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# Quantitative determination of chitosans by ninhydrin

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**Abstract**

A simple method for quantitative analysis of chitosan based on the ninhydrin reaction is described. Only the 2-amino-2-deoxy-β-D-glucopyranose (GlcN) units of chitosans were found to form coloured products with ninhydrin. This reaction of chitosans with ninhydrin was sensitive and reproducible. Linear calibration curves were obtained in the concentration range of 10–120 mg/l, depending on the chemical composition of the chitosans. The amount of colour produced per GlcN unit decreased with decreasing fraction of acetylated units ($F_A$). This imperfect stoichiometry was studied in more detail by comparing a series of β-(1 → 4)-linked GlcN oligomers. Dimer, trimer and tetramer produced 82, 67 and 61% of the colour relative to that of the monomer, respectively. The yield of almost fully deacetylated chitosan ($F_A$ = 0.01) with different molecular weights reached a constant value at 40% when the degree of polymerization increased to more than 10. Differences in reaction rates were also observed. While monomer, dimer and chitosan with $F_A = 0.6$ reacted rapidly, other chitosans did not fully complete the reaction within 30 min. Due to this behaviour, the reaction of chitosans with ninhydrin might be used for quantitative analysis only when a reliable calibration against a reference of a similar $F_A$ is available. 

*Keywords:* Quantitative analysis; Chitosan; Ninhydrin reaction; Stoichiometry; Acetylated units

## 1. Introduction

Chitosans are linear binary heteropolysaccharides composed of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN). The amount of GlcNAc, expressed as the fraction of acetylated units ($F_A$), and the molecular weight average largely determine chitosan properties, and thereby also applications. Chitosans are usually polydisperse and the molecular weight of commercial products usually range from 10 to $10^3$ kDa.

Although research activities dealing with chitosans are numerous, a generally accepted simple method for direct quantitative analysis is lacking. Most quantitative analysis of polysaccharides entails total hydrolysis to monosaccharides followed by subsequent characterisation of the monomers. The glycosidic linkages of chitosan are resistant to acid hydrolysis due to the presence of positively charged amino groups (Vårum and Smidsrød, 1998), and even the drastic alkaline conditions used for *N*-deacetylation of chitin to chitosan did not necessarily lead to significant depolymerization (Ottøy et al., 1995). This is also one of the reasons why methods for total carbohydrate analysis, such as the anthrone or phenol–sulphuric acid methods (Daniels et al., 1994), are difficult to apply. Chitosanases may be used for depolymerization of chitosan (Izume and Ohtakara, 1987), but availability and costs limit the use of enzymes. The bound chitosan can be quantified by the adsorption of anionic dyes (Roberts and Taylor, 1989). In specific experimental cases, a fluorescent or radioactive label may be used to detect and quantify the labelled chitosan.

A general method for quantitative analysis should allow quantification of chitosan not only in solution, but preferably also when bound or adsorbed to surfaces (Prochazkova et al., 1997). In addition, the procedure should be rapid, inexpensive and suitable for routine analysis of many samples.

The reaction of ninhydrin with a primary amino group will form a coloured reaction product, diketohydrindylidene–diketohydrindamine, also called Ruhemann's purple. This reaction has been known and studied for years and is extensively used for amino acid analysis. In their pioneering work, Moore and Stein (1948) developed a stable ninhydrin reagent, improved reproducibility of the method and established the reaction conditions for amino acid analysis. Later, the replacement of the toxic Methyl Cellosolve with dimethylsulfoxide led to considerable improvement in safety and colour stability (Moore, 1968). It was often observed that the amount of colour formed from a given

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Table 1
Characteristic properties of the chitosan samples applied

| Fraction of acetylated units ($F_A$) | Intrinsic viscosity, [$\eta$ (ml/g)] | Molecular weight, $M_n$ (g/mol) |
|---|---|---|
| 0.01 | 670 | 203 000 |
| 0.09 | 210 | 33 000 |
| 0.16 | 780 | 194 000 |
| 0.35 | 960 | 239 000 |
| 0.60 | 826 | 183 000 |

Fraction of acetylated units ($F_A$) was determined by $^1$H NMR spectroscopy (Vårum et al., 1991); intrinsic viscosities were measured according to Draget et al. (1992).

compound, although reproducible, did not correspond to the expected theoretical yield. Polylysine, bovine serum albumin, keratin and other proteins reacted with ninhydrin with yields of 60–70% (Friedman and Williams, 1973). Strikingly different yields were observed for some diamino acids differing only in the length of the methylene backbone (Friedman and Williams, 1974). Large differences in the reaction rate of two diastereoisomeric dipeptides reported by Yanari (1955) remain unexplained. The possible reasons for the apparent nonideal stoichiometry of the ninhydrin reaction may include slow formation of Ruhemann's purple, side reactions, colour instability as well as interfering colour (Friedman and Williams, 1974). Furthermore, a pH dependence of the yield in the ninhydrin reaction with amino acids has been reported (Lamothe and McCormick, 1972). Due to these obstacles, the ninhydrin reaction has received much less attention during the last 15 years and its application was limited mostly to quantitative analysis of amino acid.

Curotto and Aros (1993) published a study describing the use of the ninhydrin reaction for quantitative determination of chitosan as well as the percentage of free amino groups. The latter was estimated from the degree of reactivity compared to the monosaccharide GlcN assuming a stoichiometric reaction. However, this has not been proven to be the case.

Preliminary studies in our laboratory showed low colour development when the ninhydrin assay was adapted to quantify different chitosans both in solution and bound to glass. A study was therefore undertaken to obtain the information needed to establish the ninhydrin reaction as a reliable method for quantitative determination of chitosan. The dominant factors for the incomplete reaction had to be identified. In this paper, we discuss the differences in the reactivity of chitosans with ninhydrin in relation to their chemical compositions ($F_A$) as well as their molecular weights.

## 2. Experimental

### 2.1. Materials

Chitosan samples were provided by Pronova Biopolymers (Oslo, Norway) or were prepared by homogeneous *N*-deacetylation of shrimp chitin. All of them were converted into water-soluble hydrochloride salts (chitosan–HCl). The fractions of acetylated units ($F_A$) were determined by $^1$H NMR spectroscopy (Vårum et al., 1991). The number average molecular weights were calculated from Mark–Houwink–Sakurada equations (Anthonsen et al., 1993) on the basis of measured intrinsic viscosities (Draget et al., 1992). Table 1 shows the chemical compositions and the molecular weights of the chitosans used. 2-amino-2-deoxy-β-D-glucopyranose-hydrochloride (GlcN) was purchased from Sigma. β-(1 → 4)-linked oligomers of GlcN (dimer, trimer, tetramer) were purchased from Seikagaku Corp. (Tokyo, Japan), all in the hydrochloride form. Their purity was verified by GPC elution curves in our laboratory. All samples were dried in a vacuum desiccator over $P_2O_5$.

Depolymerization of chitosan with nitrous acid was performed as previously described (Allan and Peyron, 1989). After partial degradation, the degree of polymerization was determined from intrinsic viscosity measurements or by $^{13}$C NMR spectroscopy for the low-molecular weight chitosans ($DP_n < 30$). Ninhydrin was purchased from Merck and hydrindantin from Fluka.

### 2.2. Ninhydrin method

The ninhydrin reagent (Moore, 1968) was prepared as follows: 2 g ninhydrin and 0.3 g hydrindantin were dissolved in 75 ml dimethylsulfoxide (DMSO) while flushing with nitrogen, 25 ml of 4 M lithium acetate buffer, pH 5.2 (unless otherwise stated) was added (Moore, 1968), and the resulting dark red solution was further bubbled with nitrogen. The reagent was stored refrigerated in a dark bottle with dispenser. Unless stored under a nitrogen atmosphere, the reagent bleached within a week to a yellow colour in the presence of oxygen. However, the slight bleaching which occurred in the first 3 days of storage did not affect the reaction yield.

To 1 ml of chitosan solution in deionized water (in duplicate), 1 ml of the ninhydrin reagent was added from the dispenser bottle. The tubes were immediately capped, briefly shaken by hand, and heated in a covered boiling water bath for 30 min unless otherwise stated. The tubes were then cooled below 30°C in a cold water bath and the

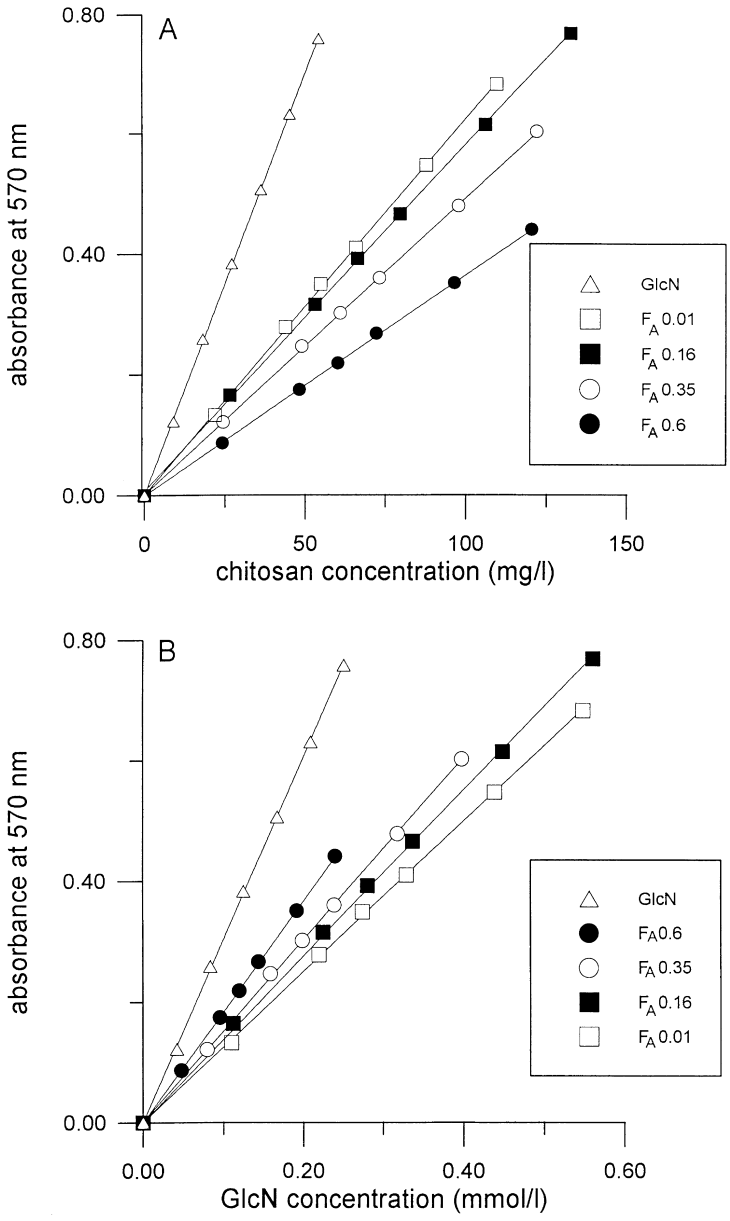


Fig. 1. Absorbance shown as a function of chitosan concentration (A) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) concentration (B) for the reaction of chitosans and monosaccharide GlcN with ninhydrin at pH 5.2 after 30 min reaction time. Reliability of regressions: $R^2 > 0.999$.

content diluted with 5 ml of 50% (v/v) ethanol/water. The solutions were then vigorously stirred on a Vortex mixer (15 s) to oxidise the excess of hydrindantin.

The absorbance at 570 nm was measured on a Medispec-III UV/VIS spectrophotometer, zero-set against a similarly treated blank of water. Absorption spectra were recorded on the same instrument.

## 3. Results

### *3.1. Reaction yield*

As the ninhydrin reaction is specific to primary amino groups, it is expected that GlcN would give a positive ninhydrin reaction, while GlcNAc should not. This was also confirmed in this study (data not shown). The colour yield, i.e. the amount of Ruhemann's purple formed per mol of amino group, obtained from the monosaccharide GlcN was approximately equivalent to that of L-leucine, often used as a reference standard in the ninhydrin reaction.

The plots of absorbance against concentration for four chitosans with different $F_A$ values gave straight lines as shown in Fig. 1A. The calibration curves were linear up to absorbance values of 0.8 and the detection limits were in the order of 10 mg/l, depending on chemical composition of the chitosan. From Fig. 1A it is also seen that the absorbance decreased with increasing $F_A$ values. In order to see if this

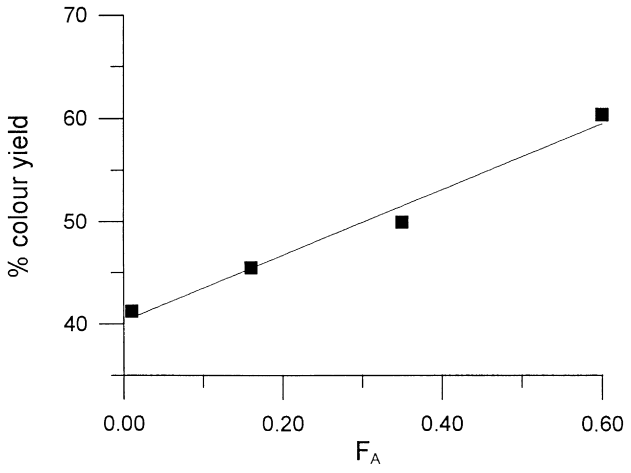


Fig. 2. Effect of fraction of acetylated units ($F_A$) of chitosan on the reactivity (% yield relatively to monomer) at pH 5.2 after 30 min reaction time. Reliability of regression: $R^2 = 0.982$.

effect is caused by differences in the content of GlcN in the chitosans, the absorbance was plotted against the calculated molar GlcN content corrected for the water loss of the glycosidic linkage, as shown in Fig. 1B. The differences in reactivity of chitosans are obvious: the higher the amount of free amino groups (GlcN units), the lower was the colour yield per mol of amino groups. Furthermore, the reactivity of all chitosans compared to the monosaccharide GlcN was low.

The fraction of acetylated units in our chitosans ranged from 0 to 0.6, and the amount of coloured reaction product formed in the reaction of chitosans with ninhydrin was expected to be directly proportional to its GlcN content, assuming that each GlcN unit can react with ninhydrin (stoichiometric reaction). However, for the almost fully deacetylated chitosan ($F_A = 0.01$), the colour yield reached only 41% of the possible theoretical yield, assuming that the monosaccharide GlcN gave the maximum obtainable colour yield assigned as 100%. Similarly, the colour yield of the chitosan with $F_A = 0.6$ was approximately 60%. As shown in Fig. 2, a linear relationship existed between $F_A$ of the chitosan and the degree of reactivity in the ninhydrin reaction. Such empirical calibration might be used to determine the $F_A$ of an unknown chitosan sample; however, the low slope and the high constant part of the linear expression make the method less accurate.

To study this imperfect stoichiometry in more detail, reactions of a series of β-(1 → 4)-linked glucosamine oligomers with ninhydrin were carried out, and the results are shown in Fig. 3. Also here, the amount of Ruhemann's purple formed per mol of the amino groups of oligomers was lower than that of the monomer. The reactivity compared to monomer clearly decreased with increasing degree of polymerization (DP) with 82, 67 and 61% yield of the dimer, trimer and tetramer, respectively.

To further examine the effect of the molecular weight on reactivity, almost fully deacetylated chitosan ($F_A = 0.01$) was degraded with nitrous acid into shorter fragments which were subjected to ninhydrin reaction. Samples obtained by $HNO_2$ degradation of chitosan were polydisperse with number average degrees of polymerization ($DP_n$) of 14, 25 and 50. As summarised in Fig. 4, there was a considerable difference in the reactivity of short GlcN oligomers (DP 2–4), while the molar colour yields of longer oligomers with $DP_n$ between 10 and 1000 remained constant (Fig. 4). When very short oligomers were prepared, the colour yield decreased due to a large portion of 2,5-anhydro-D-mannose formed at the reducing ends, which cannot react with ninhydrin (data not included).

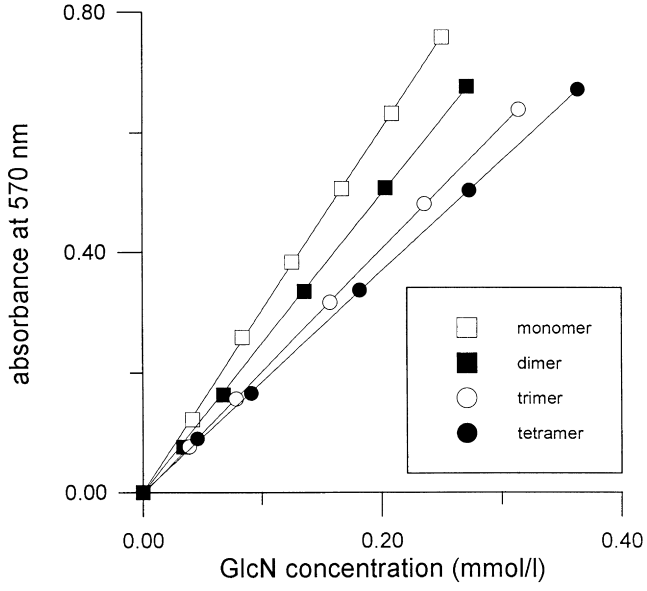


Fig. 3. Absorbance shown as a function of 2-amino-2-deoxy-β-D-glucopyranose (GlcN) concentration for reaction of monosaccharide GlcN and GlcN oligomers (dimer, trimer, tetramer) with ninhydrin at pH 5.2 after 30 min reaction time. Reliability of regressions: $R^2 > 0.999$.

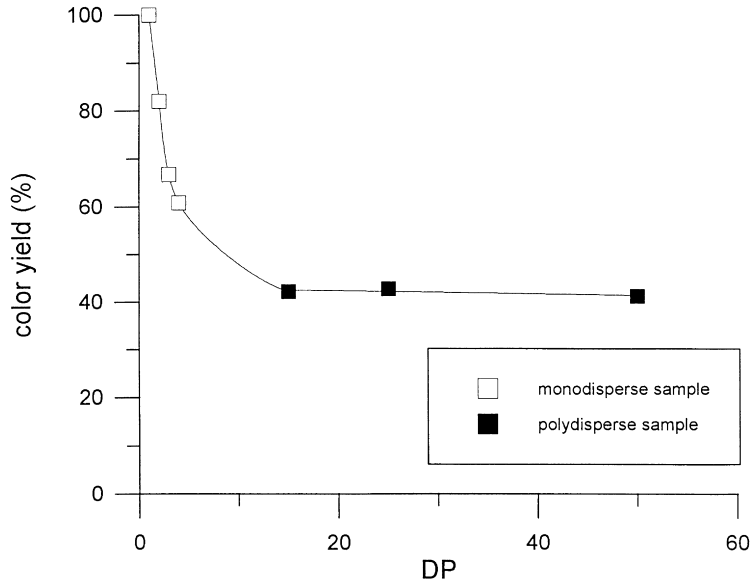


Fig. 4. Effect of degree of polymerization (DP) on colour yield (% relatively to monomer) at pH 5.2 after 30 min reaction time.

The absorbance spectra (325–750 nm) of the reaction mixture after ninhydrin reactions with GlcN, oligomers of GlcN and chitosan were identical, suggesting that the same reaction product was formed (spectra records not included). The absorption maxima at 403 and 570 nm are characteristic of diketohydrindylidene–diketohydrindamine (Ruhemann's purple).

### 3.2. Reaction kinetics

To determine whether the differences in the colour yields were a consequence of different reaction kinetics, the reaction time was varied from 10 to 40 min (see Fig. 5). The reactions of the monomer and the dimer were completed in less than 10 min and the colour yield remained stable. Similarly, the colour yields from tetramer and chitosan with $F_A = 0.6$ did not change between 20 and 40 min. However, the chitosans with $F_A = 0.01$ ($DP_n \sim 1000$ and $DP_n \sim 14$), did not fully complete the reaction within 40 min. Although most of the colour was formed during the first 10 min, a further slow increase in absorbance was observed (Fig. 5). A less evident slow increase in colour yield was also observed for chitosans with $F_A$ of 0.09 and 0.16 (data not shown). When the reaction time was extended to 120 min, the yields increased linearly up to 60 min, remaining constant after 90 min. The additional increase in colour yield after 30 min

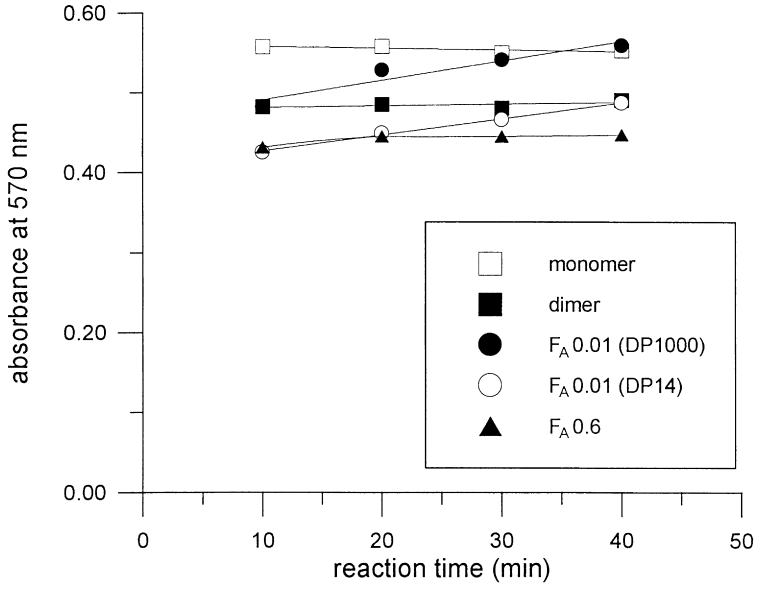


Fig. 5. Absorbance shown as a function of reaction time for reaction of monomer, dimer, chitosan $F_A = 0.01$ (DP $\sim$ 1000 and DP = 25) and chitosan $F_A = 0.6$ with ninhydrin at pH 5.2.

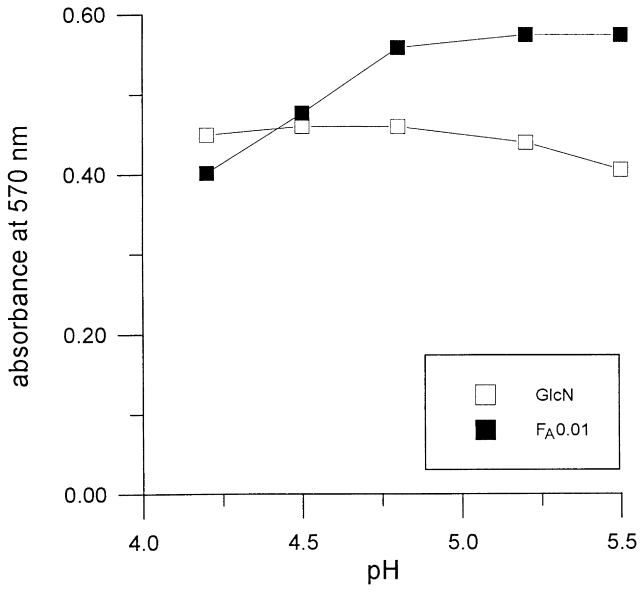


Fig. 6. Absorbance shown as a function of pH of lithium acetate buffer for reaction of 2-amino-2-deoxy-β-D-glucopyranose (GlcN) and chitosan $F_A = 0.01$ with ninhydrin after 30 min reaction time.

for chitosans with $F_A$ of 0.01 and 0.35 accounted for 15 and 8% of the total response. The lower the $F_A$ of the chitosan, the less increase was observed, indicating that the rate of the reaction of chitosans with ninhydrin was dependent on $F_A$.

### 3.3. pH optimum and colour stability

Another possible reason for the low colour yields of the chitosans compared to that of the monomer could be differences in pH optimum for the reaction. The ninhydrin reaction was carried out in lithium acetate buffer in the pH range of 4.2–5.5 (see Fig. 6). The chitosan with $F_A = 0.01$ gave highest colour yield at pH 5.2–5.5, while the pH optimum for GlcN was 4.5–4.8. This implies that, at pH 5.2, chosen for the standard assay, GlcN yielded about 96% of its maximum colour, while chitosan reacted at its optimal pH value.

Fading of colour with time was found to be very slow, after 4 h of storage in the dark more than 96% of colour was still present. No fading occurred during the first 60 min, even when exposed to indoors illumination as long as direct sunlight was avoided. After 1 h, decrease in absorbance was recorded, not exceeding 1% per hour.

### 3.4. Accuracy and reproducibility

All data points on the curves in Figs 1–6 represent a mean value of two duplicates. The reliability of regression curves in Figs 1 and 3, expressed as regression coefficient ($R^2$) was generally exceeding 0.999. The slopes of the plots for GlcN, oligomers and chitosans were reproduced within ± 3%.

## 4. Discussion

### 4.1. Reaction yield and kinetics

The reaction of chitosans with ninhydrin was accurate, reproducible and sensitive. The assay was rapid and simple, and good results were obtained both when the method was applied to chitosans in solution and chitosans bound to the glass. However, several features appeared anomalous and deserve attention: (1) the colour yields per GlcN unit of chitosans were much lower than that of monomer and varied for different chitosans (Fig. 1B and Fig. 2); (2) the colour yields of GlcN oligomers depended on DP (Figs 3 and 4); (3) differences in the reaction rate of chitosans and oligomers were observed (Fig. 5); and (4) monosaccharide GlcN and chitosan had different pH optimum for the reaction (Fig. 6).

The observation that chitosan and GlcN oligomers did not react quantitatively with ninhydrin has not been reported before, but low colour yields is a well-known phenomenon in the ninhydrin reaction. For instance, the molar colour yields of amino acids and other compounds bearing amino groups have been reported to vary from 0.03 to 1.12, expressed relatively to leucine (Moore and Stein, 1948). Some explanations for such differences have been suggested by Friedman and Williams (1974), but have rarely been established in particular cases. One of the few exceptions are amino acids, where the pH optimum in the reaction increased with the $pK_2$ and when the assay was run at the optimum pH for each amino acid, the molar yields were equivalent (Lamothe and McCormick, 1972). As the ninhydrin reaction was developed mainly for the analysis of amino acids or proteins, data for other amino compounds are lacking.

As shown in Fig. 6, a low colour yield of chitosan was observed even at its pH optimum. The $pK_a$ value of GlcN is 7.7 and for chitosan a $pK_a$ of 6.6 has been reported, irrespective of $F_A$ and DP (Anthonsen and Smidsrød, 1995). At higher ionic strength, as in the ninhydrin reaction, the long-range electrostatic effects on chitosan are reduced and the $pK_a$ value increases (Anthonsen and Smidsrød, 1995), approaching that of monosaccharide GlcN. Despite higher $pK_a$, the pH optimum for GlcN was slightly lower compared to chitosan (Fig. 6) and the pH of the assay. It is evident that the relationship between $pK_a$ and pH optimum did not seem to follow the rule for amino acids, and it follows that the pH cannot be the reason for the low yields of chitosans compared to the monomer.

Another critical factor in the ninhydrin reaction is differences in the reaction rates, which can result in slower colour formation and thus incomplete reaction. It has been shown that the reaction rate of amino acids is influenced both by the nucleophilicity of the amino group and by sterical hindrance (Friedman and Sigel, 1966). The observed reaction rates decreased with increasing $pK_2$, indicating that the nucleophilic displacement of a hydroxy group of ninhydrin by the

nonprotonated amino group is a rate-determining step. The reaction rates of substituted, sterically hindered amino acids (amino group attached to tertiary carbon) were also a function of their p$K_2$ but were several-fold lower than unsubstituted.

The difference in the reaction rates of chitosans, monomer and dimer can be explained neither as a function of p$K_a$ nor steric hindrance. Chitosans with different $F_A$ values, having all the same p$K_a$, reacted at different rates (Fig. 5). On the other hand, monomer, dimer and chitosan with $F_A = 0.6$, possibly possessing slightly different p$K_a$, reacted fast but with low yields for the latter two (Fig. 5). If some of the amino groups were sterically hindered, one would expect the reaction rate to decrease for high-molecular weight samples. Since the reaction rates of chitosan ($F_A = 0.01$) with DP $\sim$ 1000 and DP = 14 were equal, the molecular weight of samples did not influence the rates. Moreover, the high reaction rate of chitosan $F_A = 0.6$ shows that even the presence of bulky *N*-acetyl group of GlcNAc units did not hinder the accessibility of free amino groups of GlcN.

As a consequence of different reaction rates, incomplete colour formation within 30 min standard reaction time may be of importance for some chitosans. On the other hand, we have shown (Fig. 5) that the dimer reacted as fast as the monomer but with only 82% yield. Differences in the reaction kinetics of chitosans could therefore only partly explain their different colour yields, as shown in Fig. 2. If these were caused by insufficient reaction times, extended reaction times would eventually result in the same molar yield for all chitosans, irrespective of $F_A$ values. This was not observed despite the extension of the reaction time to 120 min (data not shown). Prolonged reaction times are not feasible as the Ruhemann's purple formed can be destroyed under the actual reaction conditions of 100°C and pH 5.2 (Friedman and Williams, 1974). Moreover, as heating was prolonged, significant darkening of the blank reference and side reactions in all samples occurred, indicated by a decrease in absorbance ratio at 570/470 nm, corresponding to maximum/minimum of Ruhemann's purple.

The most probable reason for the non-quantitative reaction is that some amino groups participate in a side reaction either with ninhydrin or any of the intermediates. The nature of possible side reactions is not known. The presence of several GlcN units at adjacent sites in the chain had a profound effect on the yield (Fig. 4). Possibly, some cyclic products between ninhydrin and another amino group located close to the reacting amino group may be formed. This has been postulated for several diamino acids and cystein (Friedman and Williams, 1974). If such side reactions were reversible, a slow increase in colour yield could be observed long after most of the colour was formed, depending on the stability of the cyclized product. This could explain both the $F_A$ dependence of the yield of chitosans, the low yields of GlcN oligomers and differences in the reaction rates. As the probability of neighbouring GlcN increases inversely with $F_A$, the chance for side reactions of closely located amino groups also increases, lowering the yield and the reaction rate. We have no direct evidence for such reactions, but these effects could be determined if short oligomers (e.g. DP = 2–6) consisting of GlcN and GlcNAc in different amounts were available.

### *4.2. Practical considerations and applications*

Although several features of the ninhydrin reaction seemed to be anomalous, this has only limited implications for the practical use of the method for chitosan quantification. Since chitosan samples usually range from 10 to $10^3$ kDa, the colour yield is independent on the molecular weight of the sample. The only parameter of importance remains $F_A$; however, even here the knowledge of an accurate value is not required provided a calibration curve of the unknown sample is used.

## 5. Conclusions

The reaction of chitosans with ninhydrin was rapid, sensitive and reproducible, although non-stoichiometric. The linear calibration curves were obtained in the concentration range of 10–120 mg/l, depending on the $F_A$ of the chitosan. The method may be applied for quantitative determination of chitosans, provided attention is paid to several aspects. Firstly, as the yield depends strictly upon $F_A$, calibration curves have to be made for each chitosan type analysed. Secondly, due to the non-quantitative reaction, the $F_A$ cannot be determined just by comparison to monosaccharide GlcN. The fraction of acetylated units can only be estimated when calibration against samples with known $F_A$ values are available. The molecular weight of sample did not affect the yield unless short fragments with DP $<$ 10 were analysed. The reasons for these non-stoichiometric reactions remain to be elucidated.

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