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Review

A review of several reported procedures to determine the degree of *N*-acetylation for chitin and chitosan using infrared spectroscopy

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Abstract

Several methods have been already developed to determine the degree of *N*-acetylation, DA, for chitin and chitosan. In this article, determination of the DA for chitin and chitosan by various procedures using infrared spectroscopy technique (IR) has been reviewed. The information provided in this article describes various parameters affecting the accuracy of the DA values and allows one to choose an appropriate procedure to determine the DA for chitin/chitosan samples. IR technique was used for a qualitative or quantitative evaluation of DA. Determination of several absorption ratios using only one spectrum and their evaluation by a statistical method was the best procedure for a quantitative analysis, but the procedure is relatively long and time-consuming.

Keywords: Chitin; Chitosan; Degree of *N*-acetylation; Infrared spectroscopy

1. Introduction

Chitin is considered the second most plentiful organic resource on the Earth; occurring in plants, marine invertebrate, insects, cell walls of some fungi, and microorganisms (Gorovoj & Burdukova, 1996; Knorr, 1984; Muzzarelli, 1977). The chemical structure of chitin is similar to cellulose; and illustrated in Fig. 1, along with the chemical structures of cellulose, and chitosan.

Chitin and chitosan exhibit numerous interesting physico-chemical, biological, and mechanical properties (Domard, Vårum, & Roberts, 1998; Uragami, Kurita, & Fukamizo, 2001) with great potential applications. Various properties of the two polymers are closely related to the DA. It is the most fundamental parameter influencing the properties and behaviors of the polymers. The determination of the DA for the two copolymers is essential for studying their chemical structures, properties, and structure–properties relationships. If the DA is known, many properties can be predicted.

Several methods have been already developed to determine the DA. IR spectroscopy is a relatively quick technique for a qualitative evaluation of the DA through the determination of absorption ratios. Several procedures using different absorption ratios have been already proposed for determination of the DA. IR technique was also employed for quantitative evaluation of the DA. To choose an appropriate procedure among various ones is a difficult task for researchers. A review article summarizing up-to-date literature information on determination of DA, serves as a ready reference for researchers involved in the area of chitin/chitosan characterization and thus it is a desirable. Up-to-date, no review article has been published, which takes into consideration various procedures of the DA determination for chitin and chitosan via IR technique. The objective of this study is to compare several procedures published on IR spectroscopy regarding the determination of DA. In this study, various procedures are compared for their performances and limitations as well as their advantages and disadvantages; and present various factors affecting

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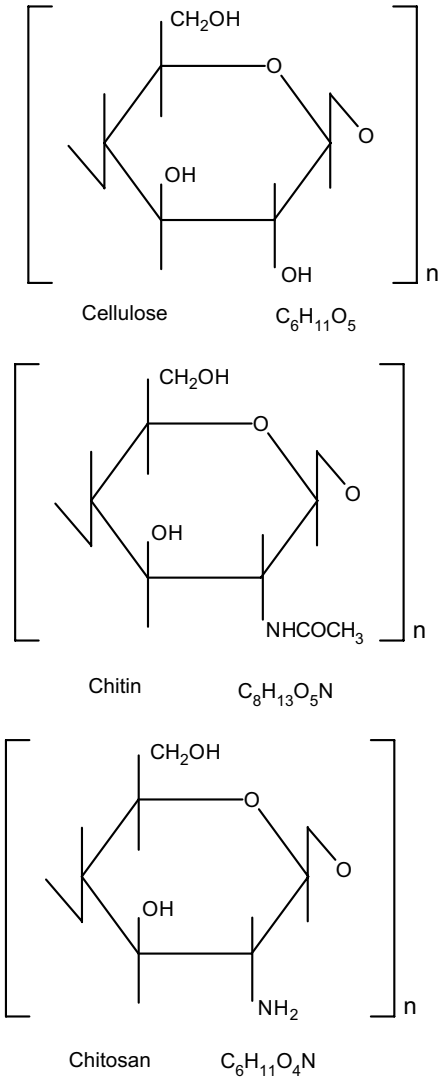

Fig. 1. Chemical structures of cellulose, chitin and chitosan.

the experimental results. The validity of DA values obtained from IR method will be also discussed.

2. Description of various procedures presented in the literature for determination of DA

The DA was determined as described in the following: (1) determination of the ratio of A_M/A_R: where A_M is the intensity of a probe band, which is a measure of *N*-acetyl or amine content; and A_R is the intensity of a reference band, having an intensity that does not change with the DA. The DA of unknown samples can be estimated by comparing the values of A_M/A_R with similar ratio of a few reference samples having a known DA; (2) creating a calibration curve by plotting the absorption ratio of chitin/chitosan samples of known DA versus their DA, where the DA of the samples were determined by IR or a reference method such as ^{1}H NMR spectroscopy. The DA of unknown samples were then estimated from the calibration curve; and (3) evaluation of several absorption band ratios by a statistical method (Duarte, Ferreira, Marvão, & Rocha, 2001).

IR spectra is generally recorded in the range of 1200–4000 cm^{-1}. The sample was prepared as a thin film made from a mixture of KBr and chitin/chitosan as solid forms or as a thin film made from casting procedure of chitin/chitosan solution. Several absorption band ratios such as A_{1560}/A_{2875}, A_{1655}/A_{2875}, A_{1655}/A_{3450}, A_{1320}/A_{3450}, A_{1655}/A_{1070}, A_{1655}/A_{1030}, A_{1560}/A_{1160}, A_{1560}/A_{897}, and A_{1320}/A_{1420} have been proposed to determine the DA (Baxter, Dillon, Taylor, & Roberts, 1992; Brugnerotto et al., 2001a; Domszy & Roberts, 1985; Dong et al., 2002; Miya, Iwamoto, Yoshikawa, & Mima, 1980; Muzzarelli, Tanfani, Scarpini, & Laterza, 1980; Sannan, Kurita, Ogura, & Iwakura, 1978; Shigemasa, Matsuura, Sashiwa, & Saimato, 1996a; Shigemasa, Matsuura, Sashiwa, & Saimato, 1996b).

Near infra-red (NIR) spectroscopy, has been also employed to determine the DA (Rathke & Hudson, 1993). The spectra were recorded from 1100 to 2500 nm (9090–4000 cm^{-1}) and second derivatives spectra have been used to determine DA. Gluscosamine and D-glucosamine hydrochloride were chosen as model compounds. A multivariate regression method has been employed to evaluate quantitatively the DA using NIR spectra (9090–4000 cm^{-1}) (Vårum, Egelandsdal, & Ellekjaer, 1995). A reference curve was constructed by plotting the predicted DA value (using NIR data) versus the DA determined by ^{1}H NMR spectroscopy.

3. Evaluation of reported results

3.1. General aspects

Chitin occurs naturally in association with proteins, organic pigments, and minerals (Muzzarelli, 1977; No, 1995). The nature and the level of impurities vary from one source to another one. Among them, proteins possess functional (amine, *N*-acetyl and carboxyl) groups similar to chitin/chitosan. IR technique detects all signals of the polymer itself and its impurities. The latter induce interference peaks in the spectra and influence the positions and intensities of some peaks. The position and shape (broadness or sharpness) of absorption bands in the spectra of chitin/chitosan samples with a certain DA value may change depending on several factors, such as type and level of impurities; source and the polymer morphologies. Prior knowledge on water content and impurities, as well as their percentages, yield in more accurate data for the DA.

Two concepts (resolution; and signal-to-noise ratio) that are important in all types of spectroscopy and are useful in the quantitative analysis of DA, are described as follows: (1) resolution: According to the Heisenberg Uncertainty Principle, no molecular system undergoing a transition between two quantized energy states that absorbs radiation at a single frequency, λ. But it absorbs a frequency of radiation with a maximum intensity at midpoint and drops rapidly its intensity at lower and higher frequencies. If a

sample has two closely spaced transition frequencies (λ_1, λ_2), then the resolving power ($\Delta\lambda$) of the spectrometer is a measure of the distance between the two transition frequencies that can be recorded as individual peaks rather than a single one; and (2) signal-to-noise ratio (S/N): In any spectrometer, there will be noise arising from random fluctuations of the electronic components. In the absence of any signal absorption, the noise produces background fluctuation, which results in a randomly varying output. For a given molecular energy transition, there will be a certain absorption coefficient, which will give the fraction of power absorbed per unit path length. If this coefficient is sufficiently small, the variation of output power is high and the ratio of S/N will be less than one and a desired signal will be lost in a high value of noise. The value of S/N can be improved by the use of: (i) high-quality stable electronics; (ii) the incorporation of various modulation methods such as beam-chopping in the infrared region; and (iii) the use of cumulative spectra techniques such as transient averaging or fast Fourier transform methods (Graybeal, 1988).

Usually a signal-to-noise ratio of 2 or 3 is acceptable. One may measure the background noise of a blank sample to determine the Relative Standard Deviation (RSD) of the response. Thus, the limit of detection equals the value of RSD multiplied by a factor 2 or 3. The smallest amount of the DA (limit of quantification) can be determined with a relatively high degree of precision. There is a correlation between the limit of quantification and the limit of detection. Generally, the limit of quantification equals to the limit of detection multiplied by a factor of 5–10 (Fischbacher, 2000; Lacroix, 1995).

Test criteria for a spectrometer such as IR are: accuracy of wavelength; signal-to-noise ratio; resolution; and linearity of the absorbance response. Usually the spectra of unknown samples are compared with a few reference spectra regarding peak positions, intensities, and RSD of the measured signal (Fischbacher, 2000).

General issues in spectroscopy methods include choosing an appropriate measuring band, choosing an appropriate reference band, and drawing a good baseline. Different values for DA were obtained by choosing different baselines for IR spectra of chitin/chitosan samples (Baxter et al., 1992; Khan, Peh, & Ch'ng, 2002; Moore & Roberts, 1980). Duarte, Ferreira, Marvão, and Rocha (2002) stressed that baselines have a considerable effect on the estimation of DA values. FTIR spectrum of α-chitin was shown in Fig. 2. Several probe and reference bands and corresponding baselines can be seen in this figure (Duarte et al., 2001). A fraction of Fig. 2 was extended and illustrated in Fig. 3a (Duarte et al., 2002). This figure shows different reference bands and corresponding baselines. A fraction of Fig. 2 was enlarged and also shown in Fig. 3b. In this figure, different probe bands and respective baselines were shown (Duarte et al., 2002).

Broadening of a peak and overlapping of two or more peaks are frequently observed in spectroscopy; and both effects induce difficulties to choose a separated and resolved absorption band. The use of sharp and well-separated peaks for the determination of the DA results in more accurate data compared to a broad and overlapped peak. To overcome the problem of broadening and shoulder effects, the following steps may be taken: (i) to enlarge the spectra in the region of the measuring band; (ii) to assign all of the absorption bands appearing in the measuring region; (iii) to calculate the sum of absorption bands lying close to the measuring band, instead of considering only a single measuring band; (iv) to calculate the ratio of $(A_M + A_X + A_Y + A_Z)/A_R$ instead of A_M/A_R, where A_X, A_Y, and A_Z are absorption bands appearing in the region of the measuring band; and (v) to plot the ratio of $(A_M + A_X + A_Y + A_Z)/A_R$ versus the DA, instead of A_M/A_R versus the DA.

IR region of an electromagnetic spectrum extends from 0.8 to 400 μm. The range of 0.8–2.5 μm (12,500–4000 cm^{-1}) is generally considered to be near-IR region; from 2.5 to 25 μm (4000–400 cm^{-1}) is known as mid-range region; and from 25 to 400 μm (400–25 cm^{-1}) is as far-IR region. Measurements can be made with concentrations of 1–10 mg per mL and up to 100 mg per mL for IR and near-IR regions, respectively. These concentrations are required to produce sufficient absorption values. Near-IR is a suitable method to identify and determine OH and NH groups (Graybeal, 1988).

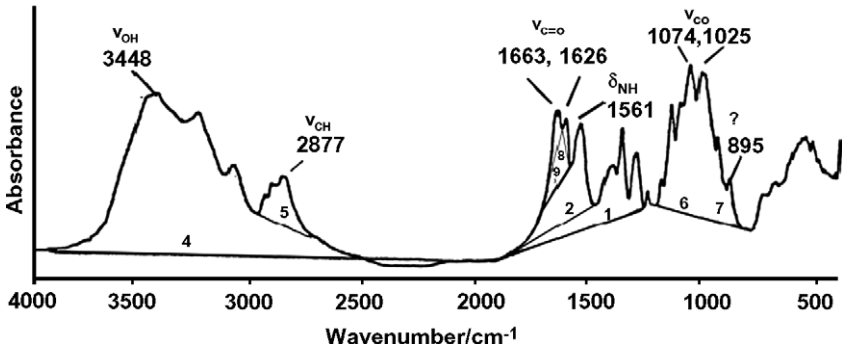

Fig. 2. Probe and reference bands and corresponding baselines for FTIR spectrum of α-chitin (reproduced from: Duarte, Ferreira, Marvão, & Rocha, 2001).

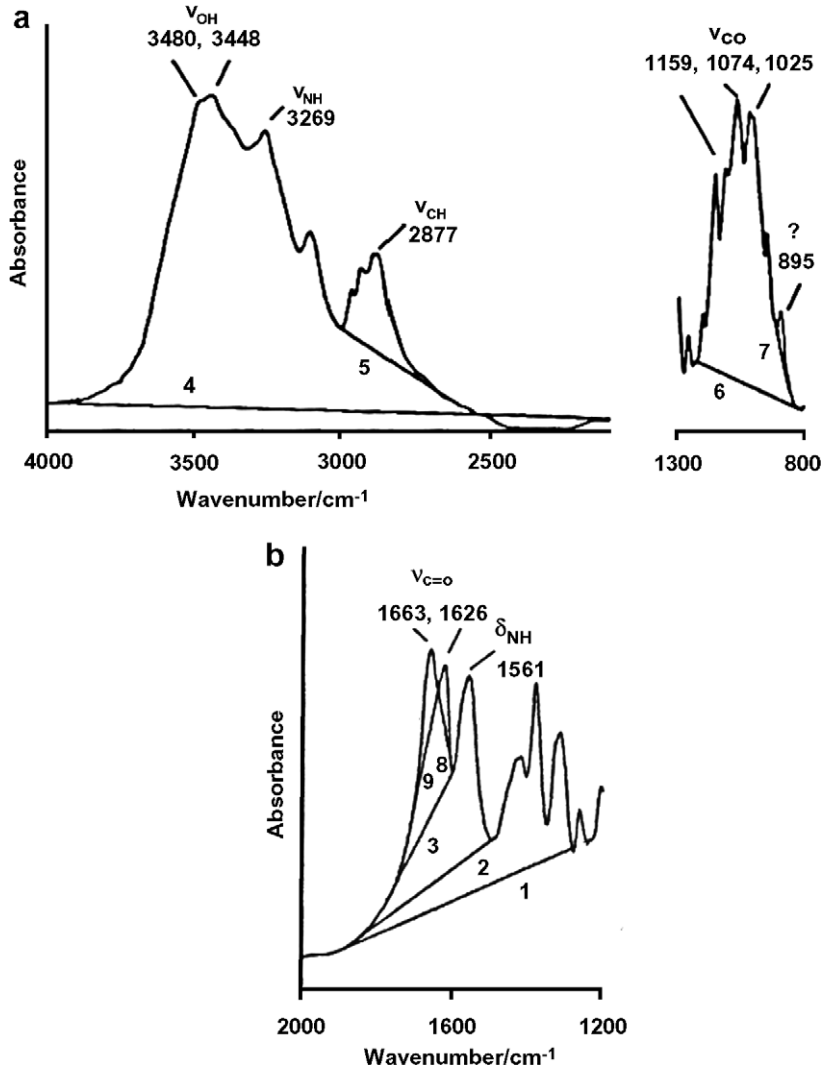

Fig. 3. (a) The extended of a fraction of Fig. 2 showing different reference bands and corresponding baselines; and (b). The enlarged of a fraction of Fig. 2 showing different probe bands and respective baselines [reproduced from: Duarte, Ferreira, Marvão, & Rocha, 2002].

Two methods proposed by Moore and Roberts (1978) and Baxter et al. (1992) are absolute methods. Their proposed equations (see Section 3.3) were set up without comparing the calculated DA values to a range of samples with known DA values. Once the equations were set up, their accuracy was checked by comparing the DA values obtained from IR technique with the DA values determined by other techniques. Generally, quantitative evaluation requires creating a calibration curve by plotting the absorption ratio versus the DA of chitin/chitosan samples with known DA. The DA of unknown samples is estimated via determination one specific absorption ratio and using a calibration curve. The calibration curve is employed to convert an absorption ratio to a DA value. A calibration curve is used to verify and validate the experimental results. If the experimental points lie close to the calibration line, this indicates that the experimental results are in good agreement with the reference data and the technique is thus validated. Some errors may arise from the reference technique. For example, Rathke and Hudson (1993) noted that the error obtained from the NIR procedure may be related to the reference (hydrobromic acid titration) method. Comparison studies have been also developed between unknown samples and reference ones instead of using a standard curve. The unknown and standard samples were similarly treated. The reference samples had DA values approximately equal to that of unknown samples. An absorption band may be chosen as an internal reference. Standard chitin/chitosan samples with different DA can be used as external references. Individual monomer of *N*-acetyl glucosamine and glucosamine or mixtures of these two monomers have been also used as external standards. The use of such references may not yield reliable data, because the mixtures of monomers are not true representatives of chitin or chitosan samples. There are inter- and intramolecular interactions between macromolecule chains in the polymers, whereas such interactions are not present in the mixtures of monomers. The responses of instruments (detectors) as a function of mole fraction of *N*-acetyl glucosamine or glucosamine may be linear for the monomer

units and their mixtures, but may not be linear for real chitin/chitosan samples. Brugnerotto et al. (2001a) constructed a calibration curve by plotting the absorption ratio as a function of the mole fraction of *N*-acetyl glucosamine using several mixtures of glucosamine and *N*-acetyl glucosamine as standard samples. A reproduced spectrum of *N*-acetyl D-glucosamine (Brugnerotto et al., 2001a) showing the position of absorption and references bands and corresponding baselines was illustrated in Fig. 4.

Some investigators have claimed that their results of the DA were reliable (see the following section); even though the criteria for selecting the standard methods are not as a validated or cannot be considered as a precise quantitative method. Considering that the accuracy is the closeness of a measured value to its true value (Lacroix, 1995); determining the DA with a high accuracy using a method is not an easy task. The DA can be estimated by comparing the results of a desirable method with the results of a validated/precise method. Some methods such as colloidal/hydrobromic acid titration and elemental analysis are less specific than other methods. Among various methods, ^{1}H NMR spectroscopy can be considered as a precise and reliable one. ^{1}H NMR spectroscopy has been chosen as a standard method by the American Standard Test Method organization to determine the DA for chitosan, and has been published (ASTM, edition 2003, test F2260-03). The choose of this technique as a standard method is due to its high sensitivity and precision, resulting in the most accurate data as well as the lowest variation of experimental results, compared to other methods.

Chemical modifications should change the IR spectra of the samples by increasing/decreasing the intensity or shifting the position of some peaks. The objective of this study was not to examine the effects of chemical modifications (such as deacetylation, acetylation, decomposition, depolymerization, and fragmentation) on specific absorption bands used to determine the DA, nor their influence on the accuracy of measurement. The effects of chemical modifications on the DA can be found elsewhere (Domard & Rinaudo, 1983; Focher, Beltrame, Naggi, & Torri, 1990; Focher, Naggi, Torri, Cosani, & Terbojevich, 1992; Kasaai, Arul, Chin, & Charlet, 1999; Kim, Kim, & Lee, 1996; Knaul, Kasaai, Bui, & Creber, 1998; Sannan, Kurita, & Iwakura, 1976).

3.2. Comparison of various internal reference absorption bands

There is no unique reference band that can be used as a reference band for the entire range of the DA. This is because the spectrum of chitin/chitosan changes as a function of the DA; the suitable reference band depends on the DA. Until now, several absorption bands have been proposed as internal reference bands as follows: the OH stretching band at 3450 cm^{-1} (Domszy & Roberts, 1985; Moore & Roberts, 1978, 1980); the C—H stretching bands (Dong et al., 2002; Miya et al., 1980; Sannan et al., 1978) within 2870–2880 cm^{-1}; the skeletal vibrations involving the C—O—C stretching band (Peniche, Elvira, & Roman, 1998; Shigemasa et al., 1996a, 1996b) at 1030 or 1070 cm^{-1}; the $-CH_2$ bending centered at 1420 cm^{-1} (Brugnerotto, Desbrières, Heux, Mazeau, & Rinaudo, 2001b; Brugnerotto et al., 2001a); the anti-symmetric stretching of the C—O—C bridge around 1160 cm^{-1} (Miya

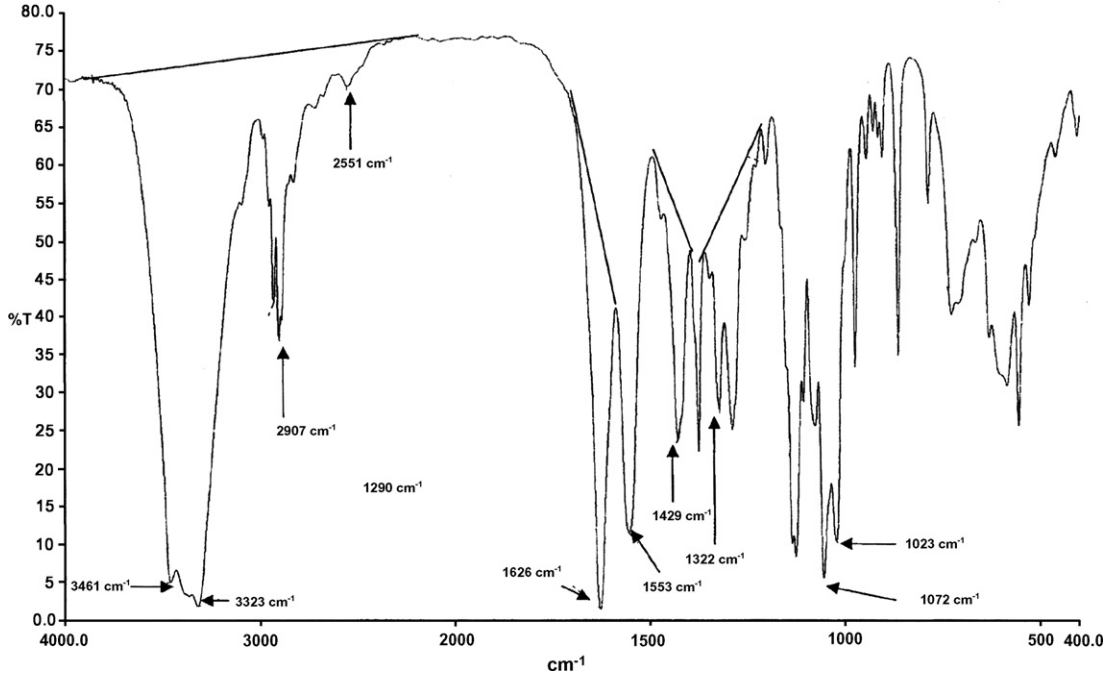

Fig. 4. A typical spectrum of *N*- acetyl D-glucosamine showing the positions of probe and reference bands and corresponding baselines [reproduced from: Brugnerotto et al., 2001a].

et al., 1980); 1315–1320 cm^{-1} (amide III band) (Qin et al., 2006); 1620–1630 cm^{-1} (–NH bending of NH_2) (Shigemasa et al., 1996a, 1996b); and 890–900 cm^{-1} (C—O—C bridge as well as glucosidic linkage) (Qu, Wirsén, & Albertsson, 2000).

Although the intensity of the O—H stretching band does not change with the DA, this band may still experience some interference bands as follows: (i) OH groups are sensitive to humidity, since chitin/chitosan is a hygroscopic material. The O—H stretching absorption band of water molecules appears in this region when the chitin/chitosan sample is not well-dried. Three absorption bands centered at 1595, 3657, and 3756 cm^{-1} were identified for H_2O vapor (Barrow, 1988). Increase in water content of the polymer sample results in an increase in the intensity of the absorption band or an increase in its width (broadening effect). The error arising from the moisture effect can be minimized by drying the sample; (ii) OH groups may be involved in hydrogen bonds (Blackwell, Minke, & Gardner, 1978; Hwang, Kim, Jung, Cho, & Park, 2003; Prashanth, Kittur, & Tharanathan, 2002; Sun, Xu, Liu, Xue, & Xie, 2003). The O—H fraction of CH_2OH groups or O—H groups of glycoside rings may be involved in intra- and intermolecular hydrogen bonds (Prashanth et al., 2002). In addition, the N—H stretching band appeared around 3270 cm^{-1} (Duarte et al., 2002); and its intensity varied with DA. Although the two bands (–OH and –N—H) are not very close to each other and have separated peaks, due to the hydrogen bonds and water effects in the chitin/chitosan samples, the two bands still overlap. As a result, a broad band was observed in the same region for O—H and N—H groups. Thus, the peaks appeared around 3450 cm^{-1} generally are not sharp. The two absorption bands (O—H and N—H stretching) are resolved and separated when the samples are crystalline and well-dried.

The C—H stretching band (2870–2880 cm^{-1}) is a complex absorption band, because several symmetric and asymmetric C—H stretching bands appear in this region as follows: C—H of glucose ring; C—H of CH_2OH group; and C—H of CH_3 attached to *N*-acetyl groups. Among various indicated C—H stretching bands, only the C—H of *N*-acetyl group is sensitive to DA, and its intensity decreases with an increase in DA (Duarte et al., 2002; Pearson, Marchessault, & Liang, 1960; Shigemasa et al., 1996a). A major advantage of the stretching band at (2870–2880 cm^{-1}) as a reference band is that its position and intensity does not change with water content or hydrogen bond. Domard and Rinaudo (1983) obtained reliable results when they used the C—H stretching band at 2867 cm^{-1} for chitosan samples with a DA < 10. These authors thus noted that the reference band of C—H at 2867 cm^{-1} was a good reference band for a DA < 10, and the reference band of O—H at 3450 cm^{-1} was a good one for a DA > 10. They obtained overestimated values using O—H band at 3450 cm^{-1}.The absorption bands at 1160 (anti-symmetric stretching of the C—O—C bridge); 1075 and 1025 (skeletal vibrations involving the C—O—C stretching); and 897 cm^{-1} are characteristic bands for chitin and chitosan macromolecules (Peniche et al., 1998), and have been also used as reference bands. The band at 897 cm^{-1} was not a sharp band and its intensity was weak. Four above-mentioned absorption bands appear in the fingerprint region, where several bands are observed in this region (Duarte et al., 2002; Shigemasa et al., 1996a, 1996b). Thus, the resolution of the absorption bands appearing in this region is low as well. Table 1 shows reference bands, their advantages and disadvantages.

3.3. Evaluation of several absorption ratios

3.3.1. (A_{1655}/A_{3450}), ($A_{1655}+A_{1625}/A_{3450}$), and ($A_{1320}/A_{3450}$) ratios

Moore and Roberts (1978) proposed the following equation for determination of the DA:

$$DA = (A_{1655}/A_{3450}) \times 100/1.33 \quad (1)$$

The Eq. (1) obtained from the plot of A_{1655}/A_{3450} versus DA. The samples were prepared from heterogeneous *N*-acetylation of chitosans as a function of time. The *N*-acetylation gradually leveled-off and the extent of *N*-acetylation as a function of time was evaluated by measuring the absorption ratio (A_{1655}/A_{3450}). The ratio of A_{1655}/A_{3450} was equal to 1.33, and zero for fully *N*-acetylated chitin and fully deacetylated chitosan, respectively. Domard and Rinaudo (1983) and Prashanth et al. (2002) used the Eq. (1) to determine DA% in the range of 10–21% and 12–15%, respectively. The reported data was in good agreement with the results obtained by ^{13}C NMR spectroscopy (Prashanth et al., 2002).

Baxter et al. (1992) proposed Eq. (2).

$$DA = (A_{1655}/A_{3450}) \times 115 \quad (2)$$

The Eq. (2) derived from the ratio of A_{1655}/A_{3450} versus DA. The analysis was conducted on chitosan samples prepared from homogeneously re-*N*-acetylated chitosan samples. The DA of the samples was determined by titration technique. The latter Data was in agreement with the DA obtained from IR via measuring the A_{1655}/A_{3450} values. Shigemasa et al. (1996a, 1996b) constructed a calibration curve by plotting the absorption ratio of $(A_{1655}+A_{1630})/A_{3450}$ versus DA, where the DA was obtained from ^1H NMR spectroscopy. These authors obtained a linear curve for the latter plot. Another calibration curve was constructed by plotting the absorption ratio versus DA, where the DA was the DA of known chitin/chitosan samples [completely acetylated chitin (DA% = 100), completely deacetylated chitosan (DA = 0), or the DA of fully *N*-acetylated chitin and fully *N*-deacetylated chitosan mixtures with known ratios]. The authors again obtained a linear curve for the ratio of $(A_{1655}+A_{1630})/A_{3450}$ versus DA. They did not provide any equation for the ratio–DA relationship. Osman and Arof (2003) reported that the position of the carbonyl band (1655 cm^{-1}) has shifted to a lower value when they used chitosan-acetic acid salt film, more probably due to interference by the absorption band of

the acetate ion at ~1600 cm^{-1}, which would tend to swamp a much weaker amide band at 1655 cm^{-1}. Acetic acid also protonates only amine groups, but not amide groups. In amorphous chitin/chitosan samples, two bands at 3474 and 3434 cm^{-1} corresponding to the vibrational stretching of hydroxyl groups were observed (Paulino, Simionato, Garcia, & Nozaki, 2006). When the latter two absorption bands appeared with a certain intensity, two bands at 1625 and 1655 cm^{-1} were also observed. Broad peaks around 3500 and 1655 cm^{-1} indicate that the hydrogen interactions are less accentuated, or hydroxyl groups are free and no interaction is present (Duarte et al., 2002; Paulino et al., 2006). Only one intense peak at 1625 cm^{-1} was observed in crystalline chitin, whereas two absorption bands, one at 1625 cm^{-1} and another one at 1655 cm^{-1} were observed for amorphous sample (Paulino et al., 2006).

Eqs. (1) and (2) were obtained from the linear relationships between the absorption ratio of A_{1655}/A_{3450} and DA. The values of 100/1.33 (75.2) and 115 are reciprocal values of the slopes of the linear curves (the absorption ratio versus DA). The Eq. (2) resulted in more reliable data for DA > 20, whereas the Eq. (1) yielded in overestimated values for DA > 20. The reasons for the differences are: (i) different baselines were used to determine the DA; (ii) the Eq. (1) proposed by assuming the leveling-off represented complete *N*-acetylation, while the leveling-off did not; and (iii) broadening and shoulder effects were observed in the region of 1625–1655 cm^{-1}. Tsaih, Tseng, and Chen (2004) applied Eq. (2) to determine the value of DA. Sabins and Block (1997) determined the DA of various chitosan samples with a good accuracy by constructing a calibration curve using the absorption ratio of A_{1655}/A_{3450}, where the DA determined by titration of hydrobromide salts of chitosan. The peak centered at 3450 cm^{-1} was shifted to a smaller value (3435 cm^{-1}) and became sharp in completely deacetylated chitosan (Mima, Miya, Iwamoto, & Yoshikawa, 1983; Mima, Miya, Iwamoto, & Yoshikawa, 1982).

Care must be taken when 1655 or 1625 cm^{-1} is used as a measuring band especially when broadening and shoulder effects are observed. It is necessary to stress that these two bands are the most important bands and appear in spectra of *N*-acetyl gluscosamine, glucosamine, chitin, and chitosan. Concerning the shoulder, Blackwell et al. (1978) noted that the structure of chitin contains two types of amide groups and both created from C═O—H—N intermolecular bonds. The reduction of DA in chitin/chitosan sample was in good agreement with a decrease in absorbance of amide I band at 1655 cm^{-1} (Sannan et al., 1976). Shigemasa et al. (1996a, 1996b) reported that the plot of A_{1655}/A_{3450} or A_{1625}/A_{3450} versus DA did not yield a single linear relationship. The non-linearity should be due to the presence of two types of amide groups in the chitin/chitosan structure. This problem may be solved by plotting a curve of $(A_{1655} + A_{1625})/A_{3450}$ versus DA. The latter plot yielded in a linear relationship and is applicable for the DA% between 50 and 100 (Shigemasa et al., 1996a, 1996b). The two bands at 1655 and 1625 cm^{-1} correspond to the stretching of C═O and C—N of amide I, respectively. These two bands (1655 and 1625 cm^{-1}) may be superimposed or may be clearly observed in all of chitin/chitosan samples (Paulino et al., 2006; Shigemasa et al., 1996a, 1996b).

Table 1
Reference bands, their performances, and limitations

Absorption band	Wave number (cm^{-1})	Advantages	Disadvantages
O—H stretching	3450	OH has a highly intense absorption band	(1) O—H of water molecule appears in this region; (2) O—H groups involve with intra- and intermolecular hydrogen bonds and result in a broad peak; and (3) N—H stretching band appears around 3300 cm^{-1} and creates an interference peak, and its intensity changes with the DA
C—H stretching	2870	The intensity of peak is significant; the band does not involve in hydrogen bonds; and water does not create any interference peak	The position of C—H stretching corresponding to *N*- acetyl groups changes with the DA
CH_2 bending	1420		Shape and intensity of the peak change with changing the crystallinity of chitosan samples through re-arrangement of hydrogen bonds at position of primary OH groups (Prashanth et al., 2002)
C—O stretching	1030		Several absorption bands appear in this region; and the peak is not clearly separated, and its intensity is weak
C—O stretching	1070		
C—O stretching of glycoside linkage	897		
Glycoside linkage / C—O—C bridge (asymmetric CO stretching)	1160		

The use of A_{1655}/A_{3450} has some other limitations as follows: (1) the absorption band around 1655 cm^{-1} is not a suitable measuring band, because OH groups of polysaccharides and water molecule appear around 1640 cm^{-1}. An increase in water content yields in an increase in absorption band at 1640 and 3450 cm^{-1} (Shigemasa et al., 1996a, 1996b), which may in turn induce interference for the amide band at 1655 cm^{-1} and O—H stretching at 3450 cm^{-1}. Another interference is indicative of presence of the NH_2 band centered at 1590 cm^{-1} (Shigemasa et al., 1996a). Deviation of the result was large for DA% > 70, due to the shoulder effect of amide I at 1655 and 1625 cm^{-1} (Shigemasa et al., 1996a). There was a large deviation in DA (for DA% > 70), since A_{1655}/A_{3450} did not yield a single linear relationship for the whole range of DA (Gail et al., 1991; Poirrier & Charlet, 2002; Shigemasa et al., 1996a).

Brugnerotto et al. (2001a) determined the DA using the absorption ratio of A_{1320}/A_{3450}. The correlation between the experimental DA values and the absorption ratio was expressed by following relations:

$$A_{1320}/A_{3450} = 0.03146 + 0.00226 \cdot \mathrm{DA} \quad (R^2 = 0.95) \qquad (3)$$

Brugnerotto et al. (2001a, 2001b) reported that the DA determined from the A_{1320}/A_{3450} ratio showed a poor correlation with the DA determined by ^1H NMR and ^{13}C NMR spectroscopy and Eq. (3).

The amide I band at (1655 cm^{-1}) is clear in the spectrum of fully *N*-acetylated chitin. Its intensity decreased with a decrease in DA and finally disappeared in completely deacetylated chitosan (Domard & Rinaudo, 1983). The peak centered at 1655 cm^{-1} was employed to determine the DA of larger than 10% (Mima et al., 1983). The intensity of the latter peak significantly decreased for the DA smaller than 10% and it was not detectable for the DA smaller than 5%.

3.3.2. A_{1560}/A_{1160}, A_{1560}/A_{897}, A_{1560}/A_{1030}, A_{1560}/A_{1070}, A_{1655}/A_{1030}, A_{1630}/A_{1030}, and A_{1630}/A_{1070} ratios

Shigemasa et al. (1996a, 1996b) constructed several calibration curves by plotting the ratios of A_{1630}/A_{1070}, A_{1655}/A_{1070}, A_{1655}/A_{1030}, $(A_{1655} + A_{1630})/A_{1070}$, $(A_{1655} + A_{1630})/A_{1030}$ versus DA, where the DA was obtained from ^1H NMR or determined from IR spectroscopy using samples with a known DA [completely *N*-acetylated chitin (DA% = 100), completely *N*-deacetylated chitosan (DA% = 0) and their mixtures having known DA]. They did not provide an equation for the ratios–DA relationships.

The absorption ratios of A_{1560}/A_{1070} and A_{1560}/A_{1030} have been also used to determine DA (Miya et al., 1980; Shigemasa et al., 1996a, 1996b). Shigemasa et al. (1996a, 1996b) have found a good linear relationship between the DA obtained from the above-mentioned ratios and the DA obtained from ^1H NMR spectroscopy for the whole range of the DA. They did not provide any equation for the above relationships. The absorption band at 1560 cm^{-1} shifted to a larger value up to 1590 cm^{-1}, when the DA value decreased from large to small values (Domszy & Roberts, 1985; Duarte et al., 2002). This is presumably due to the disruption of hydrogen bonds involving the amide group with a decrease in DA. The absorption band at 1560 cm^{-1}, corresponds to amide II (for non-protonated chitosan sample) has shifted about 10 cm^{-1} in chitosan–acetic acid salt film. This is because some interactions between acetic acid and nitrogen of the chitosan polymer have taken place (Osman & Arof, 2003).

3.3.3. A_{1320}/A_{1420} ratio

The absorption band at 1320 cm^{-1} was considered as contribution of amide III (Qin et al., 2006). Brugnerotto et al. (2001a) determined the DA using the ratio of A_{1320}/A_{1420}. The correlation between the experimental DA values and the ratio was expressed by following relation:

$$A_{1320}/A_{1420} = 0.3822 + 0.03133 \cdot \mathrm{DA} \quad (R^2 = 0.99) \qquad (4)$$

Brugneroto et al. (2001a, 2001b) reported that the DA determined from the ratio of A_{1320}/A_{1420} was in agreement with the DA determined from ^1H NMR and ^{13}C NMR spectra and Eq. (4) for the entire range of DA. This is because the intensities and positions of bands appeared at 1320 and 1420 cm^{-1} did not change with humidity and hydrogen bonds. Mima et al. (1983) have shown that the position of the absorption band around 1320 cm^{-1} was shifted to 1335 cm^{-1} and the peak became sharper when the DA decreased from 22% to 1%.

3.3.4. A_{1560}/A_{2875} and A_{1655}/A_{2875} ratios

Dong et al. (2002) constructed a standard curve by plotting the absorption ratio of A_{1560}/A_{2875} against DA, and obtained a linear relationship ($R^2 = 0.99$) for the whole range of DA. Ten chitin/chitosan samples having known DA were used to construct a calibration curve, but no equation was provided for this relationship. The author of the present work estimated the following equation using their data and linear plot:

$$A_{1560}/A_{2875} = 0.0125 \cdot \mathrm{DA} + 0.2 \quad (R^2 = 0.99) \qquad (5)$$

The use of A_{1560}/A_{2878} yielded in more reliable data for the DA in the range of 0–60% (Shigemasa et al., 1996b). The data of IR in the latter range was in agreement with the data obtained from ^1H NMR spectroscopy. The use of A_{1655}/A_{2875} yielded in accurate results, when the sample was highly acetylated (Mima et al., 1983, 1982). The authors noted that the peak at 1655 cm^{-1} was not detectable when the DA% was close to 100. The C=O stretching at 1655 cm^{-1} (amide I) and N—H bending at 1560 cm^{-1} (amide II) were not observed in the spectra of chitosan samples with DA% = 0 and 100 (Hwang et al., 2003). The intensity of the NH-bending band increased with a decrease in DA (Takai, Shimizu, Hayashi, Uraki, & Tokura, 1989).

3.4. Near infrared spectroscopy (NIR)

Rathke and Hudson (1993) proposed different absorption bands in the range of 4000–9090 cm^{-1} to determine DA% (DA% $\leqslant$ 60). Monomer sugars, *N*-acetyl D-glucosamine and D-glucosamine hydrochloride, have been used as model compounds. The absorption ratios of A_{7669}/A_{7474} and A_{6039}/A_{5342} were chosen as the best ratios for the model compounds and chitin/chitosan samples, respectively. The wavelength at 7669 cm^{-1} was chosen, because it had the highest correlation with the DA of the monomer mixtures. The intensity of the band at 7474 cm^{-1} was invariant to different samples prepared from mixtures of the monomers. The calibration curve was constructed by plotting: the absorption ratio of A_{7669}/A_{7474} versus the mole percent of *N*-acetyl D-gluscoamine. Twenty-one chitin/chitosan samples including the monomers and mixtures of monomers were employed to construct a calibration curve. The following equation was obtained (Rathke & Hudson, 1993):

$$A_{7669}/A_{7474} = 0.0111(N\text{-acetyl D-glucosamine}) - 0.6294 \quad (R^2 = 0.998) \tag{6}$$

The absorption ratio of A_{7669}/A_{7474} resulted in a high value for the linear correlation coefficient. The second calibration curve was constructed by plotting the ratio of A_{6039}/A_{5342} versus DA% in the range of 8–22% using five chitosan samples. The equation obtained from this curve was given as follows:

$$A_{6039}/A_{5342} = -0.0043 \cdot \mathrm{DA} + 0.4567 \quad (R^2 = 0.996) \tag{7}$$

There was a shift in the spectrum of chitosan around 4892 cm^{-1} compared to the spectrum of *N*-acetyl D-glucosamine (Rathke & Hudson, 1993). This is due to the presence of hydrogen bonds in the polymeric backbone of chitosan compared to its monomer units.

Vårum et al. (1995) have analyzed the data obtained from NIR spectra (4000–9090 cm^{-1}) using the multivariate regression method. A calibration curve of the DA (estimated by using NIR spectroscopy) versus DA (determined by ^{1}H NMR spectroscopy) was constructed in order to predict the DA and to optimize the accuracy. The correlation coefficient and root mean square for the plot (as a calibration curve for DA% = 0–60) were 0.985% and 3%, respectively.

3.5. Comparison of reported results

IR technique is a suitable method for a qualitative evaluation. The procedures of Duarte et al. (2002) and Vårum et al. (1995) can be used for quantitative evaluation of the DA. The advantage of the procedure that has been employed by Duarte et al. (2002) over other procedures is that several absorption ratios were employed to determine the DA using only one spectrum. The statistical evaluation of the data resulting from the combination of all the absorption ratios yields in a DA with a higher accuracy compared to only one ratio. The statistical methods were employed for the entire range of the DA for chitosan/chitin and two morphological forms of chitin (α, β). Brugnerotto et al. (2001b) compared the results of DA obtained from the absorption ratios of A_{1320}/A_{1420} and A_{1650}/A_{3450}. They obtained superior agreement for the ratio of A_{1320}/A_{1420}. The reference data for comparison of the two ratios was the DA, which was obtained from ^{1}H NMR and ^{13}C NMR spectroscopy. The absorption band ratios, their corresponding applicable DA ranges, their advantages and disadvantages are listed in Table 2. Quantitative evaluation by IR has quite a large margin of error, if a validate method is not used as a standard method. The validity of estimated DA for unknown chitin and chitosan samples can be examined from the knowledge of the DA determined by ^{1}H NMR and ^{13}C NMR spectroscopy using standard chitin and chitosan samples. The range of the DA for standard samples must cover the range of the DA for unknown samples.

In most of the results reported by research groups, linear relationships for the plots of absorption ratios versus the DA (for a limited or entire range of DA) were observed. The regression formula allows one to predict the DA from the absorption ratio data. The relationship was based on the assumption that the relationship between variables (absorption ratio versus DA) is linear. One needs to calculate regression coefficients; *A*; and *B* to predict the DA.

$$Y = A \cdot \mathrm{DA} + B \tag{8}$$

If the linear curve has been found, one can determine the slope (*A*) and intercept (*B*) from the linear plot. A correlation coefficient, *r*, provides a quantitative way to express the degree of association existing between the two variables (Spatz & Johnston, 1989). The purpose to determine the correlation coefficient is to assess the reliability of IR instruments that measure the absorption of probe and reference bands. High correlations means lots of agreement and therefore high reliability, and low correlations means lots of disagreement and therefore low reliability. If the regression equation is found with a sufficient accuracy, the residual mean square will be only due to the error mean square. The smaller the contribution of the latter error to the overall estimated variance, the stronger the correlation between the ratio and DA (Akhnazarova & Kafarov, 1982). After the regression equation has been found, it must be subjected to statistical analysis, i.e. all regression coefficients should be tested for significance in comparison with the sample standard deviation (replication error); and the equation itself should be tested for the goodness of fit. Once the values of *r* and standard deviations for both DA (S_{DA}) and absorption ratio (S_{ratio}) are computed, the slope, *A*, can be obtained by the following formula (Akhnazarova & Kafarov, 1982):

$$A = r \cdot S_{ratio}/S_{DA} \tag{9}$$

and *B* can be obtained from the following equation:

$$B = Y - A \cdot \mathrm{DA} \tag{10}$$

Table 2
The infrared absorption band ratios, their corresponding DA ranges, advantages and disadvantages

Absorption band ratio	DA	Advantages	Disadvantages
A_{1655}/A_{2870}	0–20		Broadening and shoulder effects are observed in the region of the probe band; and low resolution for small values of the DA
A_{1655}/A_{3450}	15–80	Suitable for crystalline and well-dried samples	Possible errors arising from the humidity or OH groups of polysaccharides; low resolution for small values of the DA
A_{1630}/A_{3450}	0–60		
$(A_{1655}+A_{1630})/A_{3450}$	0–100	No broadening and shoulder problem; and suitable for crystalline and well-dried samples	Possible errors arising from the humidity or OH groups of polysaccharides; and possible error for high values of the DA
A_{1560}/A_{2870}	0–60		Possible errors for high values of the DA
A_{1655}/A_{1070}	0–60		Many peaks appear in the region of the reference peak; broadening and shoulder effects are observed in the region of the probe band; low resolution for small values of the DA; and OH bending band of water molecule appears in the region of the probe band
$(A_{1655}+A_{1630})/A_{1070}$	0–100	No broadening and shoulder problem, suitable for the effect of acetylation/deacetylation process on the DA	Many peaks appear in the region of the reference peak; and possible error for high values of the DA
A_{1655}/A_{1030}	0–60		Many peaks appear in the region of the reference peak; broadening and shoulder effects are observed in the probe band region; low resolution for small values of the DA
$(A_{1655}+A_{1630})/A_{1030}$	0–100	No broadening and shoulder problem	Many peaks appear in the region of the reference peak
A_{1560}/A_{1070}	0–100		
A_{1560}/A_{1030}	0–100		
A_{1560}/A_{897}	0–100		Possible error for high values of the DA
A_{1560}/A_{1160}	0–100		Possible error for high values of the DA
A_{7669}/A_{7474}	0–60	Reliable results for small values of the DA	
A_{6039}/A_{5342}	8–22	Applicable for small values of the DA	

If a good baseline is selected and IR spectroscopy results in a highly-resolved spectrum, it is a good initial data for a quantitative analysis. Crystalline samples result in higher resolved spectra than those of amorphous samples. The sharpening behavior is consistent with the generally accepted concept that crystallization induces sharp absorption bands for IR spectra. The quantitative analysis of the DA for amorphous samples is more difficult than crystalline ones, because the former ones produce broad absorption bands. For a quantitative analysis with a high accuracy, the chief limitations of the IR method are that: (i) sophisticated procedures are required, such as the statistical evaluation of various absorption ratios; (ii) interference from the sample must be removed; and (iii) calculating the DA is long and time-consuming.

The cost of analysis is also an important parameter for routine analysis and quality control, but it is less important for research aspects. The time required for qualitative via comparison evaluation is usually short for IR.

4. Conclusions

In this article, several reported procedures to determine the DA of chitin/chitosan regarding IR spectroscopy technique, were compared and reviewed. The following conclusions were made from this study: (i) the DA is estimated by determining an absorption ratio (absorption of a probe band/absorption of a reference band); (ii) it is a flexible method, because several measuring bands or absorption ratios have been proposed to determine the DA, each one is generally applicable for a limited range of the DA; (iii) generally, it is used for a qualitative analysis. IR spectroscopy is rarely used for quantitative analysis of the DA. If one decides to make a quantitative analysis, and then, one must choose an appropriate probe and references bands and draw a good baseline. The quantitative evaluation obtained from a crystalline sample is more reliable than that of an amorphous one. It is also results in more reliable data for α-chitin than β-chitin due to the higher crystallinity of α-chitin. IR technique was employed for a quantitative analysis via: a statistical evaluation of several absorption ratios; a multivariate regression method; or determination of an absorption ratio and construction of a calibration curve (absorption ratio versus DA), where the DA of reference samples are obtained by IR or a reference method such as ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy was used to verify the validity of IR technique; (iv) IR method can be used to determine the DA

for chitin, chitosan and their derivatives, amino-sugars, hetero-polysaccharides and glycol-proteins; (v) the samples for DA measurement by IR technique were prepared from either the mixture of chitin/chitosan and KBr or from casting of chitin/chitosan solution as a film. The use of the former procedure provided a number of advantages over solution procedure such as: solvent was not required; sample preparation from the solutions was not required, where the latter procedure was time-consuming; (vi) impurities (minerals, proteins, and pigments) and water content induce interferences and difficulties to obtain accurate results. To achieve more accurate results, care must be taken to identify the peaks correspond to impurities and the sample should be dried before the DA measurement; and (vii) among various reference bands, the band at 2870 cm^{-1} (C—H stretching) is the best one, due to minimum effect of hydrogen bond and humidity on its intensity and position. This peak is relatively separated from other peaks.

Quantitative analysis through the determination of an absorption ratio in conjunction with construction a calibration curve yields in DA value with low to moderately accuracy. Determination of several absorption ratios using one spectrum and their evaluation by statistical method is the best procedure for quantitative analysis with a good accuracy. The latter procedure is relatively long procedure and is time-consuming. It is desirable to take into consideration the following major parameters for quantitative analysis: time of measurement; precision of method; and accuracy of results. In this way, ^{1}H NMR (for soluble samples) or other NMR spectroscopy is the best one for a quantitative evaluation.

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