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The use of DSC curves to determine the acetylation degree of chitin/chitosan samples

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Abstract

The use of DSC curves is proposed as an alternative method to determine the degree of *N*-acetylation (DA) in chitin/chitosan samples, based in both peak area and height of the decomposition signal. Samples with DA from 74 to 16% were prepared from a chitin commercial sample and the DA was determined by ^{1}H NMR, ^{13}C CP/MAS NMR and IR spectra. The effect of water content, heating rate, sample mass and gas flow on the DSC peaks were evaluated and optimized. Using optimized conditions a linear relationship between peak area and height with the DA could be achieved with linear correlation coefficients of −0.998 and −0.999 ($n = 7$), respectively. The calibration graphs were used to determine the DA of a commercial chitosan sample with relative errors ranging from 2 to 3% for both peak area and peak height, when compared with the DA determined by ^{1}H NMR method.

Keywords: DSC; Chitin; Chitosan; Acetylation degree

1. Introduction

Chitin is a natural, biodegradable and non-toxic polysaccharide widely spread among marine, land invertebrates and fungi. It is usually obtained from waste materials of the sea food-processing industry, mainly crab, shrimp, lobster and krill shells [1]. *Chitosan* is much less widespread in biomass being produced mainly by thermochemical alkaline deacetylation of acetamide group at the C-2 position in the 2-acetamido-2-deoxy-D-glucopyranose unit [2].

Chemically, chitin and chitosan are closely related since both are linear polysaccharides containing 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN) units joined by β(1 → 4) glycosidic bonds. They can be distinguished by their contents of the above-mentioned units [1,3] as represented in Fig. 1.

The proportion of GlcNAc in relation to the GlcN units is defined as the degree of *N*-acetylation (DA) of the biopolymer, an important parameter which provides not only its physical–chemical properties [4–6] but also its biological [7], biomedical [8] and food [9,10] applications, among others. These applications are remarkably dependent on the physical and chemical properties of the chitin and/or chitosan, such as solubility, strongly dependent of the DA. In this sense the accurate determination of the DA is a very important issue in order to define the applications of such materials.

For some authors the name chitosan means that the DA is around (or lower) than 0.5 with the predominance of 2-amino-2-deoxy-D-glucopyranose residues whose solubility in acidic medium is controlled by these units and the distribution of the acetyl groups remaining along the chain [11,12] while chitin relates to compound with DA > 0.5.

Usually, a single technique cannot be adopted to cover the full range of DA, i.e. for chitin as well as for chitosan. For chitin, due to the lack of solubility, ^{13}C CP/MAS NMR [3,4,13,14] and infrared spectroscopy [15–19] can be used. For chitosan, soluble in aqueous acidic media, other methods are available such as: potentiometric and conductometric titrations [4,13,20], ^{1}H NMR [1,3,4,11,21], gel-permeation chromatography [22], ultraviolet spectrometry [23,24] and infrared spectroscopy [15–19] among others. All these methods present advantages and difficulties to be performed.

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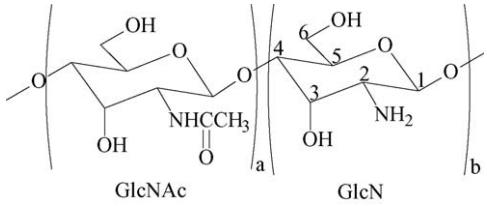

Fig. 1. Chemical structure of (a) 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and (b) 2-amino-2-deoxy-D-glucopyranose (GlcN) units joined by β(1 → 4) glycosidic bond. The a and b contents distinguish chitin from chitosan [1,3].

Thermal methods, such as themogravimetry (TG), differential scanning calorimetry (DSC) and differential thermal analysis (DTA) have emerged as powerful thermoanalytical techniques to monitor characteristic physical and chemical changes in biopolymers, including DA determination [25–29].

Kittur et al. [29] proposed the use of DSC data in order to determine the DA of the chitin and chitosan samples. The method is based on the exothermic degradation peak observed for chitin and chitosan samples, which changes in temperature, area and intensity depending on the DA. Although the authors described properly how the water behaves in these samples during the heating process, they did not issued the influence of thermoanalytical parameters and water content of the samples in the DA determination results. The water content is highly dependent on the sample treatment and conservation. In addition, they have not evaluated the reliability of their results in relation to a reference technique, such as ^{1}H NMR.

The main purpose of the present work is to investigate the dependence of the exothermic peak area and height regarding the amine (GlcN) groups decomposition in DSC curves in order to determine the DA in chitin/chitosan samples comparing the results with reference techniques. However, parameters such as heating rate, sample mass and water content should be optimized to reach this objective.

2. Experimental

2.1. Chitosan purification

The chitosan from crab shells used in this work was a commercial product of medium molecular weight (Aldrich, USA) of technical grade. The purification was attained by the dissolution of the crude commercial product (approximately 1 g) in 300 mL of dilute 0.5 mol L^{-1} acetic acid solution. The dissolution of the polysaccharide was assured by stirring the initial suspension during 18 h and precipitated in the hydrogel form by carefully adding concentrated NH_4OH. The chitosan hydrogel was washed with water until neutrality followed by ethanol. The final product was dried at 60 °C under reduced pressure and freeze dried. The sample was kept under reduced pressure in a desiccator over silica gel [1].

2.2. Chitin purification

The α-chitin from crab shells used here was also a technical grade reagent (Aldrich) which was initially demineralized in 0.25 mol L^{-1} HCl at 25°C during 15 min. The deproteinization step was performed by adding 15 mL of 1.0 mol L^{-1} NaOH per gram of dried powder, at 25 °C during 12 h under nitrogen atmosphere [3].

2.3. Heterogeneous N-deacetylation of α-chitin

Typically, 3 g of dried α-chitin powder was suspended with 70 mL of 50% NaOH (m/v) heated at 100 °C in a round-bottom flask for different reaction times from 60 to 360 min under nitrogen atmosphere. The insoluble deacetylated chitin/chitosan fractions were extensively washed with distilled water until the conductivity of the washings was close to that of the water. The excess of humidity was eliminated at 60 °C under reduced pressure overnight and freeze dried. The sample was kept under reduced pressure in a desiccator over silica gel [2].

2.4. Differential scanning calorimetry

Differential scanning calorimetry measurements were performed in a DSC-910 modulus coupled to a TGA-2100 thermal analyzer (both from TA Instruments, USA). The DSC curves were performed under dynamic nitrogen atmosphere (50–100 mL min^{-1}) using different sample mass as 2–5 mg and heating rates 5, 10 and 20 °C min^{-1}. Accurately weighed samples (±0.1 mg) were placed into a covered aluminum sample holder with a central pin hole. Indium metal (99.99%) has been used to calibrate the DSC modulus in relation to temperature and enthalpy. An empty sample holder was used as reference and the runs were performed by heating the samples from 25 up to 110° with an isothermal for 15 min. The samples were reweighed and heated from 25 up to 480 °C.

2.5. Infrared spectroscopy

The infrared spectra were recorded in an MB102 FT-IR spectrophotometer (Bomen-Michelson, Canada). The polysaccharide sample (2 mg) was dried overnight at 60 °C under reduced pressure and mechanically well blended with 100 mg of KBr in agate mortar. The pellets were desiccated for 24 h at 110 °C under reduced pressure and the IR spectra were recorded, against KBr disk as a blank, by accumulation of at least 64 scans with a resolution of 2 cm^{-1}. The intensity of maxima of the IR absorption band was determined by the baseline method [15–17].

2.6. Nuclear magnetic resonance

The solution ^{1}H NMR spectra were measured in a Brucker AMX 400 spectrometer (Germany). For these measurements, a suspension of 10 mg of chitosan samples with 1 mL of D_2O/HCl (100:1, v/v) solution was prepared and stirred for 15 h. All the measurements were performed at 70 °C, using 16 scans pulse accumulation and the LB parameter of 0.30 Hz. The spectral width and data points were 8000 Hz and 32 K points, respectively.

Solid-state ^{13}C CP/MAS NMR experiments were carried out on a Varian INOVA spectrometer at a resonance frequency of 75.43 MHz for ^{13}C nuclei (USA). Chitin samples, 200 mg, in powder form were loaded into a zirconium oxide rotor spinning at the magic angle of 4.5 kHz. The contact time and recycle delay were set at 1.8 ms and 13 s, respectively. Spectra were referenced indirectly to a zero value for tetramethylsilane (TMS).

3. Results and discussion

3.1. Preparation of chitin/chitosan samples with known DA values

The first step in the present work was the preparation of a set of samples with different, but well-known, DA values ranging from chitin to chitosan.

The packing structure of α-chitin is strongly stabilized by intra-chain, intra-sheet and inter-sheet hydrogen bonds in the three unit cell directions [3]. This structure requires high concentration of alkali and prolonged reaction time for thermochemical deacetylation leading to sparsely as well as non-uniformly accessible block copolymers of 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose residues, i.e. a chitin/chitosan samples whose DA depends on the reaction time [4]. Chitin can underwent an important alkaline hydrolysis of the β-1,4 linkage when submitted to severe deacetylation conditions. The use of an inert atmosphere such as nitrogen has been proposed to minimize this depolymerization that consequently affects the mean molecular weight [2,3].

The nuclear magnetic resonance and infrared spectra were adopted as reference techniques in determining the degree of *N*-acetylation for the samples.

For chitin, Fig. 2a, DA is calculated by the exact ratio of 1/6 between the integrated areas of the methyl-C atom of the *N*-acetyl group (δ 22 ppm) and the C2–C6 atoms (δ 50–105 ppm) of the D-glucopyranosyl ring from solid ^{13}C CP/MAS NMR spectrum [4].

For chitosan, Fig. 2b, DA is deduced from exact ratio of 1/3 between the integrated resonances of the methyl proton signal of the 2-acetamido-2-deoxy-D-glucopyranose (δ 2.04 ppm) and the proton at C-2 position in the 2-amino-2-deoxy-D-glucopyranose (δ 3.21 ppm) obtained from solution ^{1}H NMR spectrum [1].

The DA for chitin samples was also calculated by infrared spectroscopy as illustrated in Fig. 3. For this purpose, DA is determined by the baseline drawing for the intensity of the amide I band of the acetyl groups at 1655 cm^{-1} (A_{1655}) with that of the hydroxyl groups at 3450 cm^{-1} (reference band, A_{3450}) following the Eq. (1) [15,16].

$$DA = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \times 115\right] \quad (1)$$

The correct baseline for amide I band, Fig. 3, eliminate the inaccuracy of this ratio at low levels of *N*-acetylation covering the approximate range of 0–55% as described. The method is not available for samples with lower acetyl contents, DA < 55%,

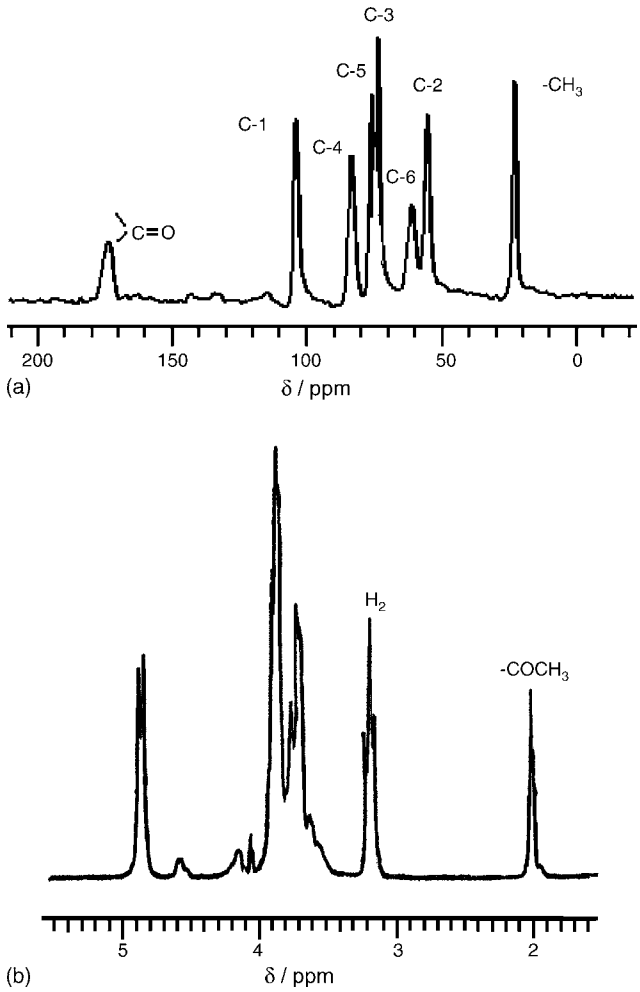

Fig. 2. (a) Typical spectra ^{13}C CP/MAS NMR of chitin and (b) ^{1}H NMR of chitosan obtained after 360 min of heterogeneous deacetylation of chitin.

in which the deconvolution of this band cannot be observed [15,16].

Although the use of empiric equations simplifies the analytical method, the invariability in sample preparation, water

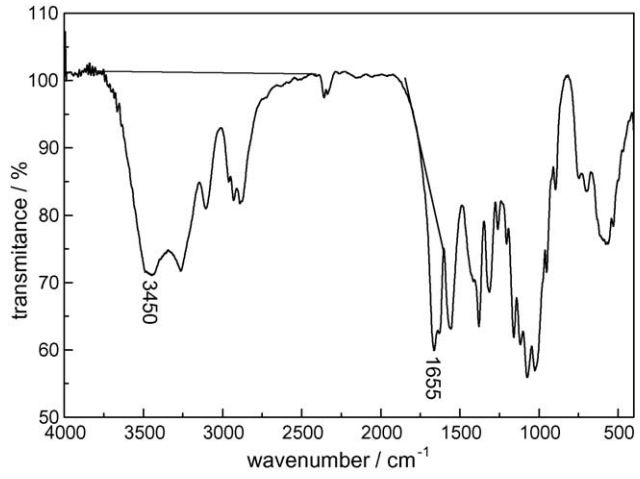

Fig. 3. Typical infrared spectrum for chitin sample. Baseline drawing for determines its acetyl content.

Table 1
Degree of *N*-acetylation determined by three techniques to chitin/chitosan samples obtained after different reaction times

Reaction time (min)	DA (%)		
	^{13}C CP/MAS NMR	^{1}H NMR	IR spectra
0.0	98.5	–	97
60.0	74.6	–	74
90.0	74.0	–	72
120.0	69.4	–	70
150.0	51.5	–	–
180.0	44.1	43.3	–
240.0	–	19.6	–
360.0	–	15.9	–

content, baseline drawing, instrument type and the experimental conditions are essential in this determination, otherwise expected accuracy is not achieved [18].

For both techniques, the values of acetyl contents showed good agreement in the chitin/chitosan samples varying the time of thermochemical alkaline deacetylation reaction in α-chitin. As presented in Table 1, the DA decreases with the reaction time.

3.2. DSC curves for chitosan and chitin

Fig. 4 depicts the DSC curves for commercial purified chitosan, DA = 15.9%, at different heating rates. In all these curves, an endothermic peak at 70 °C can be ascribed to the loss of water. The second thermal event may be related to the decomposition of amine (GlcN) units with correspondent exothermic peak at 295 °C [29]. The DSC curves at 10 and 20 °C min^{-1}, Fig. 4b and c, showed that these thermal events are present at characteristic peaks temperatures and heights that change with heating rate.

The commercial chitosan was used as a probe for evaluating the effects of gas flow (50–100 mL min^{-1}), sample mass (2–5 mg) and heating rate (5, 10 and 20 °C min^{-1}) on the repeatability of peak area and height of the exothermic event regarding the amine (GlcN) groups decomposition.

The integration of the peak area is not possible to be performed by the linear extrapolation procedure, thus the sigmoid baseline is proposed [30]. The peak height is measured by the difference between the heat flow at the peak and at the DSC curve baseline.

The influence of gas flow and sample mass on the repeatability are not significant, thus 50 mL min^{-1} and 3 mg was used, respectively, since it provides a noise free baseline. Otherwise, the heating rate did not influence the peak area, but presented a remarked influence in peak height. Although the 20 °C min^{-1} presented much higher values as expected, the resolution and repeatability are better at 5 °C min^{-1} as demonstrated by the data in Table 2. Thus, a heating rate of 5 °C min^{-1} was fixed for further experiments in order to relate the peak area and height with DA of the chitin/chitosan samples obtained in our laboratory.

However, in order to reach an acceptable repeatability, the water content should be normalized; otherwise, puzzled results are obtained despite the use of the optimized thermoanalytical parameters above. In samples highly hydrated, a broad endothermic peak due to the dehydration is expected decreasing the sample mass significantly before the amine (GLcN) groups decomposition. Under this conditions, erroneous values for both peak area (J g^{-1}) and height (W g^{-1}) has been obtained because they are related to the sample mass input at the start of the experiment and not to that anhydrous mass ideally necessary for correct determinations. Totally anhydrous samples have been hardly obtained due to the high water affinity presented by these polysaccharides, and then water normalization should overcome this difficult achieving the like-ideal condition.

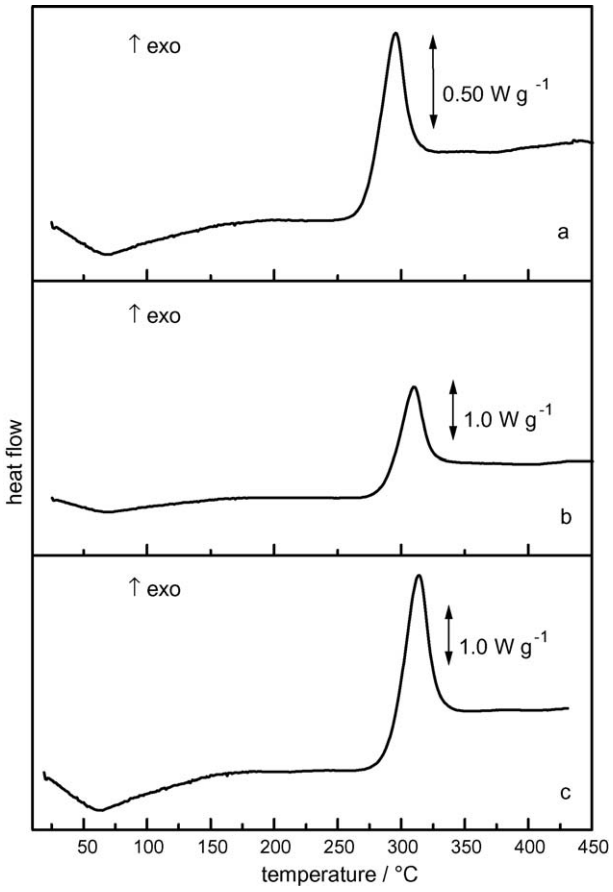

Fig. 4. DSC curves for chitosan, DA 15.9%, under dynamic nitrogen atmosphere (50 mL min^{-1}), sample mass 3 mg at heating rates of (a) 5 °C min^{-1}, (b) 10 °C min^{-1} and (c) 20 °C min^{-1}.

For the chitin/chitosan samples used here, the conservation, experimental and thermoanalytical conditions employed were adequate to reach this objective indicated by a constant ΔH for endothermic dehydration peak in DSC curves. This thermal

Table 2
Repeatability of peak area and height for chitosan, DA = 15.9%, at different heating rates

Heating rate (°C min^{-1})	Peak area (J g^{-1})[a]	Peak height (W g^{-1})[a]
5.0	207.4 ± 4.51	0.951 ± 3.22
10.0	205.1 ± 12.4	1.91 ± 11.3
20.0	208.6 ± 13.2	3.38 ± 12.7

[a] Mean ± standard deviation (n = 6).

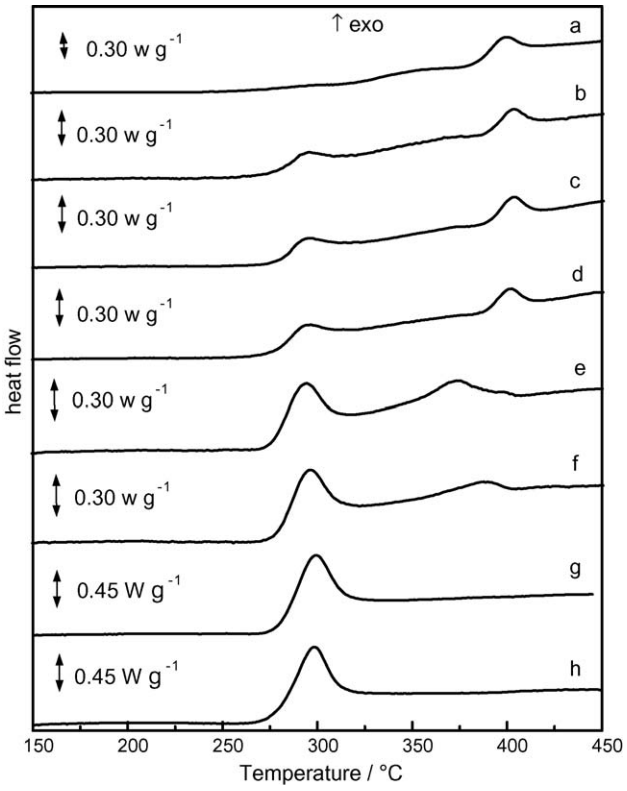

Fig. 5. DSC curves under nitrogen atmosphere (50 mL min^{-1}), sample mass 3 mg at 5 °C min^{-1} for chitin/chitosan samples obtained at different reaction times of thermochemical heterogeneous deacetylation: (a) 0 min, (b) 60 min, (c) 90 min, (d) 120 min, (e) 150 min, (f) 180 min, (g) 240 min and (h) 360 min.

event can be associated to the evaporation into free state of bound water away from the influence of polymer network due to the high temperature (110 °C) that revert to bound state on cooling (25 °C). It has been shown to remain relatively a constant event even after successive cycles of heating and cooling, typical for water associated with the hydrophilic groups in the amorphous regions of chitosan chains, i.e. non-freezing bound water [28,29].

When the chitin/chitosan polysaccharides obtained in our laboratory were analyzed under the optimized thermoanalytical conditions described above, the repeatability for peak area and height are closed to those shown in Table 2.

Fig. 5 shows the DSC curves for these samples with different DA. The thermal events are based on DSC curves for chitosan, Fig. 4, whose exothermic peak at 295 °C was properly attributed to the decomposition of the high content of amine (GlcN) groups.

The α-chitin, Fig. 5a, shows the exothermic peak at 400 °C due to the decomposition of acetyl (GlcNAc) units that predominates in this structure. For the polysaccharide obtained after alkaline deacetylation up to 60 min, Fig. 5b, two exothermic peaks are observed at 296 and 404 °C related to the thermal decomposition for amine (GlcN) and acetyl (GlcNAc) residues, respectively, characteristic for chitin/chitosan sample. Thus, the amine residues are termally less stable than acetyl ones. Similar thermal behavior has been showed for chitin/chitosan samples obtained between 90 and 180 min, Fig. 5b–f. With higher deacetylation reaction time, such as 240 and 360 min, the DSC curves are typical for chitosan samples with exothermic peaks at 299 and 298 °C, respectively, Fig. 5g and h. The exothermic peak at higher temperature is practically not observed due to the low acetyl content in these samples.

It is notorious that the peak area and peak height ascribed to the amine (GlcN) groups increase and the acetyl (GlcNAc) residues decrease while the thermochemical alkaline deacetylation proceeds. The values and temperatures for peak area and height of the exothermic event regarding to the amine (GlcN) groups decomposition are presented in Table 3. The temperature intervals used to integrate the peak areas in defined in this table.

3.3. Degree of N-acetylation and DSC curves

A plot of the DSC peak areas against the degrees of *N*-acetylation resulted in a linear relationship that obeys the Eq. (2).

$$y = 257.98 - 3.25 \times (r = -0.998;\ n = 7) \quad (2)$$

In which, y is the peak area (J g^{-1}) and x is the DA (%) of the samples we have prepared.

Similarity, a plot of DSC peak heights against the degrees of *N*-acetylation resulted in a linear relationship that obeys the Eq. (3).

$$y = 1.13 - 0.0120 \times (r = -0.999;\ n = 7) \quad (3)$$

In this case, y is the peak height (W g^{-1}) and x is the DA (%) of the samples we have prepared.

Considering a commercial chitosan as a reference sample, the interpolation of the peak area (207.4 J g^{-1}) and peak height (0.951 W g^{-1}) values on these calibration curves yields the

Table 3
DSC peak areas, peak heights and the temperatures used in these determinations regarding the amine (GlcN) residues decomposition for chitin/chitosan samples with different degree of acetylation

Degree of acetylation (%)[a]	Interval temperature (°C)	Peak temperature (°C)	Peak area (J g^{-1})	Peak height (W g^{-1})
74.3	268–312	296	20.74	0.237
73.0	267–315	295	24.31	0.258
69.7	266–317	297	27.14	0.293
51.5	265–319	294	85.76	0.511
43.7	265–323	297	114.7	0.593
19.6	264–325	299	199.5	0.896
15.9	263–328	298	204.4	0.945

[a] Mean values from different techniques when employed.

degree of *N*-acetylation in 15.6 and 14.9%, respectively, closed to that 15.9% calculated by ^{1}H NMR spectroscopy considered an absolute technique.

These results emerged the DSC as a powerful technique to determine the degree of *N*-acetylation in chitin/chitosan samples since a calibration curve has been established, independently of its composition and molecular weight due the heterogeneous alkaline treatment.

The main advantages are the possibility of determining the DA in a wide range including both chitin and chitosan forms, low sample mass usage, no need of long time solubilization, low cost in relation to NMR and higher precision in relation to IR.

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References

[1] R. Signini, S.P. Campana-Filho, Polym. Bull. 42 (1999) 159.
[2] M.L. Tsaih, R.H. Chen, J. Appl. Polym. Sci. 88 (2003) 2917.
[3] G. Lamarque, C. Viton, A. Domard, Biomacromolecular 5 (2004) 992.
[4] A. Tolaimate, J. Desbrières, M. Rhazi, A. Alagui, M. Vincendon, P. Vottero, Polymer 41 (2000) 2463.
[5] W. Wang, S. Bo, S. Li, W. Qin, Int. J. Biol. Macromol. 13 (1991) 281.
[6] M. Rinaudo, M. Milas, P. Le Dung, Int. J. Biol. Macromol. 15 (1993) 281.
[7] C. Chatelet, O. Damour, A. Domard, Biomaterials 22 (2001) 261.
[8] S.S. Koide, Nutr. Res. 18 (1998) 1091.
[9] F. Shahidi, J.K.V. Arachchi, Y.-J. Jeon, Trends Food Sci. Technol. 10 (1999) 37.
[10] L.F. Del Blanco, M.S. Rodrigues, P.C. Schulz, E. Agulló, Colloid Polym. Sci. 227 (1999) 1087.
[11] P. Le Dung, M. Milas, M. Rinaudo, J. Desbrières, Carbohydr. Polym. 24 (1994) 209.
[12] K. Kurita, M. Kamiya, S.-I. Nishimura, Carbohydr. Polym. 16 (1991) 83.
[13] L. Raymond, F.G. Morin, R.H. Marchessault, Carbohydr. Polym. 246 (1993) 331.
[14] M.G. Peter, L. Grun, H. Forster, Angew. Chem. Int. Ed. Engl. 23 (1984) 638.
[15] M. Miya, R. Iwamoto, S. Yoshikawa, S. Mima, Int. J. Biol. Macromol. 2 (1980) 323.
[16] A. Baxter, M. Dillon, K.D.A. Taylor, G.A.F. Roberts, Int. J. Biol. Macromol. 14 (1992) 166.
[17] Y. Shigemasa, H. Matsuura, H. Sashiwa, H. Saimoto, Int. J. Biol. Macromol. 18 (1996) 237.
[18] S. Sabnis, L.H. Block, Polym. Bull. 39 (1997) 67.
[19] J. Brugnerotto, J. Lizard, F.M. Goycoolea, W.A. Monal, J. Desbrières, M. Rinaudo, Polymer 42 (2001) 3569.
[20] H. Terayama, J. Polym. Sci. 8 (1986) 173.
[21] A. Hirai, H. Odani, A. Nakajima, Polym. Bull. 26 (1991) 87.
[22] S.-I. Aíba, Int. J. Biol. Macromol. 8 (1986) 173.
[23] S.C. Tan, C. Khan, T.K. Tan, S.M. Wong, Talanta 45 (1998) 713.
[24] R.A.A. Muzzarelli, R. Rocchetti, Carbohydr. Polym. 5 (1985) 461.
[25] I.G. Alonso, C. Peniche-Covas, J.M. Nieto, J. Therm. Anal. 28 (1983) 189.
[26] J.M. Nieto, C. Peniche-Covas, Thermochim. Acta 176 (1991) 63.
[27] T.G. Cárdenas, A.L. Bernal, D.H.L. Tagle, Thermochim. Acta 195 (1992) 33.
[28] J. Ratto, T. Hatakeyama, R.B. Blumstein, Polymer 36 (1995) 2915.
[29] F.S. Kittur, H. Prashanth, K.U. Sankar, R.N. Tharanathan, Carbohydr. Polym. 49 (2002) 185.
[30] J.E. Santos, E.R. Dockal, E.T.G. Cavalheiro, J. Therm. Anal. Cal. 79 (2005) 243.