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Determination of the Degree of Acetylation of Chitosans by First Derivative Ultraviolet Spectrophotometry

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SUMMARY

The degree of acetylation of chitosan can be determined in acetic acid solutions (~0·01 M) containing 1 g dry chitosan per litre by first derivative ultraviolet spectrophotometry at 199 nm. At this wavelength, the N-acetylglucosamine absorbance readings are linearly dependent on concentration and are not influenced by the presence of acetic acid. Correction factors for the contribution of glucosamine in highly deacetylated chitosans can be easily derived. Typical results for the chitosan of Euphausia superba *are: degree of acetylation, 42·6; relative standard deviation, 1·3%; confidence limits, ±0·7. This method is simpler, more precise and faster than the infrared method. Sonication of chitosan solutions leads to immediate chain degradation and to detectable deacetylation after more prolonged periods of time, especially when the pH is 1·0.*

INTRODUCTION

The current interest (Muzzarelli, 1977; Hirano & Tokura, 1982; Domard & Rinaudo, 1983; Mima *et al.*, 1983) in the use of highly

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deacetylated chitosans, for the preparation of metal ion chelating agents, textiles and pharmaceuticals, has accentuated the need for a rapid and simple instrumental method for the determination of the degree of acetylation, i.e. the percentage of acetylated units in the polymer. Such a method sought should provide accurate and precise results, especially for those highly deacetylated chitosans which cannot be adequately analyzed by existing techniques, e.g. by infrared spectrometry (Muzzarelli *et al.*, 1980), where the signals recorded are those of the *N*-acetyl group. When this group is removed by an extended deacetylation process and the degree of acetylation falls below 20%, the errors associated with the graphical reading become exceedingly large. In those cases, therefore, methods based on the reactions of the liberated amino group are preferred, among which are the colloid titration method based on the use of anionic polymers as titrants (Toei & Kohara, 1976) and the various colorimetric methods developed after the Elson–Morgan methods, such as the one by Chen & Johnson (1983). Ultraviolet spectrophotometry so far has not been used to determine chitin although attempts were made unsuccessfully by Castle *et al.* (1985) to determine chitin in amide and lithium chloride solvents by measuring absorbances. Derivative spectrophotometry is a relatively recent analytical principle and although the u.v.-visible range offers a very wide field of applications, no publication exists dealing with the determination of the degree of acetylation of chitin/chitosan by this technique.

It is therefore the purpose of the present paper to demonstrate the performance of first derivative spectrophotometry for the rapid, precise and non-destructive determination of the acetyl content of chitin/chitosan. We have associated the analytical aspects of the present research with the investigation of the effects of sonication on the chitosan molecules. The sonication of the microfibrils of chitin and cellulose was studied by Colvin (1976) in connection with the polarity of their constituent chains and resulting structures. Nothing is known about the chemical consequences of sonic irradiation. In particular, it is not known whether the *N*-acetyl group is removed. The data published by Gamzazade *et al.* (1981) and by Berkovic *et al.* (1980) on the viscosity of chitosan solutions do not include information on the effects of sonication. This information is necessary for the production of chitosan oligomers by sonic irradiation.

EXPERIMENTAL

Chitosan solutions

The chitosan powders were freeze dried to remove absorbed moisture, weighed and dissolved in dilute acetic acid to yield 0·01 M acetic acid solutions containing 1 mg of dry chitosan per ml. Solutions were also prepared with D-glucosamine hydrochloride in 0·01 M hydrochloric acid (120 mg in 100 ml) using 0·01 M hydrochloric acid as a blank, and *N*-acetyl-D-glucosamine in water or in 0.01 M acetic acid (2·5 mg in 100 ml), again using water or 0·01 M acetic acid as blanks. The sugars were supplied by Sigma Chemical Co, USA. The solutions for sonic irradiation were: hydrochloric acid solutions at pH 1·0 and 2·0; formate buffer at pH 3·0 and acetic buffers at pH 4·0 and 4·7.

Sonication

Chitosan solutions (50 ml) were subjected to sonic irradiation for five 1-min periods of time, at setting 10 in a Branson B-12 cell disruptor. During sonication, the temperature of the sample was kept constant at the desired value by using a Haake thermostat.

Viscometry

A Haake Rotovisco instrument was used, as previously described (Muzzarelli *et al.*, 1981).

Spectrophotometry

Three different spectrophotometers were used: the Kontron Model Uvikon 810, the Uvikon 860 and the Perkin–Elmer Model 550 SE. The derived spectra were obtained at a slit width of 1 nm, a scanning speed of 30 nm min^{-1} and a time constant of 4 s chart speed 10 cm min^{-1}. Far-u.v. cuvettes with 10 mm path length were used in all cases.

RESULTS AND DISCUSSION

The zero order absorption spectra of glucosamine and *N*-acetyl glucosamine show maxima at 197 and 193 nm, respectively. At 199 nm

the molar absorptivity, ϵ, of *N*-acetyl glucosamine is about 130 times higher than for glucosamine. The presence of acetic acid greatly disturbs the determination of both compounds, especially *N*-acetyl glucosamine, its contribution being particularly high around 199 nm. The first derivative spectra for *N*-acetyl glucosamine and acetic acid are shown in Fig. 1. It is apparent that small amounts of *N*-acetyl glucosamine (25 mg litre^{-1} in 0·01 M acetic acid) give signals whose maxima correspond to the lowest contribution from acetic acid (199 nm); their intensity is

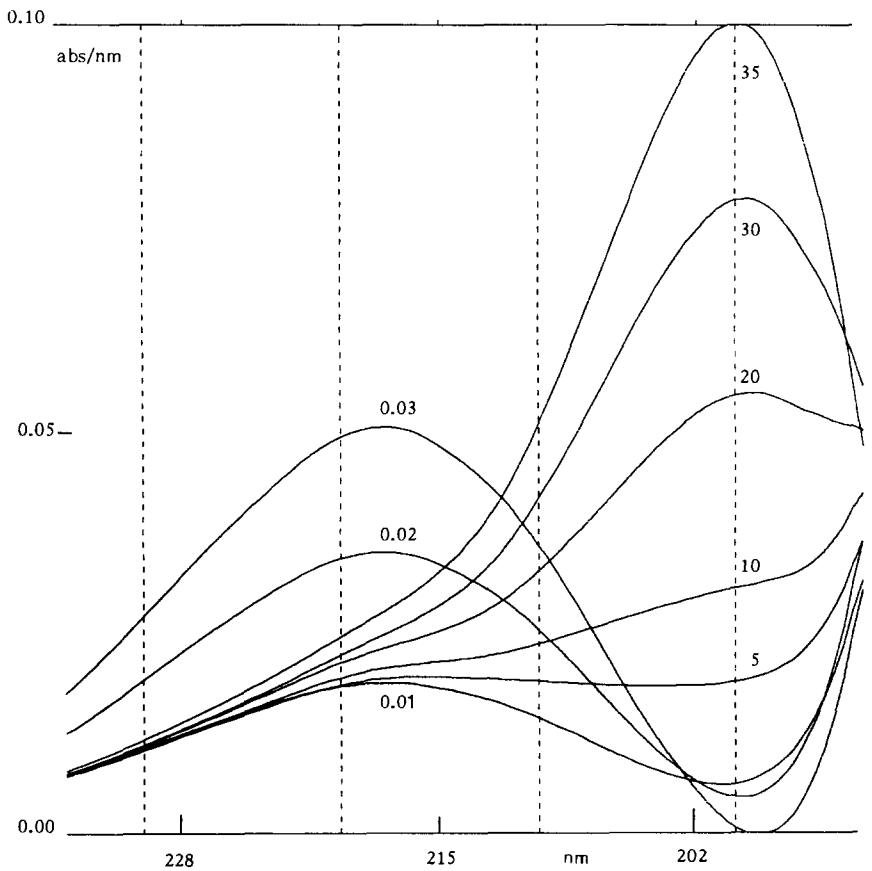

Fig. 1. First derivative spectra of 0·01, 0·02 and 0·03 M acetic acid solutions (three lower curves crossing at 202 nm), and of *N*-acetyl glucosamine at various concentrations (mg litre^{-1}) in 0·01 M acetic acid.

substantially higher than for glucosamine at the same concentration level.

When the first derivative spectra of acetic acid solutions in the range of 0·01 to 0·03 M are recorded against water, it is possible to note that all the acetic acid spectra share a common point at 202 nm, denoted as the zero crossing point. The zero crossing point is close to the *N*-acetyl glucosamine maximum on the wavelength axis; this makes the *N*-acetyl glucosamine determination independent of the acetic acid concentration in the concentration interval usually encountered in the dilute chitosan solutions.

The detection limit of *N*-acetyl glucosamine at 199 nm is 0·5 mg of *N*-acetyl glucosamine per litre, its signal height, H, being twice as large as the noise signal. To define the effect, if any, of acetic acid, on the determination of *N*-acetyl glucosamine, various aqueous mixtures of *N*-acetyl glucosamine and glucosamine were prepared and examined in the first derivative mode. The results are given in Table 1. The signal heights at 199 nm, expressed in mm, are the means of five measurements. The data show that, for varying concentrations of *N*-acetyl glucosamine, the signal heights at 199 nm are not influenced by the acetic acid present even when the latter is 300 times more concentrated than *N*-acetyl glucosamine (0·025 M ≡ 1·50 mg acetic acid ml^{-1} against 0·005 mg *N*-acetyl glucosamine ml^{-1}). Moreover, the data show that a calibration curve can be drawn easily.

Calibration curve for *N*-acetyl glucosamine

To obtain a calibration curve for *N*-acetyl glucosamine, the first derivative spectra of standard *N*-acetyl glucosamine solutions were taken for eight concentrations in the range 0·005 to 0·040 mg ml^{-1} of *N*-acetyl glucosamine in 0·01 M acetic acid. The spectrum was measured five times for each concentration, so that 40 sets of H values (y) and *N*-acetyl glucosamine concentrations (x) were obtained. The correlation coefficient between y and x was calculated to be 0·999 and a plot of y against x yielded a good straight line which passed through the origin. The slope was 3·270 mm (mg litre)$^{-1}$, with a relative standard deviation, $s/\bar{X}$, of ±2·7%. These corresponded to the confidence limits of 3·27 ± 0·081 mm (mg litre)$^{-1}$ at the 95% level. The *N*-acetyl glucosamine concentration, x, mg litre^{-1}, could be calculated from 1/slope × H, i.e.

$$x = 0{\cdot}306H \qquad (1)$$

Accuracy of the first derivative method for *N*-acetyl glucosamine was calculated by the following equation, using the data of the calibration curve:

$$\text{percentage accuracy} = (\bar{X} - \hat{X})/\hat{X}.100 \qquad (2)$$

where $\hat{X}$ is the actual concentration of *N*-acetyl glucosamine in a standard solution and $\bar{X}$ is the mean value of the *N*-acetyl glucosamine concentrations calculated by eqn (1), for each five values of *H*. As the results in Table 2 show, a satisfactory level of accuracy was demonstrated for the first derivative method at every *N*-acetyl glucosamine concentration.

TABLE 1
Effect of Acetic Acid Concentration on the Height of the First Derivative Peak of *N*-Acetyl Glucosamine

N-Acetyl glucosamine concentration (mg litre^{-1})	*Acetic acid concentration (M)*	*H value at zero crossing λ (mm)*[a]
0	0·000	0·0
	0·005	0·0
	0·010	0·0
	0·015	0·0
	0·020	0·0
	0·030	0·0
5	0·000	17·5
	0·005	17·8
	0·010	18·3
	0·015	17·0
	0·020	16·0
	0·030	17·0
10	0·000	32·0
	0·005	33·5
	0·010	33·6
	0·015	32·8
	0·020	32·6
	0·030	32·0

TABLE 1 – *contd.*

N-Acetyl glucosamine concentration (mg litre^{-1})	*Acetic acid concentration (M)*	*H value at zero crossing λ (mm)*[a]
15	0·000	50·3
	0·005	50·0
	0·010	51·5
	0·015	50·8
	0·020	49·7
	0·030	51·0
20	0·000	66·0
	0·005	65·5
	0·010	64·7
	0·015	66·0
	0·020	66·3
	0·030	65·0
25	0·000	81·5
	0·005	81·0
	0·010	82·0
	0·015	81·5
	0·020	79·8
	0·030	80·6

[a] Mean value of five duplicate measurements.

Effect of glucosamine on *H* values

The experimental data show that the presence of glucosamine in the *N*-acetyl glucosamine solutions contributes to the *H* signal, when the *N*-acetyl glucosamine to glucosamine ratio is below 0·11. When the ratio is 0·01, the *H* value is twice as high as the one due to *N*-acetyl glucosamine alone. Correction factors can be derived from the reference curve in Fig. 2, where H_1 is the peak height of pure *N*-acetyl glucosamine at zero crossing wavelength and H_2 is the peak height of *N*-acetyl glucosamine at the same concentration when accompanied by varying amounts of glucosamine.

TABLE 2
Accuracy of the Determination of *N*-Acetyl Glucosamine by First Derivative Ultraviolet Spectrophotometry

N-acetyl glucosamine concentration, mg litre^{-1}		*Percentage accuracy* $100(\bar{X} - \hat{X})/\hat{X}$
Prepared value, $\hat{X}$	*Calculated value,* $\bar{X}$[a]	
10·0	10·1	1·0
15·0	14·9	−0·7
20·0	20·2	1·0
25·0	24·8	−0·8
30·0	29·9	−0·3

[a] Mean value of five x values calculated from H values using eqn. (1).

Assay for commercial chitosan powders

Chitosan powders of various origins (0·500 g) were dissolved in 0·1 M acetic acid and brought to 500 ml. These solutions were analyzed as such or after dilution for the degree of acetylation, depending on their *N*-acetyl glucosamine contents. The results, listed in Table 3, are in agreement with those previously obtained by other techniques by the authors and by other researchers. Their precision is, however, much better. For instance, the results for the chitosan of *Euphausia superba* manufactured by Rybex are given as 42·6 with confidence limits ±0·7, instead of 42 ± 4 as determined by infrared spectrometry.

Assay for sonicated chitosan solutions

The viscosity of the sonicated chitosan solutions is in all cases lower than that of a reference solution of untreated chitosan, under the same conditions. As far as pH is concerned, the decrease of viscosity is more noticeable at pH values 1·0 and 3·0 to 4·0. At pH 2·0, in fact, the untreated chitosan solution shows minimum viscosity decrement. The viscosity decrements are temperature dependent, being larger at low sonication temperatures when pH is 2·0, 3·0 and 4·0 (Fig. 3). At pH 1·0, on the contrary, the viscosity decrease follows the opposite

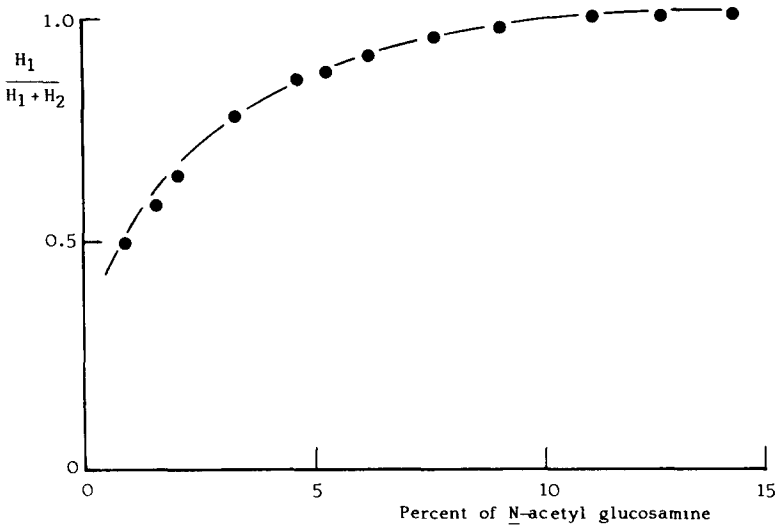

Fig. 2. Reference curve for the determination of *N*-acetyl glucosamine in the presence of defined quantities of glucosamine, percent by weight.

TABLE 3
Degrees of Acetylation of Several Commercial Chitosans Analyzed by First Derivative Ultraviolet Spectrophotometry

Chitosan	*Mean of five measurements*	*Confidence limits at 95% level*	*Relative standard deviation, %*
Bioshell, Albany, Oregon, USA	27·5	0·8	2·2
Kyowa, Tokyo, Japan	24·5	0·8	2·8
Chesapeake	27·9	0·6	1·9
Anic, Milan, Italy	26·3	0·8	2·5
Polyplate, Madras, India	23·7	1·0	3·5
Rybex, Szczneczyn, Poland	42·6	0·7	1·3
Rybex, deacetylated	4·5	0·3	6·0
Rybex, sonicated	18·4	0·8	4·2

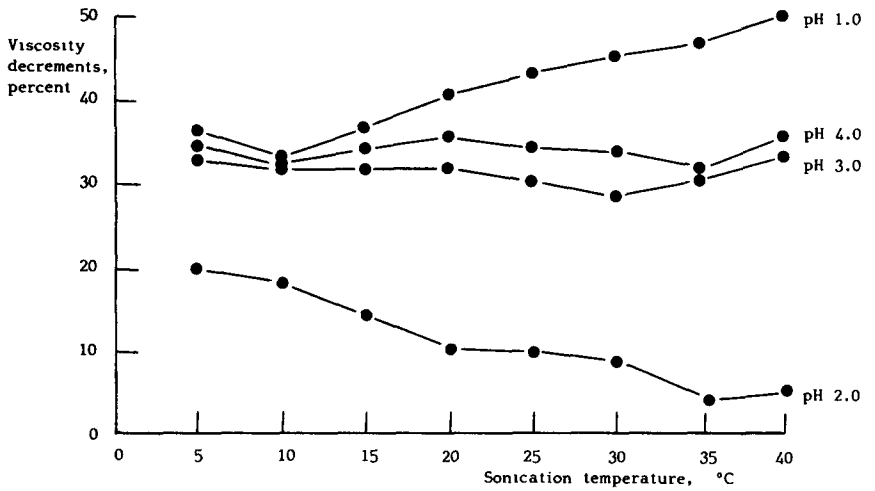

Fig. 3. Viscosity decrements (ratios between viscosity of sonicated chitosan solutions to viscosity of untreated chitosan) vs. sonication temperature, for Rybex chitosan.

trend and substantially larger decrements of viscosity are observed (Fig. 3) at high temperatures. It can be said, therefore, that the sonication exerts maximum hydrolytic action on chitosan at acidic pH values (pH 1·0) at high temperature (40°C). The reduction of molecular size consequent to sonication at pH 1·0 is accompanied by partial deacetylation. Indeed, the original degree of acetylation (42·6%) was reduced to 18·4% (Table 3) when chitosan was sonicated in acidic solutions (pH 1·0) at relatively low temperature (25°C) for 40 min. At the other pH values tested, the degree of acetylation was not appreciably altered. The best conditions for the prevention of chitosan degradation when exposed to sonic irradiation are pH 2·0 and 35°C.

CONCLUSIONS

In conclusion, it was demonstrated that first derivative u.v. spectrophotometry permits a simple and time-saving assay of *N*-acetyl glucosamine residues in chitosan which has good precision and accuracy. The method was used to detect and quantify the removal of acetyl groups

from chitosans as a consequence of ultrasonic treatment. Inherent advantages are the following:

(1) There is no need to know exactly the acetic acid concentration, within a reasonably wide range; this is important especially as, in the case of ultrasonic deacetylation, acetic acid is generated by the process and its concentration increases to an unknown extent.

(2) The spectrophotometric determinations can be carried out with water as a blank because the readings made at the zero crossing point make the determinations independent of the acid concentration. The use of water as a reference blank reduces light absorption in the reference system, thus permitting a better signal to noise ratio.

(3) Sensitive and precise measurements can be carried out in the simplest way by using commonly available spectrophotometers.

(4) Chitosan can be effectively hydrolyzed by selecting appropriate values of pH, temperatures and time (preferably 1·0, 40°C and 5 min) or, alternatively, protected from hydrolytic action when submitted to sonic irradiation by adjusting the pH value to 2·0 and the temperature to 35°C.

(5) Exposure of chitosan solutions at pH 1·0 to sonic irradiation for longer periods of time (40 min) leads to a significant lowering of the degree of acetylation.

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