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An evaluation of tritium and fluorescence labelling combined with multi-detector SEC for the detection of carbonyl groups in polysaccharides

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ABSTRACT

The carbonyl content of a pectic polysaccharide from *Sphagnum papillosum* (sphagnan) and periodate oxidised alginates was investigated using three different carbonyl labelling strategies combined with size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) and on-line fluorescence or off-line tritium detection. The labelling strategies were tritium incorporation via NaB^3H_4 reduction, and fluorescent labelling with carbazole carbonyl oxyamine (CCOA), or 2-aminobenzamide (2-AB), respectively. Carbonyl quantification was based on labelled pullulan, dextran and alginate standards possessing only the reducing end carbonyl group. As a result the carbonyl distribution in the polysaccharides could be determined. In sphagnan it was found that the carbonyl content increased with increasing molecular weight, whereas in periodate oxidised alginate the carbonyl content was as expected independent of the molecular weight. The methods proved useful for carbonyl detection in water soluble polysaccharides in general. The tritium incorporation method was preferred for alkali stable polysaccharides, while the CCOA method was most suitable for acid stable polysaccharides with low carbonyl content. The 2-AB method is applicable for all polysaccharides tested with varying carbonyl content; however, it lacks the ability to detect ketone functionalities.

1. Introduction

The quantification of carbonyl groups in populations of polysaccharide chains in solution is important because of their impact on structural and reactive properties. All polysaccharides contain one carbonyl group at their reducing end which is in equilibrium with a hemi-acetyl group, while the main cause of intramolecular carbonyl group formation in polysaccharides are by chemical oxidation either intentionally or non-intentionally. Oxidation may lead to altered chain extensions (Christensen, Vold, & Vårum, 2008; Vold, Kristiansen, & Christensen, 2006) increased reactivity and hence new possible applications (Bouhadir et al., 2001). In other cases the oxidation may be regarded as a drawback foremost since the oxidised unit is a 'hot-spot' for degradation, cellulose being the most prominent example (Potthast, Rosenau, & Kosma, 2006), but also if further reactivity is unwanted.

Intramolecular carbonyl groups introduced by oxidation are capable of existing in a variety of forms, and equilibrium occurs between the various forms in solution. In water they may exist hydrated, as acyclic aldehydes, hemi-acetals or hemi-aldals, or as combinations of these. In polysaccharides hemiacetal formation may be intermolecular as well as intramolecular (Gutherie, 1961).

Several different traditional techniques exist for measuring aldehyde and keto groups in oligo and polysaccharides (Mclean, Werner, & Aminoff, 1973; Potthast et al., 2006; Richards & Whelan, 1973). The most important traditional methods for this purpose are summarized in Table 1. The majority of these offer an estimate of the average number of carbonyl groups (weight basis). One of the most common strategies is incorporation of tritium utilizing NaB^3H_4 reduction for carbonyl detection in oligosaccharides. Borohydride reduces carbonyl compounds including aldoses and ketoses. This reaction is in general 'stoichiometric' (Mclean et al., 1973). The first step is a nucleophilic addition reaction, where $NaBH_4$ acts as a donor of a hydride ion that is attracted to the partial positive charge of the carbonyl carbon. In the second step, water protonates the tetrahedral alkoxide intermediate and yields the alcohol product. In the case of reaction with NaB^3H_4 at most one tritium atom can be bound to one carbon per carbonyl group reduction (Tinnacher & Honeyman, 2007). To our knowledge there has been only one attempt to use this method to quantify carbonyl groups in a polysaccharide (Kongruang & Penner, 2004). The main challenge using this method is potential alkaline depolymerisation because of the alkaline reaction conditions needed for borohydride stability. This is mainly an issue in polyuronides were 'internal' β-elimination can occur. Degradation due to the 'peeling reaction'

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Table 1
Common methods for determining carbonyl content in oligosaccharides/polysaccharides.

Method	Advantages/drawbacks	Refs. (method)
Colorimetric methods; e.g., copper number (Cu^{2+} (blue) → Cu^{+} (red))	+Simple ÷Sensitivity problems (det. limit = 0.3 mg ± 2%), back-oxidation. Averages only	Hodge & Hofreiter (1962).
Schiff base reactions of C=O groups with hydrazines or *O*-substituted hydroxylamines followed by; • C/N analysis • fluorescence detection	+Simple ÷Sensitivity problems with C/N analysis, fluorescence sometimes dependent on where the label is situated. Averages only	Maekawa & Koshijima (1991) Ramsay et al. (2001) Shilova & Bovin (2003)
Reduction using NaB^3H_4	+Sensitive ÷Rarly applied to polysaccharides because of possible alkaline degradation. Averages only	Mclean et al. (1973) Richards & Whelan (1973) Takeda et al. (1992)
Fluorescence/absorbance labels in combination with a separation technique; e.g., Fmoc-hydrazine, 2-aminopyridine etc.	+Data for the whole molecular weight distribution ÷Some is highly toxic, low yield, long reaction times	Praznik & Huber (2005) Röhrling et al. (2001)
Direct physical techniques; IR, NMR, etc.	+Simple ÷Poor sensitivity. Averages only	Calvini et al. (2004) Gomez, Rinaudo, & Villar (2006)

is hampered by reduction at the reducing end (Sharon, 1975). A purification step is also needed after reduction to dispose of background activity which is possibly a result of impurities in the NaB^3H_4 (Mclean et al., 1973).

Recently, a variety of fluorescence labels have been introduced for measuring carbonyl groups/reducing ends in oligosaccharides, e.g., 2-aminobenzamide (2-AB) or 9-fluorenyl-methoxycarbonylhydrazine (Fmoc-hydrazine) (Bigge et al., 2002; Zhang, Cao, & Hearn, 1991). These labels are often hydrazines or substituted hydroxylamines which bear a hydrophobic fluorescent group and can be detected at the picomolar level. The labels are connected via reductive amination (Fig. 1) or as for hydroxylamines directly via imine formation without the reduction step.

By combining carbonyl detection with size exclusion chromatography (SEC) followed by multi-angle laser light scattering (MALLS) the distribution of carbonyl groups may be obtained. In addition, estimates of the molecular weight directly provide the number of reducing ends, enabling a possibility for correction when the goal is to analyse intramolecular carbonyl groups in the polysaccharide chain. If SEC-MALLS is used to separate the labelled compounds some specific requirements are needed with respect to the label (Röhrling et al., 2002b). First it is important that the emission wavelength does not interfere with the working wavelength of the laser. Secondly the fluorescence emission wavelength should be independent on where the label is located on the polysaccharide. Finally it is important that the label does not interact significantly with the column material affecting separation and/or destroy columns after long-term use.

Overcoming the limitations mentioned above the fluorescent label carbazole carbonyl oxyamine (CCOA) has been used in combination with SEC-MALLS and on-line fluorescence detection. This strategy has been developed for measuring small amounts of carbonyl functionalities in cellulose as a function of molecular weight (Potthast et al., 2003; Röhrling et al., 2002a, 2002b). The CCOA label has the advantage of being an *O*-substituted hydroxylamine, having an increased reactivity towards carbonyl groups compared to a hydrazine and thus reduction of the double bond formed upon imine formation is not necessary. The label is however, synthesized especially for the detection of carbonyl groups in cellulose dissolved in *N,N*-dimethylacetamide (DMAc)/LiCl and is not yet commercially available (Röhrling et al., 2001). β-glucans labelled with 2-aminopyridine (2-AP) at their reducing end have also been separated with SEC in combination with RI and on-line fluorescence detection (Praznik & Huber, 2005). The method applying 2-AP however, uses a great excess of the highly toxic reagent (~2000 times carbonyl content) in order to get an acceptable conversion and the labelling procedure is not suitable for acid labile polysaccharides.

2-aminobenzamide (2-AB) represents an alternative label widely used for labelling of carbohydrates and is compatible with several chromatographic means of separation including Bio Gel P4, high-performance anion-exchange chromatography and a variety of HPLC procedures (Bigge et al., 2002; France, Cumpstey, Butters, Fairbanks, & Wormald, 2000).

The purpose of this study was to compare and evaluate three different labelling techniques in combination with SEC-MALLS in order to estimate the carbonyl content of both neutral and charged polysaccharides as a function of their molecular weight. The approaches used are labelling with tritium utilizing NaB^3H_4 reduction and labelling with the fluorescent labels CCOA and 2-AB. The 2-AB is connected via the direct reductive amination approach (Fig. 1). Both the NaB^3H_4 and 2-AB have the advantage of being commercially available.

The methods are evaluated using pullulan, dextran and alginate standards. Carbonyl groups were introduced by partial (2–8%) periodate oxidation (Aalmo & Painter, 1981). The resulting dialdehydes can in principle react with two amino-containing labels. To study the stoichiometry of the reaction with 2-AB, oligomers of alginate ($DP_n = 20$) were prepared and subjected to oxidation and labelling, and the products were studied by ^1H NMR.

These methods are also applied to determine the average amount of carbonyl groups in a pectic polysaccharide, named sphagnan, released by mild acid hydrolysis from *Sphagnum papillosum* (Ballance, Børsheim, Inngjerdingen, Paulsen, & Christensen, 2007). It has been claimed that sphagnan contains ~25 M% of a novel keto-uronic acid residue in the form of 5-keto-D-mannuronic acid (5-KMA) which could exist as either a pyranose (5-KMA*p*) or furanose (5-KMA*f*), of which the latter contains a α-keto-carboxylic acid group (Painter, 1983, 1991). 5-KMA in sphagnan was claimed to be responsible for giving *Sphagnum* moss special properties in preserving organic material. The existence of 5-KMA has been rejected (Ballance et al., 2007), but to our knowledge no exact measure of the carbonyl group distribution in sphagnan exists.

2. Materials and methods

2.1. Standards and chemicals

The pectic polysaccharide sphagnan was extracted according to the method previously described (Ballance et al., 2007). Dextran was obtained from Polymer Standard Service (PSS), Germany. Pullulan standards were supplied from Hayashibara, Japan. Alginate (LF 10/60) containing 40% guluronic acid and mannuronan were obtained from FMC Biopolymer, Drammen, Norway. Alginate containing 90% guluronic acid was prepared by epimerising polym-

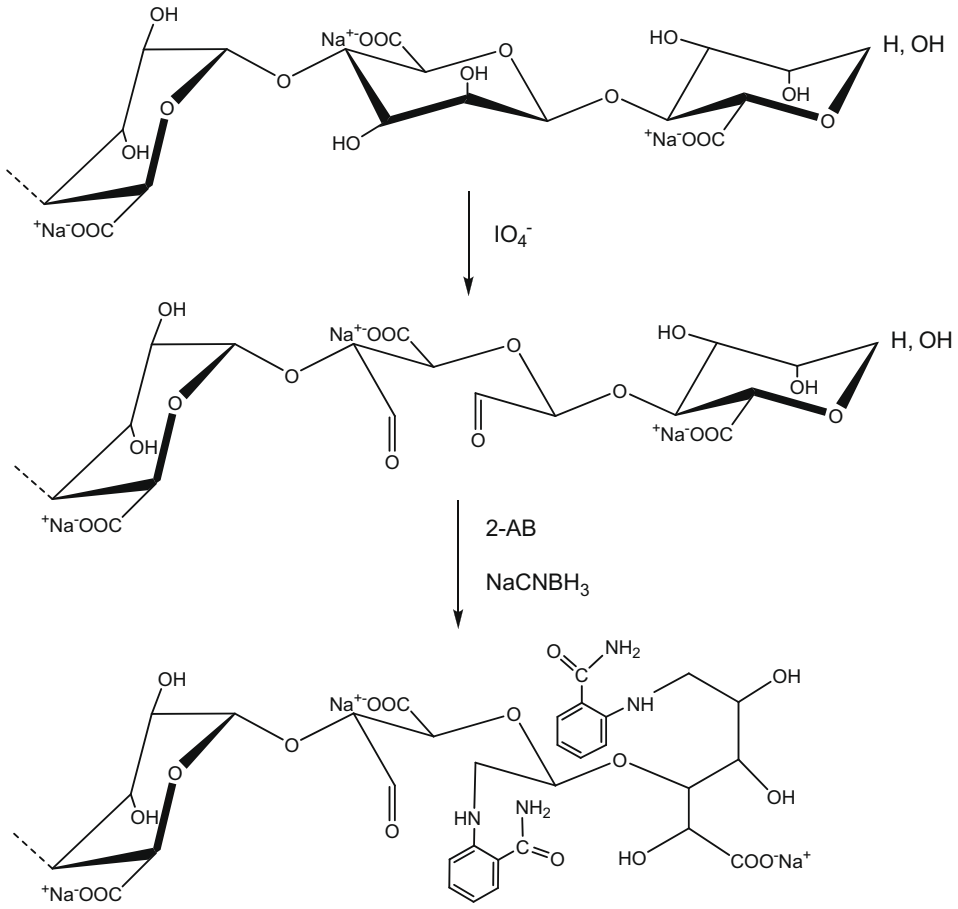

Fig. 1. Chemical structure of an alginate end fragment (. . .GMM) treated with periodate (oxidation assumed to occur randomly) followed by reaction with 2-aminobenzamide (2-AB) and $NaCNBH_3$ (direct reductive amination). Only one of the two aldehydes formed upon periodate oxidation reacts with 2-AB (see text). Abbreviations: M, β-D-mannuronic acid; G, α-L-guluronic acid.

annuronic acid with the alginate epimerase AlgE6 according to Holtan, Bruheim, and Skjåk-Bræk (2006). 100 mCi NaB^3H_4 (12.1 Ci/mmol) was obtained from Amersham Bioscience. Carbazole-9-carboxylic acid [2-(2-aminooxyethoxy)ethoxy)]amide (CCOA) was synthesized according to Röhrling et al. (2001). All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Periodate oxidation of pullulan and alginate

Pullulan and alginate were partially oxidised using sodium meta periodate. The polysaccharides were dissolved in MQ-water [deionized water purified with the MilliQ system from Millipore (Bedford, MA, USA)] to a concentration of 8.89 mg/mL. The solution was then made up with 10% (v/v) *n*-propanol (free radical scavenger (Painter & Larsen, 1973)) and MQ-water and degassed prior to the addition of 0.25 M sodium meta periodate in order to create a theoretical degree of oxidation equal to 2%, 4%, 6% and 8%. The final polysaccharide concentration was 4.45 mg/mL and the weight was corrected for 10% (w/w) water content. All pipetting and weighing was done in subdued light. The reaction was carried out at 20 °C. The monomer weight was taken to be 162 and 198 g/mol for pullulan and alginate, respectively.

2.3. Labelling with NaB^3H_4

0.3 M NaB^3H_4 (specific activity 2.5 mCi/mmol) in 0.1 M NaOH was added to ten milligram of polysaccharide dissolved in 2 mL MQ-water. The samples were set to react in the fume hood for 24 h at room temperature on a shaking devise. The reaction was then stopped by cooling the samples on ice and adding 200 μL of concentrated acetic acid and left for 1 h until all hydrogen/tritium gas had effervesced. The samples were dialysed (Medicell International Ltd., Size 4 Inf Dia 22/32″ – M.W.C.O. 12-14 kDa) against two shifts of 0.05 M NaCl and MQ-water until the conductivity was <2 μS/cm and the tritium count was equal to background (50–100 CPM). Dialysis was followed by freeze-drying.

2.4. Labelling with CCOA

2.5 mg of CCOA in 100 mM acetate buffer (pH 4) was added to ten milligram of polysaccharide dissolved in 1 mL of MQ-water. The samples containing a polyelectrolyte were pre-adjusted to pH 4. The samples were then set to react on a shaking devise for 168 h at 40 °C followed by dialysis and freeze-drying in the same way as the tritium labelled samples.

2.5. Labelling with 2-AB

Ten milligram of polysaccharide was dissolved in 3 mL of MQ-grade water, 0.1 mL 1 M 2-AB in 100% MeOH and 0.1 mL of 5 M $NaCNBH_3$ in 1 M NaOH was added in the fumecupboard. The pH was then adjusted by adding approx. 0.3 mL of 1 M acetate buffer, pH 5, to give a final pH 5.8 in the reaction solution. Addition of buffered acetate was necessary to prevent the pH from falling below five and thus preventing the undesirable formation of HCN gas. The samples were then set to react on a shaking devise for 48 h at RT. To remove excess label the samples were either dialysed in

the same way as for the tritiated samples or precipitated with 50% (v/v) isopropanol. The precipitate was centrifuged at 2559*g* for 5 min and washed with 100% isopropanol three times. The pellet were left for 24 h in the fumehood and re-dissolved in mobile phase for SEC-MALLS with fluorescence detection.

2.6. ^{1}H NMR of 2-AB labelled derivatives

^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer at 90 °C. The oxidised alginate samples for ^{1}H NMR was prepared by degrading the alginate by mild acid hydrolysis. The samples were then reduced using 0.5 M $NaBH_4$, oxidised and labelled as described above. The pullulan oligomers were obtained directly from Hayashibara, Japan. The mannuronan oligomer ($DP_n = 20$) was prepared by partial acid hydrolysis and purified using SEC. After labelling with 2-AB and removal of excess label the oligosaccharides were dissolved in D_2O to a concentration of 10 mg/mL followed by the addition of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (Aldrich, Milwaukee, WI, USA) as internal standard for the chemical shift. In the case of analysing alginate 0.3 M triethylenetetramine-hexaacetic acid (TTHA) was added in order to chelate any Ca^{2+}-ions present.

2.7. Size-exclusion chromatography with multi-angular laser light scattering (SEC-MALLS) and fluorescence or tritium detection

A part of the sample was dissolved in MQ-water and mobile phase was added so that the concentration was equal to the mobile phase on the SEC setup. Mobile phase was 0.05 M Na_2SO_4/0.01 M EDTA, pH 6. In the case were CCOA or 2-AB labelled samples were analysed the mobile phase was made up by adding 20% (v/v) acetonitrile. The flow rate was set to either 0.4 mL/min or 0.5 mL/min.

The samples were filtered through a 0.2 μm filter and injected on different combinations of Tosoh Biosep TSK 6000, 5000, 4000 and 3000 PWXL columns connected to a Dawn DSP multi-angle laser light scattering photometer (Wyatt, USA) ($\lambda_0 = 633$ nm) followed by an Optilab DSP differential refractometer (P-10 cell). In the case of fluorescence detection a Shimadzu fluorescence monitor (model RF-530) was inserted between the MALLS and the RI detector. The excitation and emission wavelengths were set to 286 nm and 340 nm, respectively, for CCOA. While for 2-AB the respective excitation and emission wavelength were set to 340 nm and 450 nm. Tritium was detected by collecting fractions after the RI detection (fraction size: 600 μL). Each fraction (500 μL) was transferred to a scintillation vial, 10 mL of scintillation cocktail (HiSafe OptiPhase 3) was added and finally the samples were counted for 5 min each on a liquid scintillation counter (Wallac 1410). Data was collected and processed using Astra version 4.90 and transferred into an excel spreadsheet for further processing. For alginate and sphagnan a specific refractive index increment (dn/dc) of 0.150 mL/g was used, while for dextran and pullulan 0.148 mL/g was used.

3. Results and discussion

3.1. Carbonyl detection using tritium labelling

The carbonyl functional groups in the polysaccharide were reduced to alcohols with NaB^3H_4 in order to incorporate one tritium atom per. aldehyde and/or keto group initially present. The reduction of oligosaccharides and polysaccharides with $NaBH_4$ is a *pseudo* first order reaction when carried out with an excess of borohydride, typically 0.5–5 M, to ensure a quick reduction and prevent alkaline degradation (Painter & Larsen, 1973). When tritiated borohydride is used the sensitivity of the assay is dependent on the ratio between tritiated to non-tritiated borohydride. A total concentration of 0.1 M NaB^3H_4 (spec. activity 2.5 mCi/mmol). in the reaction mixture was chosen since this gave sufficient sensitivity (without exceeding the limits for general radioactive disposal in Norway) and is previously shown to completely convert glucose to glucitol within 24 h at room temperature (Hansson, Hartler, Szabo, & Teder, 1969). The pH was kept equal to 12.5 in order to prevent decomposition of borohydride (Mochalov, Khain, & Gil'manshin, 1965).

The incorporation of tritium was initially measured for pullulan and sphagnan showing that reduction was nearly complete after just a few hours upon addition of borohydride. After 24 h no more tritium could be incorporated and this reaction time was chosen in subsequent experiments.

The possibilities for alkaline degradation were assayed with SEC-MALLS. However, no significant degradation of alginate (unoxidised), dextran, pectin or pullulan was detected under these conditions.

In order to determine the relative carbonyl content in an unknown sample one or more references/standards with known carbonyl content are needed. Pullulan, dextran and alginate were chosen. ^{3}H labelled samples were analysed using SEC-MALLS, and fractions (600 μL) were collected for off-line tritium counting. The chromatograms were divided into slices corresponding to the fraction size, and M_n (or DP_n) was calculated for each fraction (Fig. 2). The specific incorporation of ^{3}H (activity per μg) was inversely proportional to DP_n across the DP range, independent of the type of polysaccharide (Fig. 2B). Thus, any of these polysaccharides (and possibly many others) may serve as standards. A standard prepared at the same time as the samples must be included in all experiments since the activity of the tritiated borohydride changes both as a consequence of disintegration of the isotope and decomposition of the borohydride itself. The method is also very much dependent on a precise determination of the delay volume between the different detectors and the fraction collector.

The method was first applied to a pectic polysaccharide from *S. papillosum*, named sphagnan. According to fig. 2, the carbonyl content of sphagnan is generally higher than the standards. For example, at DP = 100 each chain contains on average one carbonyl group in addition to the reducing end, whereas at DP = 400 the corresponding number is nine (Fig. 2B). The average carbonyl content is 2.9 carbonyl groups per 100 monomers. During the extraction of sphagnan the *Sphagnum* holocellulose is treated with chlorite in order to remove lignins. It was suspected that this could introduce carbonyl groups into sphagnan. Therefore sphagnan was prepared without the chlorite bleaching step (method given by Ballance, Kristiansen, Holt, & Christensen, 2008) and measured using this method. The data in fig. 2 shows no significant difference between the two methods for preparing sphagnan, indicating that no significant amount of carbonyl functionalities was introduced during the chlorite bleaching step. Thus, a small amount of carbonyl groups, in addition to the reducing end, are indeed present in sphagnan.

The method was subsequently applied to periodate oxidised alginate and pullulan (2–8%). However, these samples were significantly depolymerised during labelling and the degree of oxidation was highly underestimated compared to the theoretical values. Although this may give interesting information about the degradation of such samples in alkali this was outside the scope of this work and further investigation of these samples were abandoned at this point.

3.2. Carbonyl detection using the fluorescent label CCOA

The fluorescent label carbonyl carbazole oxyamine (CCOA) was previously used for carbonyl detection in cellulose. Cellulose was either dissolved in 9% (w/v) DMAc/LiCl (homogenous procedure) or suspended in 20 mM zinc acetate buffer, pH 4, (heterogeneous

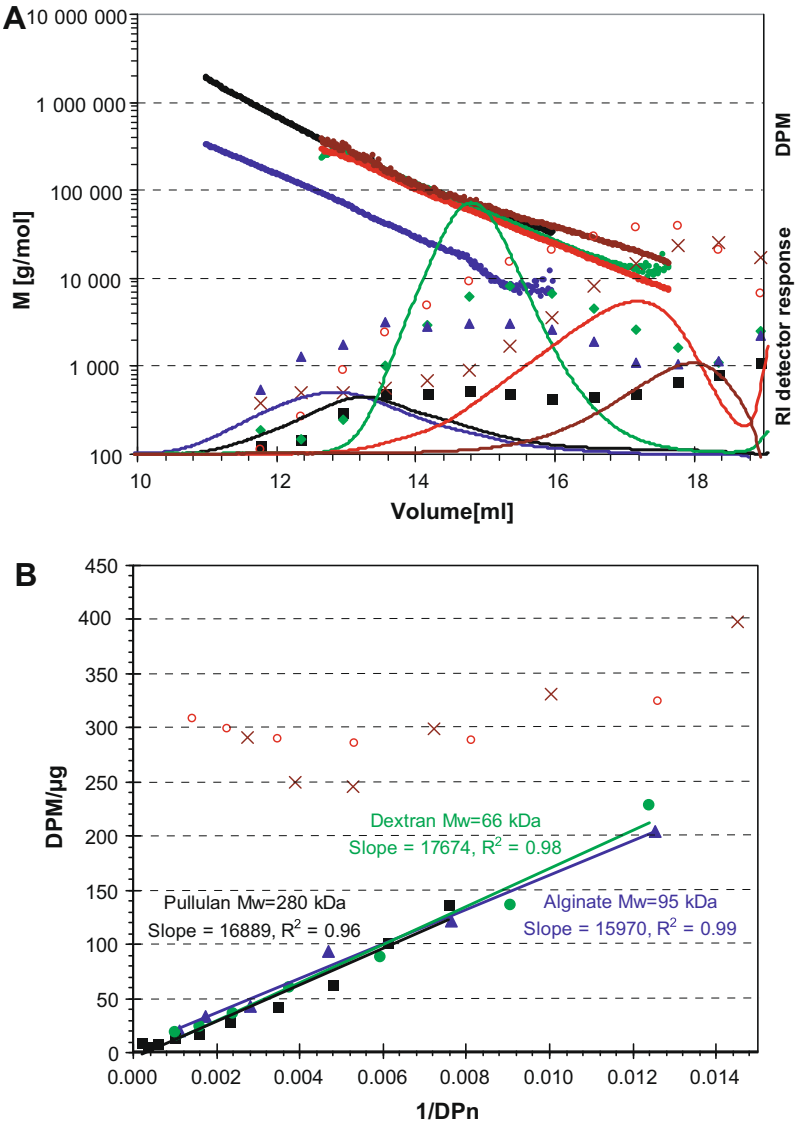

Fig. 2. Carbonyl detection using the tritium incorporation (via NaB^3H_4 reduction) method applied to alginate (90% guluronic acid) (▲), dextran (●), pullulan (■), sphagnan (X) and sphagnan not treated with chlorite (○). Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and tritium activity (DPM – disintegrations per minute) profiles. For tritium detection fractions (600 µL) was collected throughout the chromatographic profile and counted off-line. Lower figure (B): number average DP is calculated for the fractions demonstrated as spacing between the DPM data points in the upper figure. The monomer weight of pullulan/dextran, alginate and sphagnan were taken as 162, 198 and 200 g/mol, respectively. For colours, see on-line version.

procedure) in both cases followed by SEC-MALLS with fluorescence detection using 0.9% (w/v) DMAc/LiCl as mobile phase (Röhrling et al., 2002b). We wanted to adapt this method to polysaccharides labelled and analysed in an aqueous system. Pullulan, alginate, dextran and sphagnan were labelled based on the protocol for homogenous labelling of cellulose (the procedure being heterogeneous for water soluble polysaccharides), however, the zinc salt could not be included in the acetate buffer since alginate forms gels with divalent cations. Zinc was included in the buffer when assaying cellulose because it has the ability to hydrolyse lactones (Röhrling et al., 2001). Pullulan, dextran or alginate are not known to contain a significant amount of lactone structures. The structure of sphagnan, not being known in detail, might contain lactone structures. If such structures were to be present they would lead to an overestimation of the carbonyl content using this method.

The labelling of dextran and pullulan was successful, but alginate was severely degraded. It was observed, analogous to the NaB^3H_4 method, that the pullulan and dextran standards gave overlapping and near linear functions in a plot of fluorescence/RI vs. $1/DP_n$ as shown in Fig. 3B.

The separation of the CCOA labelled polysaccharides was successful although unreacted CCOA was absorbed onto the column material. This phenomenon was also described by Röhrling et al. (2002b) when DMAc/LiCl was used as mobile phase, but the interaction is likely to be stronger using an aqueous mobile phase. It is assumed that this interaction is due to the fact that unbound CCOA penetrates deep into the pores of the polyhydroxy methacrylate co-polymer network and weakly sticks to it by hydrophobic interactions. Different modifiers were tested including 20% (v/v) 2-propanol, methanol and acetonitrile to avoid this interaction. A mobile phase consisting of 10% (v/v) dimethylsulfoxide (DMSO), 0.05 M Na_2HPO_4 and 0.1% (w/v) sodium dodecyl sulfate adjusted to pH 6, previously used to successfully separate lignosulfonates at pH 10.5, (Fredheim, Braaten, & Christensen, 2002) was also tested. The best result was obtained with 0.05 M Na_2SO_4/0.01 M EDTA mobile phase containing 20% (v/v) acetonitrile. 2-propanol was equally good, but acetonitrile was preferred because of its lower

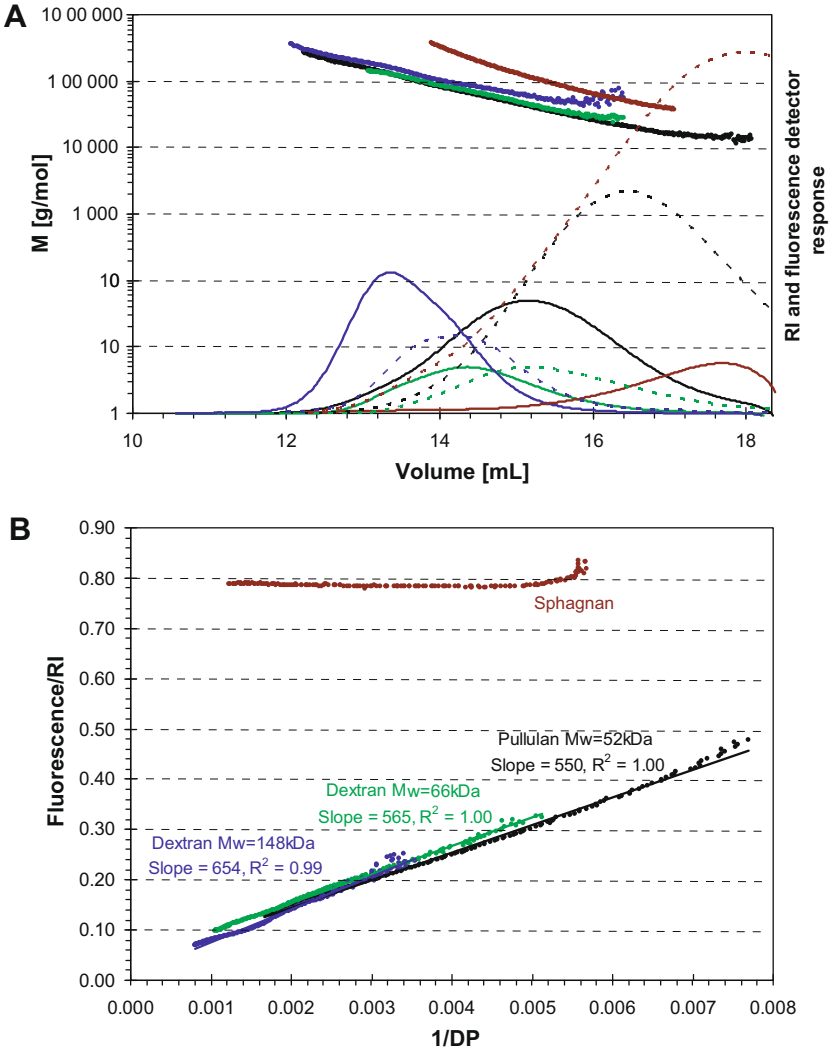

Fig. 3. Carbonyl detection using the carbazole carbonyl oxyamine (CCOA) method applied to sphagnan, pullulan and dextran. Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and fluorescence (dotted lines) profiles. Lower figure (B): monomer weight of pullulan/dextran and sphagnan are taken as 162 and 200 g/mol, respectively. For colours, see on-line version.

viscosity. The CCOA did, even with modifier present in the mobile phase, elute from the column some time after the salt peak leading to extended runtimes. The amount of unbound CCOA in the samples was however, very low since dialysis removed almost all of it.

The CCOA method was applied to sphagnan (Fig. 3). However, the carbonyl content was underestimated compared to the tritium approach resulting in an average carbonyl content of 1.4 carbonyl groups per 100 monomers. The result was corrected for a 2.6% emission background originating from the sphagnan itself. The background emission was evenly distributed at all molecular weights. It was also observed that the molecular weight was increased in the high molecular weight region of the sample when sphagnan was reacted with CCOA, reflected in an approximately 25% higher M_w and 8% higher M_n value. This phenomenon is due to partial aggregation of the CCOA labelled sphagnan sample.

Alginate and pullulan partially oxidised in the range 2–8% with periodate were also analysed, but all samples formed insoluble particles during labelling with CCOA. This is probably due to the hydrophobic character of the label which makes the polymer amphiphilic and hence difficult to dissolve in water. Since unoxidised alginate also was degraded during labelling it is reasonable to believe that the oxidised samples were degraded even more severely. The aggregates were dialysed and freeze dried, but showed no sign of dissolving in the mobile phase containing 20% (v/v) acetonitrile.

3.3. Carbonyl detection applying the fluorescent label 2-aminobenzamide (2-AB)

A third strategy was tested in order to find a method that could be used for acid or alkaline labile polysaccharides. A reference method was also needed to ensure the validity of the tritium labelling approach. 2-aminobenzamide (2-AB) is a common fluorescence label attached for reducing oligosaccharides in quantitative yields using direct reductive amination, e.g., pectic oligosaccharides (Bigge et al., 2002; Ishii, Ichita, Matsue, Ono, & Maeda, 2002). In addition it was found that the label fulfilled the demands given in the introduction and could be used in combination with SEC-MALLS. To ensure that the labelling were complete the reaction kinetics were investigated for all polysaccharides involved, see Fig. 4. The reactions could be fitted to a pseudo first order reaction with R^2 = 0.99–0.96. The figure shows that the labelling was complete after 24 h.

Oligomers of alginate and pullulan (unoxidised and periodate oxidised) were studied by ^{1}H NMR to reveal whether they were fully reactive/substituted with 2-AB or not. Fig. 5 shows the ^{1}H

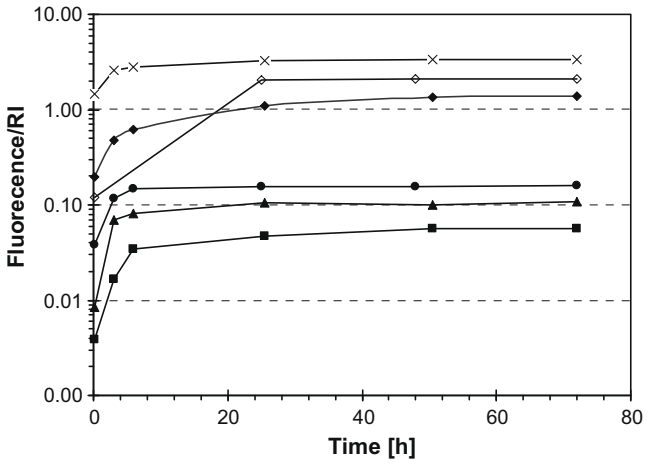

Fig. 4. Reaction kinetics for 2-AB labelling of pullulan (■), alginate (▲), mannuronan (●), sphagnan (♦), mannuronan 6% periodate oxidised (◇) and alginate (40% guluronic acid) 8% periodate oxidised (x).

NMR spectra of unlabelled mannuronan (A) and mannuronan labelled for 24 h (B). Signals from the reducing ends at 5.22 and 4.88 ppm disappear after labelling, indicating complete substitution of the reducing ends. The four protons from 2-AB appear as doublets and triplets in the 6.8–7.7 ppm region. This is analogous to the observations made by Ishii et al., 2002 for pectin oligomers. Comparing the signals from the four protons on 2-AB with the internal H-1 proton on mannuronic acid also confirmed that the oligomer was fully substituted given $DP_n = 20$. The DP_n of the mannuronan oligomer was estimated from the ^{1}H NMR spectrum by the following expression; $DP_n = (I_{M-1} + I_{M-1red\alpha} + I_{M-1red\beta})/(I_{M-1red\alpha} + I_{M-1red\beta})$ (Grasdalen, 1983) and confirmed by SEC-MALLS analysis.

Alginate oligomers reduced with 0.5 M $NaBH_4$ prior to 4% and 8% periodate oxidation were also prepared, labelled (24 h) and analysed with ^{1}H NMR, see Fig. 5C and D. The percentage of oxidation refers to oxidised residues, one residue yielding two potentially reactive carbonyl groups. The signal from one of the four detected protons on 2-AB should therefore comprise 8% and 16% of the signal intensity of the H-1 signal for mannuronic acid units, assuming that maximum one of the two potential sites on the end residues was attacked by periodate, however, it only comprised exactly half the theoretical amount. The experiment were also repeated using pullulan with $DP_n = 51$, in this case assuming no double oxidation at the α-(1-6) linkage, giving the same result (data not shown).

Since the kinetic data suggest that the labelling reaction was complete we take this as indirect evidence that the oxidised units are monosubstituted and that 2-AB is only able to react with one of the two vicinal aldehydes formed upon periodate oxidation. It is not clear whether this is due to the fact that the 2-AB cannot react with an aldehyde group when an other 2-AB molecule is located in its close vicinity or if one of the aldehydes formed upon periodate oxidation is simply not reactive towards 2-AB. When substituted hydroxylamines was reacted with cellulose with low degrees of oxidation (~1%) both aldehydes formed seems to be reactive (Potthast, Kostic, Schiehser, Kosma, & Rosenau, 2007). On the other hand when periodate oxidised alginate was substituted with a polyether via direct reductive amination only 0.12 mol polyether was incorporated per mol of uronic acid as opposed to 0.40 mol if 100% conversion was to be obtained (Carré, Delestre, Hubert, & Dellacherie, 1991).

The 2-AB labelled standards were analysed on SEC-MALLS with fluorescence detection and no degradation was observed for any of the samples analysed. Unreacted 2-AB interacts with the column material similar to CCOA, but in this case the interaction was weaker and using the buffer described earlier containing 20% (v/v) acetonitrile, 2-AB elutes off the column just after the salt peak. In an attempt to avoid this interaction completely the samples were labelled with 2-aminobenzoic acid (2-AA) as an alternative to 2-AB. It was suspected that the charge on 2-AA would minimize the interaction with the column material. However, no significant

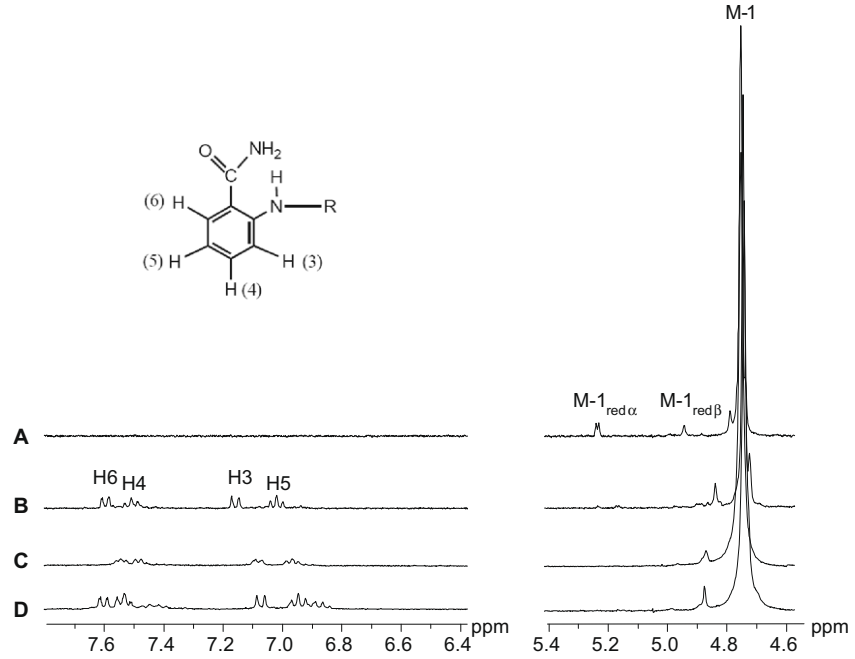

Fig. 5. ^{1}H NMR spectra of a mannuronan oligomer are shown before (A) and after (B) labelling with 2-aminobenzamide (2-AB) at the reducing end. C and D shows a 4% and 8% periodate oxidised mannuronan oligomer (borohydride reduced before oxidation), respectively, after labelling with 2-AB. All samples were labelled for 24 h. The peaks appearing in the 6.8–7.7 ppm region belongs to the four protons on 2-AB as indicated. The chemical shifts of the protons on 2-AB are assigned according to Ishii et al. (2002) and modelling using ChemBioDraw Ultra version 11.0. Abbreviation; *R* = anchor point for carbonyl group.

improvement was observed, but instead it was found that the 2-AA violated the second criteria mentioned earlier resulting in a small shift of the emission wavelength depending on where the label was located on the polysaccharide.

As for the two previous methods tested the method was able to detect the reducing end of pullulan, dextran and alginate. The additional carbonyls in periodate oxidised alginate up to 8% oxidation were also detected. Taken into account that 2-AB is only reactive towards one of the two aldehydes formed per residue upon periodate oxidation, the plot fluorescence/RI introduced earlier can be described by:

Fluorescence $\propto n_e + n_{ox}$

$$\text{Fluorescence} = A(n_e + n_{ox}) = A\left(\frac{n_0}{DP} + n_0 D_{ox}\right) = An_0\left(\frac{1}{DP} + D_{ox}\right) \quad (1)$$

RI = mass = $M_0 n_0$

$$\frac{\text{Fluorescence}}{RI} = \frac{An_0}{M_0 n_0} \cdot \left(\frac{1}{DP} + D_{ox}\right) = \frac{A}{M_0} \cdot \left(\frac{1}{DP} + D_{ox}\right) \quad (2)$$

A = constant, n_e = number of reducing end residues, n_{ox} = number of oxidised residues, n_0 = number of monomers, M_0 = monomer weight, D_{ox} = degree of oxidation (= n_{ox}/n_0).

The constant A, expressing the relationship between fluorescence intensity and number of carbonyl groups, can be found from the slope of the linear plot (A = slope M_o), while the degree of oxidation can be estimated from the intercept (D_{ox} = Intercept/(A/M_0)). A plot of the detected degree of oxidation versus theoretical degree of oxidation yielded $R^2 = 0.99$. Fig. 6 show that the linearity of the plots was reduced as the degree of oxidation was increasing. This is partly due to degradation during oxidation, but foremost the presence of small aggregates in these samples. The aggregates were present in both labelled and unlabelled oxidised samples possibly due to intermolecular cross-linking (Gutherie, 1961), since they disappear upon borohydride reduction. Although the aggregates constitute a very small part of the sample (<1%), they affect the light scattering signal to a significant extent in the high molecular weight region. Depolymerisation during periodate oxidation of alginate has been previously described and the shift in the RI-profile (Fig. 6) is mainly due to this and cannot be attributed to conformational change alone (Vold et al., 2006).

Sphagnan was also analysed using the 2-AB method resulting in an average carbonyl content that was identical to the one obtained using tritium labelling. The fluorescence signal was corrected for a background emission of 3.8% of the total intensity, originating from unlabelled sphagnan.

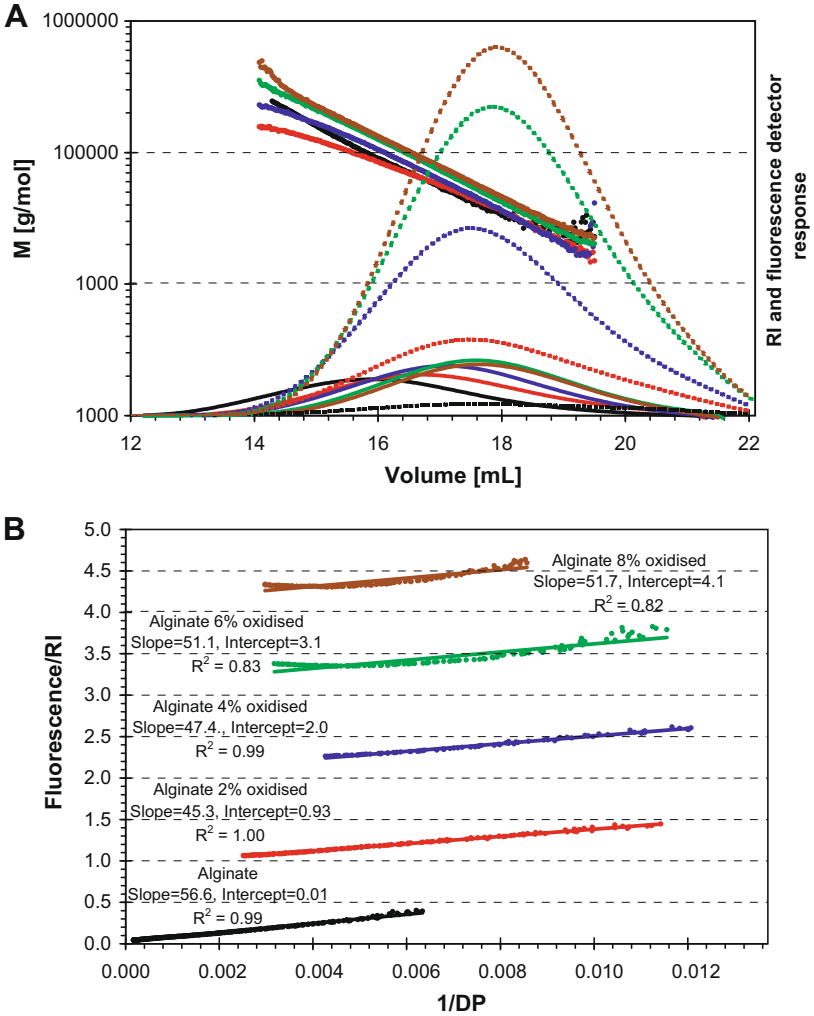

Fig. 6. Carbonyl detection using the 2-aminobenzamide (2-AB) method applied to alginate (40% guluronic acid) partially oxidised 2%, 4%, 6% and 8%. Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and fluorescence (dotted lines) profiles. Lower figure (B): monomer weight is taken as 198 g/mol for both oxidised and unoxidised alginate residues. For colours, see on-line version.

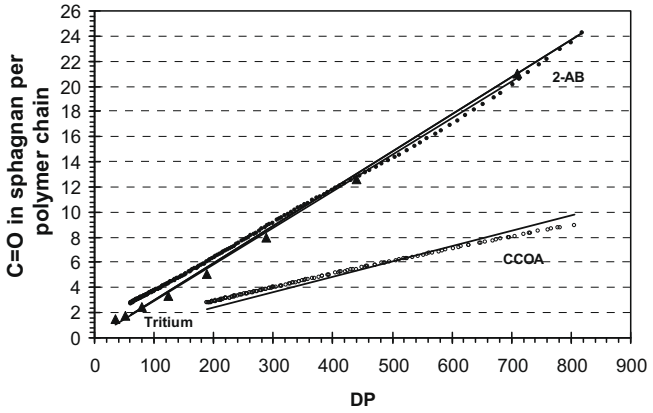

Fig. 7. The carbonyl content in sphagnan relative to a pullulan standard using the tritium (▲), 2-AB (●) and CCOA (○) approach. The data in the figure can be fitted to linear functions; slope 2AB and tritium method = 0.03 (R^2 = 0.99), slope CCOA method = 0.012 (R^2 = 0.96). The 2-AB method cannot detect ketones.

3.4. Comparison of the tritium, CCOA and 2-AB methods for determining the carbonyl distribution in sphagnan

Applying both the NaB^3H_4 and the 2-AB method to the pectic polysaccharide sphagnan an average carbonyl content of 2.9 carbonyl groups per 100 monomers was detected. Fig. 7 also shows that the two methods give the same carbonyl distribution. The CCOA method however, estimates an average content of 1.4 carbonyl groups per 100 monomers. The carbonyl profile is not only lower on average, but underestimates the carbonyl content at higher molecular weights compared to the two other methods. The reason for this could probably be found in a combination of different factors. The sample aggregates upon labelling and thus the molecular weight is overestimated. Aggregation might also affect the reaction rate between the label and the carbonyl functionalities in the polymer. If labels are closely located in space this might also lead to fluorescence quenching resulting in a lower fluorescence signal (Miller, 2005). All in all the CCOA method does not seem to be the appropriate method of choice for this sample.

The nature of the carbonyl groups in sphagnan is not known. However, it is widely accepted that aromatic amines can only react with aldehyde groups and not with ketones (Borch, Bernstein, & Durst, 1970). Since the tritium and 2-AB approach gives the same result it seems acceptable to assume that all the carbonyl groups in sphagnan are in fact aldehydes.

A novel monosaccharide, named 5-keto-D-mannuronic acid (5-KMA), was claimed to be present in sphagnan (Painter, 1983, 1991). The 5-KMA contains a keto group that should, if present, be detected by the method using NaB^3H_4, but not by the 2-AB method. The fact that the two methods give identical results implies that 5-KMA does not exist in sphagnan, which is in accordance with the recent observations made by Ballance et al. (2007, 2008).

The average carbonyl content of sphagnan was estimated by Ballance et al. (2008). Sphagnan extracted with or without the use of chlorite was reacted with either hydroxylamine or phenylhydrazine at pH 4–4.5 and the total nitrogen content was measured. The results were all similar and the *N* content of sphagnan treated with hydroxylamine was 0.63% ± 0.085 (n = 4). Given an *N* content in untreated sphagnan of 0.2% the carbonyl content was estimated to an upper limit of 5.6 carbonyl groups per 100 monomers. It was however, emphasised that the nitrogen analysis method used was on the borderline of both the sensitivity and resolution of the instrument. We believe that the methods presented here give a more precise determination of the carbonyl content in sphagnan.

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