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A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan

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Abstract

A method for the determination of the degree of deacetylation (DDA) of chitosan by ^{1}H NMR spectroscopy has been formally validated. Chitosans with DDA ranging from 48 to 100% have been used for the validation. The method is found to be simple, rapid and more precise than other known techniques like IR or titration for %DDA measurements. The precision, ruggedness, robustness, specificity, stability and accuracy of the technique are discussed in this paper.

Keywords: Chitosan; Chitin; pH-sensitive; IR, ^{1}H NMR, degree of deacetylation

1. Introduction

Chitosan is a natural polysaccharide obtained by partial deacetylation of chitin and is used in many industries such as food processing, cosmetics, waste management, water clarification, wound healing, tissue repair, drug and gene delivery. Most of the physical and chemical properties of this biopolymer depend greatly on the degree of deacetylation (DDA) and it has been repeatedly claimed in the literature that a technique to measure this parameter accurately is highly desirable. Such a technique should be fast, precise and should preferably not rely on any standard of known DDA or calibration curve obtained with another technique. Several methods have been proposed for measuring the DDA of chitosan including: titration [1,2], IR spectroscopy [3–7], UV spectroscopy [8,9], elemental analysis, circular dichroism (CD) [10], NMR spectroscopy [11–15], *N*-acetyl group hydrolysis [16] and gel permeation chromatography (GPC) [17]. Unfortunately, these techniques often show considerable discrepancies in the obtained DDA values.

IR is probably the most studied technique for DDA measurement. The method is quite fast and it can be used with insoluble chitosan via the KBr

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disc technique [3,6,15]. Over the past two decades, many authors have proposed improvements in the IR methods making use of new absorption bands and/or a new baseline for the measurement of DDA [4–6,15,18]. Other methods, when published are often compared with IR [8,10,17,19]. One issue with optical spectroscopic techniques like IR is the requirement of standards of known DDA or the use of a calibration curve obtained using another method, such as titration, which in itself is not necessarily accurate. Use of the amide I absorption band (1655 cm^{-1}) combined with the hydroxyl absorption band (3450 cm^{-1}) as a reference appears to provide the best results. Empirical equations to calculate chitosan DDA using these two bands with different baselines have been proposed [3,18]. However, as pointed out by Sabnis et al. [6], variability in sample preparation, type of instrument and experimental conditions could influence the results obtained using these equations. Sabnis et al. [6] obtained accurate results by using the method proposed by Baxter et al. [18] but they used a calibration curve obtained by titration of hydrobromide salts of chitosan. Chitosan is very hygroscopic and it must be carefully dried to eliminate moisture that could contribute to the hydroxyl band intensity and lead to incorrect DDA determination.

All techniques, except for NMR based measurements, require an accurate weighing of chitosan. Therefore, moisture needs to be eliminated carefully and the purity of the samples must be determined separately. Also, many of these non-NMR techniques are inaccurate, long or complicated to perform. In the ^1H NMR method, the amount of chitosan used does not need to be known accurately and the purity of the sample does not need to be determined as long as the impurity peaks do not overlap with the relevant peaks of chitosan. Sample preparation is simple, only a few milligrams of chitosan are required and there is no need for any calibration curve or reference sample of known DDA. The peaks used for DDA determination in this method are well resolved and the integration of these peaks is straightforward. Contrary to the claims of Domard et al. [19], ^1H NMR has been found to be precise and accurate for the quantification of high DDA, which is usually difficult to measure with conventional techniques like IR or titration. Additionally DDA can be calculated using different combinations of peaks in order to verify that the method is consistent. The liquid phase ^1H NMR technique described in this paper is only limited by the solubility of chitosan which depends on the DDA and the molecular weight of the polymer. This paper describes the validation of the measurement of the DDA of chitosan by liquid phase ^1H NMR.

2. Experimental

2.1. Materials

Chitosans with DDA ranging from 48 to 100% in powder form were prepared at Bio Syntech Canada Inc. (Montreal). Six different lots of chitosan were used in this study: PCCH00014 (DDA ~ 100%), PCCH00013 (DDA ~ 97%), PCCH00005 (DDA ~ 87%), PCCH00002 (DDA ~ 82%), PCCH00003 (DDA ~ 76%) and PCCH00024 (DDA ~ 48%). Deuterium oxide (Cat # 15,188-2), deuterium chloride 20% wt./vol. in deuterium oxide (Cat # 17-672-9) and glacial acetic acid (Cat # 33,882-6) were purchased from Aldrich Chemical.

2.2. Samples

For all tests except for accuracy, the solutions of chitosan were prepared by stirring at room temperature 10 mg of chitosan in a solution composed of 1.96 ml of D_2O and 0.04 ml of DCl and waiting about half an hour to ensure complete dissolution of the polymer. In these solutions, DCl is in excess compared with amino groups of chitosan so that the polymer is easily dissolved. For the accuracy test, solutions of chitosans of lowest and highest DDA (PCCH00024 and PCCH00014, respectively), were used. They were prepared by stirring at room temperature 30 mg of dried chitosan in a solution composed of 4.9 ml of D_2O and 0.1 ml of DCl. Test solutions were prepared by mixing these two solutions in three different volumetric ratios: 80:20, 50:50 and 20:80. For this test only, the

chitosan powders were thoroughly dried to ensure accurate calculation of the expected DDA of the three mixed samples.

2.3. NMR

^{1}H NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer equipped with a 16 bits digitizer using a Varian 5 mm Indirect Detection probe. The experiments were run at 70 °C, temperature at which the solvent (HOD) peak does not interfere with any of chitosan's peaks. After dissolution, approximately 1 ml of the chitosan solution was transferred to a 5 mm NMR tube. The sample tube was inserted in the magnet and allowed to reach thermal equilibrium by waiting 10 min before performing the experiment. The ^{1}H NMR experiment for DDA determination was a single pulse sequence with presaturation of the solvent. A 90° pulse corresponding to a pulse width of 11 μs was used. The delay before the application of the pulse was 6 s and the acquisition time was 2 s for a total relaxation time (recycle time) of 8 s between each transient. Longitudinal relaxation times (T_1's) of protons of chitosans PCCH00024 (DDA ~ 48%) and PCCH00014 (DDA ~ 100%) were measured by using the inversion recovery pulse sequence (180-τ-90°). The T_1's of chitosan protons were all found to be lower than 1.6 s ($5 \times T_1 < 8$ s) so that relaxation was complete before each pulse application in the DDA determination experiment. The hydrolytic cleavage of the acetyl groups of chitosan by dilute acid at 70 °C was found to be quite slow and, therefore, it was not necessary to use a large relaxation delay in order to quantify the amount of acetic acid resulting of deacetylation as proposed by Hirai et al. [11] who used a relaxation time of 40 s for the determination of DDA. Solvent signal suppression was achieved by saturation with irradiation from the decoupler set to the solvent resonance frequency using low power during the 6 s delay before the 90° pulse application. The decoupler was turned off just prior to the 90° pulse and during the entire 2 s signal acquisition period. The decoupler power was 1 dB (~ 0.03 mW according to Varian specifications). The spectral width was 4550 Hz, the number of data points was 18 200, the line broadening parameter was 1 Hz and the Fourier number was 32 768. The number of acquired transients was 64 corresponding to approximately 8.5 min of signal acquisition. The total time for acquisition of the data, including thermal equilibration time (10 min after reaching 70 °C) and time required to shim the magnet (5 min), was about 25 min. Prior to signal integration, a linear drift correction was applied between 1 and 6 ppm. There were no predefined boundaries for integration of the peaks. Integration boundaries were set manually by inspection of the spectrum, based on the analyst's judgment. Manual adjustment of integration boundaries was necessary since peak intensities and, therefore, integration boundaries change with DDA. No measurable inaccuracy was introduced by this manual setting since different analysts achieved very similar results (see Ruggedness test below). All spectra were obtained in triplicate and the coefficients of variation (CV) of the results were calculated using:

$$CV = \frac{\sqrt{\dfrac{\sum_{i=1}^{3}[\overline{DDA} - DDA_i]^2}{3(3-1)}}}{\overline{DDA}}$$

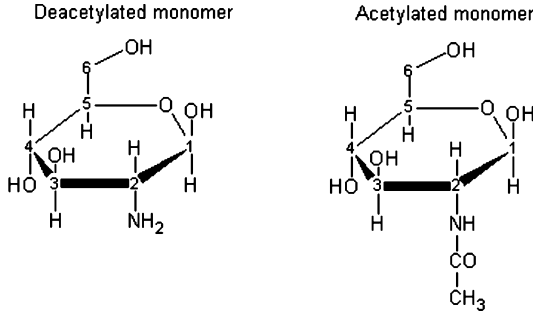

Fig. 1. Acetylated and deacetylated monomers of chitosan.

3. Results and discussion

The structures of acetylated and deacetylated monomers of chitosan are presented in Fig. 1. Fig. 2 presents the 400 MHz ^{1}H NMR spectrum of chitosan PCCH00005 (DDA ~ 87%) at 70 °C. The solvent (HOD) proton resonates at 4.67 ppm. The assignment of chitosan peaks have already been reported in the literature [11,14]. Table 1 shows the chemical shifts of chitosan protons in D_2O/DCl at 70 °C. The DDA was calculated using integrals of the peak of proton H1 of deacetylated monomer (H1-D) and of the peak of the three protons of acetyl group (H-Ac):

$$\mathrm{DDA}(\%) = \left(\frac{\mathrm{H1D}}{\mathrm{H1D} + \mathrm{HAc}/3}\right) \times 100 \quad (1)$$

For comparison, the DDA was also calculated with the method proposed by Hirai et al. [11] by using the signal from protons H2, H3, H4, H5, H6, H6′ (H2-6) of both monomers and the peak of acetyl group(H-Ac):

$$\mathrm{DDA}(\%) = \left(1 - \left(\frac{1}{3}\ \mathrm{HAc}/\frac{1}{6}\ \mathrm{H26}\right)\right) \times 100 \quad (2)$$

For DDA lower than 90%, the DDA was also calculated by using the peaks of protons H1 of both deacetylated and acetylated monomer (H1-D, H1-A):

$$\mathrm{DDA}(\%) = \left(\frac{\mathrm{H1D}}{\mathrm{H1D} + \mathrm{H1A}}\right) \times 100 \quad (3)$$

However, this equation is not suitable for high DDA because H1-A is not visible in the spectrum, as can be seen in Fig. 3. Also, use of the acetyl group peak (H-Ac) presents two advantages over

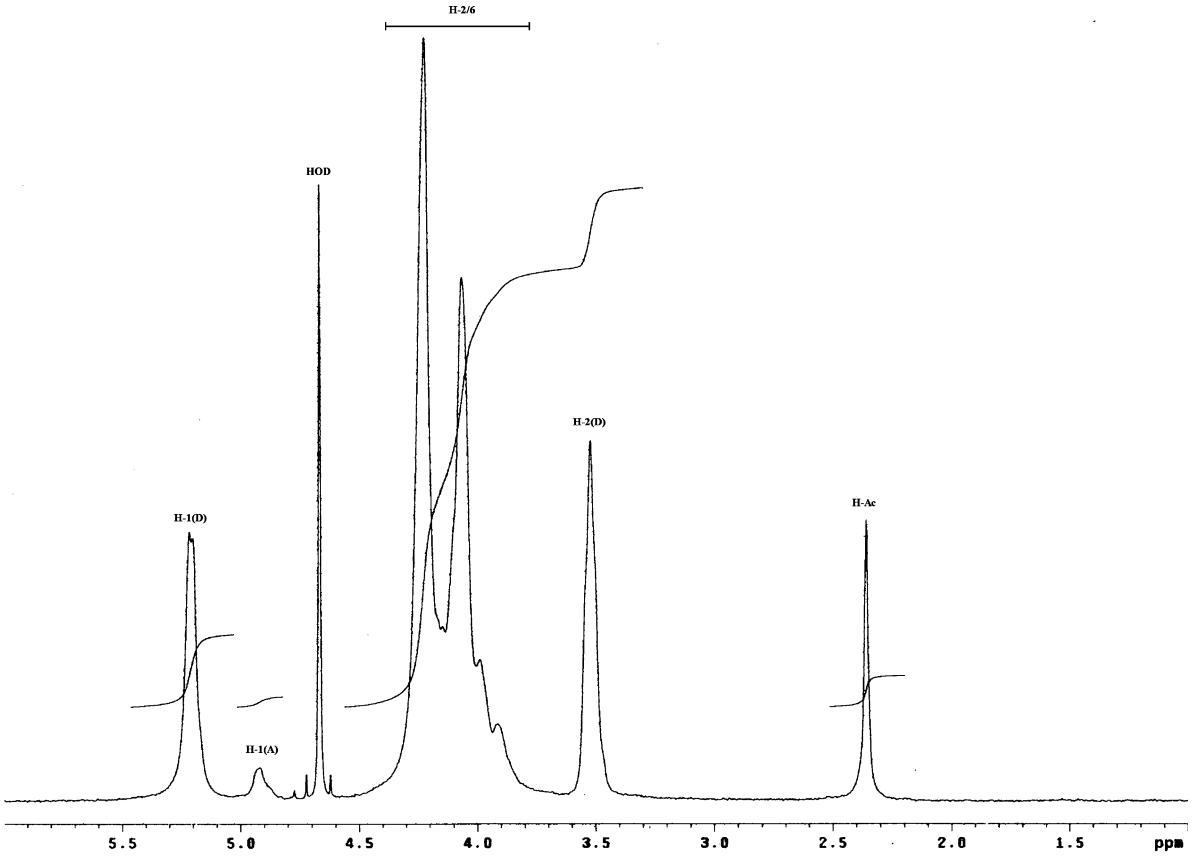

Fig. 2. Chitosan (PCCH00005) ^{1}H NMR spectrum at 70 °C.

Table 1
Chemical shifts and T_1 of protons of chitosan at 70 °C in D_2O/DCl

		Protons				
		H1-D	H1-A	H-2/6	H-2-D	Acetyl protons
Chemical shift (ppm)		5.21	4.92	3.9–4.2	3.52	2.36
T_1 (s)	DDA ~ 48%	1.12	1.12	< 1.10	1.42	1.56
	DDA ~ 100%	1.01	N/A	< 1.00	1.37	1.07

the use of the H1 peak of acetylated monomer (H1-A) (1), the H-Ac peak is three times more intense than the H1-A peak (2) and the H-Ac peak is well resolved with a flat baseline on each side of the peak. However, Eq. (3) should be used if the H-Ac peak is not well resolved due to the presence of contaminants such as acetic acid in the sample. This is the case of chitosan PCCH00024 (see Fig. 4) that contains acetic acid so that only Eq. (3) was used to determine the DDA for this sample. The evaluation of the DDA using the area of the H2 peak of deacetylated monomer as proposed by Shigemasa et al. [15] is not suitable because of the overlap with the peaks between ~3.8 and ~4.2

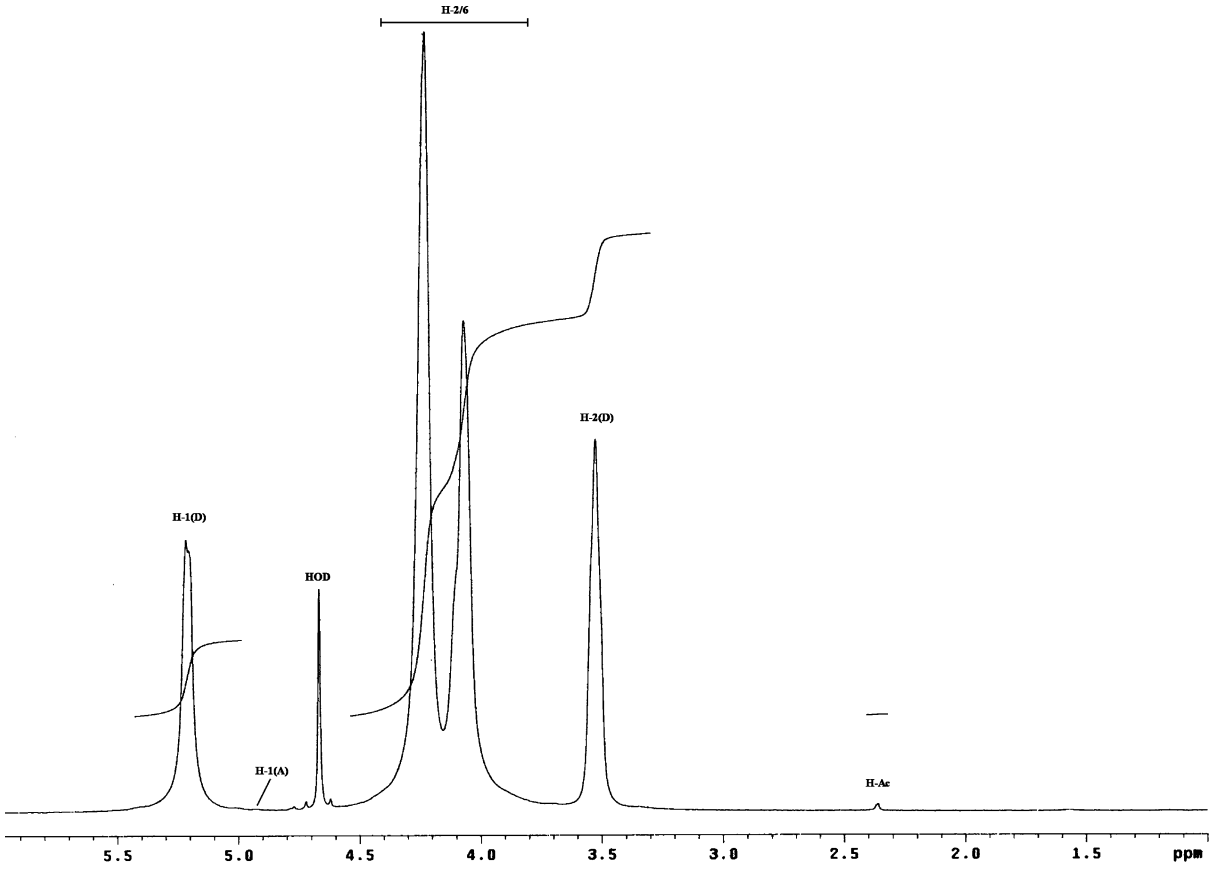

Fig. 3. PCCH00014 ^{1}H NMR spectrum at 70 °C. The small peak at 2.36 ppm originates from the acetyl protons of chitosan. The rms signal on noise ratio for this peak is aproximately 3.1.

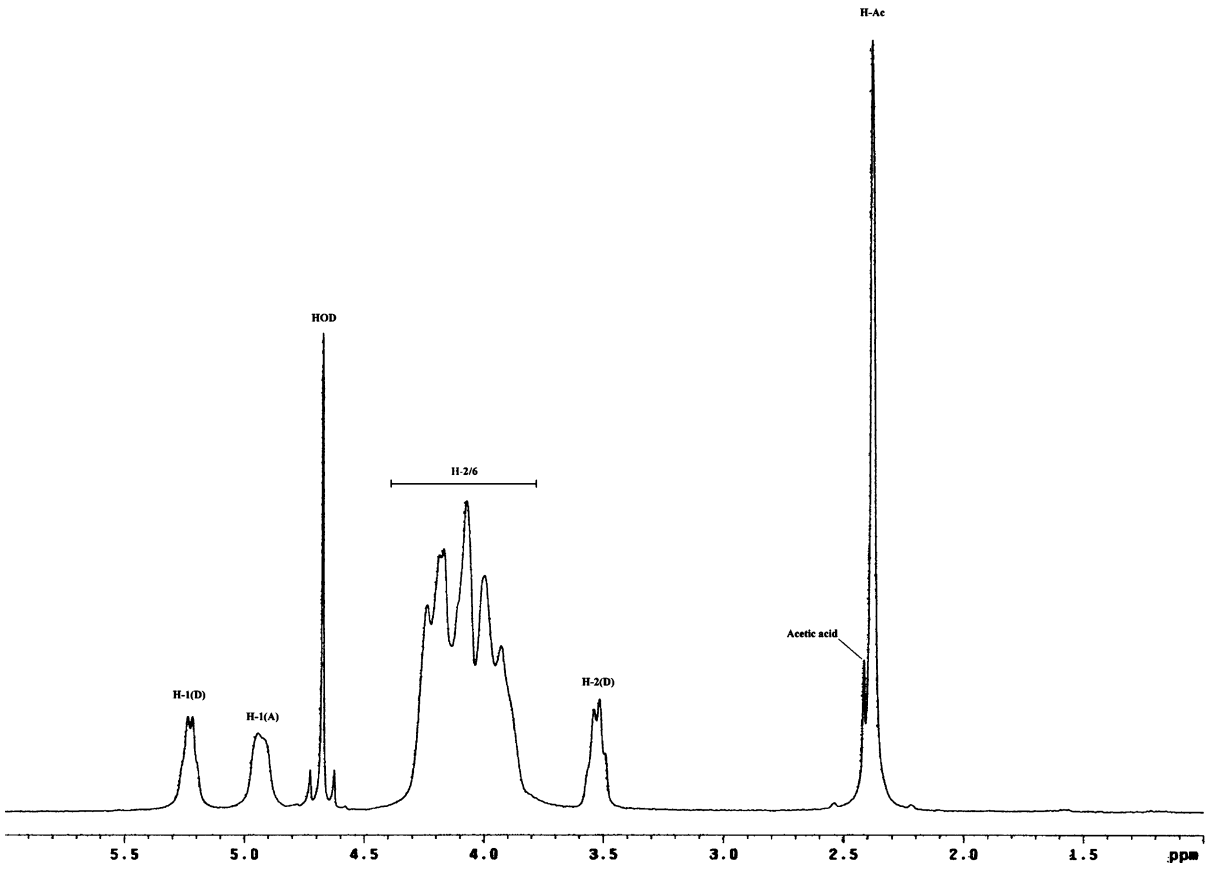

Fig. 4. PCCH00024 ^{1}H NMR spectrum at 70 °C. The peak at 2.41 ppm originates from acetic acid.

ppm. To evaluate the ruggedness of this method, three analysts contributed to this validation study and are referred to as Analyst A, Analyst B and Analyst C in the text.

3.1. Precision

Two analysts tested the precision of the method. Analyst A measured the DDA of all chitosan lots on two different days. For the second testing occasion, the same set of samples after 1 day of storage at room temperature was used. Analyst B tested the six different lots once. The results are presented in Table 2.

The %CVs of the inter-day and inter-analyst precision test were found to be less than 0.8%. The sample with the highest DDA was found to have the lowest CV and the inter-day/inter-analyst results were found to be close to each other. The results obtained by using Eqs. (1)–(3) are consistent with each other, especially for chitosan samples with high DDA. The method is, therefore, precise to determine the DDA of highly deacetylated chitosan samples (see Fig. 3). There is no significant difference between results obtained on the 1st and 2nd day of analysis by analyst A. Also the acetyl hydrolysis of chitosan in D_2O/DCl solution at room temperature was found to be negligible.

3.2. Specificity

The specificity of the method was tested by adding acetic acid to the chitosan samples. All samples were prepared fresh daily. Acetic acid protons resonate near the acetyl protons of

Table 2
Precision test results

Sample ID	DDA (%)								
	Determined using Eq. (1)			Determined using Eq. (2)			Determined using Eq. (3)		
	Analyst A 1st day	Analyst A 2nd day	Analyst B	Analyst A 1st day	Analyst A 2nd day	Analyst B	Analyst A 1st day	Analyst A 2nd day	Analyst B
PCCH00014	99.8 CV = 0.02[a]	99.7 CV = 0.02	99.6 CV = 0.05	99.8 CV = 0.02	99.7 CV = 0.01	99.6 CV = 0.05	N/A	N/A	N/A
PCCH00013	96.8 CV = 0.1	96.7 CV = 0.09	96.4 CV = 0.2	97.0 CV = 0.1	96.9 CV = 0.07	96.6 CV = 0.1	N/A	N/A	N/A
PCCH00005	87.4 CV = 0.2	87.3 CV = 0.08	86.8 CV = 0.1	88.0 CV = 0.08	88.0 CV = 0.03	87.8 CV = 0.1	87.5 CV = 0.3	87.4 CV = 0.6	87.6 CV = 0.3
PCCH00002	81.6 CV = 0.2	81.5 CV = 0.2	81.5 CV = 0.8	82.5 CV = 0.08	82.5 CV = 0.1	82.5 CV = 0.1	82.4 CV = 0.2	82.0 CV = 0.1	82.1 CV = 0.4
PCCH00003	75.9 CV = 0.04	75.9 CV = 0.4	76.2 CV = 0.6	76.9 CV = 0.05	77.0 CV = 0.1	77.3 CV = 0.1	76.7 CV = 0.1	77.1 CV = 0.3	76.9 CV = 0.5
PCCH00024	N/A	N/A	N/A	N/A	N/A	N/A	48.0 CV = 0.5	48.1 CV = 0.3	48.7 CV = 0.2

[a] All CVs given in percentage.

chitosan and are a possible product of chitosan degradation. Acetic acid is also a possible residue of chitosan manufacturing. Four solutions were tested by analyst A: two solutions of PCCH00014 (DDA ~ 100%) with 0.052 and 0.087 mmol of acetic acid, respectively, and two solutions of PCCH00003 (DDA ~ 76%) with 0.052 and 0.087 mmol of acetic acid, respectively. In the case of PCCH00014 samples, the peaks of acetic acid and acetyl protons of chitosan were well resolved and the DDA measured showed no significant difference from the DDA measured without acetic acid. In the case of PCCH00003, the two peaks could not be resolved and acetic acid peak contributed to the H-Ac term in Eqs. (1) and (2). A lower DDA is expected but the amount of acetic acid used is small compared with the concentration of acetylated monomer (the concentration of acetylated monomer is about 7 mmol) and the DDA of the two PCCH0003 solutions spiked with acetic acid showed no significant difference with the DDA found in the precision test. If a larger amount of acetic acid is present in solution, Eq. (3) should be use in order to calculate the DDA as long as the DDA is not too high.

3.3. Ruggedness

The DDAs of chitosan batches PCCH00014 (DDA ~ 100%), PCCH00005 (DDA ~ 87%), PCCH00003 (DDA ~ 76%) and PCCH00024 (DDA ~ 48%) were measured by three analysts on two different testing occasions. All samples were prepared fresh daily. The results are presented in Table 3. The results obtained by using Eqs. (2) and (3) are not presented here, but as in the precision test, the DDA's obtained by using these equations were found to be close to those obtained using Eq. (1) (except for PCCH00024 for which the DDA is only evaluated using Eq. (3)). The difference between intra and inter-user results was very small as can be seen from Table 3 showing the ruggedness of the technique. The NMR technique is reproducible and the small variation between results originates mainly from the phase adjustment and also from the integration boundaries setting.

3.4. Robustness

The robustness of the method was evaluated by varying four parameters independently: (1) the chitosan amount (2) the DCl amount (3) the temperature and (4) the number of transients. All samples were prepared fresh daily and the experiments were performed by analyst A. A variation of $\pm 20\%$ in chitosan mass or $\pm 10\%$ in DCl volume did not appreciably change the measured DDA of chitosan PCCH00013. Running the experiment at 65 or 75 °C also did not affect the measured DDA of chitosan PCCH0002. Using a different number of transients such as 128 or 256 rather than 64 also did not affect the DDA measurement of chitosan's PCCH00014 and PCCH00003 thereby indicating that this method is quite robust in terms of the above-mentioned parameters.

Table 3
Ruggedness test results

Sample ID	DDA (%) determined using Eqs. (1) and (3)[a]					
	Analyst A, 1st day	Analyst A, 2nd day	Analyst B, 1st day	Analyst B, 2nd day	Analyst C, 1st day	Analyst C, 2nd day
PCCH00014	99.8 CV = 0.04[b]	99.8 CV = 0.01	99.7 CV = 0.03	99.7 CV = 0.06	99.6 CV = 0.08	99.5 CV = 0.05
PCCH00005	87.4 CV = 0.1	87.3 CV = 0.09	87.3 CV = 0.2	87.4 CV = 0.2	86.7 CV = 0.4	87.1 CV = 0.2
PCCH00003	76.3 CV = 0.1	76.2 CV = 0.3	76.1 CV = 0.3	75.7 CV = 0.2	75.6 CV = 0.1	76.1 CV = 0.4
PCCH00024	48.0 CV = 0.5	48.1 CV = 0.3	48.7 CV = 0.2	48.6 CV = 0.4	48.6 CV = 0.7	48.2 CV = 0.7

[a] Eq. (3) was used for PCCH00024 only.
[b] All CVs given in percentage.

Table 4
Stability test results

Sample ID	Equation used	DDA (%) measured after				
		0 h	1 h	2 h	3 h	6 h
	Eq. (1)	75.7 CV = 0.1[a]	76.0 CV = 0.05	76.2 CV = 0.2	76.5 CV = 0.1	77.0 CV = 0.2
PCCH00003	Eq. (2)	76.5 CV = 0.8	77.0 CV = 0.1	77.3 CV = 0.1	77.54 CV = 0.08	78.0 CV = 0.1
	Eq. (3)	76.6 CV = 0.7	76.7 CV = 0.4	76.8 CV = 0.1	76.5 SD = 0.3	77.5 SD = 0.1
PCCH00024	Eq. (3)	47.6 CV = 0.2	48.0 CV = 0.1	48.3 CV = 0.2	48.4 CV = 0.2	48.9 CV = 0.2

[a] All CVs given in percentage.

3.5. Stability

The stability was tested by measuring the DDA after leaving a sample at 70 °C for prolonged periods of time. Chitosan PCCH00024 and PCCH00003 were tested. The samples were prepared fresh daily. The ^{1}H NMR spectrum was acquired by analyst A in triplicate after 0, 1, 2, 3 and 6 h. Results obtained are presented in Table 4. For both samples, the DDA was found to increase slightly with time because of acid hydrolysis resulting in higher DDA and higher acetic acid content in solution. The variation after 6 h was of the order of the precision of the technique. For PCCH00003, acetic acid protons and acetyl protons of chitosan peaks were not resolved and acetic acid resulting from deacetylation contributed to the H-Ac term in Eqs. (1) and (2). However, the longitudinal relaxation time of protons of acetic acid is approximately 12 s at 70 °C [11] (much longer than acetyl protons of chitosan) so that the acetic acid peak intensity was not proportional to acetic acid content because of the too short delay between each pulse (8 s). This is why the DDA measured with Eq. (2) was found to increase slightly with time. By using a longer recycle time like Hirai et al. [11], the DDA determined using Eq. (2) should not change but the total acquisition time would be much longer. The results found with Eq. (3) are supposed to give the current DDA of the sample and the biggest changes in DDA should, therefore, be noted by using this equation. However, this is not perceptible because the variation of DDA after 6 h at 70 °C is still quite small (i.e. of the order of the precision of the technique).

3.6. Accuracy

The accuracy of the technique was tested by comparing the calculated (expected) and measured DDA of samples prepared by mixing solutions of chitosan PCCH00024 (DDA ~ 48%) and chitosan PCCH00014 (DDA ~ 100%) in variable volumetric ratios: 80:20, 50:50 and 20:80. Analyst A who was unaware of the composition of the test solutions performed the experiments. The results are presented in Table 5. The expected DDA of a solution obtained by mixing the two solutions of chitosan (denoted as solutions 1 and 2) is given by the following equation:

$$\mathrm{DDA} = \frac{\frac{v_1 c_1}{\bar{M}_1}\,\mathrm{DDA}_1 + \frac{v_2 c_2}{\bar{M}_2}\,\mathrm{DDA}_2}{\frac{v_1 c_1}{\bar{M}_1} + \frac{v_2 c_2}{\bar{M}_2}} = \frac{\bar{M}_2 v_1 c_1 \mathrm{DDA}_1 + \bar{M}_1 v_2 c_2 \mathrm{DDA}_2}{\bar{M}_2 v_1 c_1 + \bar{M}_1 v_2 c_2} \quad (4)$$

where $\bar{M}_i$ is the monomer average molar weight for a chitosan with $\mathrm{DDA} = \mathrm{DDA}_i$ and is given by $\bar{M}_i = [161.2\mathrm{DDA}_i + 203.2(1 - \mathrm{DDA}_i)/100$ (161.2

Table 5
Accuracy test results

Ratio (PCCH00024:PCCH00014)	Expected DDA (%)	Measured DDA (%)[a]
80:20	59.4	59.9 CV = 0.5[b]
50:50	75.5	75.8 CV = 0.3
20:80	90.4	89.6 CV = 0.3

[a] Determined with Eq. (3).
[b] All CVs given in percentage.

and 203.2 g mol^{-1} are the molar weight of glucosamine and *N*-acetyl glucosamine, respectively), v_i is the volumetric fraction of solution i in the mixture and c_i is the concentration of chitosan (weight/volume) in solution i. The DDAs of PCCH00014 and PCCH00024 used in Eq. (4) are 99.8% (found by analyst A using Eq. (1) in the precision test) and 48.0% (found by analyst A using Eq. (3) in the precision test), respectively. The DDA of the mixtures was measured by using Eq. (3) because of the presence of acetic acid in chitosan PCCH00024. The expected and measured values were close to each other in all cases.

4. Conclusion

Liquid phase ^{1}H NMR is a very suitable method for the determination of the DDA of chitosan. The technique is found to be fast, precise, reproducible, rugged, robust, stable and requires only a small amount of chitosan. The ruggedness of the method has been demonstrated by the low inter-analyst %CV. The accuracy of the technique was shown by mixing solutions of known DDA and retrieving the expected DDA values of the mixtures. The DDA calculated by using three different combinations of peaks are very close to each other demonstrating that the technique is also internally consistent.

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