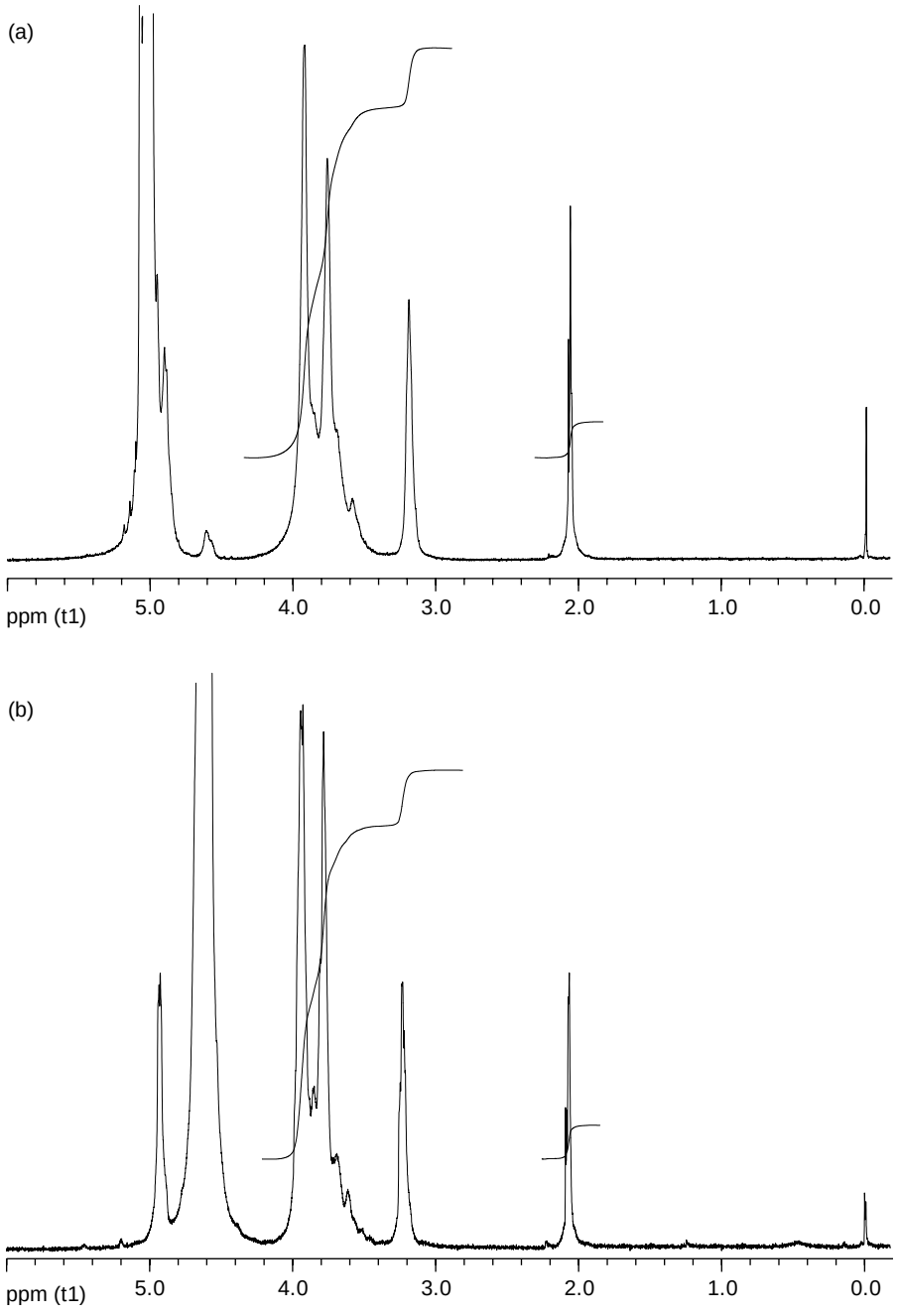


Fig. 3. $^1$H-NMR spectra of chitosan FL: (a) 27 °C, $\theta$ = 90°; (b) 70 °C, $\theta$ = 45°.

with the conditions reported, with a $\tau$ of 40 s and pulses of 45° (Hirai et al., 1991). Although, essentially the same magnetization is recovered in both cases (99.33% at 27 °C, and 99.37% at 70 °C), the NMR time for the experiment at 70 °C exceeds more than six-fold that at 27 °C, due to the longer $T_1$ and the shorter $\theta$ employed at 70 °C. Actual $^1$H-NMR spectra are shown in Fig. 2.

The DA figures at both temperatures were determined from the relative integrals of the acetyl (*N*-acetyl plus AcOH signals) with respect to the combined H2-H6 protons (Table 2).

Apart from the very significant reduction in NMR time and the advantage of working at 27 °C, the DA values obtained for each sample at both temperatures are essentially identical, confirming the advantages of recording the $^1$H-NMR spectra at 27 instead of at 70 °C.

Therefore, although it is often considered advantageous to obtain the NMR spectra of polysacharides at high temperature, our results clearly indicate that NMR spectra of chitosan in 2% DCl recorded at 27 or 70 °C are essentially identical, showing signals of comparable shape and resolution.

An additional advantage of running chitosan spectra at 27 °C for DA determination derives from the shift to lower field experienced by the HOD signal at lower temperatures. Fig. 2 shows that this signal resonates at 4.62 ppm at 70 °C and moves to 5.04 ppm at 27 °C. This separates the residual water peak from the zone of interest and allows an easier and more precise integration of the H2–H6 multiplet. This is particularly significant for chitosans with high $H_2O$ content, such as sample FL (Fig. 3).

Thus, by running the $^1$H-NMR experiment at 27 °C with adequately calibrated pulses, the time necessary to obtain accurate DA figures gets dramatically reduced. However, since pulse calibration is time-consuming and problematic for the standard user, we explored the possibility to establish parameters suitable for DA determination on standard basis, and found that after just 16 scans with non-calibrated pulses and a repetition time of 13 s, the resulting $^1$H-NMR spectra look, after a smooth exponential apodization, exactly like those obtained with calibrated pulses, affording DA values identical to those in Table 2. Under these routine conditions, the determination of the DA of chitosan by $^1$H-NMR can be carried out in less than 3.5 min, in contrast to the long experimental times required in the conditions proposed by Hirai.

## 4. Conclusion

Traditionally, the determination of the DA of chitosan by $^1$H-NMR in 2% DCl solutions was performed at 70 °C, a process that suffers from long NMR times. In this paper, it has been demonstrated that by performing the spectrum at 27 °C ($\tau = 13$ s, $\theta = 90°$), a more than six-fold decrease in NMR time is experienced and accurate DA values can be obtained in less than 3.5 min. The NMR procedure is equally accurate for chitosans with different molecular weights and DA. In addition, at 27 °C, the residual HOD signal moves away from the integration area of the spectra, and allows measurements of chitosans with high water content. We consider this approach could be of interest for other polysaccharides.

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