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Various Methods for Determination of the Degree of N-Acetylation of Chitin and Chitosan: A Review

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Chitin, chitosan, and their derivatives have been identified as versatile biopolymers for a broad range of agriculture and food applications. Up to now, several methods have been developed to determine degree of N-acetylation, DA, for chitin and chitosan. In this article, an effort has been made to review the available literature information on the DA determination. These methods are classified into three categories: (1) spectroscopy (IR, ^{1}H NMR, ^{13}C NMR, ^{15}N NMR, and UV); (2) conventional (various types of titration, conductometry, potentiometry, ninhydrin assay, adsorption of free amino groups of chitosan by pictric acid); (3) destructive (elemental analysis, acid or enzymatic hydrolysis of chitin/chitosan and followed by the DA measurement by colorimetry or high performance liquid chromatography, pyrolysis−gas chromatography, and thermal analysis using differential scanning calorimetry) methods. These methods have been compared for their performances and limitations as well as their advantages and disadvantages. The use of IR and NMR spectroscopy methods provides a number of advantages. They do not need long-term procedures to prepare samples, and they provide information on the chemical structure. ^{1}H NMR and UV techniques are more sensitive than IR, ^{13}C NMR, and ^{15}N NMR spectroscopy. The IR technique is mostly used for a qualitative evaluation and comparison studies. Conventional methods are not applicable for highly acetylated chitin. The results of the latter methods are affected by ionic strength of the solvent, pH, and temperature of solution. In destructive methods, longer times are needed for the measurements compared to spectroscopy and conventional methods, but they are applicable for the entire range of the DA.

KEYWORDS: Chitin; chitosan; degree of N-acetylation; spectroscopy; conventional; destructive

1. INTRODUCTION

Chitin is considered the second most plentiful organic resource on the Earth and occurs in plants, marine invertebrate, insects, cell walls of some fungi, and microorganisms (*1*−*3*). The word "chitosan" is used for both partially and completely N-deacetylated chitosans. The chemical structures of fully N-acetylated chitin and completely N-deacetylated chitosan are illustrated in **Figure 1**. Commercial chitin and chitosan are copolymers of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl glucosamine, GlcNAc), and 2-amino-2-deoxy-D-glucose (glucosamine, $GlcNH_2$) with β-D-(1 → 4) glycoside linkages. The solubility or insolubility of the polymers in aqueous acidic solutions/organic acids or in the mixture of salt/organic solvent such as LiCl/dimethylacetamide (*4*) has been used to distinguish between chitin and chitosan. The degree of N-acetylation, DA, has been also employed to differentiate chitin from chitosan. It is called chitin when both the DA is greater than a certain value (for example, DA > 50%) and the sample is not soluble; otherwise, it is called chitosan. The solubility or insolubility of the polymers in aqueous acidic solutions/organic acids is generally used to distinguish between chitin and chitosan rather than the use of the DA. In a particular case, when the DA ≈ 0% (completely deacetylated chitosan), the sample may be highly crystalline, and thus it is insoluble.

The solubility also depends on the degree of polymerization (molecular weight), degree of neutralization of amine groups, ionic strength of solvent, pH of chitosan solution, concentration of the polymer, and the distribution of *N*-acetyl glucosamine and glucosamine residues along the backbone of macromolecule chains. The differences in the distribution may result in random

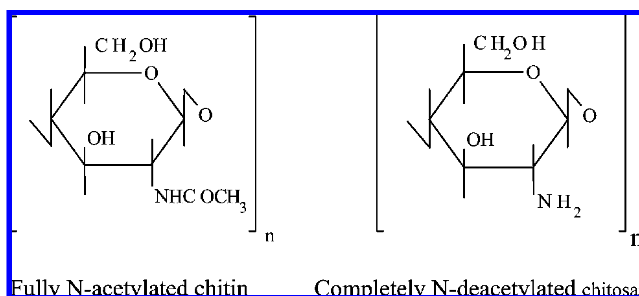

Figure 1. Chemical structures of fully N-acetylated chitin and completely N-deacetylated chitosan.

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Table 1. Various Applications of Chitin and Chitosan in Food and Agriculture

applications	references
coating and extension of shelf life for foods, particularly fresh fruits and vegetables	*22, 23, 26−33*
recovery of protein from waste foods	*34*
preparation of feed supplement from wastes of chitin and chitosan	*35*
removal of metal ions, small molecules and pesticides from water	*1, 36−38*
control of several pre and postharvest diseases	*12−18, 21, 32, 33, 39−41*
improve of seed quality	*42, 43*

or block copolymer, which would influence the solubility of the polymer.

Solubility (*5, 6*) biodegradability (*7*) aggregation properties (*8, 9*) and the value of pK_a (*10*) of chitosan depend on the proportion between N-acetylated glucosamine and glucosamine units as well as the DA. The value of pK varies within 6.46 and 7.32, and it depends on the DA (*9*). Chitosan with a smaller DA exhibits much better diverse biological properties than chitosan possessing a greater DA. The solubility increases with an increase in free amine group and/or a decrease in the DA.

Chitosan is a cationic polysaccharide, and its cationic nature in acidic medium is unique among polysaccharides. It is safe, nontoxic, and biodegradable (*11*). It exhibits diverse biological activities such as antifungal activity (*12, 13*), antibacterial activity (*14, 15*), elicitation of plant defense (*16−18*), cholesterol lowering effect (*19*), and wound-healing property (*20*). Chitosan inhibits or retard growth of Gram-positive and Gram-negative bacteria isolated from fishery products (*21*). Chitosan exhibits film and fiber forming ability (*22, 23*) and scavenging and antioxidant activities (*24, 25*). Chitosan with vast and diverse (biological, physicochemical, mechanical, and film and fiber forming) properties has been employed for various applications in food and agriculture (*1, 12−18, 22, 23, 26−43*). These applications are presented in **Table 1**.

The following conclusions can be made from **Table 1**: chitosan has potential for applications in the areas of food and agriculture as an antimicrobial agent, as an elicitor and a protector material for plants, as a controller of preharvest and postharvest diseases of horticultural commodities, as a removal material of pesticides and harmful heavy metal ions, as a reducer of water consumption, as a feed supplement, a food additive, and a food preservative, and as an extender of shelf life of fruits and vegetables.

The DA of chitin/chitosan is the most important parameter that influences their various properties including biological, physicochemical, and mechanical properties. The effectiveness and behaviors of chitin/chitosan and its derivatives have been found to be dependent on the DA. The expansion and stiffness of the macromolecular chain conformation and the tendency of the macromolecule chains to aggregate depend strongly on the DA (*44*). The determination of the DA for the two copolymers is essential for studying their chemical structures, properties, and structure−properties relationships. Knowledge on the DA is very important to maximize chitosan applications. If the DA is known, many properties and applications can be predicted. Therefore, determining an appropriate technique giving reliable data for the DA is essential and is desirable for researchers.

Several techniques have already been developed or employed to determine the DA of chitin/chitosan with different accuracies. These techniques include proton nuclear magnetic resonance, ^{1}H NMR (*45−48*); solid state cross polarization (CP)/magic angle spinning (MAS) ^{13}C NMR (*49−54*); CP/MAS ^{15}N NMR (*50, 55*); IR spectroscopy (*56−63*); near-infrared spectroscopy (NIR) (*64*); UV spectrophotometry (*65−67*); ninhydrin assay (*68, 69*); colloidal titration (*70*); conductometric titration (*51*); potentiometric titration (*53, 71*); acidic (*72*) or enzymatic (*73*) hydrolysis−colorimetry or HPLC analysis; GPC−UV analysis (*74*); elemental analysis (*44, 75*); and thermal analysis using differential scanning calorimetry, DSC (*76*).

To choose an appropriate method among various techniques is a difficult task for researchers. A review summarizing up-to-date literature information on determination of the DA serves as a ready reference for researchers involved in the area of chitin/chitosan characterization, and thus, it is a desirable. To date, no review on the various techniques of the DA determination for chitin and chitosan has been published. There is no unique technique that can measure the DA with a high precision in the entire range. For example, CP/MAS ^{13}C NMR and CP/MAS ^{15}N NMR spectroscopy can be used for chitin because of its lack of solubility. For chitosan, which is soluble in an aqueous acidic medium, some methods such as ^{1}H NMR, potentiometric and conductometric titrations, and ultraviolet spectrometry can be employed. Some techniques such as elemental analysis, acidic or enzymatic hydrolysis followed by colorimetry or HPLC analysis, thermal analysis using DSC, and IR spectroscopy may be employed for the entire range of the DA.

The objectives of this study are to describe various methods published on the DA determination, to compare them for their performances and limitations as well as their advantages and disadvantages, and to present various factors affecting the experimental results.

2. DESCRIPTION OF VARIOUS METHODS

Various methods are classified into three groups: (1) spectroscopy (^{1}H NMR, ^{13}C NMR, ^{15}N NMR, IR, near-IR, and UV); (2) conventional (various procedures using colloidal titration, conductomertric titration, potentiometric titration, and ninhydrin assay); (3) destructive (elemental analysis, acidic or enzymatic hydrolysis followed by colorimetry or HPLC analysis and thermal analysis using DSC) methods.

2.1. Spectroscopy Methods. The DA of chitin/chitosan by various types of NMR (^{1}H NMR, ^{13}C NMR, and ^{15}N NMR), IR (IR and near-IR) and UV spectrometry has been determined as described in the following: (i) 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 band or summation of several absorption bands as a reference. The intensity/intensities of the reference band(s) do not change with the DA. The DA of unknown samples is estimated by comparing the values of A_M/A_R for unknown samples with similar ratios of reference samples having known DA. (ii) Construction of a calibration curve is done by plotting the absorption ratio of chitin/chitosan samples having known DA versus their corresponding DA values. The DA of the chitin/chitosan samples was determined by the same spectroscopy technique or by a reference method such as ^{1}H NMR spectroscopy. The DA of unknown samples was then estimated using the calibration curve.

The above general description is valid for various types of NMR and IR spectroscopy. The specific description for each technique is presented as follows.

2.1.1. NMR Spectroscopy. 2.1.1.1. ^{1}H NMR Spectroscopy. Dilute solutions of chitosan samples in an aqueous acid (CD_3COOD/D_2O or DCl/D_2O) have been prepared, and their ^{1}H NMR spectra have been recorded from 0 to about 10 ppm using a proton NMR spectrometer (*45, 46, 77*). Different models of proton NMR spectrometer (fabricated by Bruker Company) have been employed to record the NMR spectra. In most of the reports, methyl protons of the $NH-CO-CH_3$ group have been selected as probe nucleus/nuclei. Different procedures have been proposed for calculation of the DA (*45, 66, 77, 78*).

2.1.1.2. ^{13}C NMR Spectroscopy. A carbon NMR spectrometer usually equipped with cross-polarization magic-angle spinning carbon NMR (CP/MAS ^{13}C NMR) has been used to record the spectra of chitin/chitosan samples (*44, 49−51, 53, 60*). Tolaimate et al. (*53*) and Duarte et al. (*60*) have modified the procedure of Ottøy et al. (*44*) through the changes in relaxation delay time and contact time. The contact time and delay time are the most variable parameters to record the CP/MAS ^{13}C NMR spectrum (*53, 60, 76*). The DA of chitosan samples was calculated from the intensity of *N*-acetyl carbon atom divided by the summation intensities of carbon atoms of the D-glucopyranosyl ring (*44, 51, 53, 60*).

2.1.1.3. ^{15}N NMR Spectroscopy. ^{15}N NMR spectra of chitin/chitosan samples in the solid state have been recorded (0−200 ppm) using a ^{15}N NMR spectrometer operating at 30 or 200 MHz (*50, 55*). Cross-polarization and strong magnetic fields have been used to increase resolution and sensitivity. The different contact times were employed to minimize errors for quantitative evaluation of the DA (*50, 55*). Two different nitrogen atoms are present in chitin/chitosan: one corresponds to amine group, and another one corresponds to the acetamide group. Thus, two major peaks have been recognized in the spectra of chitin/chitosan samples. The DA was calculated from the ratio of peak intensities [*N*-acetyl group/(*N*-acetyl group + amine group)] (*50, 55*).

2.1.2. Infrared Spectroscopy, IR. The IR region of an electromagnetic spectrum extends from 2.5 to 400 μm. The range of 2.5−25 μm (4000−400 cm^{-1}) is known as the mid-range region, and from 25 to 400 μm (from 400 to 25 cm^{-1}), it is known as the far- IR region. The IR spectrum 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 powder of chitin/chitosan sample or as a thin film made from a casting procedure of a chitin/chitosan solution. Various procedures using different absorption 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 already proposed to determine the DA (*47, 57−63, 59, 79−81*). The DA of chitin/chitosan samples has been also determined by evaluation of several absorption band ratios using a statistical method (*49, 60*). A detailed description of the various procedures of IR technique has been recently published (*82*).

2.1.3. Near-Infrared (NIR) Spectroscopy. The range of 0.8−2.5 μm (12500−4000 cm^{-1}) is generally considered to be near-IR region. Near-IR spectra for chitin/chitosan samples have been recorded from 1100 to 2500 nm (from 9090 to 4000 cm^{-1}), and their second derivatives spectra have been used to determine the DA. Gluscosamine and D-glucosamine hydrochloride have been selected as model compounds (*64*). A multivariate regression method has also been employed to evaluate quantitatively the DA using NIR spectra (*83*). A reference curve was constructed by plotting the DA estimated from NIR data versus the DA determined by ^{1}H NMR spectroscopy. A detailed description on the DA determination by NIR technique has been reviewed recently (*82*).

2.1.4. Ultraviolet Spectrometry, UV. Ultraviolet/first derivative ultraviolet spectrometry was used to determine the DA of chitosan (*65*). The maximum wavelength (λ_m) was found to be 200 ± 2 nm for *N*-acetyl glucosamine (*65, 66*). The maximum absorption wavelength has been steadily increased from 199 to 202 nm with an increase in concentration of *N*-acetyl glucosamine (*84*). The absorbance of *N*-acetyl glucosamine (at maximum wavelength) was linearly dependent on its concentration. The range of chitosan concentration was 0.01−0.03 M in CH_3COOH (*66*).

2.1.5. Gel Permeation Chromatography−Ultraviolet (GPC−UV) Analysis. This method is based on liquid chromatography analysis using an aqueous gel permeation column and UV detector (*74*). The measurement was performed using a column (Shimpack DIOL-300), an eluant of 0.01 M CH_3COOH/0.2 M NaCl, with a flow rate of 1 mL·min^{-1}, and a UV detector adjusted at 220 nm and at 23 °C (*74*). The *N*-acetyl group (acetamide) of *N*-acetyl glucosamine has an absorption band at 220 nm (*74*). The absorption peak areas are proportional to the concentrations of acetamide groups. The DA was estimated from the linear relationship between the absorbance and concentration of *N*-acetyl glucosamine residues.

2.2. Conventional Methods. *2.2.1. Titration with an Alkali.* Chitosan with a known concentration was dissolved in an organic acid. The chitosan solution ($-NH_3^+$ groups of chitosan) was then titrated with a dilute alkali solution, while the pH of the chitosan solution was measured. The variation of pH for chitosan solution as a function of added alkali volume was plotted. This plot has two inflection points. The difference between the volumes of alkali necessary for the two inflection points equals the amount of alkali that is consumed for the conversion of the amine groups into ammonium salts. The DA is determined from the amount of alkali volume consumed for the conversion. Glucosamine and *N*-acetyl glucosamine were used as reference samples with DA of 0 and 100%, respectively (*84*).

2.2.2. Titration with an Acid. A dilute alkali solution of chitin/chitosan was prepared, and its pH was adjusted to 12 at 25 °C. The latter solution was then titrated with 0.1 N HCl. The DA of chitin/chitosan was calculated from the amount of acid consumed to neutralize free amino groups of chitosan (*85*).

2.2.3. Direct Colloid Titration. A chitosan sample was dissolved in an aqueous acetic acid. The positive ammonium groups of the chitosan macromolecule were titrated directly with a known concentration of negative sulfate groups of polyvinyl sulfate potassium salt [PVSK, $(C_2H_3O_4SK)_n$, $n \geq 1500$] (*45, 70, 86−88*). Toluidine blue was used as an indicator (0.1% in alcohol). The equivalence point is identified by changing the color from blue to reddish purple and formation of flocculate/precipitate of the reacting complexes (*45*). The normality of the titer solution is defined as the number of equivalents for dissociated groups of the polymer in 1 L of solution. The amount of PVSK required for 1 g of chitosan was then calculated (*45*).

2.2.4. Conductometry. A dried chitosan sample was dissolved in an acidic solution while the solution was constantly stirred. A dilute solution of NaOH was slowly added to the initial acidic chitosan solution. The conductivitivity measurement was then performed at equilibrium and stable conditions (*51, 86*). The conductivity of the solution was plotted against added volume

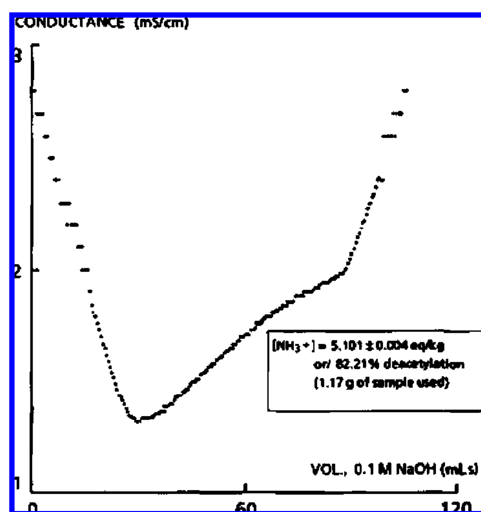

Figure 2. Variation of solution conductance versus added NaOH solution. Reproduced with permission from *Carbohydrate Research* (*51*). Copyright 1993 Elsevier.

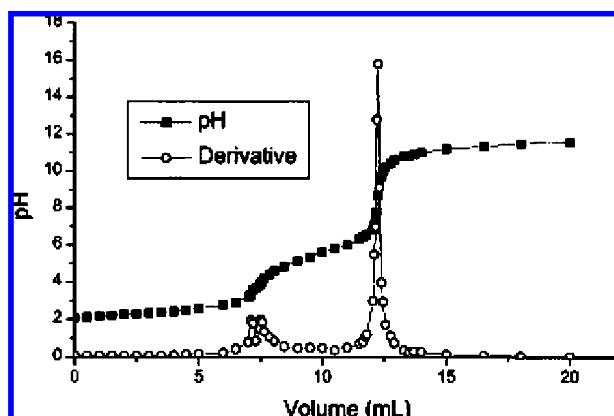

Figure 3. Variation of pH of solution versus added NaOH solution. Reproduced with permission from *Polymer* (*53*). Copyright 2000 Elsevier.

of the alkali solution (see **Figure 2**) (*51*). The first rapid descending portion corresponds to the neutralization of the free H^+ ions. The curvature at the lower end of this portion is attributed to the initial dissociation of the protonated amino groups of the chitosan sample. The first ascending portion is due to the neutralization of the protonated amino groups. A small deviation from linearity in the plot occurs during the final phase of neutralization, which coincides with the precipitation of chitosan. The final upward fraction corresponds to the increase in conductance due to an excess of added base. The number of amine groups was calculated using the equivalence point data (*51, 86*).

2.2.5. Potentiometric Titration. Chitosan was dissolved in an aqueous acidic solution with an excess amount of the acid. This solution was then titrated with 0.1 M NaOH. The procedure was initially proposed by Muzzarelli (*1*). It was then modified by Ke and Chen (*71*). The latter procedure was further modified by Tan et al. (*66*). The variation of potential or pH of the solution versus added volume of NaOH was plotted (see **Figure 3**) (*53*). A curve with two inflection points was obtained. The first and second inflection points correspond to the neutralization of the free H^+ and the neutralization of protonated amino groups by alkali, respectively (*89*). The difference between the values for the conversion of amino groups into ammonium salt allows one to determine the DA of chitosan (*66*). $AgNO_3$ solution has been also used to measure ammonium salt (*56*).

2.2.6. Ninhydrin Assay. Ninhydrin (triketohydrinedene hydrate) assay is based on determination of free amine group of glucosamine units (*68*). Different standard solutions of glucosamine ($GlcNH_2$) with known concentrations in acetic acid were prepared. Acetic/acetate buffer (pH 5.5) and ninhydrin reagent (200 mg of triketohydrindene hydrate was dissolved in 10 mL) were then added to each solution. The absorbance of each solution at 570 nm was measured. The calibration curve was constructed by plotting the absorbance of different standard solutions against their corresponding concentrations. The amounts of chitosan ($GlcNH_2$ units) in unknown samples were estimated using the calibration curve (*66*).

2.2.7. Adsorption of Free Amine Groups of Chitosan by Picric Acid. Picric acid adsorbs free amino groups of chitin/chitosan with a ratio of 1:1. In the presence of a strong base, the picric acid forms a methanol-soluble salt, which is released from the chitin/chitosan sample. Practically, a dried and finely powdered chitin/chitosan sample (5−30 mg) was placed in a small column (similar to liquid chromatography column) (*90, 91*). The amount of the chitosan sample was determined from the difference between weights of the column before and after introduction of the sample. The binding procedure between the polymer (amino groups) and picric acid was performed for 6 h after introducing 0.1 M picric acid−methanol. The bound pictric acid was then quantitatively removed from the amino group of the chitin/chitosan sample as follows: the solution of 0.1 M diisopropylethylamine−methanol was introduced into the column, allowed to stay for 30 min, and then eluted with methanol. The total eluate was then diluted to a certain volume with methanol. The concentration of diisopropylethylamine pictrate in the eluate was measured at 358 nm (Beckman DU-8B spectrophotometer). A calibration curve for the salt in methanol (in a concentration range of 0−115 μM) was linear. The DA of unknown sample was determined using amounts of chitin/chitosan and the calibration curve.

2.3. Destruction Methods. *2.3.1. Enzymatic Hydrolysis/Colorimetry or HPLC Analysis.* This method includes two steps: (i) complete hydrolysis of chitin/chitosan sample into two monosaccharides β-D-glucosamine ($GlcNH_2$) and β-D-acetylglucosamine (GlcNAc) by exo enzymes (β-D-glucosaminidase and β-D-acetylhexosaminidase) for 12 h at 40 °C (*73*) and (ii) analysis of resulting monosaccharides by colorimetric or HPLC technique. The DA was calculated from the concentration ratio $[GlcNH_2]/[GlcNH_2 + GlcNAc]$.

2.3.2. Acid Hydrolysis/HPLC Analysis. This method includes two steps: (1) digestion of chitin/chitosan sample by acid to release acetic acid and (2) analysis of acetic acid by HPLC. This method initially was developed by Holan et al. (*92*). Niola et al. (*72*) have modified the two steps of this method. Ng (*93*) and Ng et al. (*94*) have conducted a modification procedure by changing the ratio of acid/test sample. A mixture of concentrated H_2SO_4, oxalic acid, and propionic acid was used as reagent for hydrolysis. A powdered polymer with H_2SO_4 and standard mixture of oxalic acid and propionic acid were placed in a tube. The final concentration of H_2SO_4 was 2.4 M. Acid hydrolysis was conducted in vacuum at 155 °C for 1 h with a ratio of 1:4 (H_2SO_4/oxalic acid). The two acids cooperatively act. That is, H_2SO_4 depolymerizes the sample to some extent to make it accessible for the action of oxalic acid. The deacetylation process becomes complete using the latter acid (*72*). Propionic acid was used as an internal standard. Glucosamine and *N*-acetyl glu-

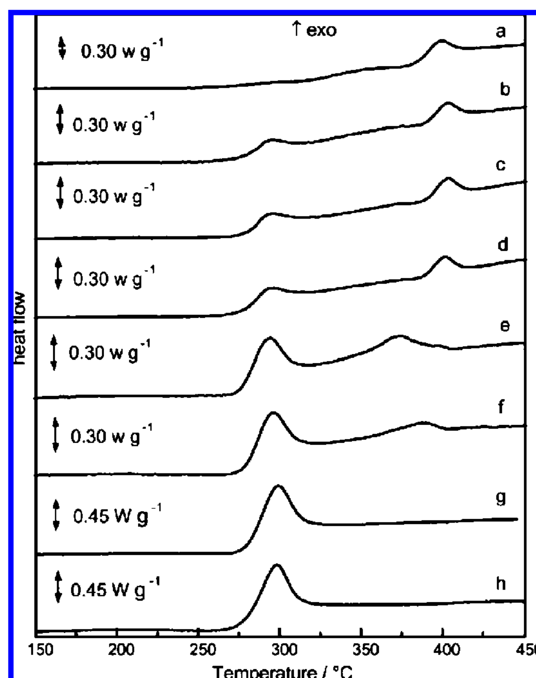

Figure 4. DSC curves under nitrogen atmosphere (50 mL·min^{-1}) and sample mass of 3 mg at 5 °C·min^{-1} for chitin/chitosan samples obtained at different reaction times of thermochemical heterogeneous deacetylation: (a) 0 min, (b) 60 min, (c) 90 min, (d) 120 min, (e) 150 min, (f) 180 min, (g) 240 min, and (h) 360 min. Reproduced with permission from *Thermochimica Acta* (*76*). Copyright 2006 Elsevier.

cosamine were determined by colorimetry (*73*) or HPLC analysis. In the HPLC analysis, the peak area was converted into a molar concentration using a standard curve. A standard curve was constructed using glucosamine or *N*-acetyl glucosamine as standard material. The DA obtained from the HPLC analysis was in agreement with colorimetry assay (*73*).

2.3.3. Elemental Analysis (EA). This method is based on determination of C, H, N, and O (*44, 75, 78, 81, 95*). Elemental analysis of the polymer sample was performed using an analyzer of CHN (Carlo-Erba, model EA1108 or higher) with a carrier gas (He at a flow rate of 100 mL·min^{-1}) at a combustion temperature of 1000 °C (*75*). The DA was calculated from the ratio of C/N (*81*). The ratio of C/N varies from 5.145 for completely N-deacetylated chitosan ($C_6H_{11}O_4N$ as repeat unit) to 6.861 for fully N-acetylated polymer ($C_8H_{13}O_5N$ as repeat unit) (*78*). The percent of N in completely N-deacetylated chitosan and fully N-acetylated chitin is 8.7 and 6.9%, respectively.

2.3.4. Thermal Analysis. This method is based on decomposition of the chitin/chitosan sample into two fragments having NH_2 and *N*-acetyl groups. The DSC technique has been used to analyze the decomposed species. The effect of water content, heating rate, amount of the chitin/chitosan sample, and flow rate of gas on the DSC results was evaluated and optimized. The DSC results for chitin/chitosan samples having different DA (16−74%) are shown in **Figure 4**. Two exothermic decomposition peaks at 296 °C (amine residues) and 404 °C (*N*-acetyl residues) were observed in the DSC thermogram. Two endothermic peaks at 70 and 110 °C corresponding to the free and bound water may be observed in chitin/chitosan samples. This is because anhydrous chitin/chitosan samples have been hardly obtained because of the hygroscopic nature of the polymers. Thus, thermal analysis by DSC results in erroneous values for the DA. Water normalization is required to eliminate the error arising from the moisture content. Under optimized conditions, a linear relationship between peak area/peak height and the DA of the test samples was obtained (*76*). Peak areas or peak heights of the signals have been used to estimate the DA (*76, 96*). The DA obtained by thermal analysis using DSC was compared with the DA determined by ^{1}H NMR and CP/MAS ^{13}C NMR methods. The data obtained from DSC were consistent with the DA determined by the two NMR techniques (*76*).

2.3.5. Pyrolysis−Gas Chromatography (Pyrolysis−GC) Analysis. The DA of chitin/chitosan sample was determined from the intensities of the characteristic products such as acetonitrile, acetic acid, and acetamide. These products were librated from *N*-acetyl groups of *N*-acetyl D-glucosamine units of chitin/chitosan by acid hydrolysis (*97*). Holan et al. (*92*) have proposed a method using GC for the determination of acetic acid. The acid was librated from *N*-acetyl groups of chitin/chitosan sample by HCl hydrolysis. Sato et al. (*97*) have used oxalic acid as an acid for hydrolysis. Oxalic acid was used to prolong the lifetime of the GC column, since the use of HCl significantly reduces the lifetime of GC column. The result of pyrolysis−GC was consistent with ^{1}H NMR, IR, and CP/MAS ^{13}C NMR spectroscopy results (*97*).

3. GENERAL ASPECTS FOR THE DA DETERMINATION

3.1. Effect of Chemical Structure, Impurities, Morphology, and Solubility of Chitin/Chitosan on the DA Measurements. Chitin naturally occurs associated with proteins, organic pigments, and minerals (*1, 98*). The nature and the level of the impurities vary from one source to another. Among them, proteins possess functional (amine and NH−C═O) groups similar to chitin/chitosan. The results of a chitin/chitosan sample with a certain DA value may change depending on the nature and level of the impurities, source, and polymer morphologies. Thus, prior knowledge on water content and the impurities would yield more accurate data for the DA.

The most complicated problem for the determination of the DA is related to the poor solubility of chitin/chitosan. Chitin with β-(1 → 4)-2-acetamido-2-deoxy-D-glucan (GlcNAc) as structural units, and β-(1 → 4)-D-glycoside linkages, forms a linear chain through the many inter- and intramolecular hydrogen bonds. The hydroxyl groups in chitin contribute strong intra- and intermolecular hydrogen bonds. In addition, both hydrophobic and hydrophilic interactions may occur between macromolecule chains. The linearity of chitin makes it easy for the molecules to produce strong intermolecular forces and results in a high degree of crystallinity (*1, 99*). The degree of crystallinity changes with the DA (*5*). Both crystallinity and DA change from one source to another through the change in the morphology of chitin (α, β) (*1*). A larger degree of crystallinity and a greater DA and a stronger intermolecular interaction reduce the solubility of the polymers in aqueous and other solvents. The distribution of *N*-acetyl groups in chitin or chitosan has a significant effect on solubility of the polymers. Chitin in nature occurs as a random or a block copolymer (*46, 94*), where the block copolymer is not soluble. The solubility of chitosan in an aqueous acidic solution depends on the degree of polymerization (molecular weight), degree of neutralization of amine groups, ionic strength of solvent, pH of chitosan solution, and concentration of the polymer. A smaller macromolecule or a smaller concentration of chitosan in solution enables one to dissolve chitin/chitosan samples with a greater DA. The latter conditions enable one to determine a wider range of the DA in solution techniques.

3.2. Amount of Sample Needed for DA Measurement. Low amount of a chitin/chitosan sample (up to 50 μg) is needed in thermal methods using either DSC or GC (pyrolysis−GC) analysis. The amount of sample for EA is reported to be between 0.5 and 2 mg (*44, 45*). Approximately 5, 300, and 200 mg of chitin/chitosan samples have been used to record ^{1}H NMR, ^{13}C NMR, and ^{15}N NMR spectra, respectively (*44, 45*). The amount of chitin/chitosan samples for the DA analysis by conventional methods generally should be larger than those of spectroscopy and destructive methods. However, the amount of samples required for the analysis depends on the sensitivity and limit of detection of the instruments. Smaller amounts of the chitin/chitosan sample are needed for the analysis using an instrument with a higher sensitivity. Generally, smaller amounts of samples for the analysis are required for instruments fabricated using high and advanced technologies.

4. EVALUATION OF REPORTED RESULTS

4.1. Evaluation of Reported Spectroscopy Methods. In the region of UV, a low concentration (as low as 0.1 mg·L^{-1}) of *N*-acetyl glucosamine (GlcNAc) or glucosamine ($GlcNH_2$) produces an absorbance greater than 0.2. A chitosan sample produces a larger absorbance in UV technique in comparison with NIR, IR, and NMR methods. In the latter methods, in order to obtain sufficient absorbance, more amounts of the sample or greater concentrations are needed. Thus, determination of the DA by UV spectrophotometry results in greater accuracy compared to NIR, IR, and NMR methods. In IR and near-IR, concentrations of 1−10 mg·mL^{-1} (IR) and up to100 mg·mL^{-1} (near-IR) are needed in order to produce sufficient absorbance.

In the UV method, a calibration curve was constructed for GlcNAc in dilute acetic acid (in the range 0.50−5.0 mg·L^{-1}) (*65*). The detectable concentration of GlcNAc in 0.01 M acetic acid was found to be as low as 0.5 mg·L^{-1} (*65, 66*). The lower concentration enables one to analyze a wider range of the DA. However, too low concentrations cannot be used, since it induces large experimental error. Tan et al. (*66*) have compared the results of four methods (^{1}H NMR, UV, ninhydrin assay, and potentiometric titration). Of the four methods, the result obtained by the first derivative UV was the closest to the actual value of the DA. Arcidiacono and Kaplan (*84*) have determined the DA of chitosan samples by three methods (first derivatives UV spectrophotometry, IR spectroscopy, and titration by a diluted NaOH solution). Khan et al. (*100*) have shown the UV/first derivative of UV method was more sensitive than two other methods (titration with acid and IR). One can conclude that the first derivatives UV spectrophotometry resulted in the most accurate data compared to the other methods.

The IR technique can be used mainly for qualitative analysis and comparison studies. It has been used for quantitative analysis of crystalline samples, since crystalline samples created sharper signals and higher resolution compared to amorphous samples. IR or NMR technique gives signals for any chemical compounds present in chitin/chitosan samples. Therefore, a large number of signals appear in IR or NMR absorption spectra. Small differences in the compositions result in significant differences in IR and NMR spectra. An intense peak corresponding to the OH group has been observed in IR and ^{1}H NMR spectra of chitin/chitosan. This is due to the hygroscopic nature of chitin/chitosan, while a chitin/chitosan sample with high water content can be used for the DA analysis by UV spectrophotometry. Chitin/chitosan frequently associated with other polysaccharides, which makes the evaluation of *N*-acetyl content by ^{1}H NMR, ^{13}C NMR, and IR more problematic and in some cases impossible. Chemical modifications such as deacetylation, acetylation, decomposition, depolymerization, and fragmentation result in changes in NMR and IR spectra by increasing/decreasing the intensity or shifting the position of some peaks.

^{1}H NMR technique has usually been employed as a standard method to calibrate other methods (*46, 59, 63*). Among various conditions proposed for determining the DA of chitosan by ^{1}H NMR (*41, 42, 59*), the procedures proposed by Hirai et al. (*45*) and Vårum et al. (*46*) have been widely accepted. The interpretation of ^{15}N NMR spectrum and evaluation of the DA measurement by NMR is simple, since it gives only two signals. The DA determination of chitin/chitosan in the presence of other polysaccharides by ^{15}N NMR spectroscopy is possible without any purification process (*55*). This is because the N nucleus is only present in chitin and chitosan. Interpretation of a ^{13}C NMR spectrum is not a difficulty because carbonyl and methyl groups are well resolved (*50*). ^{15}N NMR and ^{13}C NMR do not need a dried sample. The two techniques are not appropriate techniques for chitin/chitosan having low DA values. These two NMR techniques resulted in underestimated values for low DA values (*50, 51, 101, 102*). ^{1}H NMR and ^{13}C NMR spectroscopy may also provide information on the sequential distribution of free amine and *N*-acetyl groups (*46, 103*).

4.2. Evaluation of Conventional Reported Results. Conventional methods are applicable for soluble as well as low acetyl contents of chitosan samples. Contradictory data and conclusions for low acetyl contents of chitosans by conventional methods were given by different research groups. Raymond et al. (*51*) have compared the results of the DA determined by conductometry with the corresponding data determined by CP/MAS ^{13}C NMR and UV. The DA of chitosans having low acetyl content obtained by conductometry was consistent with the DA obtained by CP/MAS ^{13}C NMR and UV methods. This is because the amino groups of chitosan (with low acetyl contents) are accessible for titration in the conventional methods. The DA of chitosans with low acetyl content obtained from potentiometric titration was also in agreement with the result of ^{1}H NMR spectroscopy (*53*). The potentiometric colloidal titration resulted in overestimated values for the DA of <10 (*73*). The titration of chitosan solution with an alkali solution resulted in large standard deviations. This is due to the formation of viscose solution and precipitation of chitosan (*84*).

Conventional techniques require a long time for equilibrium and measurement. The results obtained by conventional methods are affected by ionic strength of solvents, pH, and temperature of solutions. The presence of the impurities, particularly proteins (ninhyrin assay, colloidal titration), may have adverse effect on the experimental results (*66, 69, 70*). The major source of error for conventional methods arises from various measuring parameters (volumes and concentrations of titrant and titer, pH, conductance, and potential). The DA determination employing picric acid, in contrast to other conventional methods, has been used for a wide range of the DA (0.25−0.99). This method is useful for highly acetylated chitin/chtosan.

4.3. Evaluation of Reported Destructive Methods. Destructive methods (EA, thermal analysis by DSC, acid or enzymatic hydrolysis followed by HPLC or spectrophotometry analysis, and pyrolysis−GC analysis) can be used for the entire range of the DA. In the HPLC and GC analyses of chitin/chitosan samples in the presence of other carbohydrates or polysaccharides, overestimated DA values may be obtained. This is due to the formation of additional acetic acid from the impurities. Excess amounts of oxalic acid had an adverse effect on the experimental result obtained from the pyrolysis−GC

Table 2. DA Ranges, Advantages, and Disadvantages for Different Spectroscopy Methods

	DA (%)	advantages	disadvantages
UV	0−50	Simple, easy-to use-apparatus, high accuracy for the DA results. High sensitive method. Humidity and mineral ions do not induce interference for the DA determination. The UV method can be used for routine analysis of the DA with a high accuracy.	Requires sample preparation. Applicable for a limit range of the DA. The accuracy is reduced with an increase in solvent concentration.
NIR	0−60	Accurate results for DA $\leq$ 60.	Possible error from adsorbed humidity.
IR	varies with different ratios	More suitable for the DA determination of crystalline samples. Several possibilities exist to choose absorption band ratios and determine the DA.	Drawing baseline is a general difficulty. Wet samples may result in less accurate data. Some absorption ratios result in less accurate data.
^{1}H NMR	0−60	Gives accurate results and some information on the distribution of the co-units.	Requires sample preparation.
^{13}C NMR	0−100	No sample preparation. Applicable for entire range of DA. Gives information on sequential distribution. No need to dry the sample.	Low sensitivity. Availability of the instrument is not universal.
^{15}N NMR	0−100	Well-resolved spectra, no sample preparation. Water as an impurity does not cause any problem. No need to dry the sample. Applicable for entire DA range. Suitable for a composite or blend of chitin/chitosan with other polysaccharides.	Possible errors if the sample contains proteins as impurities. Availability of the instrument is not universal.

Table 3. Different Methods of the DA Determination, Their Corresponding DA Ranges, and Their Performances and Limitations

method	DA range	performances	limitations
conventional	applicable for soluble chitin/chitosan	Availability of the instruments is not a problem. Easy to use the instrument and easy to perform the method. Humidity is not an interference.	Applicable only for soluble chitin/chitosan samples. Proteins and mineral ions may induce interference peak(s) and result in unreliable results.
spectroscopy (^{13}C NMR, ^{15}N NMR)	0−100	Applicable for soluble and insoluble chitin/chitosan samples. Some information on chemical structure and sequence of comonomer units may be obtained from the spectra of chitin/chitosan samples. The more sensitive instrument generally results in the higher precision. Resolution, limit of detection, and accuracy of results are improved using cross-polarization and strong magnetic fields.	The impurities of chitin/chitosan (moisture, protein, pigments, and metal ions) may create interference peaks. These techniques are not sufficiently sensitive for low values of the DA. The availability of the instrument is a limitation due to the cost, special considerations, and sophistication, especially for the instruments having higher sensitivity and stronger magnetic fields.
spectroscopy (^{1}H NMR, IR, near-IR, UV)	soluble samples	^{1}H NMR and UV techniques are more precise and result in more accurate data in comparison to other methods.	IR and UV methods usually require carefully selected chitin/chitosan reference samples (with certain the DA values).
destructive	0−100	Entire range of the DA.	Two steps are required for analysis (decomposition of chitin/chitosan and analysis of the decomposed species). Long term is required to analyze the DA.

method (*97*). The impurities do not create any difficulties for the DA analysis by HPLC and GC analysis if the impurities create separated peaks. The HPLC and GC analysis may provide information on the impurities. The variation of error in the EA method is relatively large (*38*). This is because the presence of organic materials or polysaccharides other than chitin/chitosan (as impurities) significantly changes the ratio of N/C.

5. COMPARISON OF VARIOUS METHODS

The DA range, performances, and limitations of each class (conventional, spectroscopy, and destructive) of the DA determination are given in **Table 2**. The DA range, advantages, and disadvantages for different spectroscopy methods are presented in **Table 3**. Most of methods are applicable for a limited range of DA. Conventional, UV/first derivative of UV, and ^{1}H NMR are applicable for those chitosan samples that are soluble. Among conventional methods, picric acid assay can be also used for a wide range of the DA and highly acetylated chitin/chitosan. ^{13}C NMR, ^{15}N NMR, and IR spectroscopy and destructive methods (acid or enzyme hydrolysis followed by analysis of the decomposition species by HPLC or spectrophotometry, pyrolysis−GC, EA, and DSC) have been employed for the DA in entire range, for both crystalline and amorphous samples. The combination of the three types of NMR techniques can be

used for the entire range of the DA for chitin/chitosan samples and some of their derivatives and composites. ^{1}H NMR, UV, near-IR, and conventional methods need solution preparations and cannot be used for block copolymers and highly acetylated chitin/chitosan samples. UV spectrometry is an appropriate technique for quantitative analysis of soluble samples particularly for highly deacetylated chitosans.

The accuracy and precision of the methods depend on the nature and level of impurities. The impurities induce difficulties for determination of the DA in different methods through the creation of interference, changes of the positions and intensities of some peaks in spectroscopy methods, formation of some decomposition species in destructive methods, and creation of interference species or ions in conventional methods. In some techniques, the impurities create interference and yield large variation for experimental results. To achieve more accurate results, care must be taken to identify the impurities. The samples should be dried before the DA measurement. The experimental error for the DA values obtained by some methods such as EA may be enough large (*38, 44*). Some methods such as CP/MAS ^{13}C NMR, ^{15}N NMR, and acid or enzymatic hydrolysis followed by analysis by HPLC or colorimetry yield reliable results for insoluble chitin/chitosan samples even though the samples are associated with humidity and some impurities (*44*). The result obtained from UV/first derivative of UV method was reasonably independent of protein and humidity contaminates. These impurities are not considered as interferences for the DA measurement.

The conventional and UV/first derivative methods are easy to perform, and the apparatuses are available in most laboratories for routine and research purposes. The availability of the instruments (spectroscopy, HPLC, GC, and DSC) is a limitation due to cost, special considerations, and sophistication, which render them more appropriate for research purposes.

Spectroscopy methods give information on chemical structure, sequence, and morphology, and GPC−UV method provides information on molecular weight and molecular weight distribution of chitin/chitosan samples.

Among all of the proposed methods, UV/first derivative of UV is the most sensitive. ^{1}H NMR spectroscopy has been chosen as a standard method by the American Standard Test Method organization to determine the DA for chitosan (*104*). This is due to its accurate data and low variation of experimental results. UV/first derivative of UV (*65, 66, 100*) and ^{1}H NMR (*47, 50, 53, 66, 78, 97, 105*) techniques have been used to verify the validity of several methods in different laboratories. The estimation of the DA by some methods had a large margin of error. The validity of these methods for unknown chitin/chitosan samples can be examined from the knowledge of the DA determined by UV and ^{1}H NMR spectroscopy. An analytical method should meet proper standards of accuracy and reliability. It is essential that an analytical method be supported by sufficient laboratory data to document their validity.

The time required for the DA measurements by destructive and conventional methods is longer than the time for spectroscopy methods. 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 and UV (for soluble samples) are appropriate methods for quantitative evaluation.

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