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Improved infrared spectroscopic method for the analysis of degree of *N*-deacetylation of chitosan

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Summary

Various infrared spectroscopic techniques for the analysis of degree of N-deacetylation of chitosan were evaluated for accuracy. A new method was proposed which involved development of a calibration curve using the absorbance intensities of the chitosan infrared spectrum at 1655 and 3450 cm^{-1}. The degree of N-deacetylation of various chitosan samples was then determined by using the absorption ratios [A_{1655}/A_{3450}] in the equation of the calibration curve. This method yielded results which were superior to those of any of the previously reported infrared spectroscopic methods tested herein.

Introduction

Chitin, a naturally occurring biopolymer, is a structural shell component of crustaceans and insects. The versatility of this molecule, and especially its N-deacetylated derivative, chitosan, explains its application in vastly diverse fields ranging from waste management and medicine to food processing and biotechnology (1). However, in order to exploit the utility of this biopolymer, it is imperative to determine its degree of N-deacetylation (DD), i.e. the average number of D-glucosamine units per 100 monomers, expressed as a percentage. (2). Various analytical techniques have been used in the determination of chitosan's DD (3-8). Most of these methods are of limited value since they can be used only if the polymer can be dissolved in a suitable aqueous solvent. In effect, only if the DD of chitosan is approximately 70% or greater can these methods be used. More universal analytical methods are those in which the sample preparation does not necessitate dissolution of chitosan in any solvent. Such methods include the infrared (i.r.) and near i.r. methods of analysis. The near i.r. method for the determination of the DD was developed by Rathke and Hudson (2). This method requires reference samples of known DD. It can also be used for analyzing the mixtures of monomeric sugars present in chitosan. Although the ruggedness of the near i.r. technique has yet to be established for this application, the i.r. technique has been explored by several researchers (5,9,10) since it was first proposed in 1978 by Moore and Roberts (11).

In this study, we report the development of a new method of analysis of chitosan's DD that combines the techniques used in previously reported methods (5,9,10) in order to obtain more accurate results. A brief description of each of the earlier methods is given below:

Method A (9). This method involved obtaining an i.r. spectrum of the polymer in the 800 to 4000 cm^{-1} range. The intensities of the i.r. absorption band maxima at 1550 and 2878 cm^{-1} were then determined by the baseline method. The i.r. spectra of chitosans of known DDs (determined by a reference method) were obtained in this manner. The DDs of chitosan samples were then obtained from a standard curve prepared by plotting the absorbance ratio of the amide II band at 1550 cm^{-1} and the C-H band at 2878 cm^{-1} against the known DD of each of the chitosan reference materials. Our

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preliminary experiments utilizing this method of analysis yielded suboptimal results when compared to a reference method of analysis [described in the experimental section].

Method B (5). The i.r. method developed by Domszy and Roberts (5) utilized the hydroxyl group of the glucosamine unit of chitosan — present in acetylated as well as deacetylated monomeric units — as an internal standard instead of the C-H band utilized by Sannan *et al.* (9). Also, Domszy and Roberts have pointed out that the intensity of the 2878 cm^{-1} C-H absorption band at a given DD is dependent on the molecular weight of the polymer. Therefore, inaccuracies are inevitable when analyzing a heterodisperse biopolymer such as chitosan. Domszy and Roberts compared the intensity of the amide I band of the acetyl groups of chitosan at 1655 cm^{-1} with that of the hydroxyl groups at 3450 cm^{-1} and proposed the following equation to determine the DD:

$$DD = 100 - [(A_{1655}/A_{3450}) \cdot 75.2] \qquad \text{Eq.1}$$

Method C (10). The method proposed by Domszy and Roberts was modified by Baxter *et al.* (10) by proposing a different baseline computation for the amide I band at 1655 cm^{-1}. The absorption ratio was then calculated in a manner identical to that employed in method B. The DD of the chitosan sample was then calculated by the empirical equation (Eq. 2):

$$DD = 100 - [(A_{1655}/A_{3450}) \cdot 115] \qquad \text{Eq.2}$$

Although the use of empirical equations considerably simplifies the analytical method, the variability in sample preparation, analytical technique, type of instrument, and the experimental conditions are likely to influence the outcome significantly.

Experimental

The sources of biomedical grade chitosans obtained for this study are listed in Table 1. Anhydrous diethyl ether, diethyl ether, deionized water (HPLC grade),

Table 1: Specific chitosans used in this study

Supplier/source	Lot # (DD*)
MMP, Inc. Mountainside, NJ	**8009** (91.80 ± 1.39)
Nova Chem Ltd. Nova Scotia, Canada	P32 (85.74 ± 0.25) SAN10 (79.61 ± 0.67) **SAN9** (79.32 ± 0.18) P15SAN (77.52 ± 0.40)
Sigma Chemical Co. St. Louis, MO	**83H0620** (84.05 ± 0.86)
Duquesne Univ. Pharmaceutics Laboratories Pittsburgh, PA	P15 (73.30 ± 1.07) **P40** (79.46 ± 1.40) Prepared from chitin [Lot 724283G] donated by Protan, Drammen, Norway

* DDs for bold-faced lots were determined titrimetrically (the reference method) while other lots were assessed by the proposed i.r. method, as described in the *Experimental* section; mean ± s.d. for 3 replicates

hydrobromic acid (9 M), methanol, phenolphthalein solution and potassium bromide were used as supplied by Fisher Scientific, Fair Lawn, NJ. Sodium hydroxide solution (0.0982 N) was supplied by Aldrich Chemical Co., Milwaukee, WI.

Reference method for determination of the DD of chitosan (5). Hydrobromic acid, 0.2 M, was prepared from 9 M acid by diluting 22.2 mL of the concentrated acid to 1 L with deionized water. Freshly prepared 0.2 M hydrobromic acid solution was added

(100 mL) to chitosan (0.5 g) in a 250 mL flask and the dispersion stirred until the chitosan was dissolved (~ 1 hr). Concentrated hydrobromic acid (50 mL) was then added to the chitosan solution while stirring vigorously. The resultant white slurry was centrifuged at 2000 rpm for 30 min. The supernatant was discarded and the precipitate washed several times with a 1:1 mixture of methanol and diethyl ether. This slurry was then filtered through a sintered glass filter funnel (medium porosity). Residual moisture in the precipitate was removed by resuspending it in *anhydrous* diethyl ether, and then stirring the suspension for six hours. After final filtration, the precipitate was dried *in vacuo* for 12 hours to yield the dry, white hydrobromide salt of chitosan. The dried HBr salt was accurately weighed and then dispersed in deionized water. Dispersion volume was adjusted to 100 mL and the mixture sonicated for 10 min. to dissolve the salt. Phenolphthalein indicator solution (~0.15 mL) was added to 20 mL of this chitosan HBr solution and the solution then titrated against 0.0982 N standard sodium hydroxide solution. This titration was performed in triplicate. The moles of neutralized alkali corresponded to the moles of hydrobromic acid present which in turn corresponded to the moles of glucosamine units of chitosan initially present in the solution, thus facilitating the calculation of the DD of the specific chitosan sample.

Sample preparation and infrared analysis. Approximately 40 mg of chitosan (particle size ≤125 μ), previously dried *in vacuo*, were mixed and triturated with 120 mg of potassium bromide and then dried at 80°C in a hot air oven for at least 12 hours before analysis. Approximately 40 mg of this blend were filled in an i.r. press and the compact subjected to compression for at least 30 min. to make a transparent disk. The i.r. spectrum was then scanned — while the disk was still in the punch cavity — using an FT infrared instrument (model 1605, Perkin-Elmer, Norwalk, CT) scanning between 400 and 4000 cm^{-1}. Three such disks were prepared for each sample of chitosan.

Proposed new method of determination of DD. The proposed i.r. spectroscopic method involves the determination of absorbances at 1655 cm^{-1} (the amide I band), as a measure of the N-acetyl group content, and at 3450 cm^{-1} (the hydroxyl band), as an internal standard to correct for film thickness or for differences in chitosan concentration, as employed by Domszy and Roberts (5) and Sannan *et al.*(9). However, our procedure involves the generation of a standard curve using samples with a known DD. The relationship of absorbance ratio to DD is then utilized to determine the DD of unknown chitosans.

Four chitosans of known DD were selected as standards for generating the calibration curve. A typical i.r. scan is shown in Figure 1. The amide band absorbance is given by

$$\log_{10}(DF/DE) = (A)_{amide} \qquad \text{Eq.3}$$

and,

$$\log_{10}(AC/AB) = (A)_{hydroxyl} \qquad \text{Eq.4}$$

DF, DE, AC, and AB are defined in Figure 1. The absorbance ratio for each chitosan standard was calculated in accordance with Eq. 5:

$$\text{Absorbance ratio} = \frac{(A)_{amide}}{(A)_{hydroxyl}} \qquad \text{Eq.5}$$

Subsequently, the absorbance ratio, as a function of the DD of the chitosans determined titrimetrically, was graphically and mathematically evaluated to obtain the standard curve.

Chitosan samples were analyzed for their DD by calculating their i.r. absorbance ratios, using Eq. 5, in conjunction with the standard curve in order to determine the corresponding DD.

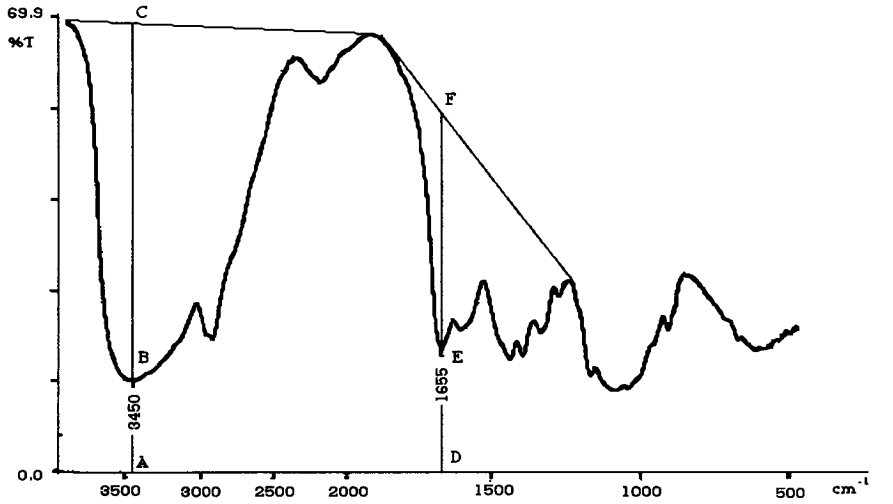


Figure 1. *Typical infrared scan of chitosan*

Results and discussion

Proposed method. In this procedure, the absorbance ratio [A_{1655}/A_{3450}] was utilized to generate the standard curve using the chitosan standards whose DDs were determined titrimetrically (see Table 1). The DDs of the samples were interpolated from the standard curve thus obtained (Figure 2) using the equation of the standard curve:

$$DD = 97.67 - \left[26.486 \bullet \left(A_{1655}/A_{3450}\right)\right] \qquad \text{Eq.6}$$

This linear relationship had a coefficient of determination, r^2, of 0.976 (n = 12) and a standard error of the estimate, SEE, of 0.86.

The DDs of four chitosan samples evaluated using this method were contrasted with the corresponding values obtained by the reference method. A good correlation (Eq. 7) was observed between the results obtained by the test method, i.e. the proposed new method, and the reference method (Figure 3):

$$\text{Reference DD} = 1.033 \bullet \text{Test DD} - 1.455 \quad r^2 = 0.889 \qquad \text{Eq.7}$$

The standard error of the prediction, SEP, was 1.49 (n = 12).

The above results indicate that the use of the amide I band at 1655 cm^{-1} along with the hydroxyl band at 3450 cm^{-1} yields acceptable results. Miller and Stace (12) employed a comparable procedure to evaluate the ratio of ethylene to vinyl chloride in polyethylene-vinyl chloride copolymer. Nonetheless, since the presence of free water may interfere with analysis by contributing towards the intensity of the hydroxyl band, steps must be taken to rid the sample of any residual moisture. Baxter *et al.*(10) have noted that the hydroxyl band (3450 cm^{-1}) of the i.r. spectrum must have a transmittance of ≥10% for superior assay sensitivity. This can be achieved by selecting (a) a finely powdered chitosan, and (b) a low ratio of chitosan to KBr for the preparation of chitosan-KBr pellet. Furthermore, sample preparation, type of instrument used, conditions may influence the analysis. Unquestionably, continued reliance on an empirical equation without regard for variations in experimental conditions is unacceptable. The proposed method provides a more rational, universal approach to resolving a vexatious issue.

Figure 2. Standard curve for determination of DD by the proposed method

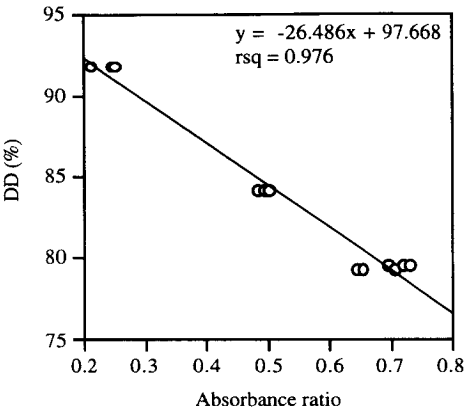
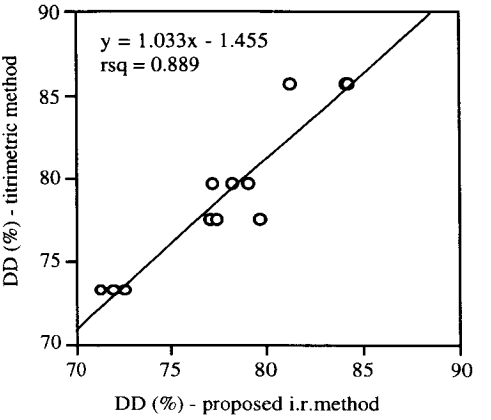


Figure 3. Comparison of chitosan DDs: titrimetric method vs. proposed i.r. method



Conclusions

Accurate estimates of the degree of N-deacetylation of a chitosan specimen can be made by the determination of i.r. absorption at 1655 and 3450 cm^{-1} (and the corresponding i.r. absorption ratio $[A_{1655}/A_{3450}]$) in conjunction with a standard curve obtained for chitosans with a known degree of deacetylation as determined by titrimetry.

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