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A rapid method for the determination of the degree of *N*-acetylation of chitin–chitosan samples by acid hydrolysis and HPLC

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ABSTRACT

A simple and rapid method for the quantitative determination of *N*-acetyl groups in chitin–chitosan samples consists of an acid hydrolysis conducted at high temperature with a mixture of sulfuric and oxalic acids. The acetic acid formed is determined by spectrophotometry at 210 nm after separation from the reaction mixture by HPLC. The method is valid for the entire range of degrees of acetylation and the results compare fairly to those of other techniques.

INTRODUCTION

Chitin[1], a (1 → 4)-2-acetamido-2-deoxy-β-D-glucan commonly found as the tough polymer matrix of crab and shrimp shells, has a combination of biological activity and physical properties that has stimulated renewed attention for its many potential applications. Chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan] obtained by partial *N*-deacetylation of chitin, has been extensively studied in recent years[2]. The physical and chemical properties of chitin and chitosan are very different and, therefore, the precise knowledge of the degree of *N*-acetylation (da) is crucial in order to characterize any chitin deacetylation process as well as any chemical modification.

The da can be determined by IR spectroscopy[3,4], titration[5], pyrolysis–GLC[6], first derivative UV spectrophotometry[7], NMR spectroscopy[8,9], thermal analysis[10], circular dichroism[11], enzymatic hydrolysis[12], and elemental analysis. Given the solubility properties of chitin–chitosan samples, some of these methods are inap-

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plicable for highly deacetylated chitosan samples, or for highly acetylated chitin. In some methods, the samples have to be purified to remove the fraction of insoluble material. Moreover, most methods present several drawbacks regarding either analysis time, cost, or accuracy.

A simple method for the determination of the acetyl content is acid hydrolysis and subsequent quantitative determination of the acetic acid produced by GLC. This approach has been successfully used by Holan et al.[13] for the determination of chitin in biological samples and by Solar et al.[14] for the determination of acetyl groups in wood and related products. The former method consists of a 4 M HCl hydrolysis during 16 h at 96–100°C, and the latter employs 4 M oxalic acid at 155°C during 1 h. The method of Holan et al.[13] is time consuming and the use of HCl significantly reduces the life of the column which has to be repacked after 150 injections. Recently, it was found that this limiting number can be increased up to 1000 when capillary columns are used[15]; the analysis time, however, remains the same. As wood and related products are known to give overestimated acetyl values due to the formation of additional acetic acid from sugar decomposition when strong mineral acids are employed[16,17], Solar et al.[14] proposed the use of oxalic acid and an increase of the hydrolysis temperature up to 155°C. As far as we know, this method, which has the advantage of being faster than that of Holan et al.[13], has never been reported for chitin–chitosan samples. Since we found that direct application of the method of Solar et al.[14] to chitin–chitosan samples failed, we had two different objectives: (a) to develop an acid hydrolysis procedure that is rapid, accurate, and easy to carry out; and (b) employ HPLC instead of GLC in order to overcome the constraint of column longevity.

EXPERIMENTAL

Materials.—Chitin and chitosan standards were obtained from Sigma Chemical Co., Saint Louis, MO, USA. Chitin C3641 (lot no. 75F-7015) was in powder form, and chitin C3387 (lot no. 84F-7072) and chitosan C3646 (lot no. 122F-0883) were as flakes; all three were derived from crab shells. Deacetylated chitins, obtained from chitin C3387 via a proprietary techniques using a Thermo-Mechano-Chemical treatment[18], were also characterized (samples identified as TMC-CHIT). 2-Amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, and all other chemicals were analytical grade from Aldrich Chemical Co., Milwaukee, WI, USA.

General methods.—Acid hydrolysis was conducted in Vacuum Hydrolysis Tubes (internal volume, 5 mL), placed in a heating module (Pierce Chemical Co., Rockford, IL, USA). The identification and quantitative determination of acetic acid were performed on a high performance liquid chromatograph, Varian Model 5000 (Varian, Montreal, PQ, Canada). The system consisted of a 300 × 7.8 mm column HPX 87H (H^+) cation-exchange resin (Bio-Rad Laboratories, Mississauga, ON, Canada) and a UV detector. The eluant was filtered (0.45 μm); 5 mM H_2SO_4, flow rate 0.8 mL min^{-1}, pressure 6.7 MPa, temperature 23°C, injection volume 10 μL, and the detection was carried out at 210 nm. All data were acquired, stored, and processed on a microcomputer with the aid of a software program Maxima 820 (Waters, Milford, MA, USA). The calibration curve used different amounts of acetic acid. The eventual degradation of reagents was monitored with a Shimadzu Total Organic Carbon Analyzer Model TOC 500 (Shimadzu, Montreal, PQ, Canada).

Determination of the degree of N-*acetylation (da) in chitin and chitosan.*—The da was determined by titration[5], by the picric acid method[19], by elemental analysis, and by ^{13}C NMR spectroscopy. For this last method, the analyses were carried out on a Bruker WM-250, on freeze-dried samples, by use of magic-angle spinning, at 45.67 MHz. The da was evaluated by comparison of the integrated areas of the methyl group signal in relation to the signal of C-1 of the sugar residue. For elemental analysis, the da could be related to the percentage of nitrogen, after the proportion of inorganic material had been determined by weighing the residue obtained after 1 h at 600°C, by use of eq *1*,

$$\mathrm{da}(\%) = \left(\frac{8.695 - \%\mathrm{N}}{1.799}\right) \cdot 100 \qquad (1)$$

where 8.695 is the percentage of N in fully deacetylated chitosan, 1.799 the difference between 8.695 and 6.896 (percentage of N in fully acetylated chitin), and %N is the percentage of N calculated on the organic fraction of the analyzed material.

Acid hydrolysis and HPLC procedure.—A weighed amount (M) of dried, finely powdered polymer (~ 10 mg) was placed in a Vacuum Hydrolysis Tube with 0.5 mL of 12 M H_2SO_4 (final molarity 2.41 M) and 2 mL of the standard mixture (6.3 mg of oxalic acid dihydrate and 0.5 mL of propionic acid completed to 100 mL with distilled water). The tube was closed and placed in the heating module preheated at 155°C. After 1 h, the tube was placed in ice–water for at least 2 h, and then allowed to reach room temperature. The mixture was filtered (0.45 μm) and 10 μL was injected in the HPLC instrument.

The amount of acetyl groups liberated (m_x) was calculated according to eq *2*,

$$m_x = K \cdot \frac{A_x}{A_{is}} \cdot m_{is} \qquad (2)$$

where K is the response factor, A_x and A_{is} are the areas of the acetic and propionic acid (internal standard) peaks, respectively, and m_{is} is the amount of internal standard. The percentage of N-acetylation was calculated according to eq *3*

$$\mathrm{da}(\%) = \frac{161X}{43 - 42X} \cdot 100 \qquad (3)$$

where $X = m_x/M'$; $M' = M - m_i$ (m_i = mass of inorganic material); 161 is the mol wt of a 2-amino-2-deoxy-D-glucose unit; and 43 the mol wt of an acetyl group.

RESULTS AND DISCUSSION

According to Holan et al.[13], the fraction (y) of the liberated acetyl groups over the total acetyl groups in the sample should be 0.96 when the hydrolysis is conducted at 155°C during 1 h. Employing sulfuric instead of hydrochloric acid (as HCl is not suitable to HPLC), we were not able to obtain this value. Table I shows the results obtained when hydrolysis was conducted with 2.41 M H_2SO_4 at 155°C for 1 h. together with the results obtained by ^{13}C NMR spectrometry. It appears clearly that this modification of the Holan et al.[13] procedure underestimates the degree of acetylation, especially at higher da values. This procedure resulted, however, in no prejudicial sugar residue degradation since no acetic acid was detected after the analysis of pure 2-amino-2-deoxy-D-glucose carried out using the modified procedure.

With the temperature set at 155°C, an increase of the H_2SO_4 concentration from 2.41 to 3.6 M provided no improvement. The fraction, y, slightly increased (2%), but the difference can be attributed to a degradation of the sugar residue leading to acetic acid formation, as found via a parallel analysis using 2-amino-2-deoxy-D-glucose. Moreover, since the general mechanism for amide hydrolysis has, as the rate-determining step, the attack of a water molecule on the conjugate acid of the amide[20], this suggests that the only effect of increasing acid strength is to decrease the activity of the water[21].

In order to avoid the possible degradation of carbohydrates to acetic acid, Solar et al.[14] developed a method for wood and related materials where acetyl groups are liberated by acidolysis with oxalic acid at high temperature, the acetic acid formed being determined by GLC. It was found that direct application of this method to chitin–chitosan samples led to very incomplete deacetylation. For example, the da of chitin C3387 and chitosan C3646 were evaluated to be 27 and 9%, respectively, whereas ^{13}C NMR spectroscopy gave 74 and 25%, respectively (Fig. 1).

The limited accessibility of acetyl groups present in chitin–chitosan towards oxalic acid was assumed to be the cause of this underestimation rather than the fact that the samples are *N*- instead of *O*-acetylated as 2-acetamido-2-deoxy-D-glu-

TABLE I

Determination of the da (%) of chitin–chitosan samples following acid hydrolysis[a] and HPLC as compared with ^{13}C NMR spectroscopy

Sample	Acid hydrolysis–HPLC[b]	^{13}C NMR spectroscopy
Chitin C3641	79 ± 2	95
Chitin C3387	66 ± 2	74
TMC-Chit A	43 ± 2	48
Chitosan C3646	21 ± 2	25

[a] With 2.41 M H_2SO_4 at 155°C for 1 h. [b] Without oxalic acid.

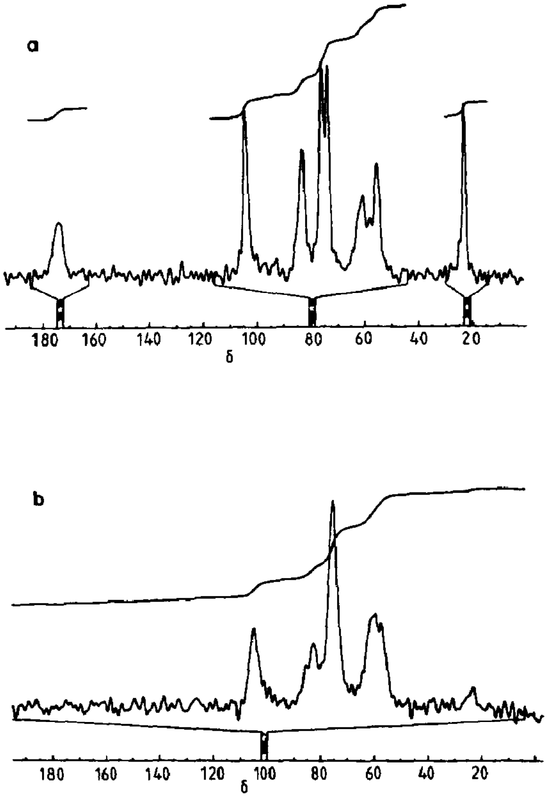

Fig. 1. CP/MAS ^{13}C NMR spectra of (a) chitin C3387 (da = 74%) and (b) chitosan C3646 (da = 25%); δ 24 (CH_3), 56 (C-2), 62 (C-6), 75 (C-5,3), 85 (C-4), 105 (C-1), and 175 (CO).

cose was completely deacetylated under the same conditions. In order to increase this accessibility, the influence of the reaction time on y was studied and proved to have little effect on the deacetylation (Fig. 2). The temperature effect was also studied but 155°C appeared to be a maximum in order to avoid carbonization of the sample. Ball milling showed little effect on the deacetylation yield (data not shown).

Examples of the use of a combination of two acids are numerous in organic chemistry. For example, the production of furfural is increased by 15% when two acids are used together instead of either alone[24]. Thus, when acid hydrolysis of chitin C3387 and chitosan C3646 was conducted at 155°C for 1 h with 1:4 (v/v) 12.05 M H_2SO_4–M oxalic acid, the da had the expected values. It is likely that the two acids act cooperatively, that is, H_2SO_4 depolymerizes the sample to some extent as to make it accessible to the action of oxalic acid, which completes the deacetylation.

In order to determine the optimal reaction time, a kinetic study of the deacetylation of a chitin sample was performed. Deacetylation was complete within 60 min at a temperature of 155°C, and longer reaction times induced a degradation that

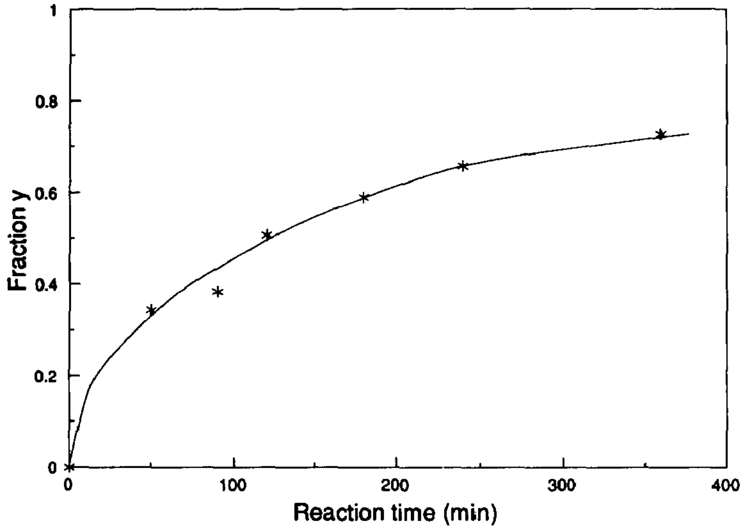

Fig. 2. Deacetylation of chitin C3387 with oxalic acid according to the method of Solar et al.[14]. Fraction *y* of the liberated acetyl groups over the total acetyl groups in the sample as a function of the reaction time.

manifested itself by the appearance of a carbon residue on the hydrolysis tube walls (Fig. 3). The reaction was faster for chitosan samples having a da ~ 20%, but the degradation began almost at the same time (75 min); 60 min should then be considered as an adequate reaction time for both chitin and chitosan samples.

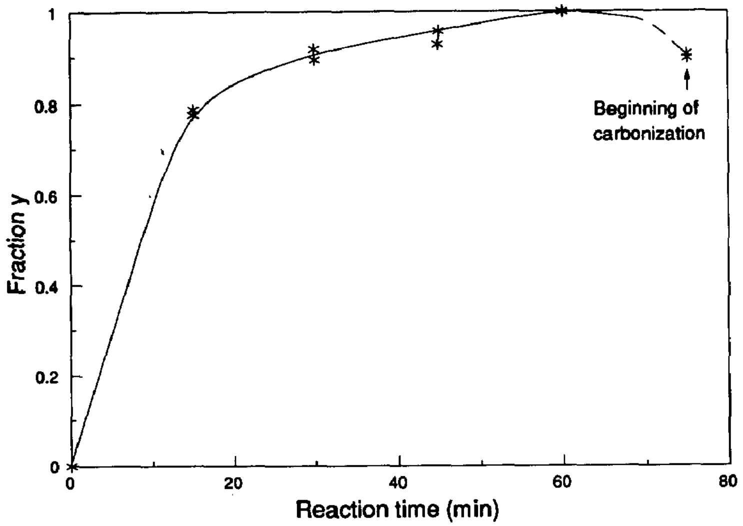

Fig. 3. Optimization of the proposed method. Influence of the reaction time upon the fraction *y* of the liberated acetyl groups over the total acetyl groups in chitin C3641.

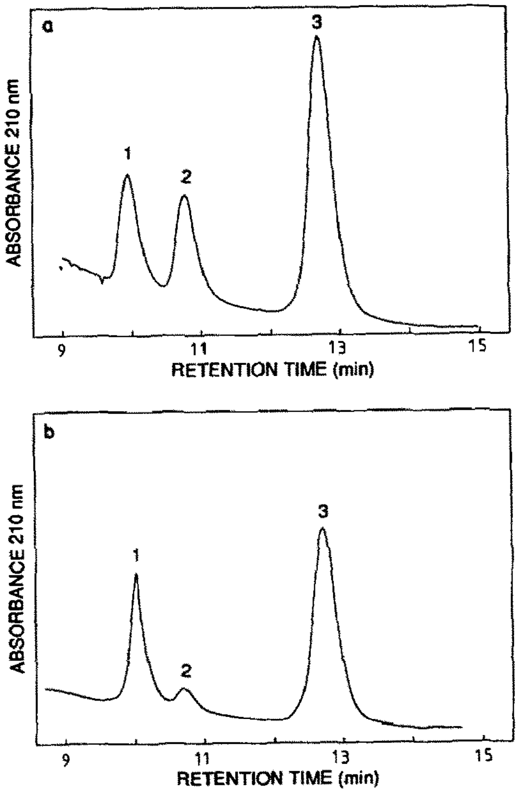

Fig. 4. Chromatograms of (a) chitin C3387 and (b) chitosan C3646 reaction mixture: (1) formic acid, (2) acetic acid, and (3) propionic acid.

Figure 4 shows the HPLC chromatograms of the reaction mixture obtained after treatment of chitin C3387 and chitosan C3646. Formic, acetic, and propionic acid were eluted in this order, formic acid being a product of oxalic acid degradation. In order to improve the resolution of the first two peaks, the flow rate was varied and 0.8 ml min^{-1} was found to be the minimum in a Van Deemter graph. Despite this optimization, there was still some overlapping. The skim ratio was fixed so that the unskimmed mode of integration is used by the software program.

Because of the simultaneous use of high temperature and high acidity, a possible degradation of the reagents and of the product that could lead to the formation or degradation (or both) of acetic or propionic acid was considered. As expected, HPLC revealed that, under the conditions used, neither acetic nor propionic acid led to any other soluble product. In order to establish if there was formation of any volatile compounds, the carbon content of known concentration solutions of both acids was controlled at different temperatures and H_2SO_4 concentrations (Table II). Again, no degradation was detectable. Furthermore, the analysis of 2-amino-2-deoxy-D-glucose at different concentrations evidenced that no degradation of the sugars in the form of acetic acid had taken place.

TABLE II

Carbon analysis of acetic and propionic acid solutions in the presence or absence of 12.05 M H_2SO_4 at different temperatures [a]

Acid	Conditions	Temperature (°C)	
		25	155
Acetic	Without H_2SO_4	194	190
	With H_2SO_4 (1 h)	204	196
Propionic	Without H_2SO_4	243	242
	With H_2SO_4 (1 h)	246	254

[a] Results expressed in ppm.

The da over a wide range of chitin and chitosan samples, as determined by our method, compare adequately with those obtained by ^{13}C NMR spectroscopy, titration, the picric acid method, and elemental analysis (Table III). The data spread (calculated upon an average of three experiments, two injections each) is within the accepted deviations for GLC. Moreover, the linearity of the method has been established by analyzing different mixtures of chitin C3641 and fully deacetylated chitosan C3646 (data not shown).

In conclusion, the present microanalysis method shares the advantage of validity and precision over the complete range of degrees of acetylation with the highly sophisticated ^{13}C NMR method, but without the intrinsic drawbacks of the latter, mainly, the cost of the instrument and its complexity. Other techniques, such as titration, depend on the sample solubility making them valid only for da < 50%. With the proposed method, once the sample is well characterized for its humidity and inorganic material content, the da can be obtained within 4 h, including

TABLE III

da determination of chitin–chitosan samples by acid hydrolysis–HPLC, ^{13}C NMR spectroscopy, titration, picric acid method, and elemental analysis

Sample	Acid hydrolysis–HPLC	^{13}C NMR spectroscopy	Titration	Picric acid	Elemental analysis
Chitosan C3646	23 ± 2	25	28	18	23
Chitosan C3646 (fully deacetylated [b])	0 ± 2	0	[a]	[a]	[a]
Chitin C3387 (practical grade)	73 ± 3	74	[c]	[a]	76
Chitin C3387 (purified [d])	91 ± 4	90	[c]	93	[a]
Chitin C3641	94 ± 4	95	[c]	97	94
TMC-Chit 1	21 ± 2	24	26	28	[a]
TMC-Chit 2	32 ± 2	34	39	36	[a]
TMC-Chit 3	49 ± 2	51	[c]	50	[a]

[a] Not determined. [b] Complete deacetylation was carried out according to the method of Rinaudo and Domard[22]. [c] Not applicable. [d] Purification was carried out according to the method of Skujins et al.[23].

preparation and cooling time. Of course, several samples, up to six in our case, could be prepared simultaneously and analyzed in a row. There is a certain latitude allowed for the HPLC analysis since the reaction mixture was demonstrated to be stable for over a week when stored at 4°C. The special tubes employed for this study can be substituted with less expensive, flame-sealed ampoules, and the heating module with a gas chromatograph oven. The calibration is simple and reliable, and the interpretation of the results is straightforward. The only requirement is a suitable HPLC column since the acetic acid must be separated from the other acids prior to its quantitative determination.

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