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Determination of the degree of acetylation (DA) of chitin and chitosan by an improved first derivative UV method

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Abstract

An economical and accurate determination of degree of acetylation (DA) for highly acetylated chitin has always been a challenge for researchers dealing with chitin and chitosan. A new protocol for the first derivative UV method using concentrated phosphoric acid as a solvent for highly acetylated chitin was developed in this study. The solvent was proposed based on thorough investigation of the effects of associated reactions including chain degradation, monomer dehydration, and oxazolinium ion formation. The reproducibility and performance of the new protocol was evaluated using commercial samples and the results showed the DA values of both chitin and chitosan could be determined accurately by a single analytical technique in less than 3 h.

Keywords: Degree of acetylation; Chitin; Chitosan; First derivative UV method; Method development

1. Introduction

Interest in application of chitin and its derivative chitosan in the food industry and biomedicine is constantly increasing (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). The degree of acetylation (DA) is a key parameter that influences the physico-chemical properties of chitin and chitosan, such as solubility, chain conformation (Berth & Dautzenberg, 2002), electrostatic properties (Sorlier, Denuziere, Viton, & Domard, 2001) and biological properties of chitosan films (Chatelet, Damour, & Domard, 2001). Numerous analytical methods, including Fourier transform infrared spectroscopy (FTIR) (Duarte, Ferreira, Marvao, & Rocha, 2002; Van de Velde & Kiekens, 2004), high performance liquid chromatography (HPLC) (Frederic, Nuria, Chornet, & Vidal, 1993), nuclear magnetic resonance (NMR) (Duarte, Ferreira, Marvao, & Rocha, 2001; Lavertu et al., 2003), titration (Jiang, Chen, & Zhong, 2003; Raymond, Morin, & Marchessault, 1993) and ultraviolet–visible (UV) adsorption spectroscopy (Muzzarelli & Rocchetti, 1985) have been proposed to precisely determine its value. However, all the methods have some limitations. For example, expensive instruments are needed in case of FTIR, NMR and HPLC methods, extensive sample preparation is required for HPLC, or accuracy is insufficient in titration and IR procedures. The first derivative UV method developed in late 1980s, offered a simple and fast measurement of DA value with good accuracy and precision (Muzzarelli & Rocchetti, 1985). Although zero order of UV spectra can be used for DA measurement as well (Hsiao, Tsai, Chen, Hsieh, & Chen, 2004), the first derivative of the spectra is less affected by the background noise and impurities and has been suggested as a standard method for routine determination of DA of chitosan (Tan, Khor, Tan, & Wong, 1998). Several modified first derivative UV methods have been proposed to improve the convenience and accuracy of the measurement (Liu, Wei, Yao, & Jiang, 2006; Pedroni, Gschaider, & Schulz, 2003). However, those methods use diluted acetic or hydrochloric acid to dissolve chitosan prior to analysis.

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This unavoidably limits the determination scope to highly deacetylated chitosan samples only, due to their solubility in diluted acids.

For highly acetylated chitin, concentrated phosphoric acid has been proposed as a good solvent and the UV determination appears to be valid in the whole range of DA (Hsiao et al., 2004). Additionally, the results obtained by this method are well correlated with a solid state ^{13}C NMR method, so far the best but the most expensive method for the DA analysis (Hsiao et al., 2004). However, polysaccharides generally undergo complex reactions under the conditions of concentrated acids and heat. For example, cellulose can be hydrolyzed into glucose and consequently converted into 5-hydroxymethylfurfural (HMF) and levulinic acid by acid catalyzed dehydration (Girisuta, Janssen, & Heeres, 2007). Compared to these well investigated reactions of cellulose, less information is available regarding the effects of hot concentrated acid on chitin and chitosan. One study has reported the formation of HMF from fully deacetylated chitosan after nitrous acid depolymerization (Tommeraas, Varum, Christensen, & Smidsrod, 2001). Apparently, the formation of HMF can introduce errors in the DA measurement because both acetyl-glucosamine and glucosamine can be converted to HMF.

An intermediate glucofuranosyl oxazolinium ion of acetyl-glucosamine and similar products have been found in chitin solutions in concentrated phosphoric acid and anhydrous hydrogen fluoride (Bosso, Defaye, Domard, Gadelle, & Pedersen, 1986; Vincendon, 1997). The intermediate ion is not stable and can be hydrolyzed into monosaccharide phosphate in diluted acids (Bosso et al., 1986; Vincendon, 1997). Apparently, the formation and hydrolysis of such intermediate ions affect the DA measurements due to blocking or liberating acetyl group (Fig. 1). In addition, chitin, chitosan, and acetyl-glucosamine may undergo acid deacetylation what would result in underestimated DA (Gizatulina, Chebotok, Novikov, & Konovalova, 2005).

Therefore, without thorough investigation of these possible reactions, DA determination method employing concentrated phosphoric acid as a solvent for chitin and chitosan should be used with caution. The objectives of this study were (1) to evaluate the effects of chemical reactions associated with utilization of phosphoric acid as a solvent on the DA determination by first derivative UV method, and (2) to improve the method so it can be used for quick and accurate determination of whole range of DA values.

2. Experimental details

2.1. Materials and instruments

Acetyl-glucosamine (GlcNAC), D-glucosamine hydrochloride (GlcN) and 85% phosphoric acid were purchased from Sigma (St. Louis, MO). Chitin and chitosan samples were provided by Primex (Primex, Iceland). A Shimadzu 2010 (Shimadzu, Columbia, MD) double beam UV–vis spectrophotometer was used to collect the UV spectra of standards and samples under scan mode in the range of 400–190 nm. Sampling interval and slit width were both set at 1.0 nm. Far UV cuvettes with 10 mm pathway length were used for all samples. UV Probe software (Shimadzu) was applied to calculate the first derivative spectra in the range of 190–220 nm.

2.2. Standard preparation and formation of standard curve

Standard solutions of GlcNAc and GlcN were prepared in 0.85% phosphoric acid at concentrations of 0, 10, 20, 30, 40 and 50 μg/ml. The calibration curve was made by plotting the first derivative UV values at 203 nm (H_{203}) as a function of GlcNAc and GlcN concentration.

2.3. Sample preparation and the DA determination

Chitin and chitosan samples were ground using Thomas Wiley Mini-Mill with sieve #40 (Thomas Scientific, Swedesboro, NJ) and stored in desiccators at room temperature until analysis. A 3-step procedure was used to prepare a sample for the DA determination (Fig. 2). Aliquots of 100 ± 10 mg chitin or chitosan were heated in 20 ml 85% phosphoric acid for 40 min at 60 °C with constant stirring. After 40 min, when chitin/chitosan was completely dissolved, 1 ml clear solution was taken and diluted to 100 ml with deionized water. The dilution was necessary to get the chitin/chitosan concentration to the range detectable by a spectrophotometer. The diluted solutions were incubated at 60 °C for 2 h prior the UV measurement. This was considered as a standard method. These parameters were applied in all experiments if not otherwise explained.

2.4. DA calculation method

The degree of acetylation of chitin and chitosan samples was calculated as:

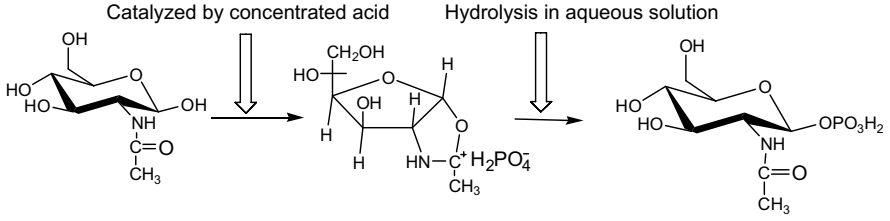

Fig. 1. Formation and hydrolysis of glucofuranosyl oxazolinium ion from acetyl-glucosamine. *Adapted from (Vincendon, 1997).

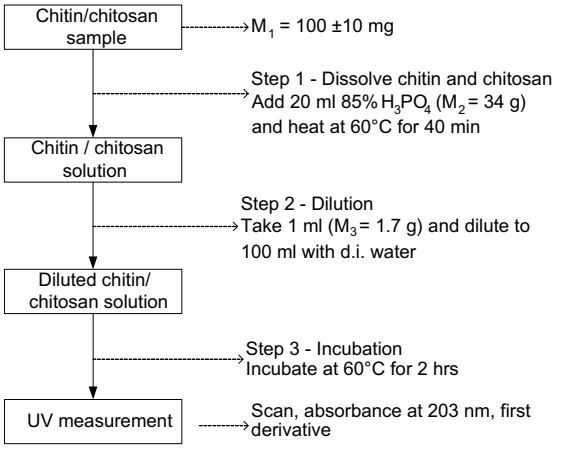

Fig. 2. Diagram of the three-step procedure for determination the degree of acetylation (DA) values for chitin and chitosan.

$$\mathrm{DA}(\%) = \frac{\frac{m1}{203.21} \times 100}{\frac{m1}{203.21} + \frac{m2}{161.17}}$$

where: $m1$ is the mass of acetyl-glucosamine in 1 ml chitin/chitosan solution (Step 2; Fig. 2), calculated from the calibration curve by the corresponding H_{203}; $m2$ is the mass of glucosamine in 1 ml chitin/chitosan solution (Step 2; Fig. 2), calculated as $m2 = M - m1$. The mass of chitin/chitosan (M) in the 1 ml solution (Step 2; Fig. 2) was calculated as: $M = (M_1 \times M_3)/(M_1 + M_2)$, where M_1 is mass of solid chitin/chitosan sample taken for analysis (100 ± 10 mg; Fig. 2); M_2 is mass of 20 ml 85% phosphoric acid (Step 1; Fig. 2); and M_3 is mass of 1 ml chitin/chitosan solution in concentrated phosphoric acid (Step 2; Fig. 2).

2.5. Effects of chitin/chitosan solubilization on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60 °C for 10–40 min. After heating, the samples were diluted as explained earlier and incubated at room temperature for 0, 1, 2, 3 and 4 h before UV scans.

2.6. Effects of oxazolium ion formation on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60 °C for 40–180 min. After heating, the samples were diluted, incubated at room temperature for 0, 8 and 24 h before UV scan.

2.7. Effects of oxazolinium ion hydrolysis on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60 °C for 40 min. After heating, the samples were diluted with d.i. water (1–100 ml), and incubated at room temperature or 60 °C for 0–24 and 0–5 h, respectively before UV measurements.

2.8. Effects of dehydration reactions on DA determination

Chitin and chitosan samples were dissolved in conc. phosphoric acid and heated at 60 or 80 °C for 40, 60, and 100 min. After the heating, 5 ml sample solutions were diluted to 100 ml with d.i. water and the UV scans were immediately taken.

2.9. Effect of concentrated phosphoric acid on acid deacetylation reaction

Acetyl-glucosamine samples (100 ± 10 mg) were dissolved in conc. phosphoric acid and heated at 60 °C for 40 min. After heating, the samples were diluted with d.i. water (1–100 ml), and incubated at 60 °C for 2 h before UV measurements. The recovery rate was calculated as the ratio of determined and originally used acetyl-glucosamine amounts.

3. Results and discussion

3.1. Standard spectra and calibration curve

The principle of the first UV derivative method for the DA determination is based on the absorbance intensity of acetyl group in chitin or chitosan (Muzzarelli & Rocchetti, 1985). Although acetyl-glucosamine absorbs in the range of 190–220 nm, the minimum interferences from glucosamine on the first derivative of acetyl-glucosamine was between 202 and 208 nm (Fig. 3). By plotting the first derivative UV values at those wavelengths against the concentrations of GlcNAc to perform linear regression analysis, the best linear regression was obtained at 203 nm. Thus, the first derivative value at 203 nm was chosen for DA measurements in this study and was represented by the symbol of H_{203}. A similar wavelength selection was made in a previous study where 202 nm was chosen when acetic acid was

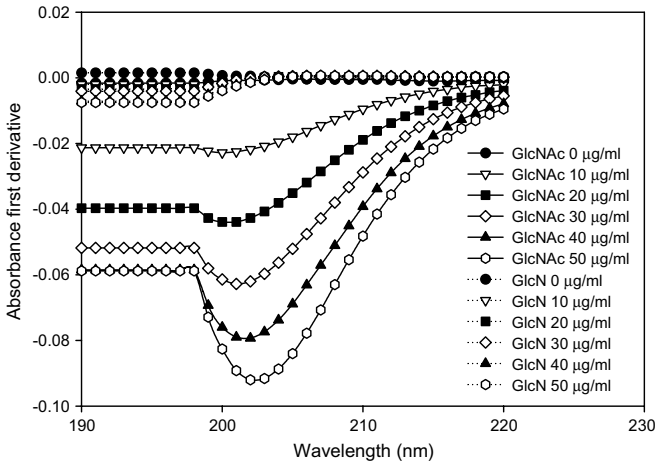

Fig. 3. First derivative UV spectra of acetyl-glucosamine (GlcNAc) and glucosamine (GlcN) standards at concentrations ranged from 0 to 50 µg/ml.

used as a solvent (Muzzarelli & Rocchetti, 1985). A plot of H_{203} values against the concentrations of *N*-acetyl-D-glucosamine in the range of 0–50 μg/ml resulted in a good linear regression with the R^2 value of 0.996 (Fig. 4). The H_{203} of glucosamine was also plotted against its concentration in the same graph clearly showing that the presence of glucosamine at the tested concentrations did not impose noticeable interference to the calibration curve of acetylglucosamine (Fig. 4).

3.2. Effects of chitin/chitosan solubilization on DA determination

Although chitin/chitosan samples visually appeared to be dissolved in conc. phosphoric acid after a few minutes or so by heating at 60 °C (Fig. 2, Step 1), the determined DA values of the samples heated for 10 min were reasonable only if the UV scans were taken immediately after dilution (Fig. 2, Step 3). If the diluted samples were incubated for an hour or longer, appearance of white haze was observed, and the resulting DA values were significantly lower (Fig. 5). For samples dissolved by heating for 20 min, the DA values were correctly determined in samples incubated up to 1 h after dilution. If the chitin samples were heated for 30–40 min in conc. phosphoric acid, the samples stayed clear under conditions examined in this study, and the DA values were the same regardless of how long the diluted samples were incubated (Fig. 5).

These results indicated that if the chitin was not completely dissolved in conc. phosphoric acid, it re-aggregated and precipitated out in diluted solutions causing underestimation of DA. Therefore, unless all samples can be analyzed in the exact time immediately after dilution (what is possible only if there is no more than one sample), minimum of 30 min of heating is necessary to achieve an accurate DA determination.

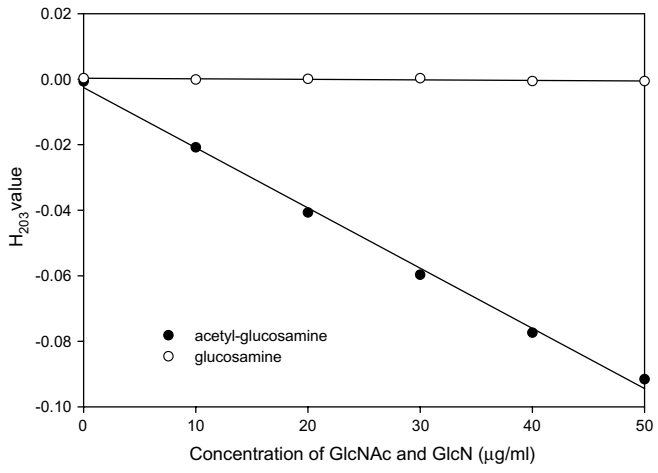

Fig. 4. Plot of H203* values (the first derivative value at 203 nm) against concentrations of glucosamine (GlcN) and acetyl-glucosamine (GlcNAc).

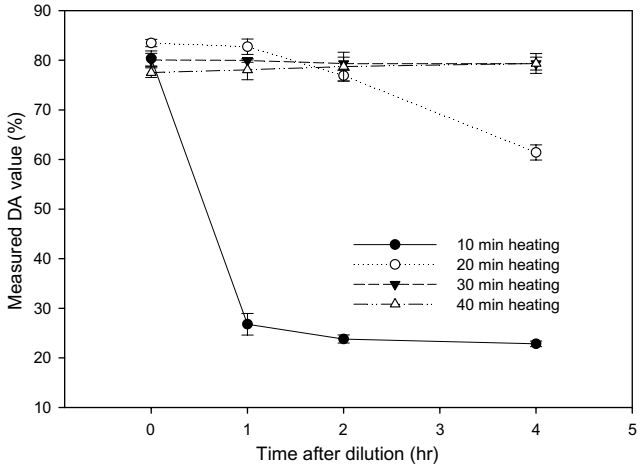

Fig. 5. Variation of degree of acetylation (DA) values determined for the same chitin sample dissolved by heating at 60 °C for 10–40 min and incubated for 0–4 h after dilution of the chitin solution (values are represented as means ± standard deviation, $n = 3$).

3.3. Effects of glucofuranosyl oxazolinium ion formation and hydrolysis on DA determination

The DA values determined after various length of heating at 60 °C (to help dissolve chitin or chitosan) and different times of incubation after dilution are shown in Fig. 6. The determined DA values for the samples which UV scans were taken immediately after dilution decreased as the time of heating increased over 40 min. This was probably caused by the formation of oxazolinium ions during prolonged heating of chitin/chitosan in conc. phosphoric acid. The oxazolinium ion blocked the acetyl-group (Fig. 1) which resulted in significant reduction of absorbance at 203 nm and, consequently, underestimated DA value. On the other hand, if the measurements were taken after prolonged incubation of diluted samples (8 and 24 h), the determined DA values were higher than those obtained immediately after

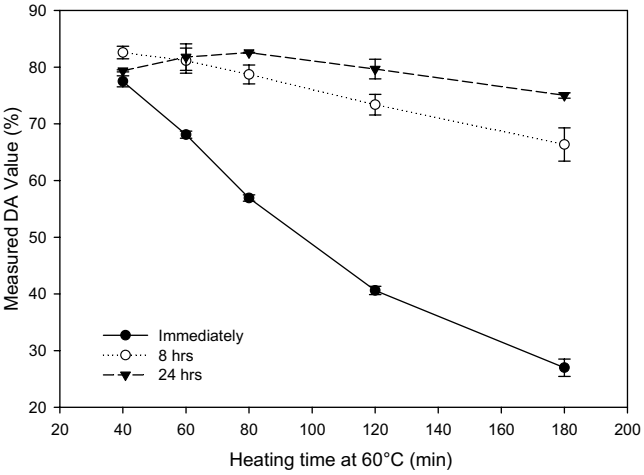

Fig. 6. Degree of acetylation (DA) values of chitin measured immediately, 8 and 24 h after dilution (values are represented as means ± standard deviation, $n = 3$).

dilution. This was probably the result of hydrolysis of the oxazolinium ions in aqueous solutions over time and liberation of the acetyl-groups (Fig. 1). Hsiao et al. made similar observations (Hsiao et al., 2004). However, they oversimplified the fact by saying that the 'sample inhomogeneity' was the reason for the inconsistent results without recognizing potential reactions during the analysis.

The hydrolysis of oxazolinium ions was accelerated by incubating the diluted chitin solution at elevated temperature, what significantly reduced analysis time. It can be seen from Fig. 7 that if the diluted chitin solutions were kept at 60 °C, the determined DA values achieved the highest value after 2 h (89.8%, for this sample) while the highest DA value of the samples incubated at room temperature was obtained after 4 h, and the value was still significantly lower (79.3%) compared to the values determined after 2 h-incubation at 60 °C.

3.4. Effects of possible dehydration reactions on DA determination

The UV spectra of chitin samples in diluted aq. phosphoric acid are shown in Fig. 8. The samples prepared by dissolving chitin at 60 °C for 40 and 60 min had the characteristic peak at 203 nm relevant to absorbance of the acetyl group. However, if the samples were heated for 100 min, a new peak appeared at 285 nm. This peak was also present in all the samples heated at 80 °C. The area of the peak increased with the temperature and heating time. The maximum absorbance at 285 nm is characteristic for the 5-hydroxymethylfurfural (HMF), a dehydration product of hexoses, including glucosamine and acetyl-glucosamine (Jun, Shao, Ho, Koetter, & Lech, 2003). As the formation of HMF can significantly reduce the number of acetyl-glucosamine units and/or altered the glucosamine/acetyl-glucosamine ratio in chitin or chitosan molecules, its formation during the assay (Step 1: dissolving of polymers, Fig. 2) must be avoided. Therefore, solubilization of chitin and chitosan in conc. phosphoric acid must

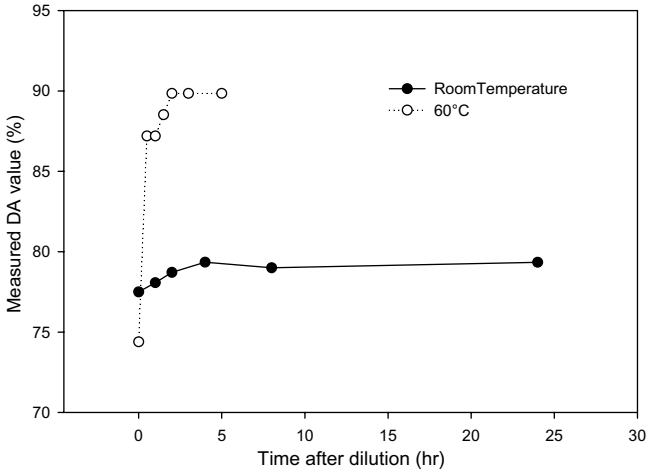

Fig. 7. Effect of incubation temperature (room temperature and 60 °C) on determined degree of acetylation (DA) value.

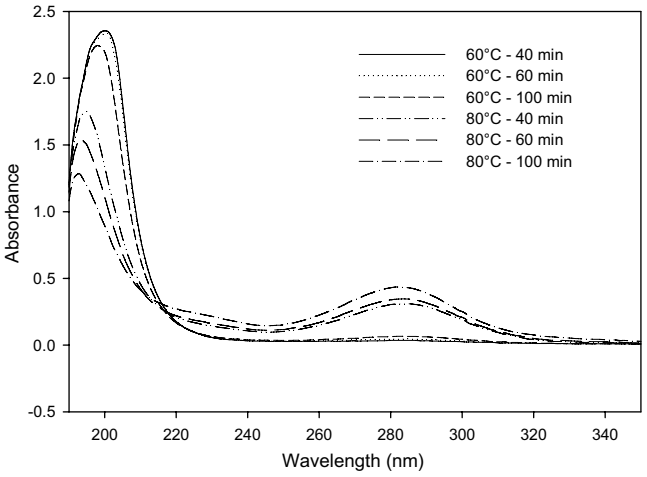

Fig. 8. UV spectra of chitin in diluted phosphoric acid after heating.

be achieved at temperatures not higher than 60 °C for no longer than 60 min.

3.5. Effect of concentrated phosphoric acid on acid deacetylation reaction

The recovery rates of acetyl-glucosamine from concentrated phosphoric acid at various concentrations were over 90% (data not shown) suggesting that acid deacetylation of acetyl-glucosamine occurred at a low level. Therefore, we believe that the acid deacetylation of chitin in concentrated phosphoric acid would be very slow and with no significant impact on the DA determination. Furthermore, it has been reported that acid deacetylation rate of chitin and chitosan in hydrochloric acid decreased when the acid concentration increased over 29.8% (Gizatulina et al., 2005).

3.6. Validity and reproducibility of the method

An accurate and rapid determination of DA values by the proposed first derivative UV method must satisfy two requirements: (1) a complete solubilization of chitin and chitosan in conc. phosphoric acid without significant loss of the monomers due to formation of HMF; (2) a full availability of acetyl groups by complete release of the oxazolinium ions in a short time. These two requirements can be met by dissolving chitin and chitosan samples in conc. phosphoric acid at 60 °C for 40 min and incubating the diluted solutions at 60 °C for 2 h. The reproducibility of new protocol was tested using commercial chitin and chitosan products with known DA values and the results are presented in Table 1. As it can be seen, average error was generally less than 8% and the results correlated well with the values obtained from the manufacturer.

4. Conclusion

An improved first derivative UV method is proposed based on the evaluation of effects of chemical reactions

Table 1
The degree of acetylation (DA) values of commercial samples

Sample	Nominal DA*	Determined DA***
(1) Chitosan	19	20.2 ± 0.1
(2) Chitosan	29	30.3 ± 0.2
(3) Chitosan	39	32.5 ± 0.8
(4) Chitin	85**	88.7 ± 2.6
(5) Water soluble chitosan	Unknown	9.1 ± 0.6

* Provided by manufacturer.
** Determined by FTIR method (Duarte, Ferreira, Marvao & Rocha, 2002).
*** Values are represented as means ± standard deviation ($n = 3$).

possibly associated with the assay. Using the conc. (85%) phosphoric acid as a solvent for both chitin and chitosan, heat treatment at 60 °C for 40 min to enhance solubilization, and incubation of diluted solutions at 60 °C for 2 h, the method enables determination of DA in the whole range. The assay is simple and accurate, does not require expensive equipment, can be performed in less than 3 h, and what is most important, allows use of the same procedure for analysis of both chitin and chitosan.

Acknowledgement

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