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# Degree of acetylation of heteropolysaccharides

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**Abstract**

The acetyl groups in polysaccharides and glycoproteins have been determined using 4 N HCl at 120 °C for acid hydrolysis. Acetic acid and hexosamine were determined by high-performance cation-exchange chromatography with UV detection and high-performance anion-exchange chromatography with pulsed amperomeric detection, respectively. The method compares well with other procedures and shows an additional advantage of being able to analyze for hexosamine in the same hydrolyzate, thus permitting the degree of acetylation of hexosamine-containing biopolymers to be determined directly without correction for additional components in the material of interest.


*Keywords:* Acetyl groups; Polysaccharides; Chitin; Chitosan

## 1. Introduction

Glucosamine and to a lesser extent other hexosamines are ubiquitous in nature, occurring in biopolymers, such as chitin and glycoproteins. Most commonly the amino group of the hexosamines is acetylated and the determination of the acetyl content is of interest. Many procedures have been described for this determination, involving spectroscopy (UV [1,2], IR [2–6] and NMR [7–9]), dye absorption [10] and analysis of released acetic acid [11]. Each method has its advantages and disadvantages, depending upon the nature of the material of interest, such as solubility, composition, and the availability of appropriate analytical equipment.

One of the most generally applicable procedures involves the acid hydrolysis of the acetamido group and the determination of the released acetic acid. This methodology has been re-examined for a variety of starting materials.

## 2. Materials and methods

2 - Amino - 2 - deoxy - D - glucose (GlcN), 2-amino-2-deoxy-D-galactose (GalN), 2-acetamido-2-deoxy-D-glucose (GlcNAc), L-fucose (Fuc), and D-mannose (Man) were obtained from Pfanstiehl (Waukegan, IL), sodium glucuronate (GlcA) from Sigma Chemical Co. (St. Louis, MO); sodium pyruvate from Nutritional Biochemical Corporation (Cleveland, OH); ovalbumin from Fisher Scientific (Pittsburgh, PA); amino acid standard mixture from Beckman (18 components, Part No. 338088, Fullerton, CA). Extracellular polysaccharides of *Erwinia chrysanthemi* species Ech6 and CU643 were produced in our laboratory as described elsewhere [12]. Chitin C3641 (lot no. 35H7015) and chitosan C3646 (lot no. 98H0504), which were derived from

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crab shell, were obtained from Sigma. All other chemicals were of analytical grade.

*Acid hydrolysis of carbohydrate.*—The carbohydrate-containing material was hydrolyzed in two ways.

(a) The solid material (2–5 mg) in a vial (2 mL) for analysis was stirred in 4 N HCl (1 mL) for 6 h at 120 °C. The reactants were mixed, sonicated to remove trapped air, and flushed with He gas before placing in the heating block. The final reaction solution was cooled in an ice bath and filtered (0.25 μm Nylon Acrodisc, Gelman). The resulting filtrate was analyzed by high-performance liquid chromatography (HPLC) with and without addition of the internal standard (propanoic acid).

(b) The material was hydrolyzed with $H_2SO_4$–oxalic acid for 1 h at 155 °C as described elsewhere [11].

The hydrolyses were conducted either in vacuo using a vacuum hydrolysis tube (Pierce Chemical Co. cat no. 29560, Rockford, IL) or in a vial that had been sonicated and sparged with helium as described in (a) above.

*HPLC analysis for acetic acid.*—Acetic acid in the hydrolyzate (10 μL) was analyzed by high-performance cation-exchange chromatography on a Dionex BioLC (Dionex Corp., Sunnyvale, CA, USA) system, consisting of a cation-exchange analytical column (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm, Hercules, CA), a guard column (4.6 × 30 mm), and a UV detector. The sample was eluted from the column with 5 mM $H_2SO_4$ at a flow rate of 0.5 mL $min^{-1}$ and the eluate monitored at 210 nm.

In the analysis of ovalbumin, the hydrolyzate was mixed with propanoic acid and the amino acids and peptides were removed with a spin column (2 mL, AG50-X8 $H^+$: Bio-Rad). The eluate and washings were pooled and analyzed for acetic acid.

*Determination of hexosamines.*—The hydrolyzates (50 μL) were diluted with water (1.5 mL) and evaporated on a Speed Vac, which was repeated twice. The dried material (equivalent to 25–50 μg of starting material) was reconstituted in water (1 mL) containing internal standard (50 μg of 2-amino-2-deoxygalactose) and analyzed on a PA1 CarboPac column (4 × 250 mm) by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex BioLC as described earlier [13,14]. The elution was performed either isocratically with 16 mM NaOH or 16 mM NaOH for the first 30 min, then a gradient of 50–220 mM NaOAc in the presence of 40 mM NaOH for another 30 min.

*$^1H$ NMR spectroscopy.*—Chitosan C3646 (132 mg) in 0.1% HCl (70 mL) was gently shaken overnight at room temperature. The clear solution was filtered (0.45 μm) and the filtrate freeze-dried. The dried material was dissolved in $D_2O$ and freeze-dried again. This was repeated twice. The $^1H$ NMR spectra were recorded with a Bruker AMX-600 NMR spectrometer at 65 °C. The chemical shifts were referenced to acetone (2.225 ppm) as an internal standard.

Extracellular polysaccharide of *E. chrysanthemi* strain Ech6 [12] was dissolved in $D_2O$ containing 2% NaCl and freeze-dried. Proton exchange was completed by repeated evaporation from $D_2O$. The $^1H$ NMR spectra were obtained at 25 °C.

## 3. Results

The acidic decomposition of sugars produces a complex mixture of furans, humins and acids [15,16]. The nature of these products, which include simple acids, complicates the analysis of acetic acid unless great care is taken to effect a clean separation from other components (Fig. 1). It is clear that acidic or other products, which were not identified, eluting around the acetic acid peak will cause an overestimate of the degree of acetylation in the compound of study. Several elution profiles were studied to overcome this difficulty. For example, the modification of the sulfuric acid eluant by adding acetonitrile (10%) enhanced the chromatographic separation of aliphatic acids, such as propionic and butyric acids. However, under these conditions some of the degradation products eluted very close to the formic, acetic, and propanoic acids, which consequently interfered with their quantitation, resulting in a 10–20% overestimation of acetate. Elution with 5 mM sulfuric acid

was therefore adopted, but the column needed to be cleaned and regenerated routinely.

The acid degradation products of all the monosaccharides of interest are very complex and very similar in composition. The example of mannose is shown (Fig. 1). However, the nature of the monosaccharide determines the relative distribution of products; the most significant component from fucose was observed at 11 min; from mannose at the 17 min (formic acid); from glucosamine at the 21 min.

As can be seen from the summary of results (Table 1), hydrolyses with HCl or by the methods of Niola et al. [11] give comparable data. The need to use specialized hydrolysis tubes is not evident; sparging with helium in a suitably capped vial gives the same results. The internal standard used in these studies in the Dionex procedure for hexosamine was 2-amino-2-deoxy-D-galactose, which obviously would need to be changed if this sugar were present in the unknown sample. There is a small but significant quantity of acetic acid

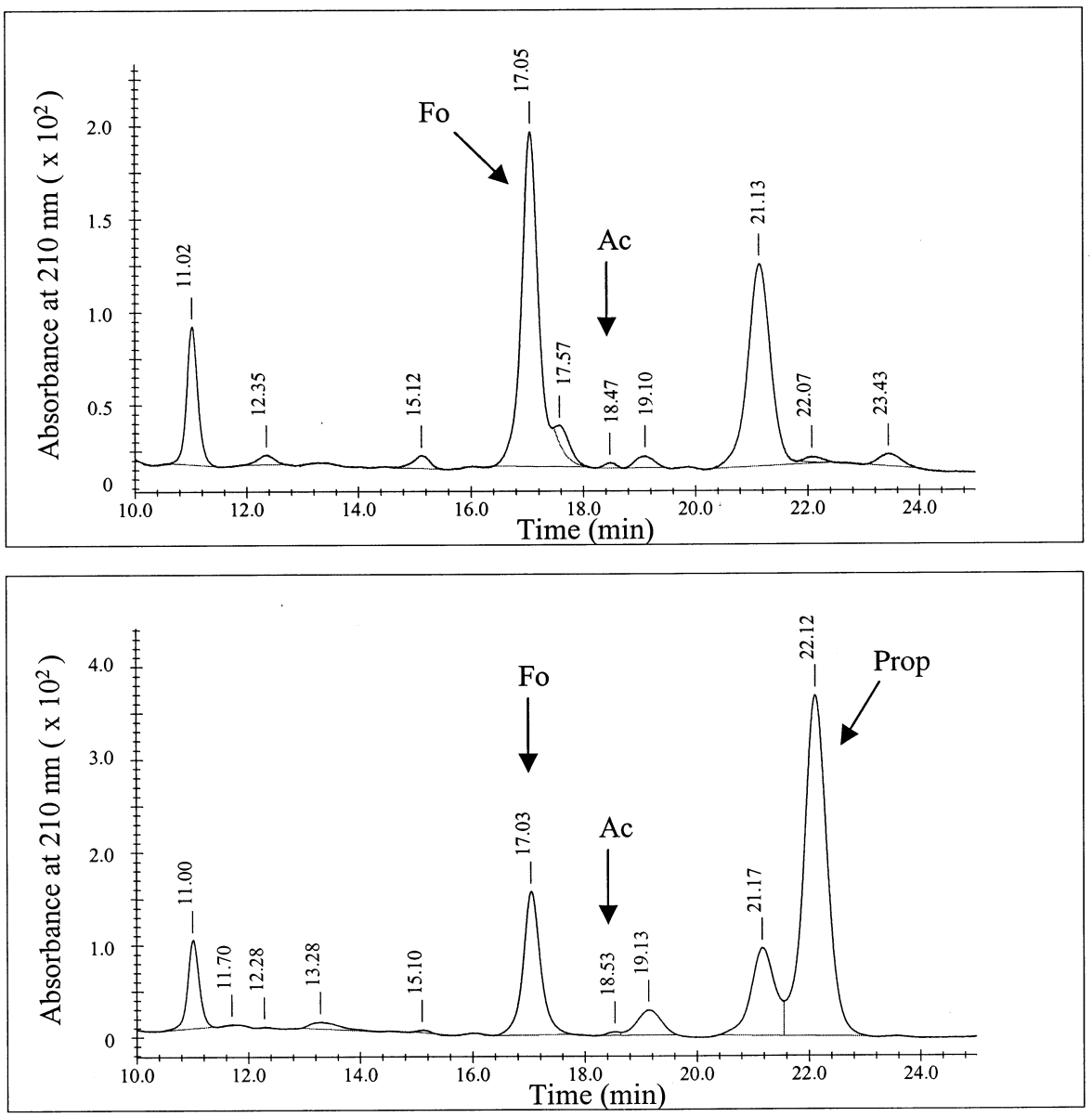


Fig. 1. HPLC analyses of mannose degradation by two different acids with or without an internal standard (propanoic acid). Top panel: 4 N HCl, 120 °C, 6 h; bottom panel: in vacuo with $H_2SO_4$–oxalate 155 °C, 1 h. Fo, Ac, and Prop denote formic acid, acetic acid and propanoic acid, respectively.

Table 1
HPLC determination of acetic acid generated from carbohydrates by acid hydrolysis

| Materials | Mole AcOH/mole starting material [a] | | | Mole AcOH/mole glucosamine |
|---|---|---|---|---|
| | $H_2SO_4$–oxalate, 155 °C, 1 h | | 4 N HCl, 120 °C, 6 h | 4 N HCl, 120 °C, 6 h |
| | In vacuo | He gas sparged | He gas sparged | He gas sparged |
| GlcN | 0.01 | 0.00 | 0.02 | |
| GlcNAc | 1.04 | 1.06 | 1.06 | |
| Man | 0.01 | 0.02 | 0.01 | |
| Fuc | 0.02 | 0.03 | 0.03 | |
| GlcA | 0.01 | 0.01 | 0.01 | |
| Pyruvate | 0.00 | 0.00 | 0.00 | |
| CU643 [b] | 0.12 | 0.14 | 0.09 | |
| Ech6 [b] | 0.89 | 0.88 | 0.84 | |
| Ovalbumin | nd | nd | nd | 1.84 [c] |
| Chitin | 0.83 | 0.88 | 0.80 | 0.94 |
| Chitosan | 0.17 | 0.17 | 0.16 | 0.19 [d] |

[a] Based upon weight of dried material; nd, could not be determined without previous introduction of ion-exchange chromatography.
[b] Based on the repeating unit of hexasaccharide.
[c] Hydrolyzate was subjected to cation-exchange chromatography (spin column (2 mL) of AG50W-X8 $H^+$) and expressed after subtraction of acetic acid derived from acid degradation of sugar residues.
[d] HCl salt prepared after solubilization in 0.1% HCl and freeze-drying.

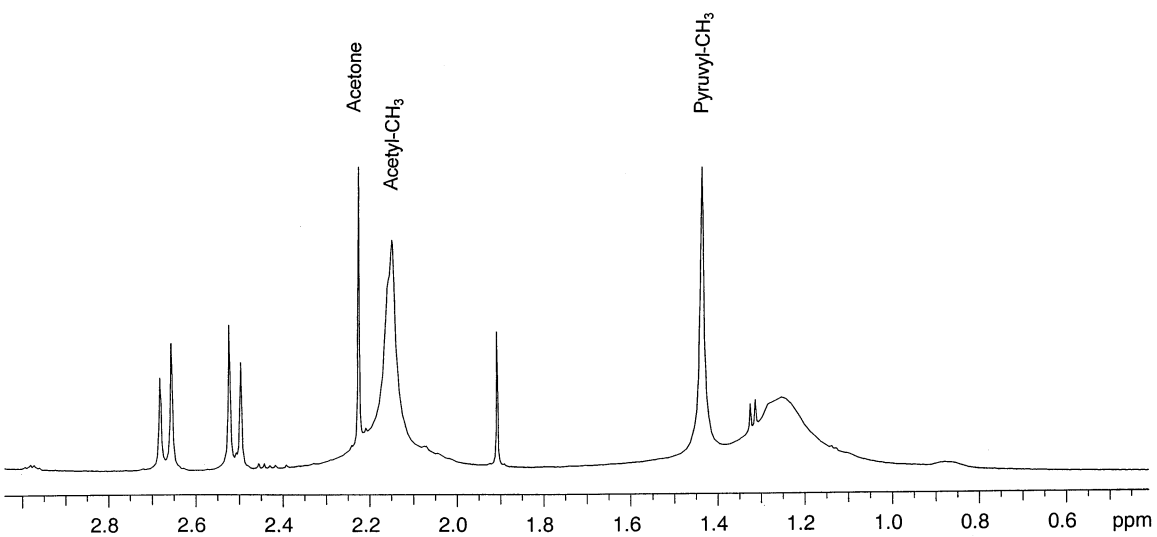


Fig. 2. 1D $^1H$ NMR spectrum (600 MHz) of Ech 6 (2.3 mM in terms of the repeating unit molecular weight 1044) in $D_2O$ containing 2% NaCl, 25 °C. Acetone (internal standard) set to 2.225 ppm; pyruvyl-$CH_3$ at 1.435; acetyl-$CH_3$ at 2.148 ppm.

produced from the sugar components of the heteropolysaccharides, which must be taken into account when expressing a degree of acetylation in these polymers. Examples of this are seen in the analyses of two extracellular polysaccharides from *Erwinia* spp., CU643 [17] and Ech 6 [12]. Both were examined by $^1H$ NMR. The 1D $^1H$ NMR analysis of CU643 indicated no O-acetyl groups, but upon analysis some acetic acid was detected after acid hydrolysis. Expressing the results in terms of the hexasaccharide repeating unit, CU643 (repeat unit $M_w$ 940), the acetic acid produced corresponded to that which would be predicted from the decomposition of the component monosaccharides. Ech 6 (repeat unit $M_w$ 1044) gave a value that, when corrected for the decomposition of the monosaccharide constituents, corresponded to 0.85 mol of acetyl group for each repeat unit. Re-examination of the 1D $^1H$ NMR of Ech 6 (Fig. 2) clearly indicates the resonance of both

acetate (2.148 ppm) and pyruvate (1.435 ppm) methyl groups, which are present in a 0.8:1.0 ratio, in agreement with the chemical analysis and suggesting that not all the repeat units are acetylated.

Because the acetyl group is linked to glucosamine residues in the chitin or chitosan polymers, the determination of both acetic acid and glucosamine from the same acid hydrolyzate is desirable in the expression of the degree of acetylation. This excludes the need for the analyses of any possible interference from components in the starting materials, such as moisture or salts. Other polysaccharides can be determined by the phenol–$H_2SO_4$ procedure [12] for appropriate correction of the small amount of acetic acid that they produce. Chitin C3641 was completely hydrolyzed within 2 h (Fig. 3), but to determine any possible incomplete hydrolysis, the hydrolyzate was further analyzed using a different gradient of NaOH and sodium acetate as described earlier [17]. No significant peak other than glucosamine was detected, indicating the absence of oligosaccharides. Equimolar amounts of acetate and glucosamine were produced from 2 to 5 h of hydrolysis, after which time a slight decrease ($< 5\%$) in the ratio was observed. The mole ratio of acetate to glucosamine after 6 h of hydrolysis was 0.94, which differs from the value (0.80) based on the weight of the starting materials, presuming pure chitin (Table 1).

Chitin and highly acetylated chitosan are insoluble in simple solvents. Chitosan C3646 is soluble in 1% acetic acid, 0.1% trifluoroacetic acid, or 0.1% HCl. The resulting partially purified chitosan C3646 (HCl salt) indicated that its degree of acetylation is lower by 12% when based on the initial weight rather than that based on the glucosamine content (Table 1). This chitosan, DCl salt, lends itself to analysis by NMR. The results (Fig. 4) gave 16.0% acetylation from the ratio of the responses of the anomeric proton of GlcNAc (0.19) to that of the total anomeric protons of GlcN (1.00) and GlcNAc (0.19), 19.3% acetylation by comparing the ratio of the acetyl protons (0.69) to the total anomeric protons or, by using the intensity of all the ring protons as proposed by Vårum et al. [8], the degree of acetylation is 17.8%. An average of these NMR determinations (17.3%) is in agreement with the chemical method (18.7%).

Analysis of glycoproteins presents the difficulty of the presence of large amounts of amino acids and small peptides compared with the acetic acid in the hydrolyzate. It was necessary to remove these protein degradation

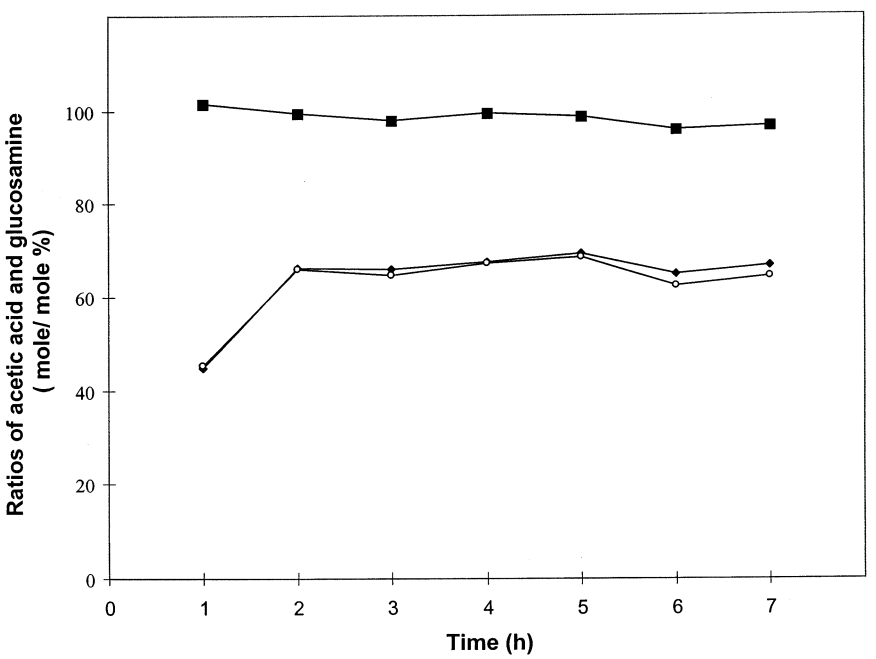


Fig. 3. Glucosamine and acetic acid released with time by hydrolysis of chitin C3641 with 4 N HCl, 120 °C. ○, mol% of acetic acid–GlcNAc based on the weight of the starting material; ◆, mol% of glucosamine based on the weight of the starting material; ■, ratio (mol:mol%) of acetic acid to glucosamine.

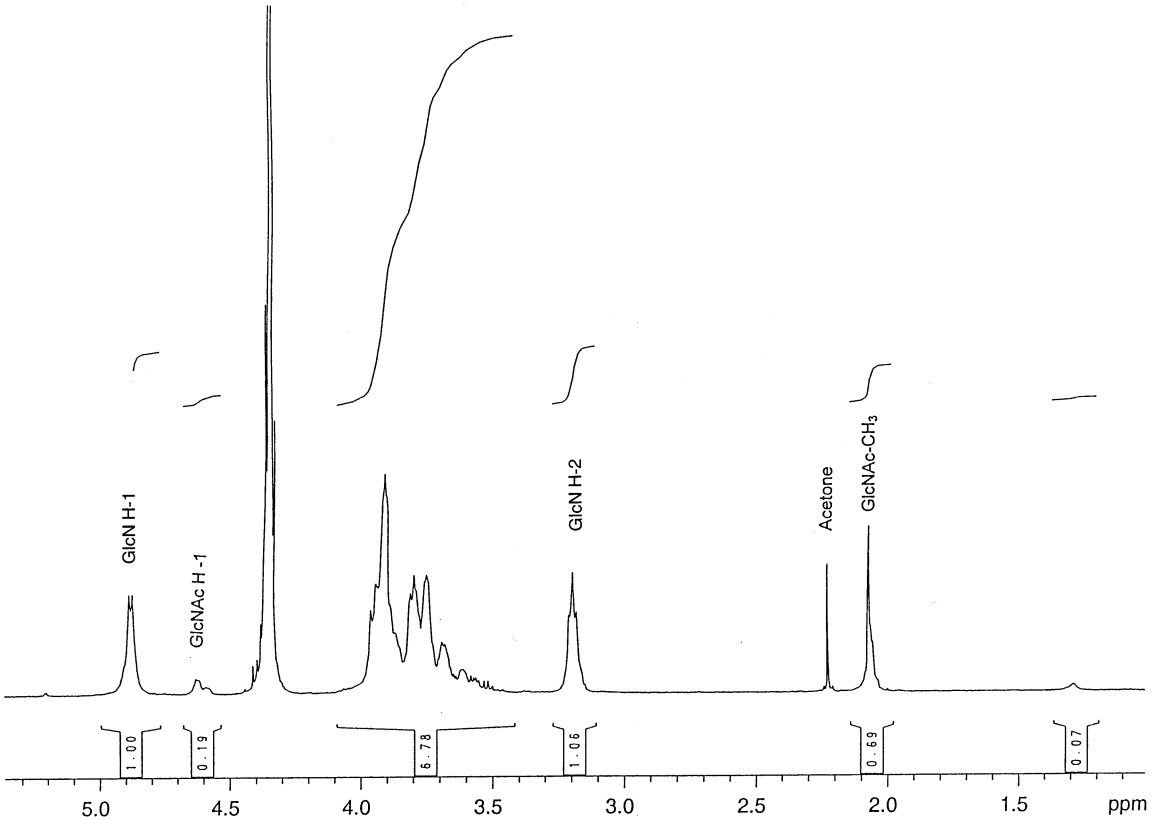


Fig. 4. $^1$H NMR spectrum (600 MHz) of chitosan C3646, DCl salt (12.5 mM with respect to glucosaminyl residue) in $D_2O$, 65 °C. Acetone (internal standard) set to 2.225 ppm; GlcN H-1 and H-2 at 4.875 and 3.191 ppm, respectively; GlcNAc H-1 at 4.608 ppm; acetyl protons of GlcNAc at 2.068 ppm.

products by cation-exchange chromatography (spin column, 2 mL of AG50W-X8, $H^+$) before the analysis of acetic acid. The result for ovalbumin, a glycoprotein that contains on the average 2 mol of *N*-acetylglucosamine and 5 mol of mannose, indicated that 0.34 mol excess of the expected acetic acid (1.5 mol) was produced, taking into account that produced by the mannose and the acetylation of the protein moiety, as N-terminal N-acetylated glycine [18,19]. This might suggest some O-acetylation of the protein moiety or acetic acid may be formed from the decomposition of the protein, which was not studied further, except to note that treatment of amino acid standard mixture containing 18 components did not produce acetic acid.

Occasionally the determination by Dionex of neutral sugars, such as Gal and Man, in partially purified chitin is complicated by partial hydrolyzates of the chitin containing glucosamine oligomers, which are removed $>90\%$ by cation-exchange chromatography as described in the ovalbumin before analysis.

## 4. Discussion

It is well known that sugars decompose in acidic conditions [20], the rate depending upon the pH, temperature and monosaccharide in question. This is recognized in the quantitative analysis of heteropolysaccharides, where the rate of hydrolysis of the glycosidic bonds and the rate of decomposition of the monosaccharides released determine the optimum conditions required to give the maximum yield of each monosaccharide.

The same considerations apply when one analyzes for substituents, such as acetate, pyruvate or sialic acid groups. Fortunately, the latter two substituents are released under such mild conditions (5–50 mM $H_2SO_4$, 80–120 °C, 1 h) that few glycosidic bonds and O-acetyl groups are cleaved and the release of monosaccharide components is minimal. Acetamido linkages are more resistant and require strong acid at elevated temperatures, under which conditions most monosaccharides significantly decompose, including to a

small extent the hexosamines, which are the usual sugars carrying the acetyl groups. However, in complex biopolymers, *O*-acetyl groups are not uncommon and N-acetylation of amino acid residues in proteins and glycoproteins is also possible.

One of the more recent methodologies for the chemical determination of the degree of acetylation of chitin and chitosans [11] does not address the applicability of the method to heteropolysaccharides.

The significant advantage of using HCl for hydrolysis of acetamido groups is the ability to use the same hydrolyzate directly for hexosamine determination. Hydrolysis with sulfuric acid complicates this analysis and must be removed prior to chromatographic analysis for hexosamine.

It is clear that the methods of NMR and acidic hydrolysis of carbohydrate-containing materials give comparable results for the degree of acetylation. Complications arise, however, when the sample is not soluble in simple solvents, or mixtures (impure samples) give interfering signals in the NMR, or non-carbohydrate components hide the true value of acetylation. Some correction for these difficulties is obtained for hexosamine-containing biopolymers by also analyzing for hexosamine in the same hydrolyzate, since this type of sugar most frequently carries the acetyl group. It is critical, however, that the acetic acid be clearly separated from closely associated products by whatever means the final determination is made.

The determination of both acetate and hexosamine, particularly from purified chitin or chitosans, can give the degree of acetylation without other fundamental analyses, such as elemental analysis, moisture, inorganic or organic materials.

The application of the method to crude chitins and fungal mycelia, which are frequently contaminated with sugars, nutrient media components and glycans, presents problems due to secondary reactions that often lead to lower recovery of hexosamine [21] and higher yields of acidic products. In such situations it is advantageous to perform preliminary purification by thorough washing of the material with water or aqueous ethanol and even dilute alkali if these do not remove materials of interest.

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