Further investigation on the degree of deacetylation of chitosan determined by potentiometric titration.

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ABSTRACT

The degree of deacetylation (DDA) of various low molecular weight chitosan (LMWC) species as the hydrochloride and free base (amine form) was determined by direct and back potentiometric titration, respectively. The DDA values obtained for the chitosan hydrochloride by direct titration were greater than 93% for all oligomers tested (Molecular weight (Mwt) between about 1.3 to 30.0 kDa). However, the DDA values obtained for chitosan amine oligomers using back titration were significantly lower, especially for the relatively high molecular weight (30.0 kDa) chitosan amine oligomers. Furthermore, after using the back titration method, greater DDA values were obtained for the same samples of chitosan amine after the chitosan solution had been heated to 60°C before titration. In addition, the DDA values showed a significant decrease with increased concentration for a given chitosan oligomer. Although the effects of hydration time, ionic strength and method specific behavior were not explicitly studied and therefore cannot be entirely ruled out, the results from this study might be attributed to the conformation of changes of chitosan in solution that lead to more inter/intra attractive forces in the case of chitosan amine. The DDA values obtained by the potentiometric method were also compared with those obtained by the FDUV and IR spectroscopic methods. Thus, subject to the caveats mentioned above, DDA values obtained for chitosan amine by the back titration method should be used with caution since the results are significantly lower than those obtained by direct titration, FDUV spectroscopy or FTIR.

INTRODUCTION

Chitosan is a partially deacetylated derivative of chitin, a polysaccharide found in the shells of crustaceans. When the degree of deacetylation (DDA) of chitin exceeds 50% it becomes soluble in acidic aqueous solutions. Chitosan is a copolymer of N-acetylglucosamnie (acetylated unit) and glucosamine (deacetylated unit), that is 2-acetamido-2-deoxy-D-glucopyranose (GIcNAc) and 2-amino-2-deoxy-D-glucopyranose (GIcN) units, respectively, joi-

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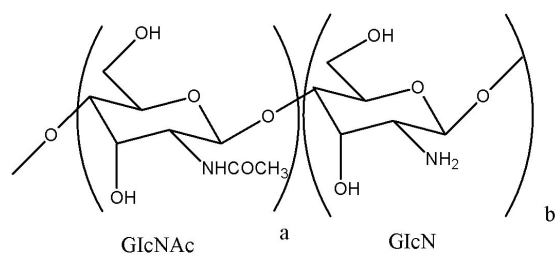
Figure 1 (a) 2-acetamido-2-deoxy-D-gluco-pyranose **(b)** 2-amino-2-deoxy-D-glucopyranose

ned by a β(1-4) glycosidic bond shown in Figure 1. Low molecular weight chitosan (LMWC) may be produced by acid depolymerization, oxidative degradation, or enzymatic hydrolysis (1) of high molecular weight chitosan.

The DDA is likely an important chemical characteristic that may be related to the performance of chitosan in different applications, for example the ability to chelate metal ions, acid-base characteristics, sorption properties, self aggregation, solubility and biodegradability (2). The process of deacetylation involves the removal of acetyl groups from chitin leaving free amino groups (-NH$_2$) to which the chitosan properties may be ascribed (2). The method of purification and reaction conditions (3-4) may affect the DDA value of chitosan. Therefore, it is essential to use an accurate analytical method for the determination of chitosan DDA value since this value represents a critical parameter when chitosan is used as a drug-carrier in different drug delivery systems (5). The most widely used methods for the determination of the DDA for chitosan include first derivative UV-spectroscopy (FDUV) (6-7), IR spectroscopy (8-9), ^{1}H NMR spectroscopy (10-12) and potentiometric titration (13-14). However, other methods have also been used, such as gel-permeation chromatography (15-16) and thermal methods (differential scanning calorimetry and thermogravimetry) (17-18). The FDUV spectroscopic method is a compendial method in the British Pharmacopeia (BP). IR spectroscopy is a rapid and easy method, not

affected by most organic and inorganic impurities but suffers from problems associated with the baseline definition and the need for very well dried samples. The best accuracy achieved by this technique was about ±2% (19). The NMR technique is costly and not always available in laboratories.

Potentiometric titration was introduced (13) as a simple and low cost technique for the determination of chitosan DDA. Potentiometric titration is advantageous because of its applicability for turbid, colored and dilute solutions, as well as, its adaptability to automation. Using a direct pH-potentio-metric titration, one equivalence point is obtained and the DDA values are determined by calculating the number of moles of sodium hydroxide corresponding to the number of moles of ammonium (-NH$_3^+$) ions present which is the same as the number of moles of GIcN units present in the chitosan sample (20). In the back titration method, an accurate excess of HCl is added to a known amount of chitosan amine and the remaining amount of HCl is back titrated with a standard NaOH solution. The resultant titration curve shows two equivalence points, the first one corresponds to the excess HCl, while the second corresponds to the protonated chitosan. Thus, the difference would correspond to the free deacetylated amino groups that form the GIcN portion of the chitosan (19).

The variation in results obtained using potentometric titration was further investigated with the objective of understanding the source of the variations and thus how to avoid them.

The results were also compared to those obtained by FDUV and IR spectroscopic methods.

MATERIALS AND METHODS

Materials

High molecular weight Chitosan (250 kDa) was obtained from Hongjo Chemical Company

Ltd., China Batch No. HJ040411-CH, and ethanol (96%) was obtained from Hayman Company Ltd, UK. Analytical grade hydrochloric acid (37%) and sodium hydroxide (NaOH) were obtained from Merck, Germany. Distilled deionized and carbon dioxide-free water was used for the preparation of solutions.

Equipment

A freeze drier (Hetopower dry PL 9000, Thermo Fisher Scientific, Inc., Waltham, MA, USA), Sine-wave vibro viscometer (SV-10/SV-100, A&D Company, Ltd., Japan) and high speed centrifuge (Sorval Super Speed RC2-B, Norwalk, CT, USA) were used. A pH-electrode (Mettler DL-111, Mettler Toledo, UK), a memotiter (DL67) and Karl-Fischer titrator (DL38) from Mettler Toledo, UK were used for the potentiometric titration. A Beckman Coulter-04 640i ultraviolet spectrophotometer (USA) and FTIR spectrometer were used for spectrophotometric studies (PerkinElmer, Germany).

Preparation and molecular weight determination of various LMWC oligomers

LMWC hydrochloride oligomers with different molecular weights were prepared by the acid hydrolysis of high molecular weight chitosan. Changing the hydrolysis reaction time yielded oligomers with different molecular weights. 10 grams of the high molecular weight chitosan was dissolved in 830 ml 0.1M HCl and then 170 ml of concentrated HCl (37%) was added to adjust the final concentration of HCl to 2M and 1% (w/v) chitosan. Chitosan solution was vigorously stirred (1000 rpm) with heating under reflux at a temperature of 100°C. The reaction was allowed to take place for a defined period of time (reaction time), the reaction mixture was then allowed to cool down and 96% ethanol was added to precipitate the hydrochloride salt of chitosan oligomers. The precipitated chitosan salt was centrifuged at 4000 rpm for 15 minutes, then dried using the freeze dryer at a drum temperature of -85°C, shelf temperature of 10°C, and operating under a pressure of 20 Pa. The drying time was

around 4 hours and to ensure the removal of all excess hydrochloride acid, the precipitated chitosan was washed several times with ethanol, and the pH of the washing ethanol was monitored during the washing process until the pH was around the neutral range (6.9-7.1).

Finally, the chitosan oligomers produced were stored in dry vials at room temperature (25°C).

The viscosity average molecular weight of the prepared chitosan oligomers was determined by preparing aqueous solutions of 1%, 3%, 5%, 7% and 10% (w/v) for each chitosan oligomer of lower molecular weights (<18 kDa, or prepared with reaction times greater than 2 hours). However as a result of solubility limitations for the high molecular weight samples (30 kDa and 18 kDa), their viscosity values were determined by preparing aqueous solutions of 1%, 2%, 3%, 4%, and 5% (w/v). The viscosities of the prepared samples were measured at 25°C using the Sine wave vibro viscometer. The relative and reduced viscosities were then determined and used to calculate the viscosity average molecular weight.

LMWC amine oligomers of different molecular weights were prepared by taking about 1 g of the known viscosity average molecular weight chitosan hydrochloride previously prepared and dissolving it in 15 ml distilled deionized water. 20 ml of 2 M NaOH were added and stirred for 30 minutes. In order to ensure that the procedure for preparing the chitosan amine would not cause any change in the molecular weight of chitosan, it was measured after each preparation. No changes were found (i.e., chitosan of the molecular weights 1.3 kDa, 6 kDa, 13 kDa, and 30 kDa gave molecular weights of 1.3 kDa, 6 kDa, 13 kDa, and 30 kDa, respectively after preparing them as chitosan amines). The chitosan amine precipitate was filtered and washed with carbon-dioxide free water until neutralization, followed by washing with ethanol. The final material was freeze-dried and kept at room temperature in desiccators over silica gel and protected from light.

Determination of DDA for LMWC oligomers
Potentiometric titration

Both direct and back potentiometric titrations were carried out as a means for the determination of DDA values for LMWC oligomers. A Mettler automatic titrator was used and the end-point was determined by plotting the first derivative curve ($\Delta E/\Delta V$) versus volume of NaOH. The effects of hydration time and ionic strength of the chitosan solutions were not investigated as potential contributory factors toward influencing their DDA values as determined by both potentiometric titration (direct and reverse) methods. Additionally no samples of chitosan hydrochloride or chitosan amine were analyzed using both methods as a cross-check.

FDUV spectroscopy

FDUV method used here was that given in the British pharmacopeia (22), except that 0.1 M HCl was used as a solvent, instead of water, to prepare both the test and reference samples. Three replicates of DDA determination were carried out for each chitosan sample.

FTIR spectroscopy

About 20-30 mg of dried powder and 60 mg of KBr were mixed and triturated using an agate mortar and pestle for about 10 minutes. Approximately 30 mg of the mixture was used to make the KBr disks using a hydraulic press at a pressure of 80 tons for 60 seconds. Infrared spectra were recorded using the FTIR spectrometer at a frequency range of 4000-400 cm^{-1}. DDA-values were calculated using equation 1 (23):

$$DDA = 100 - [(A_{1655}/A_{3450}) \times 100/1.33)] \qquad \text{Eq. 1}$$

Where, A_{1655} and A_{3450} are the absorbencies at 1655 cm^{-1} (N-acetyl content) and 3450 cm^{-1} (assigned to the hydroxyl group) respectively. A_{3450} was used as an internal standard to correct for differences in the chitosan concentration in the KBr disk. Duplicate IR measurements were made for each sample.

Effect of heat on DDA

The heat effect on the DDA was investigated by adding an excess amount of standard HCl solution (0.5 M) to known quantities of chitosan amine and the sample solutions heated for 5 minutes at 60 °C in sealed glass bottles. The samples were cooled to room temperature then back titrated against 0.01 M NaOH solution. The pH-glass electrode was washed with acetic acid aqueous solution (pH~2) and carbon dioxide-free water after each run to avoid chitosan precipitation at the surface of the glass membrane. This is a critical step to ensure the reproducibility of pH-measurements. Duplicate measurements were performed on each sample solution.

RESULTS AND DISCUSSION

Preliminary investigation on the preparation and molecular weight determination of LMWC hydrochloride oligomers

To prepare chitosan oligomers with different molecular weights using high molecular weight chitosan as the starting material, the reaction (depolymerization) time was set at various time intervals (1, 2, 3.5, 4.5, 6, 8, 11, 17 and 24 hours). The chitosan concentration was kept at 1% (w/v), and the reaction was carried out in 2 M HCl solution. Chitosans of relatively high molecular weight obtained at hydrolysis times shorter than one hour, or those having relatively low molecular weights obtained at hydrolysis times longer than 24 hours were out of the scope of this work. The viscosities of chitosan oligomers produced were measured at a series of concentrations and the relative (η_{rel}) and reduced (η_{red}) viscosities for each were calculated according to the following relationships:

$$\eta_{rel} = \frac{\eta_{sample}}{\eta_{water}} \qquad \text{Eq. 2}$$

$$\eta_{red} = \frac{\eta_{rel} - 1}{C_{sample}} \qquad \text{Eq. 3}$$

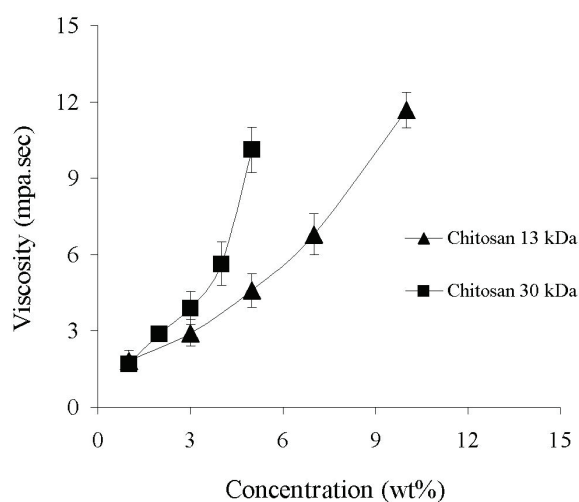where, C is the sample concentration %(w/v).

Consequently the intrinsic viscosities of the samples were determined by plotting η_{red} versus concentration where the intercept on the y-axis is the intrinsic viscosity. The molecular weight of chitosan was determined according to the Mark-Houwink equation:

$$[\eta] = k\, M^a \qquad\qquad \text{Eq. 4}$$

Where, $[\eta]$ is the intrinsic viscosity, M is the viscosity average molecular weight, and k and a are constants for a given solute-solvent system and temperature with the values of 5.8×10^{-4} and 0.69, respectively (24-25).

In reference (24) the exponent a is a function of the pH, ionic strength, and the degree of acetylation. The degree of deacetylation of all prepared grades of chitosan was initially calculated using the FDUV method and it was close to 100% thus the degree of acetylation (DA) is zero, leading to constant values of a and k and they were close to the values stated above. On the other hand, reference (25) discusses the values of 0.69 and 5.8×10^{-4} for a and k, respectively for LMW chitosan ranges in the molecular weight investigated here.

The standard deviations of three replicates of viscosities measured at the same concentration of 13.0 kDa and 30.0 kDa were always less than

1% in each case (shown in Figure 2) indicating the repeatability of the method used for the viscosity measurement. Figure 2 also shows that the viscosities of chitosan oligomers up to 30.0 kDa are indicative of their molecular weights. Tthere were difficulties when using chitosan oligomers of molecular weights greater than 30.0 kDa due to solubility problems.

Table 1 shows the molecular weights of LMWC oligomers obtained from the depolymerization of the high molecular weight chitosan in 2M HCl at different time intervals. The table shows the SD calculated for six trials indicating a high degree of repeatability of the molecular weights determined.

Table 1 Depolymerization reaction time required to produce different molecular weights of chitosan HCl

Depolymerization Reaction Time (hr)	Average Molecular Weight kDa ± (SD)
1	$30.0 \pm (6.3\times10^{-3})$
2.0	$17.1 \pm (7.0\times10^{-4})$
3.5	$13.0 \pm (1.5\times10^{-3})$
4.5	$9.3 \pm (7.6\times10^{-3})$
6.0	$8.7 \pm (2.1\times10^{-3})$
8.0	$6.0 \pm (1.8\times10^{-3})$
11.0	$4.7 \pm (2.2\times10^{-3})$
17.0	$3.6 \pm (15\times10^{-3})$
24.0	$1.3 \pm (2.8\times10^{-3})$

The depolymerization kinetics of polymers is usually approximated by the difference in their (inverse) molecular weight at time (t) versus that at time zero. Using this relationship, a straight line can be obtained as shown in Figure 3.

Figure 2 Viscosities of sample solutions comprising chitosan of different molecular weight
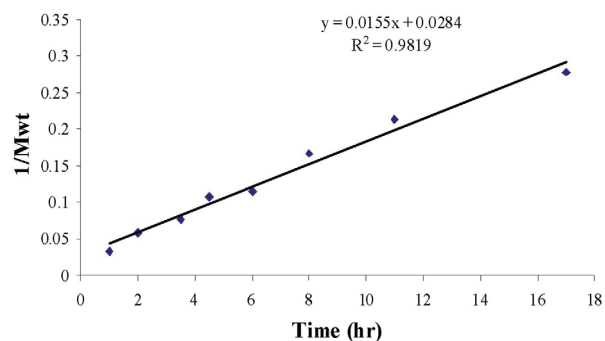
Figure 3 (1/Mwt) of chitosan versus time

Determination of DDA by direct and back potentiometric titrations

Both direct and back pH-potentiometric titrations were investigated as a means for the determination of DDA values for LMWC oligomers.

For direct titration, accurately weighed quantities of chitosan hydrochloride samples were dissolved in carbon dioxide-free water and potentiometrically titrated with 0.01 M NaOH solution that had been already standardized against a standard potassium hydrogen phthalate (KHP) solution. The titration followed the reaction scheme shown in Figure 4.

The end-point was determined by plotting the first derivative ($\Delta E/\Delta V$) versus volume of NaOH. The water content of each sample was determined by Karl-Fischer titration (21). The degree of deacetylation was calculated according to equations 5 and 6:

$$\% \ NH_2 = (CV \times 16)/(W \times (100 - \% \ H_2O) \qquad Eq. \ 5$$

$$\% \ DDA = (\% \ NH_2 / 9.94) \times 100 \qquad Eq. \ 6$$

Where C and V correspond to the concentration and volume of the standard sodium hydroxide solution, $\%H_2O$ is the percentage of water in each sample, 16 is the mass of 1 mol of NH_2 and 9.94% is the theoretical percentage of NH_2 in a glucosamine unit. Duplicate measurements were carried out on each sample.

In back titration, a known amount of chitosan amine (deactylated chitosan) is dissolved in a known excess of 0.5 M HCl (standardized against a standard solution of sodium carbonate) and the excess HCl is back titrated against 0.01 M NaOH standard solution. The water content of each sample was also determined by Karl-Fischer titration and the degree of deacetylation was determined using equations 5 and 6:

$$NH_2(\%) = \frac{(C_1V_1 - C_2V_2) \times 16 \times 100}{w(100 - \%H_2O)} \qquad Eq. \ 7$$

Where, C_1 and C_2 are the molar concentrations of HCl and NaOH, respectively, V_1 and V_2 are the volumes (liter) of HCl and NaOH, respectively; w is the mass of the sample (g). Again, duplicate measurements were carried out on each sample.

The DDA values using both direct and back potentiometric titrations are summarized in Table 2. There was almost no difference in DDA values (93.3 to 94.5 kDa) determined for chitosan hydrochloride oligomers of molecular weights within the range of 1.3 to 30 kDa when the direct titration method was used. In addition, the DDA value for each oligomer was greater than 93% indicating an almost complete deactylation for all molecules of the chitosan oligomer. As the strong base (OH^-) was added, it reacted readily with H^+ of the ammonium ions regardless of the molecular weight or the conformation of LMWC hydrochloride.
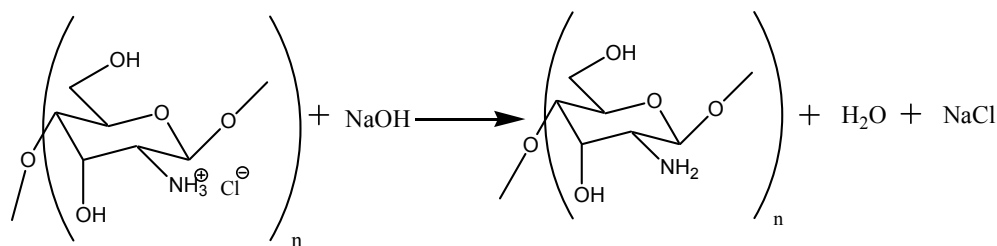

Figure 4 Titration reaction scheme

Table 2 %DDA values of chitosan obtained by the direct and back titration methods

Chitosan HCl Mwt (kDa) [a]	% DDA ± RSD(n = 6)	
	Direct	Back titration
1.3	93.5 ± 0.7	77.1 ± 0.8
6	93.8 ± 0.6	77.3 ± 0.6
13	93.3 ± 0.8	78.8 ± 0.7
30	94.5 ± 0.6	70.6 ± 0.8

[a]Chitosan concentration was about (1.3 ± 0.1 mg/ml) for all chitosan oligomers

However, the DDA values obtained by back titration for the same chitosan oligomers (70.6 to 78.8 kDa) were found to be lower than their direct titration counterparts (Table 2). These results would suggest that the deprotonation step leads to molecules with free amino groups (-NH$_2$) which would be more susceptible to intra/inter attractive forces in the solutions of low molecular weight of chitosan amine, and fewer of these groups would be available for a reaction with the HCl titrant used in the back titration. Thus, the relatively low DDA value determined by the back titration for the chitosan oligomer of 30 kDa could be ascribed to the folded conformation structure of this chitosan which might affect the ability of amino groups to accept the proton (H$^+$) from the acidic medium forming ammonium chloride salt. However, the lower molecular weight chitosan molecules (MW ≤13.0 kDa) would have a more extended conformation that may enhance the possibility for the exposure of more amino groups, thus there may be a better chance of the involvement of more amino groups in the acid-base reaction during the potentiometric back titration method.

This conformational change has also been reported (26) with the low and high concentrations of LMW chitosan, where in low concentrations chitosan exists as the extended form while in higher concentrations it adopts more contracted form. Other studies (27) showed that chitosan has a conformation structure in solution that could affect its

chemical reactions. The gross conformation of LMWC molecules in solution could be of spherical shape, random coil or rod shape, depending on several factors such as, molecular weight, DDA value, solvent, temperature and pH of the solution. In spite of the extensive discussions on the influence of DDA value on the chain, this effect is still in dispute and contradictory reports have been published in the literature.

Consequently, attention should be paid to the DDA values obtained by the back potentiometric titration method in as much as they might be less than the anticipated values. This finding confirms the earlier report (2) that DDA values for chitosan are highly dependent on the analytical method employed and consequently the method should be stated when reporting the DDA values of chitosan oligomers. Jiang *et al.* (28) calculated the DDA for chitosan polymer based on two potentiometric titration functions. They calculated the required volume of titrant (NaOH) to reach the first equivalence point, since at this point chitosan polymer starts to precipitate and affect the accuracy of the pH electrode. However, these approaches are too complicated for routine use in laboratories for rapid determination of DDA. In the present work the precipitation of chitosan oligomer at the first equivalence point is low since the samples have relatively low molecular weights and dilute solutions of HCl and NaOH were used (28).

Effect of heat on the DDA values determined by potentiometric titration

The back titration referred to above was carried out at room temperature. In order to investigate effect of temperature on the DDA values obtained by this method, the back titration was repeated using fresh LMWC samples with the same molecular weights as those titrated at the room temperature but after dissolving them in excess HCl solution and heating for 15 minutes at 60°C (from the development of the polymerization process of the high molecular

weight chitosan, no depolymerization was observed under these mild conditions) followed by cooling before conducting the titration. The results are summarized in Table 3.

Table 3 Effect of heating on the %DDA values determined by the back titration

Chitosan HCl Mwt (kDa) [a]	% DDA ± RSD
1.3	83.0 ± 0.7
6	86.5 ± 0.7
13	83.5 ± 0.7
30	72.8 ± 0.6

[a]Chitosan concentration was about (1.3 ± 0.1 mg/ml) for all chitosan oligomers

It is obvious from Table 3 that heating for a short period (~ 15 min) resulted in a significant increase (about 8.5% on average) of the DDA values obtained for the chitosan oligomers of ≤13.0 kDa, while for 30.0 kDa chitosan, it had a relatively lower effect (about 3%) on the DDA values obtained. In general, this increase in DDA values could be attributed to a more extended chitosan confirmation. As discussed above, this would be expected to occur at higher temperatures, but would be less significant with high molecular weight oligomers. It is obvious that heating allows the chitosan conformation to be extended in a direction that allows the $-NH_2$ groups to be more accessible to the interaction with protons. Increasing the heating period for about 50 minutes at about 70 °C did not show any significant change compared to the heating period of 15 minues.

Effect of concentration on the DDA values determined by potentiometric titration

DDA values were determined for three different concentrations of chitosan hydrochloride (Mwt 13.0 kDa) using potentiometric titration. The DDA values obtained for 0.4, 0.8 and 8.0 mg ml^{-1} were 90.6 ± 1.0, 91.1 ± 0.6 and 81.2 ± 0.6 kDa, respectively. This trend has also been observed by others (19) showing the DDA (%) values of chitosan for three different concentrations

(0.51, 1.03, 1.58 mg/ml) to be 86.6, 81.8 and 81.1, respectively.

Comparison of DDA values obtained by various experimental methods

The DDA values of chitosan amine and chitosan hydrochloride determined by the FDUV spectroscopic method were found to be around 100% (Table 4) which indicates that the method is superior for the determination of DDA for chitosan. The results obtained here are comparable to those obtained earlier (2). However, although this method gives 100% DDA values, the absorbance measured at the short wavelength of 202 nm would be prone to the fluctuation of the light source.

The DDA values for chitosan hydrochloride samples were also determined by the FTIR spectroscopic method. The absorbance measurements were made at 1620 cm^{-1} and 3420 cm^{-1} that correspond to the amide and hydroxyl bands, respectively. The results are summarized in Table 4.

Table 4 Chitosan %DDA ± RSD obtained using different spectroscopic techniques

Chitosan HCl Mwt (kDa)	FDUV Chitosan HCl	FDUV Chitosan amine	FTIR Chitosan HCl
1.3	100.8 ± 0.1	100.7 ± 0.2	89.8 ± 0.7
6	100.1 ± 0.1	100.0 ± 0.5	81.6 ± 0.6
13	99.9 ± 0.4	100.0 ± 0.6	85.4 ± 0.4
30	100.0 ± 0.7	100.2 ± 0.9	83.5 ± 0.7

It is obvious that the DDA values determined by this method vary within a range of 81.6% to 89.8% for the different molecular weights studied. The random variation in the DDA values determined by this method may be attributed to the dependence of IR technique on the baseline definition. Using different baselines would contribute to a variation in the DDA values. IR measurements were conducted for chitosan amine samples and their DDA values are similar to those obtained from chitosan hydrochloride samples. Moreover, chitosan is hygroscopic in nature, therefore, low-moisture content in samples would also

contribute to the hydroxyl band that affects the DDA values. Furthermore, sample preparation, type of instrument and conditions may influence the results (2).

The DDA values obtained by various methods are shown in Table 4. It is obvious from Tables 2 and 4 that the potentiometric direct titration method gave consistent results and achieved DDA values as high as 93% for the chitosan hydrochloride oligomers. Thus, the potentiomtric titration results are the closest to those of the FDUV method (BP method). Consequently the direct potentiometric titration method appears to work well irrespective of the chitosan concentration. It is easy, quick and does not require any sophisticated equipment or tedious training, lending it to the adoption of online testing of the DDA during the LMWC depolymerization process.

CONCLUSION

The potentiometric titration is considered an easy and quick method for the determination of DDA for chitosan. Although the hydration time and ionic strength were not investigated in this study, their effects on DDA results obtained by the two potentiometric methods cannot be discounted. It is postulated that the presence of free amino groups within the structure of LMWC would enhance the formation of intra/inter attractive forces which would lead to a decrease in the DDA values determined by the back titration method. Although this study did not subject the same material to both potentiometric methods of analysis, the results suggest the DDA values obtained by the back titration method are always significantly lower than anticipated. Heating the chitosan solution prior to the titration step should be taken into consideration when reporting the DDA values for any chitosan sample. Heating of chitosan amine up to 60°C had a significant effect on the DDA values determined for chitosan of molecular weight of ≤13.0 kDa. Furthermore, solutions having relatively high concentrations

of LMWC may give lower than anticipated DDA values compared to solutiong having relatively low concentrations of the same oligomer.

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