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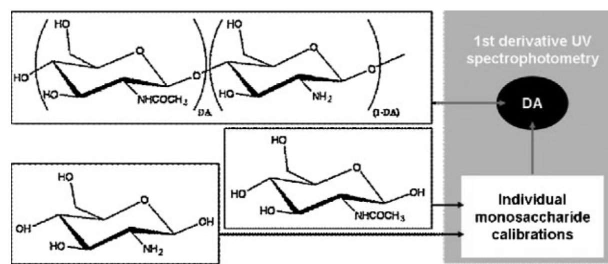
Straightforward Determination of the Degree of *N*-Acetylation of Chitosan by Means of First-Derivative UV Spectrophotometry

Ricardo M. P. da Silva,* João F. Mano, Rui L. Reis

First-derivative UV spectrophotometry is shown to be a reliable method for the determination of the degree of *N*-acetylation of chitosan samples. A mathematical expression is derived that allows to determine the DA directly from the mass concentration of a chitosan solution and the first derivative of its UV spectrum at 202 nm, thus eliminating the need for empiric correction curves for highly deacetylated samples. A procedure is proposed for the accurate mass determination of the hygroscopic chitosan. The proposed approach facilitates the routine determination of the DA, especially when using potent multiwell microplate readers, which allow hundreds of samples to be measured in just a few minutes.



Introduction

Chitin is a structural polysaccharide that can be found in the cell wall of fungi and in the exoskeleton of most invertebrates, of which crustaceans are currently the major source of chitin for the industry.[1] This polysaccharide is similar to cellulose, both in function, chemical structure and abundance; chitin is likely to be one of the most abundant natural macromolecules in the biosphere. Besides its central importance as a building and structural material in a great number of biological organisms, chitin is winning a central role as a raw material in engineering and emergent technologies, in great part owing to its soluble derivative, chitosan. Given its natural abundance, it promises to be one of the future, important sources of renewable materials. In fact, more or less successfully, chitin and chitosan have been proposed for a broad range of industrial applications, including wastewater treatment, food, agriculture, cosmetics, biotechnological processes and separation technologies,[2] as well as, for medical applications, such as biomaterials[3] and tissue engineering.[4] It can be also used as a precursor to produce other materials through chemical modification.[5]

Chitin is poly[β-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-D-glucopyranose]. *N*-deacetylation processes give origin to a series of copolymers varying in the relative amounts of its comonomers, β-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-D-glucopyranose (GluNAc) and β-(1 $\rightarrow$ 4)-2-amino-2-deoxy-D-glucopyranose units (GluN).[6] The molar fraction of the GluNAc units is defined as the degree of *N*-acetylation (DA). Chitosan is the most *N*-deacetylated part of this series of copolymers. Although the criteria that defines what is chitin or chitosan based on the DA value is somewhat controversial, chitosan is often

R. M. P. da Silva, J. F. Mano, R. L. Reis
3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
E-mail: ricardosilva@dep.uminho.pt
R. M. P. da Silva, J. F. Mano, R. L. Reis
IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

Macromol. Chem. Phys. **2008**, *209*, 1463–1472
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WILEY InterScience DISCOVER SOMETHING GREAT
DOI: 10.1002/macp.200800191

regarded as the soluble copolymers in dilute acidic solutions, converse to the insoluble chitin. Much more relevant than the details of the nomenclature, both the physicochemical[7–9] and biological properties[10] of these copolymers are strongly dependent on the DA. For instance, dependence on the DA has been shown for properties such as: the supramolecular aggregation[7] and the pK_a;[8] the crystallinity[9,11] and the mechanical properties of these biopolymers films;[11] the cellular uptake, and the *in vitro* cytotoxicity of its molecules and nanoparticles.[10] Therefore, chitin/chitosan biopolymers can only be correctly defined once their DA is known. The determination of this parameter is apparently only a simple analytical problem but actually has been revealed to be a rather complicated issue. A myriad of methods have been proposed and tuned over the past two decades, but all of them possess inherent drawbacks and limitations. The portfolio of methods includes techniques such as Fourier-transform infrared (FTIR) spectroscopy,[12-14] potentiometric titration,[15] elemental analysis[15,16] ultraviolet (UV) spectrophotometry,[16–18] ninhydrin assay,[19] conductometric titration[20] and a range of nuclear magnetic resonance (NMR) spectroscopic methods, both in the liquid[6,21,22] and solid state,[23–25] just to refer to most common.

Solid-state methods that do not require the material's dissolution present the advantage of being applicable over the entire range of DA values, both for chitin and chitosan. Solution methods are limited to soluble samples and, by definition, can only be applied to determine the DA of chitosan (approximately for DA < 0.60).

The most widely used solid-state method for the DA determination is FTIR spectroscopy, but some bands depend on intricate associations with the typical hydrogen-bonding networks different for each chitin polymorphic form.[12] A great number of different bands and baselines have been suggested in the literature, but important variations can often be found in the respective results.[12] This fact makes the selection of suitable bands and baselines quite problematic. Statistical studies comparing the vast number of proposed bands and baselines combinations have been employed to assist in that selection based on robust criteria.[13] Despite its drawbacks, FTIR spectroscopy has been often preferred because it is a quick, user-friendly and low-cost method, but mostly because it can also be applied to insoluble chitin. Nevertheless, the construction of a specific calibration line for each particular isolation and *N*-deacetylation procedure may be necessary to obtain reliable values of DA[13] The calibration requires the use of standards previously assessed for the DA, which, in the case of insoluble samples, is normally done using solid-state ^{13}C NMR spectroscopy as a reference method.[12–14] Solid-state NMR spectroscopy methods are powerful tools for determining the DA of chitin and chitosan. ^{15}N cross polarization/magic-angle spinning (CP/MAS) NMR spectroscopy has been used to evaluate the *N*-acetyl content in the case of a complex association of chitin with other polysaccharides. The combination with ^{13}C CP/MAS NMR spectroscopy also allowed the determination of the chitin content in structural polysaccharides in fungus.[25] NMR spectroscopy methods are often referred as the gold-standard techniques and are employed to calibrate other techniques or to assess their accuracy.[12–15,18–20] NMR spectroscopy does not require the use of external standards, it provides a simultaneous checking for the presence of some impurities and structural information can be inferred. The experimental parameters should be carefully adjusted in order that the signal is proportional to the concentration of the all of the sample nuclei. Comprehensive studies on the adjustment of the NMR spectroscopy assay parameters have been reported both for liquid ^{1}H NMR spectroscopy[21,22] and solid-state ^{13}C CP/MAS NMR spectroscopy.[24] Unfortunately, the related costs and complicated technical considerations hinder its widespread use as a routine technique at the industrial scale and in non-specialized laboratories.

Amongst the solution methods, 1st-derivative UV spectrophotometry presents several advantages. It was conceived by Muzzarelli and Rocchetti to provide accurate and precise results in a simple and fast way for highly deacetylated chitosan, which is difficult to analyze by techniques that record the signals of the *N*-acetyl group.[17] Furthermore, this method is insensitive to the acetic acid concentration, under reasonable limits (a typical residue from the manufacturing process). The use of water as a reference blank reduces light absorption in the reference system, thus permitting a better signal-to-noise ratio.[17] It also tolerates the presence of remaining traces of protein contaminants;[18] its calibration does not rely on other determinations of the DA of standard samples; and it only requires very small amounts of sample, simple reagents and instrumentation. The main disadvantages of the method are the requirement of an accurate determination of the weight, particularly difficult for the highly hygroscopic chitosan samples, and the need of using an empiric correction curve for DA < 0.11,[17] due to the contribution of the GluN to the 1st-derivative signal.

The GluN and GluNAc present two far-UV chromophoric groups that contribute in a simple additive way to the total absorbance of the material at a particular wavelength, since they do not interact within the polymer in a manner that would affect absorption of UV radiation.[16] The amide group of the GluNAc presents a relatively strong signal ($\pi \rightarrow \pi^*$) with a maximum below 200 nm, which is the base of the most UV methods for the determination of the DA. In turn, although giving a relatively weak signal, GluN also absorbs UV light in this region, which may interfere with

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DOI: 10.1002/macp.200800191

the measurement of low DA samples. Based on this evidence Liu et al.[16] derived a linear relationship between the absorbance divided by the total molar concentration of the monomers and the DA. The main advantage of such an approach is the absence of the necessity for corrections at lower DA. In our approach, we derived a similar Equation that combines the advantages and robustness of the 1st-derivative method, with the exact mathematical description of the DA as a function of the 1st derivative of the absorbance at 202 nm and the mass concentration of the polysaccharide solutions. A solid criterion for the absorbance range within which the method remains valid was also defined. We believe that the proposed procedure, combining the accuracy and precision of 1st-derivative UV spectrophotometry with a straightforward determination of the DA, constitutes a breakthrough in the reliable determination of the DA of chitosan samples at a large industrial scale, especially if taking into consideration the currently available, potent, multiwell microplate readers that can allow the fast measurement of hundreds of samples in just a few minutes.

Experimental Part

Purification and Characterization of Chitosan

Chitosan raw materials from crab shells were purchased from Sigma-Aldrich (USA). The chitosan samples were purified once in an amount sufficient to perform all of the experimental work reported, by reprecipitation in sodium hydroxide. First, the chitosan was dissolved in an aqueous acetic acid solution (1%) at ≈1% (w/v). The insoluble material was removed by filtration with Whatman® ashless filter paper (20–25 μm). The obtained clear solution was precipitated adding a NaOH solution (final pH ≈ 8). The formed white gel was sieved to remove the exuded liquid and thoroughly rinsed with distilled water, until no changes in the pH were detected. The chitosan gel was further washed with ethanol, freeze-dried, ground to powder and dried at 60 °C overnight. Two different chitosan raw materials varying in the degree of *N*-acetylation were labeled according to their nominal DA values as DA05 and DA20 (see Table 1). The average molecular weights were found to be 790 kDa for DA05 and 770 kDa for DA20 by viscometry in CH_3COOH (0.5 M)/$NaCH_3COO$ (0.2 M), according to Mark-Houwink theory ($k = 3.5 \times 10^{-4}$; $a = 0.76$).[26]

Preparation of Chitosan Samples with Several DA by Selective *N*-Acetylation

The DA20 chitosan sample (5 g) was dissolved in 1% (w/v) of aqueous acetic acid (50 mL). A variable volume of acetic anhydride was mixed with 50 mL of ethanol, added slowly to the chitosan solution and stirred overnight. The ratio of the acetic anhydride to the chitosan GluN units was adjusted to obtain samples with different DA. The solutions were precipitated with acetone, followed by diethyl ether and dried under vacuum. The acetylated samples were then neutralized in 1 N NH_4OH aqueous solution, thoroughly washed with distilled water and freeze-dried. The resultant sponges were milled with liquid nitrogen and the obtained flakes were dried at 80 °C under reduced pressure. The Fourier-transform infrared (FTIR) spectra of the *N*-acetylated chitosan were recorded using an IRPrestige 21 FTIR spectrophotometer (36 scans, resolution 4 cm^{-1}) from solvent cast films, previously neutralized in 1 N NH_4OH in water/methanol (1:3) and thoroughly dried under vacuum.

Thermogravimetric Analyses (TGA)

In one of our previous works,[27] we found that chitosan membranes contain residual moisture irrespective of the drying

Table 1. Degree of *N*-acetylation (DA) from 1st-derivative UV spectrophotometry and liquid-phase ^1H NMR using Equation (16)–(18); along with moisture content (MC). The coefficient of variation, $CV = (\sigma/DA) \times 100$, where σ is the standard deviation of three measurements.

Sample ID[a)]	UV		^1H NMR spectroscopy [Equation (16)]		^1H NMR spectroscopy [Equation (17)]		^1H NMR spectroscopy [Equation (18)]		MC
	DA	CV	DA	CV	DA	CV	DA	CV	%
		%		%		%		%	
DA05	0.067	2.0	0.049	6.9	0.052	2.7	0.101	40.6	7.3
DA20	0.216	1.3	0.187	4.8	0.200	5.4	0.251	6.4	7.1
DA30	0.326	0.8	0.279	2.0	0.314	4.0	0.388	11.9	8.2
DA40	0.415	3.2	0.372	1.0	0.435	3.6	0.516	6.9	4.5
DA50	0.514	0.0	0.506	3.6	0.526	1.5	0.543	5.2	6.2
DA60	0.613	0.6	0.556	1.4	0.592	2.2	0.617	3.8	10.0

a)Samples DA30, DA40, DA50 and DA60 were *N*-acetylated from the DA20 sample.

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procedure. The hypothesis that this residual moisture could be caused by a fast water uptake from the atmosphere would make the accurate determination of the chitosan weight a tricky procedure. The water content of the chitosan samples was estimated by TGA (TA Instruments, model TGA Q500), immediately after being weighed for the UV determination of the DA. The thermograms were obtained under an atmosphere of flowing nitrogen. The chitosan powder (4–10 mg) was first heated at a 10 °C · min^{-1} ramp, which was followed by an isothermal step of 20 min at 110 °C to assure complete dryness of the samples. The moisture content MC (%) was considered to be the weight loss at that time point. The temperature program also included a cooling-down period, under the same nitrogen stream. Then, the dried sample was exposed to the air atmosphere and weighed again at preset time periods, by temporarily closing the TGA apparatus furnace. This procedure allowed us to estimate the time necessary for the dried chitosan materials to recover the initial water content when exposed to atmospheric moisture.

First-Derivative UV Spectrophotometry

The monosaccharides, GluN (D-glucosamine hydrochloride, > 99%, *M* = 215.6 Da) and GluNAc (>99%, *M* = 221.2 Da), were purchased from Sigma-Aldrich. The GluNAc was stored in the freezer and kept in a vacuum desiccator for at least 2 h before use. One stock solution of acetic acid (AcOH, 0.1 M) was prepared and another 0.01 M AcOH stock solution was obtained by diluting the previous one. Standard monosaccharide solutions were prepared dissolving each sugar powder in 0.01 M acetic acid at several molarities, in the range of 3×10^{-3} to 0.2 M for GluN and from 0.01×10^{-3} to 1×10^{-3} M for GluNAc.

For the determination of the DA, the chitosan samples were dried over vacuum at 80 °C. The dried chitosan powder uptakes the water from the atmosphere at a rate that is high enough to provoke water-content shifts within two consecutive weightings of the same sample, as previously verified by means of thermogravimetric analysis (TGA). For this reason, the chitosan samples were previously conditioned at the atmospheric moisture for 10 min, and subsequently weighed. After that, the chitosan samples were subjected to TGA to determine the water content, as described above. The accurately weighed (10.0 mg) chitosan samples were dissolved in 2.00 mL of 0.1 M AcOH and diluted 10-fold with distilled water to obtain a final AcOH concentration of 0.01 M (chitosan was not dissolved directly in 0.01 M AcOH, since it would be more time consuming). When required, further dilutions were performed using the 0.01 M AcOH stock solution to keep the AcOH concentration at this value. All of the solutions, including the 0.01 M AcOH, were prepared from the same 0.1 M AcOH stock solution. All of the spectra (range 200–240 nm, step 1 nm) were recorded with a Bio-Tek® Synergy™ HT microplate reader in a 96-well quartz plate from Hellma® using 300 μL of each solution. The empty quartz plate was read before each set of experiments and subtracted from measured spectra to attenuate possible differences in the residual absorbance, arising from an eventual drift in the plate thickness, scratches or occasional dirtiness. Distilled water was used as a blank. All of the measurements were performed at least in triplicate.

Determination of the DA by ^1H NMR Spectroscopy

Three different solutions of each chitosan sample were prepared by stirring 10 mg of chitosan in 1 mL of 0.4% (w/v) DCl in D_2O solution at room temperature. In order to minimize the *N*-deacetylation, catalyzed by the presence of deuterium chloride, only freshly prepared solutions were used. The ^1H NMR spectra were acquired in a Varian Unity Plus (300 MHz) spectrometer at 70 °C: the temperature at which the solvent signal (HOD) does not interfere with the chitosan peaks. The acquisition (64 transients) started after 10 min, which was considered to be enough to reach thermal equilibrium. The pulse repetition delay of 6 s, and the acquisition time of 2 s, were set to assure complete relaxation of the nuclei before each pulse application. This procedure (repetition time of 8 s) guarantees that the relative intensities of the resonances correlate with the exact number of nuclei originating that signal.[22]

Results and Discussion

Preparation of Chitosan Samples with Different DA over the Entire Solubility Range

The chitosan samples (both purified raw materials (DA05 and DA20) and *N*-acetylated samples) are depicted in Table 1 and cover the entire DA range of the polysaccharide solubility. The ratio of acetic anhydride to GluN units was chosen in order to get samples with differences of around 10% between each DA value. The use of aqueous alcoholic acetic acid solutions for the reaction of the chitosan with carboxylic anhydrides has been shown to avoid the *O*-acylation side reaction.[28,29] The selective *N*-acetylation was confirmed by FTIR spectra, where absorption bands typical of the *O*-acetyl groups were absent, ≈1 750 (C=O) and ≈1 240 cm^{-1} (C–O),[28] in addiction to those assigned to the *N*-acetyl groups at ≈1 655 (C=O) and ≈1 560 cm^{-1} (N–H).

The molecular weight was reported to not vary considerably if the *N*-acetylation is performed under mild conditions,[8] similar to the ones used in this study. For this reason and since the molecular weight should obviously not influence the DA measurement, it was only determined for the original purified raw materials.

Determination of the First Derivative of the Monosaccharides Molar Absorptivities

The high absorbance of the acetic acid at the working concentration disturbs the determination of both GluN and GluNAc residues, when using the zero-order UV spectra (see Figure 1a). The 1st-derivative spectra of the AcOH solutions, reported in Figure 1b, share a common point at around 202 nm for concentrations from 0.005 M up to 0.03 M, designated as the zero crossing point by

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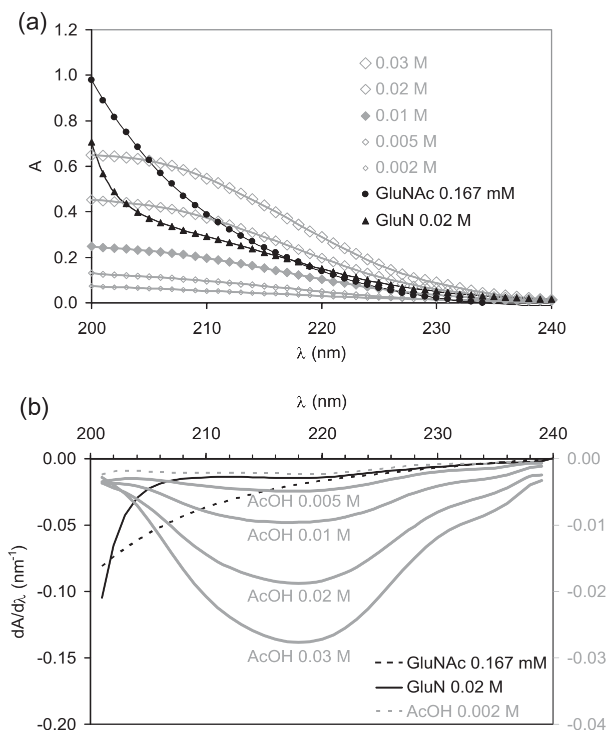

Figure 1. (a) Zero-order and (b) first-derivative UV spectra of the acetic acid (AcOH) solutions with different concentrations (grey) and of the monosaccharides standards (black) dissolved in 0.01 M AcOH (closed dots). Each spectrum represents the average of three independent data sets.

Muzzarelli and Rocchetti.[17] In this sense, at the zero crossing point, the determination of the monosaccharides concentration should be relatively insensitive to fluctuations in the acetic acid concentration. It also corresponds to a stronger signal of the monosaccharides, if compared to the quite-low contribution of the acetic acid, as can be observed in the Figure 1b.

The monosaccharides individual calibration curves were easily drawn through a linear regression between the concentration and the 1st-derivative UV signal arising either from GluNAc (Figure 2) or GluN (Figure 3). This can be deduced from the Beer-Lambert law for diluted solutions, which correlates the concentration (C) with absorbance (A), for a given wavelength (λ):

$$A(\lambda) = \varepsilon(\lambda) l C \tag{1}$$

where ε is the molar absorptivity and l is the optical path length. Since both l and C are independent of the wavelength,

$$\frac{dA}{d\lambda} = \frac{d\varepsilon}{d\lambda} l C \text{ or } \frac{dA}{d\lambda} = \varepsilon'(\lambda) l C \tag{2}$$

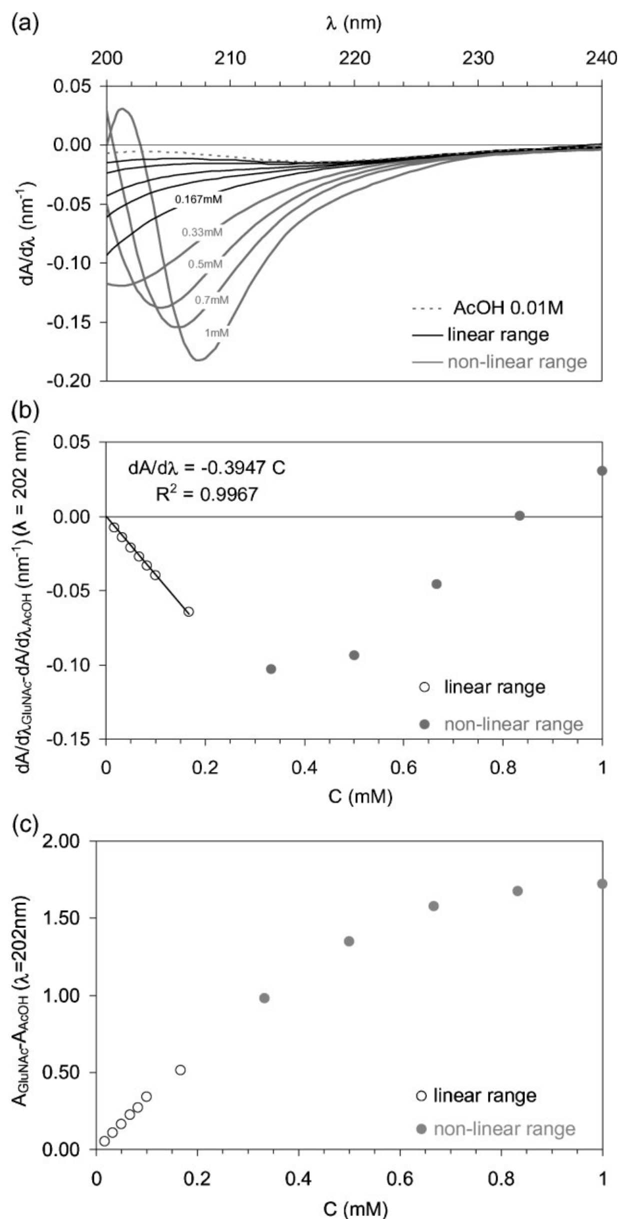

Figure 2. (a) First-derivative UV spectra of GluNAc at several concentrations (0.0167 to 1×10^{-3} M) dissolved in 0.01 M AcOH; (b) first-derivative and (c) zero order UV spectral values at $\lambda = 202$ nm (after subtracting the contribution of the 0.01 M AcOH) as a function of the GluNAc concentration. Each spectrum represents the average of three independent data sets.

It should be noticed that the acetic acid gives also a signal at $\lambda = 202$ nm, thus the expressions should be corrected by

$$A - A_{\mathrm{AcOH}} = \varepsilon\, l C \tag{3}$$

$$\frac{dA}{d\lambda} - \left(\frac{dA}{d\lambda}\right)_{\mathrm{AcOH}} = \varepsilon'\, l C \tag{4}$$

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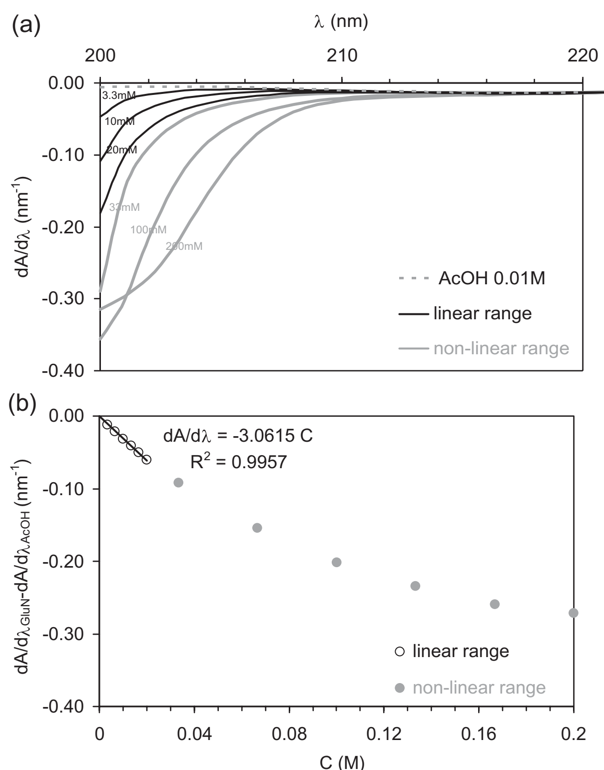

Figure 3. (a) First-derivative UV spectra of GluN at several concentrations (3.33×10^{-3} to 0.200 M) dissolved in 0.01 M AcOH; (b) first-derivative UV spectral values at $\lambda = 202$ nm (after subtracting the contribution of the 0.01 M AcOH) as a function of the GluN concentration. Each spectrum represents the average of three independent data sets.

In Figure 2c it is possible to observe that the GluNAc concentration (0.167×10^{-3} M) above which the Beer-Lambert law lost validity (Equation (1) and (3) with ε no longer independent on the concentration), matches the same limit value observed for Equation (4) (Figure 2b), as expected. The validity limit is regarded as the concentration above which the linear correlation is lost ($r^2 < 0.99$). This linearity limit is not a problem concerning the estimation of the GluN residues (20×10^{-3} M), because this limit is never reached in the determination of the DA, even considering the maximum concentration of the chitosan used and a theoretical DA of 0 (maximum GluN molar fraction). On the other hand, the 1st-derivative spectra of GluNAc suffer a continuous peak deviation as the concentration increases above the linearity limit (Figure 2a) and, as a consequence, the $dA/d\lambda$ at $\lambda = 202$ nm reaches a minimum at around 0.4×10^{-3} M and then starts to increase again (Figure 2a and 2b). Therefore, one single measurement of $dA/d\lambda$ at $\lambda = 202$ nm could be assigned to two very different GluNAc concentrations. This would pose a difficulty in determining the DA of chitosan without ambiguity and would require the analysis of the entire spectrum. In order to ensure that the GluNAc concentration is within the linearity range, one may alternatively use the following practical criterion:

$$(A - A_{\mathrm{AcOH}}) < 0.5 \tag{5}$$

Since the GluNAc absorbance at 202 nm is an increasing monotonic function of the concentration (Figure 2c), each absorbance value is unequivocally assigned to one concentration value of the GluNAc. In this way, it is still possible to only use the spectral region in the neighbourhood of 202 nm (few data points) for the routine determination of the DA of chitosan samples.

Denoting the molar absorptivities of GluNAc and GluN as ε_a and ε_g, respectively, a linear regression of the experimental data gives $\varepsilon'_a l = -394.7$ and $\varepsilon'_g l = -3.061$ $\mathrm{M}^{-1} \cdot \mathrm{nm}^{-1}$.

The optical path length (l) was estimated (approx. 0.93 cm) from the geometrical features of the microplate wells and the solution volume (300 μL) added to each well. The solution shape was considered to be roughly cylindrical, disregarding the meniscus concavity.

Determination of the DA by Means of 1st-Derivative UV Spectrophotometry

The method is based on the assumption that the molar absorptivities of both the GluN (ε_a) and GluNAc (ε_g) chromophoric groups do not change when they are covalently bound through β-(1 → 4) glycosidic linkages. Being so, the monosaccharides contribute in an additive way to the total absorbance, which in the presence of acetic acid can be expressed as:

$$A = \varepsilon_a l C_a + \varepsilon_g l C_g + \varepsilon_{\mathrm{AcOH}} l C_{\mathrm{AcOH}} \tag{6}$$

with concentrations (C) in $\mathrm{mol \cdot L^{-1}}$. Since the optical path length (l) and the concentration (C) are independent on the wavelength, differentiating Equation (6) gives:

$$\frac{dA}{d\lambda} - \left(\frac{dA}{d\lambda}\right)_{\mathrm{AcOH}} = \varepsilon'_a l C_a + \varepsilon'_g l C_g \tag{7}$$

The DA, defined as the molar fraction of the GluNAc units, can be expressed as the ratio between the GluNAc concentration and the total monosaccharides concentration (C_t):

$$\mathrm{DA} = \frac{C_a}{C_a + C_g} = \frac{C_a}{C_t} \tag{8}$$

Macromol. Chem. Phys. 2008, 209, 1463–1472
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DOI: 10.1002/macp.200800191

Combining Equation (7) and (8) and rearranging, it follows that:

$$\frac{1}{C_t}\left[\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right]=\left(\varepsilon_a' l-\varepsilon_g' l\right)DA+\varepsilon_g' l \tag{9}$$

This equation is the basis of the method proposed herein. It is interesting to notice that the method developed by Muzzarelli and Rocchetti[17] is a special case of this equation. If fact, since $|\varepsilon_a'| \gg |\varepsilon_g'|$, Equation (9) can be simplified to

$$\frac{1}{C_t}\left[\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right]\approx(\varepsilon_a' l)DA+\varepsilon_g' l \tag{10}$$

If DA is not too small (those authors found that for $DA > 0.11$ the GluN does not interfere with the GluNAc determination), it can be further simplified, giving

$$\left[\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right]\approx(\varepsilon_a' l)DA\, C_t=\varepsilon_a' l\, C_a \tag{11}$$

For $DA < 0.11$, Muzzarelli and Rocchetti[17] proposed a correction curve.

The molar concentration of both pyranosyl units (C_t) within a chitosan sample cannot be achieved without knowing the DA. Thus, it is more convenient to express the copolymer concentration in terms of solute mass ($\overline{C}_t$) in $g \cdot L^{-1}$, which is defined experimentally. These two concentration values are related by the next equation:

$$\frac{\overline{C}_t}{C_t}=(M_a-M_g)DA+M_g \tag{12}$$

where M_a and M_g are the molecular weights of the GluN and GlunAc units within the copolymer. Combining Equation (9) and (12) gives:

$$\frac{1}{\overline{C}_t}\left[\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right]=\frac{\left(\varepsilon_a' l-\varepsilon_g' l\right)DA+\varepsilon_g' l}{(M_a-M_g)DA+M_g} \tag{13}$$

and, rearranging,

$$DA=\frac{\varepsilon_g' l-\frac{M_g}{\overline{C}_t}\left(\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right)}{\frac{M_a-M_g}{\overline{C}_t}\left(\frac{dA}{d\lambda}-\left(\frac{dA}{d\lambda}\right)_{AcOH}\right)-\left(\varepsilon_a' l-\varepsilon_g' l\right)} \tag{14}$$

The first derivatives of the molar absorptivities were determined in a separate calibration experiment. Also, the molecular weight of the monosaccharides within the copolymer are easily calculated ($M_a = 203$ and $M_g = 161$). Therefore, Equation (14) allows a straightforward determination of the DA, knowing the 1st-derivative UV spectral signal of the chitosan solution at 202 nm, at a suitable concentration. The results obtained for the different chitosan samples are depicted in Table 1. The determination of the mass concentration ($\overline{C}_t$) requires accurate measurement of the chitosan weight. Since chitosan is very hygroscopic, concerns can be raised regarding the accuracy of this measurement. This issue was addressed by means of TGA and is detailed in the following section.

Determination of the Moisture Content of Chitosan

In order to ensure an accurate measurement of the chitosan mass, the uptake of water from the atmosphere for thoroughly dried chitosan samples was assessed under similar conditions used to weigh the samples. It was found that the completely dried chitosan samples (after a TGA drying cycle) recover their initial moisture content in less

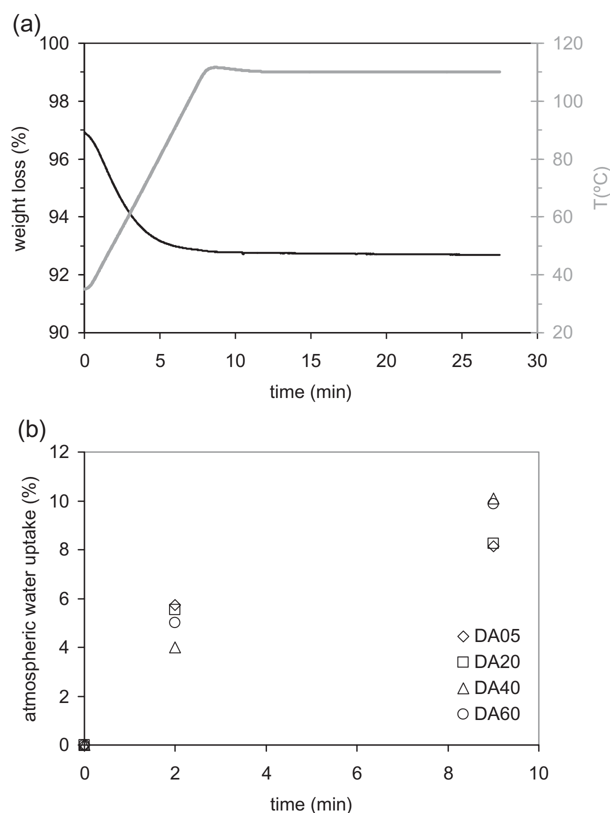

Figure 4. (a) Thermogram of DA05, conditioned in a room atmosphere for 10 min and accurately weighed for the UV-spectra determination; (b) water uptake (ratio between the weight increment and the initial dry weight) of the samples exposed to the room atmosphere over different times after a TGA drying cycle.

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than 10 min (see Figure 4b), when exposed to the room atmosphere. A longer exposure time point would be required to show that the typical equilibrium plateau has been already reached. Nevertheless, the total recovery of the initial moisture content was considered as evidence that exposure to room atmosphere for 10 min gives a value that, for our purposes, was close enough to the equilibrium. The fast atmospheric-water uptake, calculated as the ratio between the weight increment and the initial dry weight, is enough to provoke water-content shifts within two consecutive weighings of the same sample. Based on this result, an alternative procedure was adopted. Thoroughly dried samples were conditioned in an air atmosphere for around 10 min and accurately weighed (W). After that, the moisture content MC (%) of the samples was determined by TGA (see Figure 4a and Table 1) and the mass concentration was corrected according to the following expression:

$$\overline{C}_{\mathrm{t}} = \frac{W \times (1 - \mathrm{MC}(\%)/100)}{V} \tag{15}$$

Although the difficulty of determining the accurate weight of a chitosan sample is often considered one of the drawbacks of the UV-spectrophotometry-based methods, we found that the DA value is relatively insensitive to a small drift in the moisture content. We simulate an absolute error of $\pm 1\%$ in the moisture content of our samples and encountered a relative error associated to the DA of $\pm 1.3\%$. Besides, the proposed procedure should provide reliable chitosan weight determinations.

Comparison with the DA as Determined by Liquid-Phase ^{1}H NMR Spectroscopy

Proton chemical shifts (δ), relative to 3-(trimethylsilyl)-propionic acid, were assigned as reported in the literature.[21] Data for the sample DA30 are:

^{1}H NMR (D_2O, DCl): $\delta = 2.05$ (s, H^{Ac} of GluNAc), 3.20 (s, H^2 of GluN), 3.6–4.0 (m, H^2 of GluNAc and H^3, H^4, H^5, H^6, $H^{6'}$ of both monomers), 4.61 (s, H^{1a} of GluNAc) and 4.90 (s, H^{1g} of GluN) (see Figure 5).

The comparison between the DA values calculated using different combinations of peaks can be used as an indicator of the consistency of ^{1}H NMR. We have used several methods to calculate the DA. The method proposed by Hirai et al.[21] makes use of the peak areas from the H^2, H^3, H^4, H^5, H^6, and $H^{6'}$ protons to estimate the sum of both the monomers and the signal arising from the acetyl group protons (H^{Ac}) to the amount of GluNAc:

$$\mathrm{DA} = \frac{\mathrm{H}^{\mathrm{Ac}}/3}{(\mathrm{H}^2 + \mathrm{H}^3 + \mathrm{H}^4 + \mathrm{H}^5 + \mathrm{H}^6 + \mathrm{H}^{6'})/6} \tag{16}$$

The method proposed by Lavertu et al.[22] uses the peak from the H^{1g} proton to estimate the amount of GluN and the signal from the acetyl group protons (H^{Ac}) to estimate the amount of GluNAc:

$$\mathrm{DA} = 1 - \frac{\mathrm{H}^{1\mathrm{g}}}{\mathrm{H}^{1\mathrm{g}} + \mathrm{H}^{\mathrm{Ac}}/3} \tag{17}$$

We have also calculated the DA using a combination of the two previous equations:

$$\mathrm{DA} = 1 - \frac{\mathrm{H}^{1g}}{(\mathrm{H}^2 + \mathrm{H}^3 + \mathrm{H}^4 + \mathrm{H}^5 + \mathrm{H}^6 + \mathrm{H}^{6'})/6} \tag{18}$$

The results obtained from these different calculation methods show that, although ^{1}H NMR spectroscopy is regarded as having a good internal consistency, the determination of DA may be somewhat systematically affected by the choice of the peaks to be used in that calculation and in the way those peaks are combined to estimate the GluN and GluNAc quantities. None of the calculation methods make use of the peak assigned to the H^{1a} proton of the GluNAc, because of its lower intensity for low DA values and, simultaneously, due to its proximity to the HOD signal. These equations represent the possible combinations of the other different sets of well-resolved peaks (excluding H^{1a}).

The coefficient of variation, which stands for the ratio between the standard deviation and the averaged DA value, was found to be considerably lower for the UV determination, confirming the higher precision of the method proposed in this work. The DA values achieved for the different chitosan samples using the 1st-derivative UV-spectrophotometry method were plotted against the values achieved for the same samples using each one of the calculation methods based on the ^{1}H NMR spectra (Figure 6). Ideally, this representation should give a straight line with slope 1 and y intercept 0. In this respect, Equation (17) gives the best match with the UV method. However, no irrefutable conclusions can be drawn with respect to the accuracy just

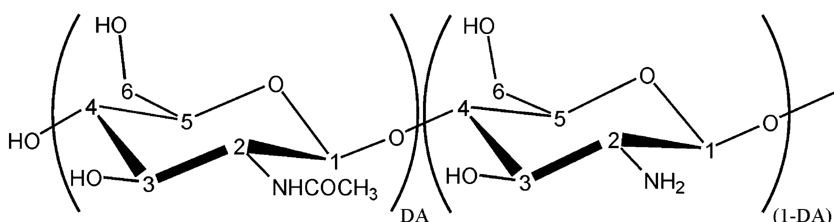

Figure 5. Chemical structure of chitosan.

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DOI: 10.1002/macp.200800191

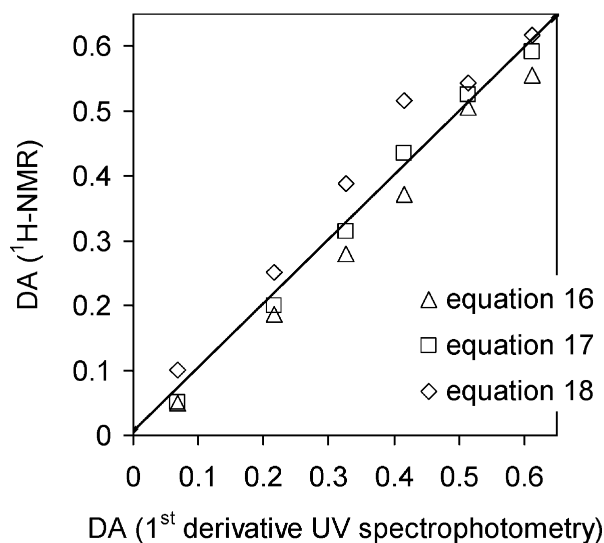

Figure 6. DA obtained by the proposed UV spectrophotometric method (Equation (14)) vs. DA calculated from the ^{1}H NMR data (Equation (16)–(18)). The straight line represents the linear regression between the DA determined by the UV method and the average of each of the three different experimental values obtained for the same sample by ^{1}H NMR spectroscopy ($y = 0.9868\ x + 0.0063$; $R^2 = 0.993$).

based on this result, because we do not know which equation gives the most-accurate results from the ^{1}H NMR data, especially when the reasons behind the discrepancy between the results are not known and logical criteria cannot be established to disregard some of the equations.

On the other hand, although the average between the three methods is solely an algebraic combination of the three equations and does not ensure that a value closer to the actual DA is obtained, the correlation between the averaged ^{1}H NMR DA values and the UV method is noteworthy. The respective linear regression gives a correlation factor $R^2 = 0.993$ and a straight line with slope 0.986 and y intercept 0.0063. It should be noticed that 1st-derivative UV spectrophotometry and liquid-state ^{1}H NMR spectroscopy are independent techniques, in the sense that the calibration of the former does not rely on the DA values of chitosan standards obtained from other techniques. Moreover, the ^{1}H NMR technique did not require any calibration. Hence, the good correlation between both techniques constitutes a strong indication of the good accuracy of the proposed 1st-derivative UV-spectrophotometry method over the whole DA range of the chitosan solubility, if a reliable measure of the chitosan weight is undertaken.

Conclusion

First-derivative UV spectrophotometry is a robust, accurate and precise technique for the determination of the DA of soluble chitosan samples. It presents several advantages, such as: it is relatively tolerant to the presence of residual acetic acid and protein contaminants; it only requires a small amount of sample, simple reagents and equipments; and the high value of the molar absorptivity 1st derivative of the GluNAc should ensure a good accuracy in the determination of the GluNAc residues, even at very-low concentrations (high DA). We derived a mathematical expression that describes the DA as a function of the 1st-derivative signal at 202 nm and the mass concentration of the polysaccharide solution, avoiding the use of empiric correction curves for the determination of the DA of highly deacetylated samples. The values of the DA for several chitosan samples over the entire range of the copolymer solubility confirmed the good precision of the method with typical coefficients of variation around 1%. Comparison with an optimized ^{1}H NMR spectroscopy determination reiterates the expected fine accuracy of 1st-derivative UV spectrophotometry.

Acknowledgements: This work was partially supported by the *Portuguese Foundation for Science and Technology* (*FCT*), through funds from the POCTI and/or FEDER programmes and through the scholarship SFRH/BD/6862/2001 granted to Ricardo M. P. da Silva. This work was carried out under the scope of the *European NoE EXPERTISSUES* (NMP3-CT-2004-500283) and also partially supported by the European Union-funded STREP Project *HIPPOCRATES* (NMP3-CT-2003-505758). The authors thank *Elisa Pinto* for her kind help and advice on setting up the ^{1}H NMR instrumentation conditions.

Received: February 11, 2008; Revised: April 7, 2008; Accepted: April 9, 2008; DOI: 10.1002/macp.200800191

Keywords: acetylation; chitin; chitosan; deacetylation; NMR; UV spectrophotometry

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Macromolecular Journals
DOI: 10.1002/macp.200800191