Available online at www.sciencedirect.com

SCIENCE DIRECT®

Carbohydrate Polymers 61 (2005) 29–38

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Determination of chitosan in wood and water samples by acidic hydrolysis and liquid chromatography with online fluorescence derivatization

M. Eikenes[a,*], M. Fongen[a], L. Roed[b], Y. Stenstrøm[c]

[a]Department of Wood Technology, Norwegian Forest Research Institute, Høgskoleveien 8, N-1432 Aas, Norway
[b]Amersham Health AS, P.O. Box 4220 Nydalen, N-0401 Oslo, Norway
[c]Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway, P.O. Box 5003, N-1432 Aas, Norway

Received 1 September 2004; revised 7 February 2005; accepted 10 February 2005
Available online 1 April 2005

Abstract

In recent years chitosans have been investigated as a natural chemical for wood preservation against fungal decay, and chitosan in aqueous solutions has been used in impregnation studies. To evaluate the retention of chitosan after an impregnation process and to evaluate the fixation of chitosan in wood a method for determination of chitosan in wood and water samples has been developed based on acidic hydrolysis of chitosan to glucosamine followed by online derivatization by *o*-phthalaldehyde, chromatographic separation and fluorescent detection. For wood samples the method was linear up to 45 mg g^{-1} chitosan in wood and had a recovery of 86%. The yield of chitosan in water was 87% at 1% (w/v) concentration.

Keywords: Acidic hydrolysis; Chitosan; Glucosamine; Quantification; Wood preservation

1. Introduction

Chitosan is a (1-4)-linked hetero polysaccharide consisting of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose derived from chitin [(1-4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose]. Chitin is mainly processed from crustacean shells by a two step process: (1) treatment with mineral acid to remove lime, and (2) alkaline hydrolysis to remove proteins and de-acetylate the amide function. In addition chitosan is found in squid pens, insects and fungi. The rate of conversion from chitin to chitosan is described by the fraction of acetylated monomer [F_A], which may influence the solubility and conformation of the polymer.

Chitosans have been investigated for a wide spectrum of usages: photography, cosmetics, artificial skin, ophthalmology, water engineering to capture metals from waste-water and for colour removal from textile mill effluents, paper finishing, solid-state batteries, drug-delivery systems, as cell stimulation material, antibacterial agents, blood anticoagulants and as fat trappers (Kumar, 2000 and references therein). Furthermore, several reports on the use of chitosans as wood preservatives against wood decaying fungi have been made (Frederiksen, 2001; Alfredsen, Eikenes, Solheim, & Militz, 2004; Eikenes, Alfredsen, Christensen, Militz, & Solheim, in press). In addition, the use of chitosans to enhance the efficacy or to reduce the leachability of other wood preservatives has been investigated (Kobayashi & Furukawa, 1995a,b; Lee, Furukawa, & Sakuno, 1992, 1993).

Several techniques have been used to determine chitosan. C/N analysis could be utilised for estimation of the mass of chitosan in wood, but biased results can be expected when fixation of chitosan in the wood is provided by nitrogen-containing compounds (Mikalsen, Ørstavik, Falck, & Steinstø, 2001).

Chitosans can be degraded to the glucosamine monomer by hydrolysis, and there are several reports on the determination of glucosamine by chromatographic, colorimetric and fluorimetric techniques, or a combination of these. Tsuji, Kinoshita and Hoshino (1969a,b) determined hexosamines (e.g. glucosamine) in aqueous solutions by colorimetric determination after an acidic hydrolysis. Roseman and Daffner (1956) determined the concentration

---

* Corresponding author. Tel.: +47 6494 9170; fax: +47 6494 2980.
E-mail address: morten.eikenes@skogforsk.no (M. Eikenes).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2005.02.006

of glucosamine by acetylating followed by photometric detection of *N*-acetyl glucosamine. Hagen (1993) reported an HPLC method for quantification of muramic acid and glucosamine from bacterial cell walls with pre-column derivatization with phenyl-isothiocyanate and UV detection.

Recently, Prochazkova, Vårum and Østgaard (1999) reported a colorimetric method for quantification of chitosans in aqueous solutions based on reaction with ninhydrine. This method did not involve any pre-treatment (i.e. hydrolysis) of the solution, but the method was highly dependent on the fraction of acetylation (F_A) of the chitosan in question.

In the present work a selective and sensitive method for the determination of chitosan was developed using acidic hydrolysis and online derivatization with *o*-phthalaldehyde in a liquid chromatographic system with fluorescent detection. The method was optimized and evaluated for wooden and aqueous samples, and the use of the method was demonstrated.

2. Experimental

2.1. Wood and chitosan materials

2.1.1. Preparation of chitosan for the experiments

Industrial chitosan flakes (110 g) were dissolved in 3.8 L of deionised water by adjusting the pH to 5.5 with acetic acid. After the pH was stabilized at 5.5 (i.e. all chitosan was dissolved), 10 mL of 30% hydrogen peroxide in 200 mL deionised water was added slowly during vigorous stirring. The reaction solution was then heated to 50 °C for 1 h. After the reaction mixture was cooled down to room temperature, the pH was adjusted to 10 by adding an aqueous solution of sodium hydroxide. Then 8 L methanol (p.a. quality) was added to aid precipitation of chitosan. The precipitated chitosan solution was then centrifuged. The precipitated chitosan was washed several times with methanol and water (2:1) until the pH was neutral, and then washed one time with deionised water and stored at −20 °C until it was lyophilized.

2.1.2. Preparation of impregnated and non-impregnated wood for the experiments

Wood samples of Scots pine (*Pinus sylvestris* L.) sapwood (10×5×30 mm) were placed in a reaction vessel and covered by 10 mm glass spheres. A 1% (w/v) solution of chitosan dissolved in deionised water by adjusting the pH to 5.5 with acetic acid was then added. The wood samples were then impregnated with the chitosan solution by evacuating the vessel to 40 mbar for 30 min and applying air pressure of 8 bar for 2 h. The samples were then wiped with a paper tissue before the weight was recorded for control of uptake. The samples were then allowed to dry at the bench. The wood samples were then ground to fine powder in a Retch mill, and the combined powder was then homogenized.

2.1.3. Characterization of chitosan

The chemical compositions of the chitosan was determined by potentiometric titration: chitosan (200 mg) was dissolved in 20 mL 0.1 mol L^{-1} hydrochloric acid overnight and then transferred to the titration vessel by 30 mL deionised water The chitosan solutions ($n=3$) were titrated on a Radiometer autotitrator with a 0.100 mol L^{-1} sodium hydroxide solution, and the amount of sodium hydroxide used between the two endpoints was recorded. The results from the titration were confirmed by ^{1}H-NMR spectroscopy with a Varian Gemini 300 NMR instrument (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991).

The average molecular weight of the chitosan was determined by gel filtration. The sample was dissolved in the solvent of the chromatographic system (described below) at approximately 0.1% (w/v) and filtered before injection (0.45 μm).

An Agilent 1100 series chromatographic system equipped with an HP 35900E interface with a Merck RI-71 Differential Refractometer was used for the gel filtration system. A series coupled TosoHaas TSKgel PWH Guard-column (7.5 mm ID×75 mm cm), TSKgel G5000 PWXL (7.8 mm ID×300 mm) and TSKgel G3000 PWXL (7.8 mm ID×300 mm) were eluted with a 0.4 mol L^{-1} ammonium acetate/acetic acid buffer system at pH 4.5 at a flow rate of 0.45 mL min^{-1} at 50 °C. The chromatographic system was calibrated with 13 different Pullulan standards ranging from 1000 Da to 4900 kDa with a third order polynomial fitted standard curve. The elugrams were analysed with Agilent GPC Data Analysis Software (Rev. A.02.01).

2.2. Method description

2.2.1. Hydrolysis of chitosan in wood samples

Ground wood powder (100 mg) was added to 2.0 mL centrifuge tubes with screw caps and rubber sealing. Then 1.5 mL of an aqueous solution of 6.0 mol L^{-1} hydrochloric acid and 2 mmol L^{-1} homocysteic acid (internal standard) were added. The samples were agitated on a whirl mixer and stored at ambient temperature overnight. The samples were then agitated again and heated to 100 °C for 48 h on a heating block placed in a fume cabinet. For the first 8 h the samples were agitated every second hour. The samples were agitated again after 48 h and stored at 4 °C until the samples were diluted 500 times by volume with deionised water and analysed for the content of glucosamine.

2.2.2. Hydrolysis of chitosan in water samples

Water samples containing chitosan (0.500 mL) were added to 2.0 mL centrifuge tubes with screw caps and rubber sealing together with 1.0 mL of an aqueous solution containing 9 mol L^{-1} hydrochloric acid and homocysteic acid as internal standard. The concentration of the internal standard was dependent on the dilution factor for HPLC analysis, and was chosen so that the diluted sample had a concentration of internal standard of 4 μmol L^{-1}. The water samples were then agitated by whirl mixer and stored at ambient temperature overnight. The samples were then mixed well again and heated to 100 °C on a heating block placed in a fume cabinet for 24 h. The solutions were then mixed again and stored at 4 °C until diluted and analysed for their content of glucosamine.

If the dilution was 20 times or less, an aqueous solution of 0.25 mol L^{-1} sodium hydroxide was used to dilute the samples.

2.2.3. Preparation of o-phthalaldehyde (OPA) solution

To a 50 mL volumetric flask 270 mg *o*-phthalaldehyde (OPA) was dissolved in 5 mL methanol. Then 200 μL 2-mercaptoethanol was added and diluted to the final volume with 0.4 mol L^{-1} borate buffer at pH 9.5. The solution was then filtered (0.45 μm, Non-Sterile Acrodisc, Gelman Sciences), transferred to 2 mL dark coloured HPLC vials purged with Argon. The vials were then immediately purged with argon and sealed with snap caps with rubber septa. The solutions were stored in the dark at 4 °C.

2.2.4. Preparation of eluent for HPLC

To 1 L volumetric flask 2.05 g sodium acetate (0.025 mol) and 5.25 g citric acid monohydrate (0.025 mol) were added and dissolved in 900 mL deionised water. The pH was adjusted to 5.3 with 50% (w/v) sodium hydroxide before the solution was diluted to the final volume and filtered (0.45 μm).

The eluent for the HPLC was prepared by mixing seven volumetric units of the buffer solution with three volumetric units of methanol (HPLC quality). The eluent was degassed by purging with helium ($>99.996\%$) for 5 min.

2.2.5. HPLC method

An Agilent 1100 series liquid chromatograph (Agilent technologies, Palo Alto, CA, USA) equipped with a Shimadzu RF-551 fluorescence detector (Shimadzu, Duisburg, Germany) was used for separation at 50 °C on a Zorbax Eclipse XDB-C8 (4.6×75 mm, 3.5 μm particle size, Agilent technologies) with an analytical guard column Eclipse XDB-C8 (4.6×12.5 mm, Agilent technologies). The eluent (see above) was continuously purged with helium, and the flow was set to 2.0 mL min^{-1}.

Prior to injection the compounds were derivatized automatically by the Agilent 1100 series auto injector/sampler (Agilent technologies) by mixing 5 μL of sample with 10 μL of OPA derivatization agent just prior to injection, and the product was detected after chromatographic separation using $\lambda_{\text{excitation}}=340$ nm and $\lambda_{\text{emission}}=445$ nm. Peak height was used for quantification by means of an internal standard (4 μmol L^{-1} homocysteic acid) and a seven point calibration with glucosamine ranging from 0 to 4 μmol L^{-1}. The standard equation was obtained by a linear regression of the ratio of the height of the internal standard and the glucosamine peaks versus the ratio of the amount of internal standard and glucosamine in the standard samples. The regression coefficient was typically better than $r=0.999$.

2.2.6. Stability of OPA during repeated injections of a control sample

The chromatographic system was set up to inject a control sample containing 4 mmol L^{-1} internal standard (homocysteic acid) and 4 mmol L^{-1} glucosamine every 4 min for 10 h (150 injections). Every ten injections were made from each of 15 separate vials containing uncontaminated control solution (as above).

2.3. Optimization of hydrolysis

2.3.1. Hydrolysis factor screening

To perform a screening for significance of factors believed to have influence on the yield in the hydrolysis of chitosan in wood a reduced factorial design with eight experiments (2^{6-3}) and two centre points were set up using JMP statistical software (version 5.0.1a). Each experiment was replicated twice ($n=3$). The 10 different experiments ($n=3$) were performed with the parameters given in Table 1. The general procedure was: wood powder containing 1% (w/w) chitosan was added to a 2.0 mL centrifuge tube with screw cap. Then 1.5 mL of

Table 1
Factors and levels of factors in the 2^{6-3} reduced factorial design for screening of the effect of the parameters in acidic hydrolysis of chitosan in wood samples

Experiment number	Design pattern	Hydrolysis time (h)	Hydrolysis temperature (°C)	HCl concentration (mol L^{-1})	Amount wood sample (mg)	Agitation	Pre-storage
1	− − − − + +	4	70	1	25	Yes	Yes
2	− − + + − −	4	70	6	100	No	No
3	− + − + − +	4	100	1	100	No	Yes
4	− + + − + −	4	100	6	25	Yes	No
C1	000011	12.5	85	3.5	62.5	No	No
C2	000012	12.5	85	3.5	62.5	No	Yes
5	+ − − + + −	21	70	1	100	Yes	No
6	+ − + − − +	21	70	6	25	No	Yes
7	+ + − − − −	21	100	1	25	No	No
8	+ + + + + +	21	100	6	100	Yes	Yes

hydrochloric acid (concentrations in Table 1) with 2 mmol L^{-1} homocysteic acid as internal standard was added. The samples were mixed on a whirl mixer, and then either stored overnight at ambient temperature and then heated to 100 °C or heated directly after mixing. The samples were mixed immediately before heating, and after 1, 2 and 3 h. The samples that were heated for 21 h were agitated again after 19 h. The samples were stored at 4 °C until HPLC analysis after dilution 1:500 with deionised water.

2.3.2. Hydrolysis temperature, time and hydrolysis acid

Further optimization of the method was performed by analysing the yield of glucosamine after acidic hydrolysis of 100 mg wood powder containing chitosan. Three aquatic hydrolysis systems were used: 6 mol L^{-1} hydrochloric acid at 100 °C and 6 mol L^{-1} sulphuric acid at 100 and 120 °C. For each hydrolysis system three hydrolysis times were tested; 8, 24 and 48 h, in total nine experiments which were replicated twice ($n=3$).

To each 2.0 mL centrifuge tube with screw cap 100 mg wood powder containing 1% (w/w) chitosan were added. Then 1.5 mL of the 6 mol L^{-1} acid were added with 2 mmol L^{-1} homocysteic acid as internal standard, agitated by whirl mixer and stored at ambient temperature overnight. The samples were agitated before being placed on the heating block. The samples were agitated every second hour for 8 h, and once at the end of the heating. The samples were stored at 4 °C until they were filtered, diluted 500 times by volume with deionised water and analysed for their content of glucosamine by HPLC.

2.3.3. Wood samples: hydrolysis time

To each of 45 wood samples with 1% (w/w) chitosan in 2.0 mL centrifuge tubes were added 1.5 mL 6 mol L^{-1} hydrochloric acid with 2 mmol L^{-1} homocysteic acid as internal standard. The samples were agitated by whirl mixer and stored at ambient temperature overnight. The samples were agitated again and heated to 100 °C on a heating block. The samples were agitated every 2 h for 8 h, except for those samples heated for 4 h only. Five samples were taken out of the heating block after 4, 8, 16, 24, 32, 36, 48, 56 and 72 h ($n=5$). The samples were agitated before storage at 4 °C until further processing. The samples were filtered and diluted with deionised water 500 times by volume and analysed by HPLC.

2.3.4. Water samples: hydrolysis time

A 1% solution (w/v) of chitosan was prepared by dissolving 2.05 g chitosan (97.2% dry weight) in a 200 mL volumetric flask with deionised water and acetate/acetic acid to buffer the solution to pH 4.6, and then diluted to the final volume with deionised water. In a 200 mL volumetric flask 10 mL of this solution of chitosan were added together with 0.218 g homocysteic acid and diluted to the final volume with deionised water. To each 2.0 mL centrifuge tube with screw cap, 500 μL of this solution were added together with 1.0 mL 9 mol L^{-1} hydrochloric acid. The samples were then hydrolysed at 100 °C in a heating block for 4, 8, 16, 24, 32, 36, 48, 56 and 72 h. For each sampling time four replicates were used ($n=5$), giving a total of 45 samples in the experiment. The hydrolysed samples were then stored at 4 °C until analysed for glucosamine by HPLC.

2.4. Evaluation of method

2.4.1. Recovery of chitosan

The recovery of chitosan in chitosan impregnated wood samples (100 mg, $n=19$) and milled wood samples (100 mg, $n=18$), spiked with increasing amount of 1% (w/v) chitosan solution, was analysed. Untreated wood samples were used as control. The samples were allowed to dry at ambient temperature before hydrolysis and quantification of glucosamine as described above. The hydrolysis conditions were as described above: 6 mol L^{-1} hydrochloric acid, with pre-storage overnight and heating at 100 °C for 48 h with initial agitation four times every second hour. The hydrolysed samples were then stored at 4 °C until analysed for glucosamine by HPLC.

2.5. Use of method

2.5.1. Leaching of chitosan from impregnated wood samples

Wood samples impregnated with chitosan were subjected to leaching by a modified EN84 (1997). The wood samples of 5×10×30 mm were placed in separate glass test tubes of 16×150 mm. One glass sphere (10 mm) was then placed on top of the wood sample and 7.5 mL deionised water were added to each test tube. The wood samples were then impregnated with water by applying 40 mbar vacuum for 30 min and subsequently stored at ambient temperature and pressure overnight. The wood samples were then taken out of the test tubes and placed in new test tubes with 7.5 mL deionised water. This was repeated eight times. All water samples were stored at 4 °C until further processing.

2.5.2. Quantification of chitin in fungal infected wood

Forty untreated control samples of Scots pine (5×10×30 mm) from a decay test with the basidiomycete *Poria placenta* (Eikenes, Alfredsen, & Solheim, 2004) were ground to fine powder and analysed for chitin by the method described above. The relative mass loss (mass loss during decay divided by initial dry weight) ranged from 20.6 to 57.9% with an average of 39.6%. Nine untreated wood samples that had been placed on Petri dishes containing sterile growth medium, which were used for corrections of change in weight during the decay period, were also included.

2.6. Data handling and statistics

The supplied chromatography software (Chemstation, Agilent technologies) was used for determining peak height and calibration. JMP 5.0.1 (SAS Institute, Cary, NC, USA) was used for the generation of the experimental design, and multiple regression analysis were conducted. Calculations from glucosamine content to mass of chitosan were performed by using the *Average Mass of Monomer*

$$\mathrm{AMM} = F_{\mathrm{A}} \times \mathrm{MW}_{N\text{-Ac Glc}} + [1 - F_{\mathrm{A}}] \times \mathrm{MW}_{\mathrm{Glc}}$$

where F_A is fraction of acetylation (i.e. *N*-acetyl glucosamine), $\mathrm{MW}_{N\text{-Ac Glc}}$ is the molecular mass of *N*-acetyl glucosamine minus one unit of water (221.21–18.02 = 203.19) and $\mathrm{MW}_{\mathrm{Glc}}$ is the molecular mass of glucosamine minus one unit of water (179.17-18.02 = 161.15). In our case where $F_A = 0.198$, AMM = 169.5.

Calculations for F_A by titration were performed by the following equation

$$F_{\mathrm{A}} = \frac{\mathrm{Glc}}{\mathrm{Glc} + \frac{C - (\mathrm{Glc} \times \mathrm{MW}_{\mathrm{Glc}})}{\mathrm{MW}_{N\text{-Ac Glc}}}}$$

where Glc is the molar amount of glucosamine units in the chitosan (which equals the amount of hydroxide used in the titration), and *C* is the net weight of chitosan (g).

3. Results and discussions

3.1. Chitosan characteristics

The average molecular weight (M_w) was determined to be 76 kDa (average of two runs) by gel filtration. The elution curves were quite broad with a polydispersity of 3.16 (M_w/M_n). The F_A was determined to be 0.198 ($n = 3$, RSD = 0.46%) by titration, which were confirmed by ^{1}H NMR spectroscopy ($F_A = 0.19$, $n = 3$, RSD = 10.5%).

3.2. Chromatographic considerations

A representative chromatogram is shown in Fig. 1. The peak from the product of the internal standard and the OPA reagent eluted at 0.55 min followed by the glucosamine main product at 1.1 min (Fig. 1). The peak at 1.8 min is probably the other anomer of the OPA-glucosamine product. A supposed by-product from the OPA reagent eluted at 2.7 min. Peak height was used for quantification. The total time between injections was 3 min, with a total throughput of 20 samples per hour. The injection and derivatization step took 1.5 min, so the injection cycle started 1.5 min before injection (during previous run). The response for both homocysteic acid and glucosamine was linear to just above 4 μmol L^{-1}. The correlation coefficient for the calibration curve was typically better than $r = 0.999$. A quality control sample was run after every 10 samples to ensure a valid and stable system.

Repeated injection of a quality control sample in the HPLC system showed a severe decrease of yield of glucosamine with increasing number of injections (results not shown, but similar to repeated control sample in Fig. 2). To further evaluate this result an experiment was carried out with injections of a separate quality control sample after every 9 injections of a repeated control sample (Fig. 2). The yield of glucosamine in the separate control samples showed no decrease with increasing number of injections. From these results it is clear that the samples are contaminated during the withdrawal of sample by the auto-sampler needle. To avoid problems with contamination all further analysis were performed only once from each sample vial. There is no evidence that the results are due to the fact that OPA solution is degraded over time.

To ensure good repeatability of the quantification of chitosan in the HPLC system the derivatization step was performed online using the autosampler of the chromatographic system. The total time of the operation of withdrawal of sample and derivatization solution was approximately 1.5 min. Zelles (1988) reported that the fluorescent yield of the *o*-phthalaldehyde glucosamine decreased rapidly with increasing length of incubation time, and set the incubation time to 2 min. In our system, at 50 °C, the total incubation time before chromatographic separation was well below this. Furthermore the pH of the sample may influence the fluorescence yield. Zelles (1988) reported that the maximum fluorescence yield of *o*-phthalaldehyde glucosamine was obtained at pH 8.5 and above.

The OPA derivatization reagent was buffered at pH 9.5 with a 0.4 mol L^{-1} borate buffer. To ensure that the amount of hydrochloric acid from the sample did not exceed 80% of the buffer capacity, samples that were diluted 20 times or less were diluted with 0.25 mol L^{-1} sodium hydroxide.

The detection limit was estimated (signal to noise ratio of 3) as 0.1 μmol L^{-1}.

3.3. Optimization of acidic hydrolysis

The experiments on optimizing the yield of glucosamine were performed on chitosan impregnated wood samples. The chitosan was chosen so that the average molecular weight was within the range used in other experiments. The concentration for the impregnation was set to 1% (w/v), which was also in the range used in the other experiments.

3.3.1. Screening of optimization parameters

The optimization of the hydrolysis system was performed in a 2^{6-3} reduced factorial design with hydrolysis time and temperature, hydrochloric acid concentration, amount of wood sample, agitation and pre-storage as the parameters investigated. The experimental data for

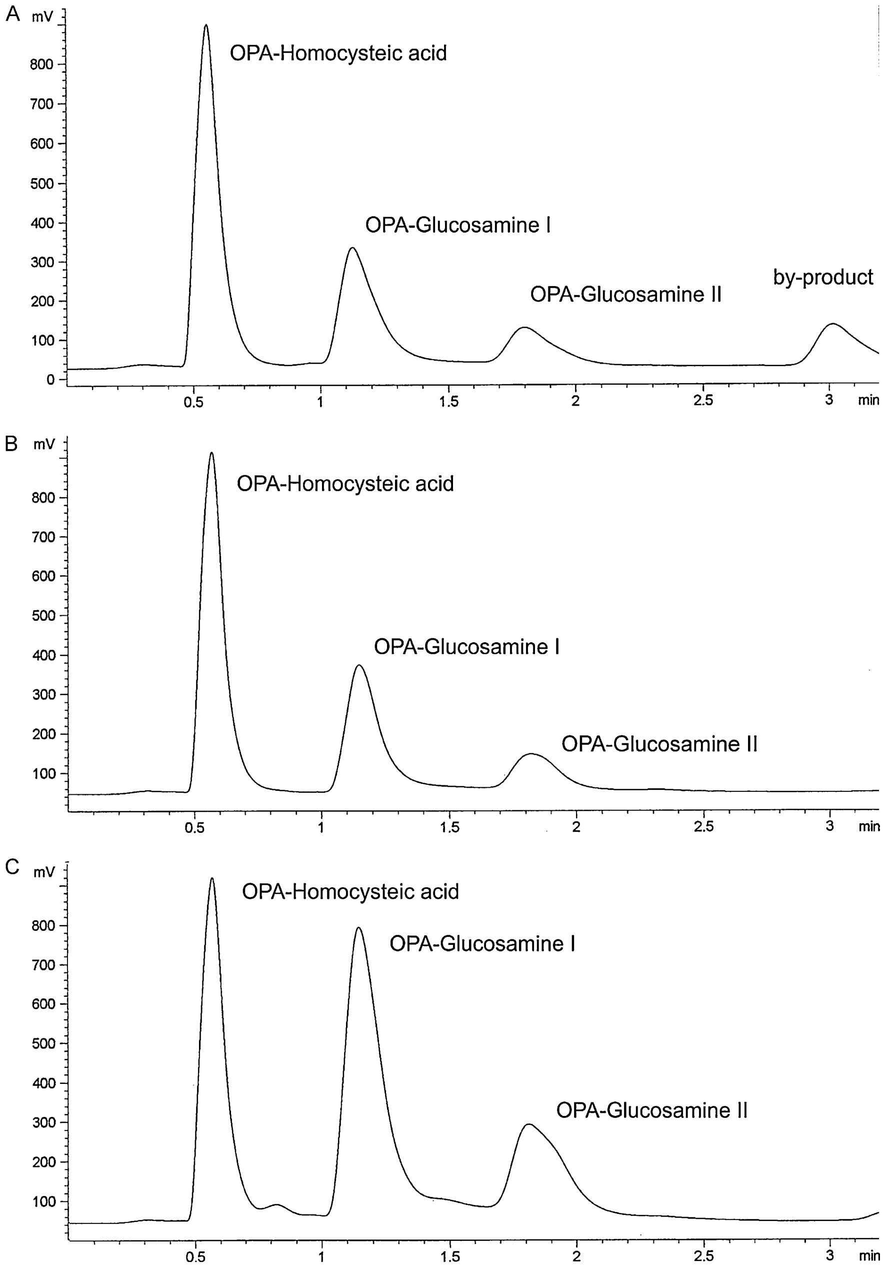

Fig. 1. Typical chromatograms for the HPLC analysis of glucosamine. The figure shows a chromatogram of a standard glucosamine solution derivatized with an OPA reagent that had been stored for 1 day at 4 °C (A), a freshly made OPA reagent (B) and a typical run of a hydrolyzed sample with a freshly made OPA reagent (C). The first peak represents homocysteic acid and the second peak represents glucosamine.

the screening experiment are shown in Table 1 and the results are presented in Fig. 3. The multiple regression analysis (Table 2) showed that all factors clearly have a significant effect on the yield of glucosamine ($p < 0.0001$, DF = 1). Furthermore, the factor profiling analysis showed that all factors gave the higher yield at the higher level (not shown), i.e. all parameters should be set to high. Due to confounding factors, the only second order interaction that could be resolved in this experiment was the interaction of hydrolysis time with hydrolysis temperature, which was also significant ($p < 0.0001$, DF = 1).

Table 2 shows that the intercept of the experiment was 2.25 mg g^{-1} wood (i.e. the estimated result when all factors were set to the average of the high and low values in the experiment). The parameter that gave the highest effect was hydrochloric acid concentration; increasing the concentration from the low to the high level (from 1 to 6 mol L^{-1}) gave an increased yield of 2.84 mg g^{-1} wood (two times the value found in Table 2), closely followed by the hydrolysis temperature (from 70 to 100 °C), which gave an increase of 2.64 mg g^{-1} wood. Agitation gave an increase of 1.74 mg g^{-1} wood. The second order interaction between

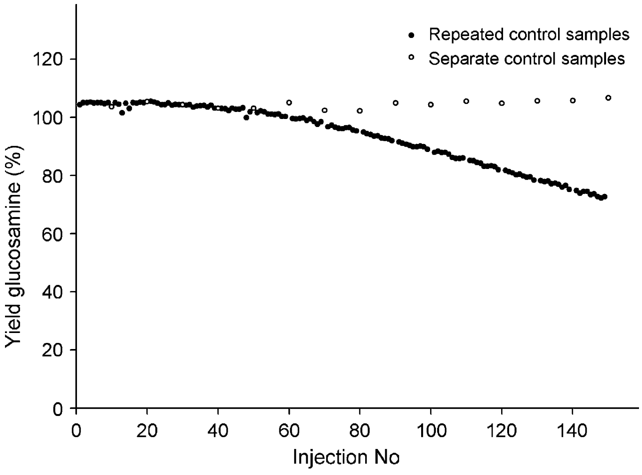

Fig. 2. Yield of glucosamine versus injection number.

hydrolysis time and temperature gave an increase of 1.00 mg g^{-1} wood. All other factors gave an increased yield of 0.70 mg g^{-1} wood or more on going from the low to the high value.

3.3.2. Optimization of hydrolysis

Further trials on optimization of the method were performed by testing three different aquatic hydrolysis systems: 6 mol L^{-1} hydrochloric acid at 100 °C and 6 mol L^{-1} sulphuric acid at 100 °C and 120 °C. The results from the experiment are presented in Fig. 4. Sulphuric acid at 120 °C gave quite a high yield after a short time. It seems that chitosan is unstable under these conditions, and the yield rapidly decreased by 50% after 24 h and by 90% after 48 h. Hydrolysis at 100 °C for both hydrochloric acid and sulphuric acid gave a higher yield after 24 h than after 48 h. As was the case for sulphuric acid at 120 °C, the chitosan yield decreased from 24 to 48 h for sulphuric acid at 100 °C.

Table 2
Parameter estimates from the multiple regression analysis of the 2^{6-3} reduced factorial design

Term	Parameter		Probability > |t|
	Estimate	Standard error	
Intercept	2.25	0.0662	<0.0001
Hydrolysis time	0.78	0.0726	<0.0001
Hydrolysis temperature	1.32	0.0726	<0.0001
HCl concentration	1.42	0.0726	<0.0001
Amount wood sample	0.35	0.0726	<0.0001
Agitation [yes]	0.87	0.0662	<0.0001
Pre-storage [yes]	0.45	0.0649	<0.0001
Hydr. time × hydr. temperature	0.50	0.0726	<0.0001

The intercept is an estimate of the yield when all factors are set to the average of the high and low values, and the parameter estimates for each factor must be multiplied by 2 in order to get the effect on the yield when going from the low to the high value.

For hydrochloric acid the maximum yield in these experiments was after 48 h. A multiple comparison test (Tukey HSD, $\alpha = 0.05$) on the differences in mean yields showed that hydrolysis with hydrochloric acid at 100 °C for 48 h gave a significantly higher yield than the rest (Table 3).

Sulphuric acid has higher viscosity than hydrochloric acid. The addition of sulphuric acid with internal standard was therefore more subject to variation than when using hydrochloric acid. Furthermore the centrifuge tubes (polypropylene) degraded over time at 120 °C. Due to these facts, hydrochloric acid was chosen for further use, even though the hydrolysis step then took quite a long time.

3.3.3. Optimization of hydrolysis time

The final experiments on optimization of the hydrolysis system were performed by testing the yield of glucosamine

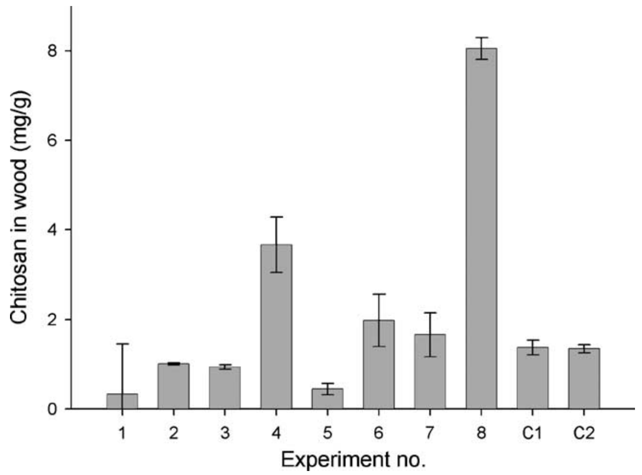

Fig. 3. Results from screening experiments on factors believed to influence yield of chitosan in acidic hydrolysis of wood impregnated with chitosan ($n = 3$, error bars represent 95% confidence interval). Data on the experiments are given in Table 1.

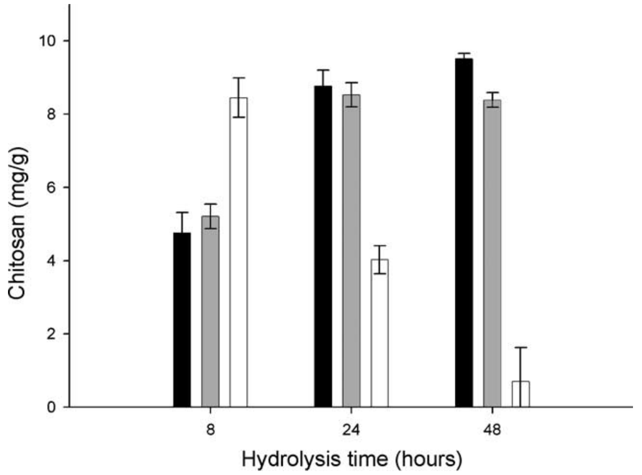

Fig. 4. Yield of glucosamine from hydrolysis of chitosan in wood with 6 mol L^{-1} hydrochloric acid at 100 °C (black bars) and 6 mol L^{-1} sulphuric acid at 100 (grey bars) and 120 °C (white bars) for 8, 24 and 48 h ($n = 3$, error bars represent 95% confidence interval).

Table 3
Least Square Means Differences for the yield of glucosamine (mg/g wood) with different hydrolysis systems

Hydrolysis system (6 mol L^{-1})	Hydrolysis time (h)						Least sq. mean
Hydrochloric acid 100 °C	48	A					9.51
Hydrochloric acid 100 °C	24		B				8.76
Sulphuric acid 100 °C	24		B				8.52
Sulphuric acid 120 °C	8		B				8.45
Sulphuric acid 100 °C	48		B				8.39
Sulphuric acid 100 °C	8			C			5.21
Hydrochloric acid 100 °C	8			C			4.76
Sulphuric acid 120 °C	24				D		4.03
Sulphuric acid 120 °C	48					E	0.70

Values not connected by the same letter are considered to be significantly different (Tukey HSD, $\alpha = 0.05$).

with different hydrolysis times on both wood and water samples containing chitosan. The hydrolyses were performed with 6 mol L^{-1} hydrochloric acid at 100 °C on 100 mg wood sample (1 mL water sample) with agitation (for the wood samples) and pre-storage at ambient temperature overnight.

Table 4
Least Square Means Differences for the yield of glucosamine (mg/g wood) with different hydrolysis times (6 mol L^{-1} hydrochloric acid at 100 °C) of wood samples containing chitosan

Hydrolysis time (h)							Least sq. mean
48	A						10.31
56	A	B					9.44
36	A	B	C				9.37
32	A	B	C				9.23
72		B	C				9.07
24			C				8.31
16				D			6.44
8					E		3.65
4						F	2.60

Values not connected by the same letter are considered to be significantly different (Tukey HSD, $\alpha = 0.05$).

3.3.3.1. Wood samples. The results from the optimization experiment for chitosan in wood samples are presented in Fig. 5. The yield of glucosamine increased with time up to 48 h, and then decreased slowly. A multiple comparison test (Tukey HSD, $\alpha = 0.05$) on the differences in means showed that the yield after 48 h was not significantly higher than the yield after 56, 36 or 32 h, but significantly higher than after other times (Table 4). Since the optimum yield seemed to be after 48 h of heating and because this was convenient for further processing, this was chosen for all further hydrolysis of wood samples containing chitosan. The concentration of chitosan used was within the range used in other experiments.

3.3.3.2. Water samples. The results from the optimization experiment for chitosan in water samples are presented in Fig. 6. The relative yield of glucosamine increased with time up to 32 h and decreased thereafter. A multiple comparison test (Tukey HSD, $\alpha = 0.05$) on the differences in means showed that there was no significant difference in yield between samples that were heated for 32, 24 and 36 h (Table 5). The yields after 32 and 24 h were, however, significantly higher than the rest (except 36 h), so 24 h of heating was then chosen for convenience for all further hydrolysis of water samples containing chitosan. The average yield of the water samples after 24, 32 and 36 h

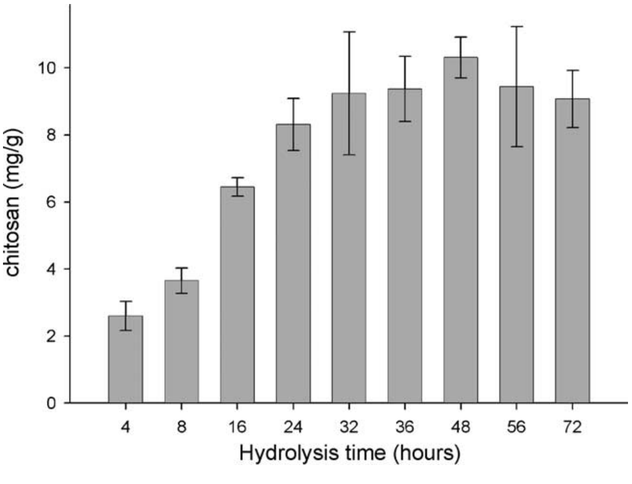

Fig. 5. Yield of glucosamine from hydrolysis of chitosan in wood with 6 mol L^{-1} hydrochloric acid at 100 °C with different hydrolysis times ($n = 3$, error bars represent 95% confidence interval).

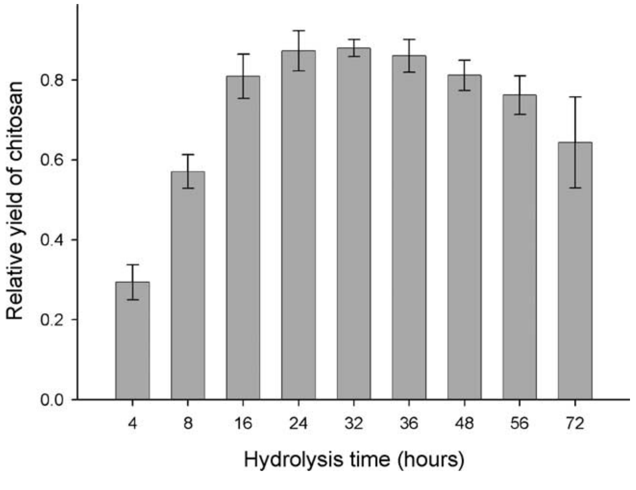

Fig. 6. Yield of glucosamine from hydrolysis of chitosan in water samples with 6 mol L^{-1} hydrochloric acid at 100 °C with different hydrolysis times ($n = 3$, error bars represent 95% confidence interval).

Table 5
Least Square Means Differences for the relative yield of glucosamine with different hydrolysis times (6 mol L^{-1} hydrochloric acid at 100 °C) of water samples containing chitosan

Hydrolysis time (h)							Least sq. mean
32	A						0.880
24	A						0.873
36	A	B					0.861
48		B	C				0.811
16		B	C				0.809
56			C				0.762
72				D			0.644
8					E		0.571
4						F	0.294

Values not connected by the same letter are considered to be significantly different (Tukey HSD, $\alpha = 0.05$).

was 87% (RSD = 2.3%, $n = 15$). The concentration of chitosan used was within the range used in other experiments. However, a dilution of high concentration samples may be helpful prior to hydrolysis.

3.4. Evaluation of method

3.4.1. Recovery of chitosan in wood samples

The results from the quantification of chitosan in wood samples spiked or impregnated with chitosan are presented in Fig. 7. For the impregnated samples there was increased variation with increased spiking of chitosan. For all samples with a theoretical yield of chitosan of 45 mg g^{-1} wood or less, the average yield was 87% (RSD = 8.9%, $n = 15$). The theoretical yield was calculated by using the uptake of 1% (w/v) chitosan per mass unit wood during impregnation corrected for water content of the wood powder used. For the samples that were not impregnated but spiked with chitosan solution there was less variation in the recovery of chitosan, with an average of 84% (RSD = 13%, $n = 17$) of theoretical yield.

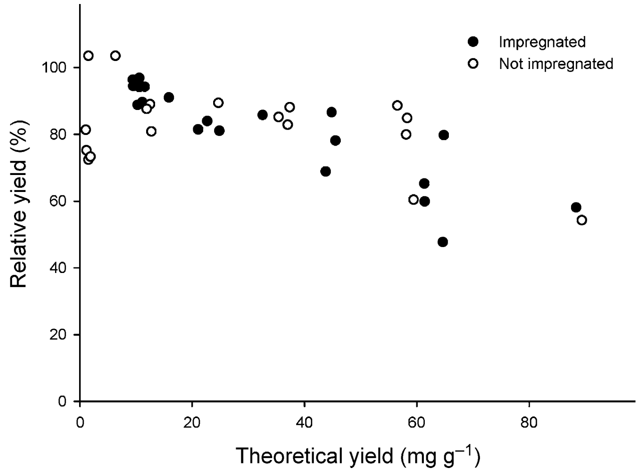

Fig. 7. Relative yield of chitosan in spiked Scots pine wood samples, both impregnated (1% (w/v) chitosan) and not impregnated.

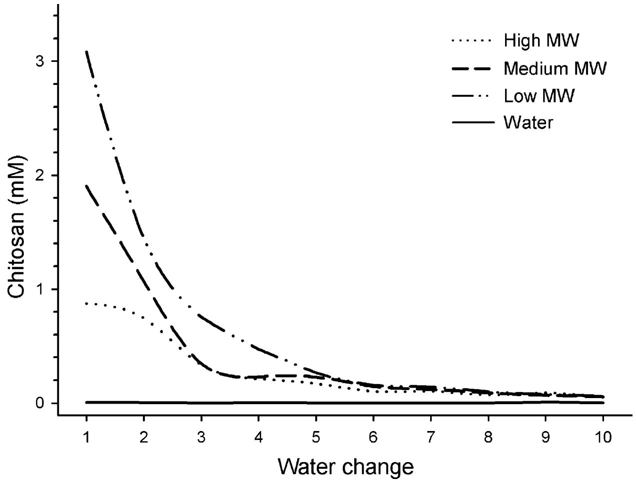

Fig. 8. Results from a leaching experiment of chitosan impregnated wood samples (Eikenes et al., in press). The results are presented as the average concentration ($n = 6$) of chitosan in 10 subsequent water changes.

3.5. Use of the method

3.5.1. Leaching of chitosan from impregnated wood samples

The results from the analysis of the leaching water clearly showed that the lower the average molecular weight of chitosan used for impregnation of wood samples, the higher the amount of leaching occurred (Fig. 8). Most of the chitosan that were able to leach out of the wood samples were leached after a few days. After 10 days the leaching rate were low and only very little chitosan still leached out of the samples.

3.5.2. Quantification of chitin in fungal infected wood

The results from the quantification of chitin in Scots pine decayed by *P. placenta* (as described in Eikenes et al. (in press)) are presented in Fig. 9. Wood samples not infected

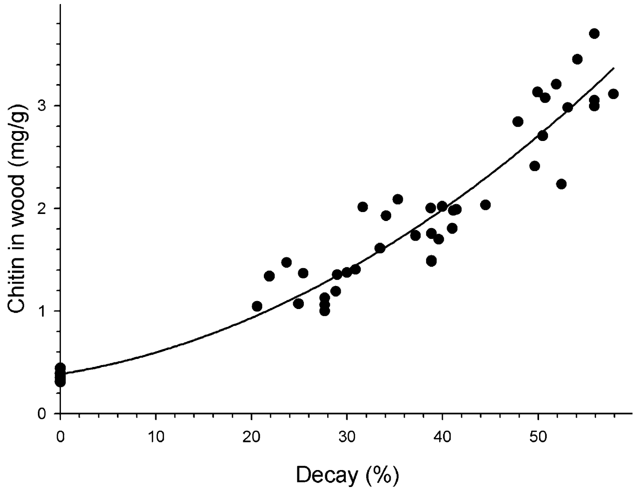

Fig. 9. Results from the quantification of chitin in wood samples of Scots pine decayed by *P. placenta* in an 8 week decay trial (Eikenes et al., in press). The results are presented as relative mass loss versus chitin content. The polynomial regression had a correlation of $r = 0.9622$.

by fungi had a content of *N*-acetyl glucosamine of 0.37 mg g^{-1} wood ($\pm 14\%$ RSD, $n=9$). The samples were chosen so that the range and distribution of the decay were maximized. The second order polynomial fit had a correlation of $r=0.9622$. This is in agreement with others (Braid & Line, 1981), and shows that the method is well suited to also quantifying chitin in wood decayed by fungi.

4. Conclusions

The results in these experiments were obtained on chitosan with an average molecular weight of 76 kDa and an F_A of 0.198. Further experiments may be needed to evaluate the validity of the hydrolysis steps for chitosans with other properties (i.e. different molecular weights and F_A) or in other matrix. However, the results from the chitin assay of fungal degraded wood states that the method is well suited also for this purpose, and indicate that the method may also be well suited for chitosans with other characteristics.

The chromatographic method was rapid and by the use of online derivatization we ensured a high degree of repeatability. The method is sensitive to degradation of OPA-solution, so care should be taken in this sense. Problems with degradation of the fluorescent product between the analytes (i.e. homocysteic acid and glucosamine) and *o*-phthalaldehyde are avoided by using online derivatization. The hydrolysis of the samples is quite time consuming, but the amount of labour is reasonable.

Acknowledgements

The authors would like to thank Sigrun Kolstad, Erik Larnøy, Gabriele Remedios and Eva Grodås for their laboratory work, Ivar Feste for providing the wood samples and MultiMar AS for providing the chitosan. Nicholas Clarke is thanked for revising the English. The Research Council of Norway is gratefully acknowledged for providing funding for this work.

References

Alfredsen, G., Eikenes, M., Solheim, H., & Militz, H. (2004). Screening of chitosan against wood deteriorating fungi. *Scandinavian Journal of Forest Research*, *19*(Suppl. 5), 4–13.

Braid, G., & Line, M. (1981). A sensitive chitin assay for the estimation of fungal biomass in hardwoods. *Holzforschung*, *35*, 10–15.

Eikenes, M., Alfredsen, G., Christensen, B., Militz, H., & Solheim, H. (in press). Comparison of chitosan with different molecular weights as possible wood preservative. *Journal of Wood Science*.

Frederiksen, O. (2001). Fungicid fra rejer (in Danish). Teknologisk Institut, Denmark: 29 pp.

Hagen, S. (1993). High-performance liquid chromatographic quantitation of phenylthiocarbamyl muramic acid and glucosamine from bacterial cell walls. *Journal of Chromatography*, *632*, 63–68.

Kobayashi, T., & Furukawa, I. (1995a). Optimum conditions for the formation of chitosan–metal salts and their fixation in wood. *Journal of Antibacterial and Antifungal Agents*, *23*(5), 263–269.

Kobayashi, T., & Furukawa, I. (1995b). Wood-preserving effectiveness of chitosan–metal salts against wood decaying fungi. *Journal of Antibacterial and Antifungal Agents*, *23*(6), 343–348.

Kumar, M. (2000). A review of chitin and chitosan applications. *Reactive & Functional Polymers*, *46*(1), 1–27.

Lee, J.-S., Furukawa, I., & Sakuno, T. (1992). Microdistribution of elements in wood after pre-treatment with chitosan and impregnation with chrome–copper–arsenic preservative. *Mokuzai Gakkaishi*, *38*(2), 186–192.

Lee, J.-S., Furukawa, I., & Sakuno, T. (1993). Preservative effectiveness against *Tyromyces palustris* in wood after pre-treatement with chitosan and impregnation wid chromated copper arsenate. *Mokuzai Gakkaishi*, *39*(1), 103–108.

Mikalsen, M., Ørstavik, A.R., Falck, R.A., & Steinstø, E. (2001). Preservative composition and kit and method for its production and use. Norwegian patent application No. 20013007, 21 pp.

Prochazkova, S., Vårum, K., & Østgaard, K. (1999). Quantitative determination of chitosans by ninhydrin. *Carbohydrate Polymers*, *38*, 115–122.

Roseman, S., & Daffner, I. (1956). Colorimetric method for determination of glucosamine and galactosamine. *Analytical Chemistry*, *28*(11), 1743–1746.

Tsuji, A., Kinoshita, T., & Hoshino, M. (1969a). Microdetermination of hexosamines. *Chemical & Pharmaceytucak Bulletin*, *17*(1), 217–218.

Tsuji, A., Kinoshita, T., & Hoshino, M. (1969b). Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-methyl-2-benzothiazolone hydrazone hydrochloride. *Chemical & Pharmaceytucak Bulletin*, *17*, 1505–1510.

Vårum, K., Anthonsen, M., Grasdalen, H., & Smidsrød, O. (1991). Determination of the degree of *N*-acetylation and the distribution of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydrate Research*, *211*, 17–23.

Zelles, L. (1988). The simultaneous determination of muramic acid and glucosamine in soil by high-performance liquid chromatography with precolumn fluorescence derivatization. *Biology and Fertility of Soils*, *6*, 125–130.