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Determination of the degree of N-acetylation (DA) of chitin and chitosan in the presence of water by first derivative ATR FTIR spectroscopy

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ABSTRACT

A new method for the determination of the degree of N-acetylation (DA) of chitin and chitosan is described using first derivative diamond ATR FTIR spectroscopy. Applying the derivative values of the amide III band at 1327 cm^{-1} and the CH deformation band of the N-acetyl group at 1383 cm^{-1} as measure of the N-acetyl content of the sample in relation to the derivative value of the bridge oxygen vibration at 1163 cm^{-1} as internal standard, a linear correlation to the results of first derivative UV spectroscopy was obtained and confirmed by elemental analysis and Raman spectroscopy. The described method allows the determination of the degree of N-acetylation of chitosan and chitin in the presence of water thus making drying procedures unnecessary.

1. Introduction

Chitosan, which is partially deacetylated chitin, is obtained by alkali treatment of chitin at high temperatures. Due to the protonation of the free amino groups, chitosan is soluble in diluted acids. Thus, compared to chitin it is much easier to handle and has a lot of interesting properties, e.g. biocompatibility and biodegradability. The variety of applications for chitosan ranges from waste water treatment to pharmaceuticals. During the last years especially the use of chitosan as a component in drug delivery formulations has gained much attention (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004).

The behavior of this polymer in solution and as complexing partner for biologically active substances, e.g. peptides or proteins, in pharmaceutical formulations depends significantly on its degree of N-acetylation (DA), representing the molar fraction of N-acetylated units in the polymer chain. Thus, an accurate, rapid and low cost method for the determination of the DA is required.

This particular analytical task is rather challenging, as can be seen from the extensive number of different methods that have been published during the last decades ranging from titrimetric techniques to UV spectroscopy (Khan, Peh, & Ching, 2002; Tan, Khor, Tan, & Wong, 1998), NMR spectroscopy (Heux, Brugnerotto, Desbrières, Versali, & Rinaudo, 2000), circular dichroism (Domard, 1987), differential scanning calorimetry (DSC) (Guinesi & Cavalheiro, 2006). UV spectroscopic methods depend in general on the sample solubility and thus are often limited to chitosan samples with DA < 50%. Based on the UV first derivative method previously developed by Muzzarelli and Rocchetti (1985), Wu and Zivanovic (2008) have overcome this problem by developing a first derivative UV spectrophotometric method for the DA determination using phosphoric acid as solvent. Destructive methods, e.g. elemental analysis, afford the determination of DA values in the entire range.

IR spectroscopy has been widely applied for the determination of the DA (Kasaai, 2008; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996). Therefore, the absorbance of a DA sensitive probe band (MB) relative to a DA independent reference band (RB) must be calibrated against standard DA values. A wide range of probe and reference bands with divers baselines have been suggested but none of them could actually give accurate values throughout the entire DA range of 0–100%. An additional disadvantage of the published IR spectroscopic methods for determination of DA is their requirement of a very low water content in the samples.

In this paper, we present a new method for DA determination of chitosan using first derivative diamond ATR FTIR spectroscopy that makes for the first time the DA determination by IR spectroscopy in the presence of water possible and thus even allows the DA determination of chitosan hydrogels.

2. Materials and methods

2.1. Materials and instruments

D-Glucosamine (GlcN), N-acetyl-glucosamine (GlcNAc), chitin (from shellfish) and low molecular weight chitosan (50–190 kDA)

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were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), 85% phosphoric acid from Fisher Scientific (Loughborough, UK), acetic acid anhydride from Grüssing GmbH (Filsum), ammonia (31%) from VWR International S.A.S. (Briare, France) and ethanol as well as acetic acid was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). All other materials were of analytical grade.

A Specord S 100 B (Analytik Jena) single beam UV/VIS spectrophotometer was used to collect the UV spectra of chitosan and monomer samples in the range of 188–400 nm.

For ATR FTIR spectroscopy, an Impact 400 D FTIR spectrometer (Nicolet) with a Golden Gate Single Reflectance Diamond ATR (10500 series) unit was applied. The necessary calculations were accomplished using software from OMNIC.

For all Raman spectroscopic measurements, a Bruker RFS 100 spectrometer was used. Excitation was carried out with a laser at 1064 nm.

Elemental analysis was carried out using an Euro EA 3000 CHNS-O Elemental Analyzer from Eurovector Instruments & Software (Italy).

2.2. Preparation of partially N-acetylated chitosan

Low molecular weight chitosan (50–190 kDa) was partially N-acetylated following the method of Freier, Koh, Kazazian, and Shoichet (2005). Chitosan (DA = 10%) was dissolved in 2% acetic acid under stirring. To the resulting viscous solution, an equal volume of ethanol and varying amounts of acetic acid anhydride were added. The resulting chitosan films with varying DA values were dried for 7 days prior to deprotonation with a mixture of ammonia (31%)/methanol/water (10/225/15 (v/v)) followed by intensive washing with distilled water.

Chitin was partially deacetylated applying the method of Mima, Miya, Iwamoto, and Yoshikawa (1983). For this purpose, 2 g of chitin were suspended in 20 ml of 47% aqueous NaOH under stirring at 110 °C for 30 min under a nitrogen atmosphere. Subsequently, the suspension was washed to neutrality with distilled water. The procedure was repeated several times with a part of the product to obtain samples with lower DA values.

One part of each prepared chitosan sample was freeze dried and another part was submerged into distilled water for 7 days prior to IR measurements.

2.3. UV spectroscopic determination of the DA

First derivative UV spectroscopy (FDUV) was employed according to Wu and Zivanovic (2008). 25 ± 2 mg chitosan were dissolved in 5 ml of 85% phosphoric acid at 60 °C for 40 min. 1 ml of the resulting solution was diluted to 100 ml with distilled water and incubated at 60 °C for 2 h prior to measurements. The determination of the degree of N-acetylation was carried out by using the first derivative UV value at 203 nm after calibration with monomer mixtures.

2.4. DA determination by first derivative Raman spectroscopy

Several solid chitosan samples were filled into glass capillaries and introduced into the laser beam (wavelength 1064 nm). The collected spectra (1000 scans) were smoothed employing 51 point Savitzky-Golay-algorithm and the first derivatives of the spectra were calculated. Afterwards first derivative values at 2936 cm^{-1} (MB) and at 2868 cm^{-1} (RB) were determined and the ratios MB/RB were calibrated against results of first derivative UV spectroscopy.

2.5. DA determination by elemental analysis

The elemental composition of chitin and chitosan samples was determined using Euro EA 3000 CHNS-O Elemental Analyzer from Eurovector Instruments & Software. The DA value of each sample was calculated from its carbon/nitrogen ratio.

2.6. Determination of the DA by first derivative ATR FTIR spectroscopy

Solid chitosan samples were clamped onto the ATR crystal (diamond) using sapphire to generate a high pressure. The collected spectra (64 scans) were smoothed employing 11 point Savitzky-Golay-algorithm and the first derivatives of the spectra were calculated. The first derivative ATR IR values at 1383 cm^{-1} (MB1), 1327 cm^{-1} (MB2) and 1163 cm^{-1} (RB) were determined and the ratios (MB1 + MB2)/RB were calibrated against the results of first derivative UV spectroscopy.

3. Results and discussion

3.1. First derivative UV-spectroscopy as reference method

First derivative ATR IR spectroscopy, as described in this paper, represents an absolute method for the determination of the DA of chitin and chitosan. However, to establish a correlation between the directly determined first derivative IR intensity ratio and the DA of the chitosan sample a non-recurring calibration against a reliable reference method is necessary.

The first derivative UV spectroscopic method (FDUV) developed by Wu and Zivanovic (2008) was most suitable. The problem of solubility has been overcome by using concentrated phosphoric acid as solvent, making the entire range of DA accessible to the determination. Furthermore a calibration with the monomers N-acetylglucosamine and D-glucosamine is possible. The required amount of chitosan is small and the values resulting from this method exhibit a very small standard deviation. UV and first derivative UV approaches for DA determination are considered to be very accurate (Kasaai, 2009). The method shows a very good reproducibility but the determination takes some hours and the sample cannot be recovered. Even though the DA determination is carried out in solution, diligently dried chitosan samples are required because the exact anhydrous mass of the sample must be known.

3.2. DA determination by first derivative ATR FTIR

A part of the same samples was freeze dried and the absorbance ratios of the amide II-band at 1552 cm^{-1} to the CH– stretching band at 2878 cm^{-1} were determined in analogy to the method of Sannan, Kurita, Ogura, and Iwakura (1978) for transmission IR spectra. Fig. 1 shows these absorbance ratios plotted against the DA values determined by first derivative UV spectroscopy. A linear relationship is obtained, but the dispersion of the values is high. The disadvantage of this method is its interference liability to water.

From Fig. 2 it can be seen that the IR absorption of water affects the CH– stretching band of chitin as well as the amide I and amide II band. Furthermore it can be seen that the absorbance of water is constant for wavenumbers smaller than 1500 cm^{-1}. This observation suggested the possibility of a water independent determination of the DA by first derivative IR spectroscopy since such a constant absorbance should not disturb the first derivative IR spectrum of chitosan.

Fig. 3A illustrates the DA sensitive changes in the considered region of the first derivative ATR FTIR spectrum. After systematic investigations, the bridge oxygen stretching band at 1163 cm^{-1} in the first derivative IR spectrum has been found to be the most

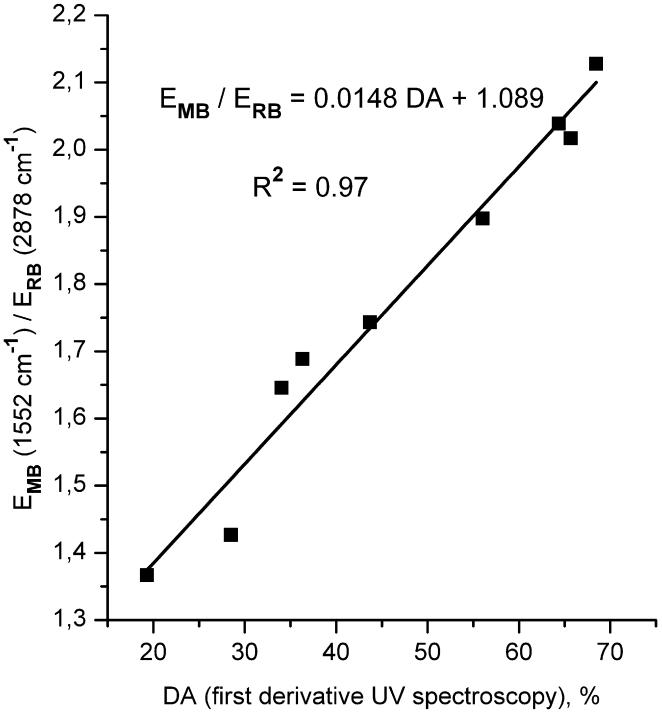

Fig. 1. Absorbance ratio between the amide II band and the CH– stretching band vs. DA values determined by FDUV spectroscopy.

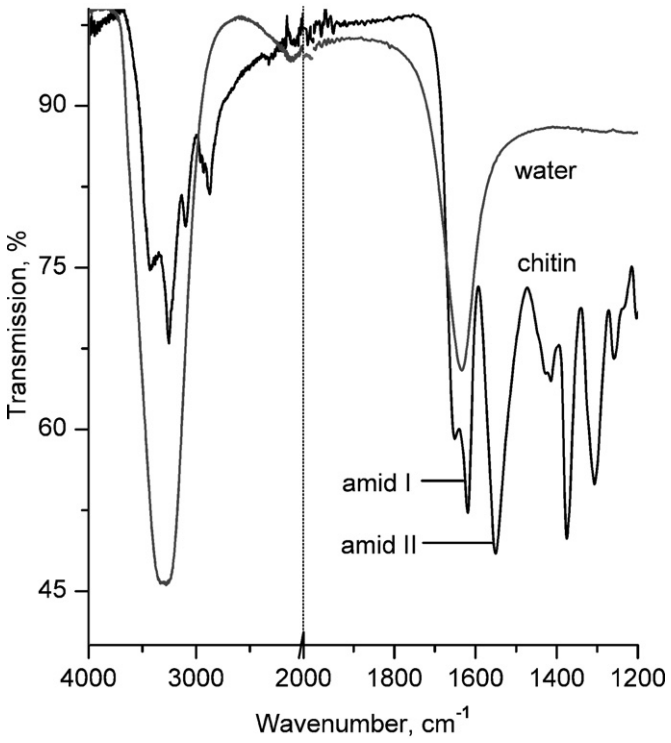

Fig. 2. Comparison of the ATR FTIR spectra of chitin and water, showing their interference.

adequate reference band (RB) and the CH– deformation band at 1383 cm^{-1} (MB1) and the amide III band at 1327 cm^{-1} (MB2) turned out to be the best probe bands. The mentioned CH– deformation is related to the methyl group of the N-acetyl group, thus making it ideally suited for the determination of DA.

In Fig. 3B, the band ratio (MB1 + MB2)/RB is plotted against the DA values resulting from first derivative UV spectroscopy (FDUV). Obviously, there is a good linear correlation between both methods over the entire DA range from 0 to 80%. The dispersion of the values allows a determination of the DA with a standard deviation smaller than 2.5% for all samples. Fig. 4 shows the values determined for water-swollen chitosan samples in comparison to dried samples. Apparently, the measurement is not disturbed by water.

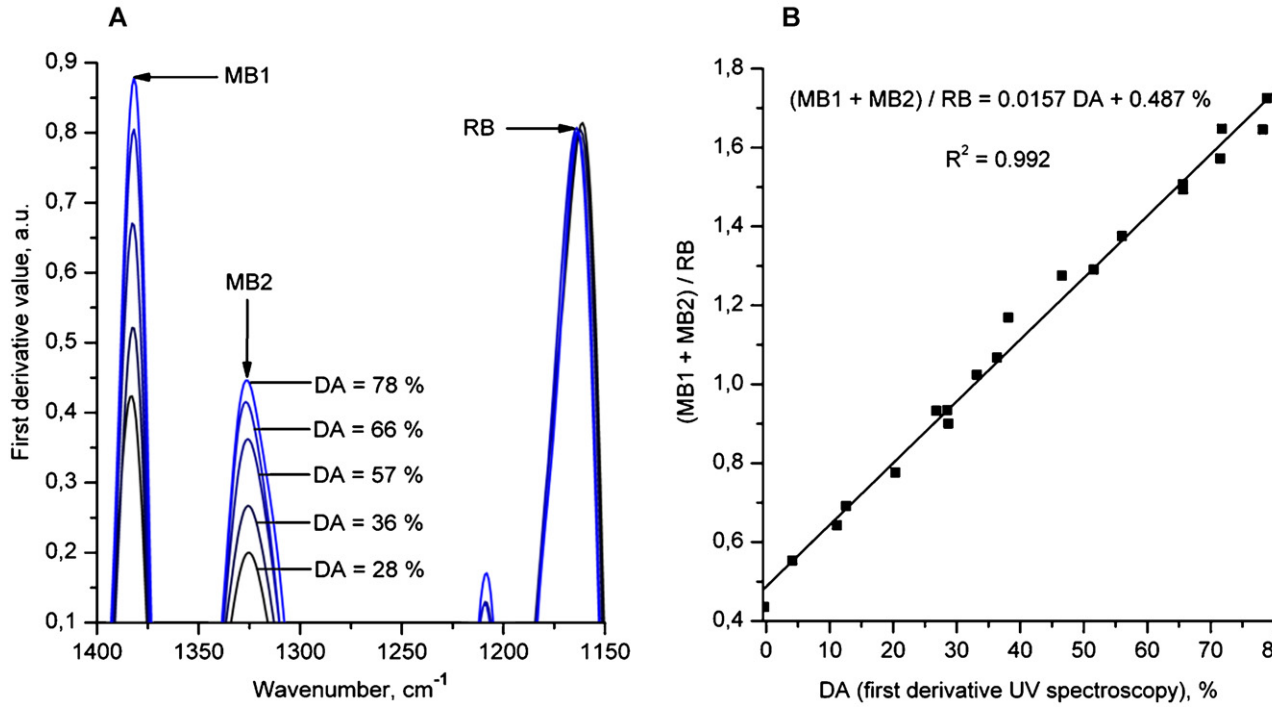

Fig. 3. (A) First derivative ATR FTIR spectra of chitosan samples with differing DA values. (B) Calibration plot of the first derivative ATR FTIR spectroscopic DA determination method.

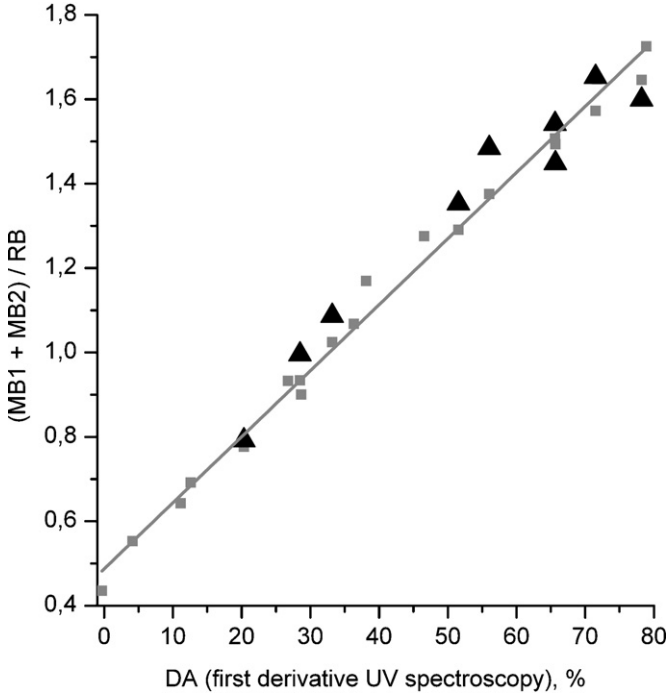

Fig. 4. Values of water-swollen chitosan samples (black) in comparison to the calibration line determined with dried samples (grey).

The development of a reliable method for the determination of the degree of N-acetylation using ATR FTIR spectroscopy was the priority objective. All eligible IR spectroscopic methods for this special analytical task which were published so far, applied transmission IR spectroscopy. Brugnerotto et al. (2001) advanced the view that the ATR technique was not appropriate to determine the DA of chitosan because of the poor resolution which can be achieved due to non-uniform contact with the ATR crystal. As evidence for this poor resolution they mentioned that both peaks of the amide I-band of α-chitin fused to a single one in the ATR spectrum. These considerations were directed at ATR spectra using ZnSe as ATR crystal.

However, diamond as ATR crystal has a much higher hardness and thus allows to use high pressure to ameliorate the contact surface. Fig. 5 shows an ATR spectrum of α-chitin with diamond as ATR crystal. Both peaks of the split amide I band are clearly visible.

Since a wavelength dependency of the depth of penetration has to be considered by using the ATR technique, the published equations for DA determination by transmission techniques with

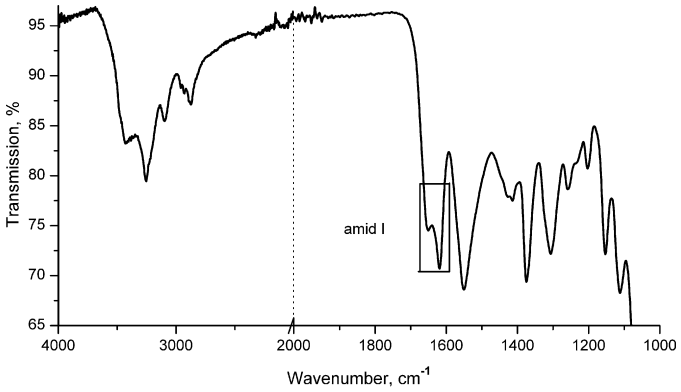

Fig. 5. Diamond ATR FTIR spectrum obtained for α-chitin showing split amide I band.

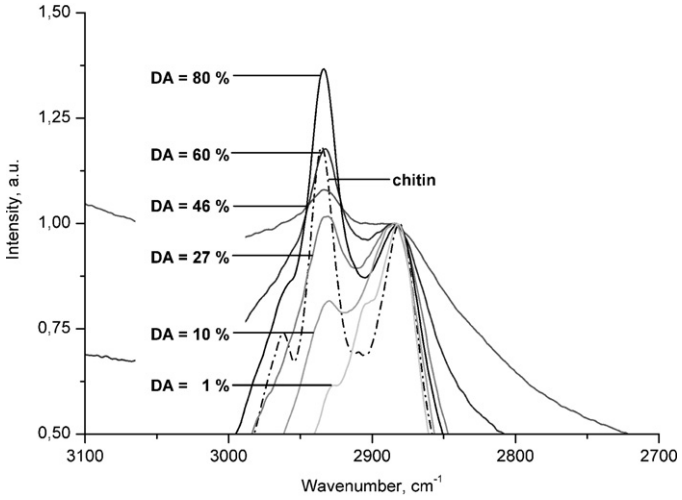

Fig. 6. Raman spectra of chitin and chitosan samples with differing DA values.

samples in KBr pellets cannot be used. Nevertheless, the principle of the methods compiled for transmission IR spectroscopy can be transferred after separate calibration for the ATR spectra.

Samples which were prepared by partial deacetylation in suspension exhibited a very good correlation to the results of first derivative UV spectroscopy, however, only when they were ground with a swing mill before measurement. Without this procedure, these samples exhibited lower DA values. This phenomenon could be ascribed to the surface sensitivity of the ATR technique. Before grinding the IR beam interacts just with the outer shells of the chitin or chitosan sample, which get faster deacetylated in the NaOH suspension; after grinding an average value is obtained.

As mentioned before, one aim was to develop a method for DA determination that is independent of the water content of the chitosan samples. Water is an essential part of the crystalline structure of chitosan and consequently could not be removed completely without losing its biologically interesting properties. It is of special importance since most pharmaceutical applications use chitosan hydrogels. However, water disturbs most determination methods due to its IR absorption or because the exact anhydrous mass is needed for the determination.

With the first derivative ATR FTIR technique neither the anhydrous mass is needed since the bridge oxygen stretching band is used as internal standard, nor does the IR absorption of water disturb the determination because the spectral range of the water absorption is avoided.

Kasaai (2009) mentioned in his review on DA determination methods that the main disadvantages of IR spectroscopic methods are the interference liability to the water content of the samples and the difficulty of drawing baselines in the IR spectra. The presented first derivative ATR FTIR approach overcomes both problems because the drawing of baselines is unnecessary and water does not disturb the measurement.

3.3. First derivative Raman spectroscopy

As a second approach to a water independent determination of DA values, we took Raman spectroscopy into consideration. Fig. 6 illustrates the changes in the Raman spectra of some chitin and chitosan samples with differing DA values. The depicted wavenumber range demonstrates the increase of the band at 2936 cm^{-1} in relation to the band at 2881 cm^{-1}. Both bands are related to CH– stretching vibrations but just the band at 2936 cm^{-1} changes with DA because it contains the symmetric CH_3 – stretching mode of the N-acetyl group.

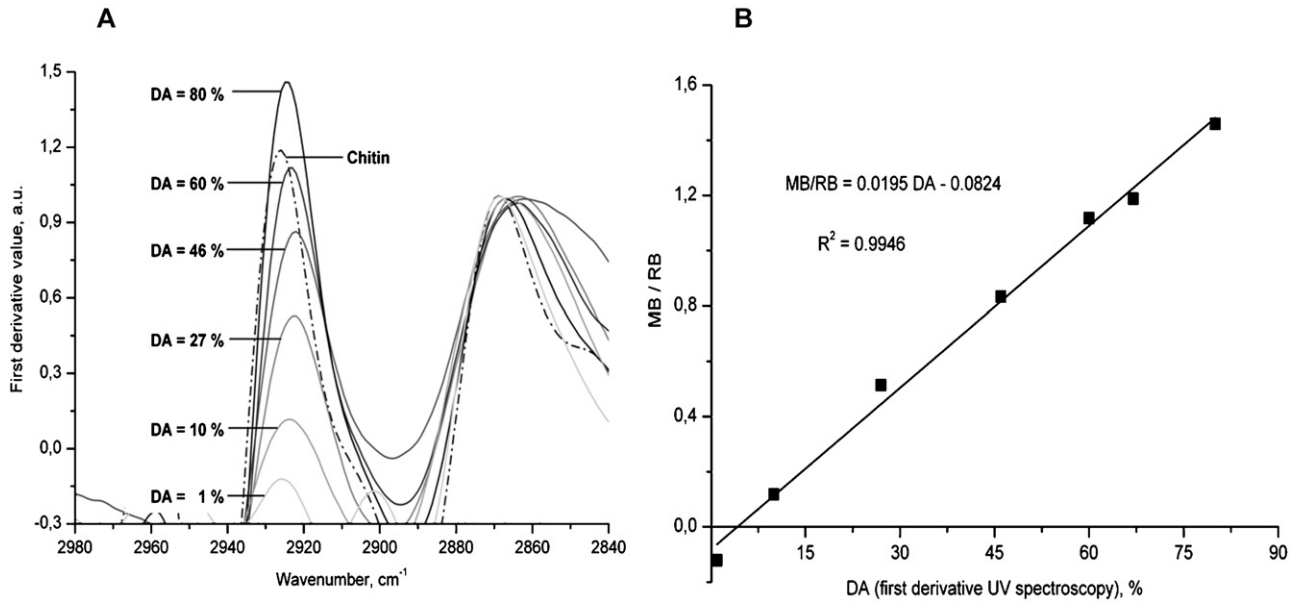

Fig. 7. (A) First derivative Raman spectra of chitin and chitosan samples with differing DA values. (B) Calibration plot of the first derivative Raman spectroscopic DA determination method.

Fig. 7A shows first derivative Raman spectra of the same samples in the spectral range of the CH– stretching vibrations. For the mentioned reasons, the band at 2924 cm^{-1} changes with DA while the band at 2868 cm^{-1} stays constant. Applying the common principal of DA determination by IR spectroscopy we used the first derivative value of the band at 2924 cm^{-1} as a probe band (MB) and the value of the band at 2868 cm^{-1} as a reference band (RB). The ratio MB/RB plotted against DA values determined by first derivative UV spectroscopy is shown in Fig. 7B. Obviously, a good linear correlation is obtained. To investigate the reproducibility, a threefold measurement of the purchased low molecular weight chitosan was performed. The resulting DA value of 10.2% could be determined with a standard deviation of 0.9%.

Due to the very weak absorbance of water in Raman spectroscopy, the suggested band ratio affords a further possibility for water independent DA determination of chitosan samples. The CH– stretching bands are by far the most intensive bands in the Raman spectra of chitin and chitosan, thus exhibiting the best signal to noise ratio. But Raman spectroscopy is in general less sensitive than ATR FTIR spectroscopy. In our special case, this problem caused a prolonged measurement time of nearly an hour per single measurement.

In Figs. 6 and 7 it can be seen that the Raman spectrum of chitin deviates in some respects from the spectra of the other samples. The bands are very narrow and the maxima are slightly shifted. These deviations are presumably caused by the high crystallinity of parts of the sample and therefore a reliable DA determination by this method has to be considered as at least uncertain.

The results of Raman spectroscopy agree well with first derivative UV spectroscopy thus affording a further approach to DA determination. However, first derivative ATR FTIR spectroscopy is preferable due to the less elaborate equipment which is needed, and due to its higher sensitivity.

3.4. Correlation to elemental analysis

The results of first derivative IR spectroscopy, first derivative Raman spectroscopy, first derivative UV spectroscopy and elemental analysis are shown in Table 1. Apparently, there is a good correlation between all four methods. Elemental analysis represents an absolute method and consequently shows the reliability of the first derivative ATR spectroscopy.

The DA value obtained for the commercial chitin sample is surprisingly low. But the industrial recovery of chitin from shellfish requires alkali treatment for the deproteinization of the raw material and this harsh chemical treatment can cause extensive deacetylation. Actually Teng, Khor, Tan, Lim, and Tan (2001) published similar values for the degree of N-acetylation of chitin samples although they avoided the alkali treatment by using

Table 1
Comparison of the DA values of chitosan samples determined by first derivative ATR FTIR (FDIR), first derivative UV (FDUV), elemental analysis (EA) and first derivative Raman spectroscopy (FDRa).

Sample	DA values with standard deviation			
	DA (FDIR) (%)	DA (FDUV) (%)	DA (EA) (%)	DA (FDRa) (%)
Deacetylated sample 1	3.3 ± 1.3	4.1 ± 1.1	0.1 ± 0.4	0.2
Deacetylated sample 2	12.1 ± 1.4	12.3 ± 1.2	9.8 ± 0.6	[b]n.d.
Deacetylated sample 3	28.2 ± 1.0	28.5 ± 0.5	30.2 ± 4.2	[b]n.d.
N-acetylated sample 1	26.9 ± 0.8	26.8 ± 0.5	24.7 ± 0.7	29.6
N-acetylated sample 2	47.6 ± 2.1	46.6 ± 2.7	42.8 ± 1.7	44.0
N-acetylated sample 3	59.8 ± 2.3	58.4 ± 1.3	58.7 ± 0.3	62.6
Commercial chitosan	9.7 ± 1.3	9.3 ± 0.9	9.1 ± 2.4	10.2
Commercial chitin	67.9 ± 2.4	[a]63.5 ± 0.6	67.2 ± 0.8	63.3

[a] The chitin sample had been stirred for 30 min in boiling water followed by diligent drying prior to UV measurement to accelerate desolvation of the sample.
[b] Not determined.

proteolytic enzymes released by fungal mycelia for the purification of chitin from shellfish.

4. Conclusions

First derivative ATR FTIR spectroscopy offers a new powerful tool for the determination of the degree of N-acetylation (DA) of chitin and chitosan. The determined DA values correlate well with the results of first derivative UV spectroscopy and elemental analysis. The shown accuracy is similar to that of first derivative UV spectroscopy over the entire investigated range of DA values, whereas sample preparation is much easier and less time consuming for ATR FTIR spectroscopy. It is also of advantage that ATR FTIR spectroscopy is a non-destructive technique and requires low sample amounts.

In contrast to the conventional IR spectroscopic methods for DA determination, the first derivative ATR FTIR approach avoids the drawing of baselines and allows the measurement of chitosan and chitin samples with high water content, thus allowing the DA determination of chitosan hydrogels of pharmaceutical interest.

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