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Simultaneous determination of substituent patterns in partially acid hydrolyzed O-Me/O-Me-d_3-cellulose and quantification of the obtained oligomers by HPLC-ESI-MS

Julia Cuers [a], Inga Unterieser [a], Walther Burchard [b], Roland Adden [c], Marian Rinken [d], Petra Mischnick [a,e,*]

[a] Technische Universität Braunschweig, Institut für Lebensmittelchemie, Schleinitzstr. 20, 38106 Braunschweig, Germany
[b] Albert-Ludwig-University of Freiburg, Institute of Macromolecular Chemistry, 79104 Freiburg, Germany
[c] Dow Wolff Cellulosics GmbH, Research & Development, August-Wolff-Str. 13, 29699 Bomlitz, Germany
[d] Dow Deutschland Anlagengesellschaft mbH, Werk Stade, Bützflether Sand, 21683 Stade, Germany
[e] KTH Stockholm, Fibre and Polymer Technology, Teknikringen 56–58, SE-10044 Stockholm, Sweden

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ABSTRACT

Substituent patterns in oligosaccharide derivatives obtained from methyl cellulose were determined up to DP10 by electrospray ionization mass spectrometry employing separation of the oligomer fractions by HPLC. Oligosaccharides were labeled with *meta*-aminobenzoic acid after perdeuteromethylation and partial hydrolysis of methyl cellulose, enabling simultaneous quantification according to DP by HPLC/UV. Control of the HPLC-method was performed with a defined oligomer mixture obtained from β-cyclodextrin.

Results from LC-ESI-MS are discussed in comparison with those from syringe pump injection and compared to a calculated pattern for a random distribution. Programing of instrumental parameters optimized for each DP and avoidance of competition of successively eluting analytes in the electrospray process allowed extension of the established method for determination of the substitution pattern of cellulose derivatives along the polymer chain from DP5 to DP10 and thus a significant gain of information.
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1. Introduction

The physicochemical properties of polysaccharide derivatives as cellulose ethers are influenced by their molar mass distribution, the type of substituents, and the substituent distribution in the glucosyl unit, along and over the polymer chains.[1] For better understanding of the relationship between the chemical structure of cellulose ethers and their properties, the substituent pattern has to be investigated carefully. Different methods for examinations on the monomer level, the glucose, are well established.[2] However, to obtain further information about heterogeneities along and over the chains, oligomers obtained by partial depolymerization have to be analyzed. Due to the complexity of analytes, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry (MS) have become key techniques in this field.[3–7]

For statistic evaluation of substituent patterns it is necessary to obtain data of high accuracy, though quantitative analysis by mass spectrometry is a critical endeavor. Evaluation of the substituent pattern by MS has been successfully applied to different cellulose ethers[4,6–8] and other 1,4-glucans[9] in the past, but in all cases the obtained data is limited to a maximum DP of 4 or 5 due to the discrimination effect during ionization and ion transfer. The different ionization efficiencies resulting from the diversities in size and chemical structure of the analytes provoke that the relative signal strength can vary significantly. In ESI, parameters like surface activity, electrophoretic mobility, and cation coordinating properties of the analytes in interaction with the solvent and instrumental parameters play an important role for the ion yield.[10–12] Competition of homolog analytes in mixtures can cause discrimination of especially the high molecular weight representatives.[13] Due to their higher mobility, small molecules will most likely be enriched at the surface so the probability for them to reach the next droplet generation is higher than for larger molecules with the same chemical structure.[11] In ESI-ion trap (IT)-MS, the target mass, which is related to the trap drive, has the strongest effect on the ion yield, however it is not easy to find adjustments which allow a quantification over a wide m/z range.[14]

To avoid potential competition during the ionization process in ESI, oligosaccharides can be separated by high-performance liquid chromatography (HPLC) prior to ESI-MS. Moreover the coupling of LC and MS bears the advantages of programing optimized instrumental MS measurement parameters for each DP within one run and automatization for high sample throughput.

Additional information can be obtained by simultaneously running HPLC/UV of chromophor-labeled analytes, which provides the molar amounts of the oligomer fractions. Therefore reductive

* Corresponding author. Fax: +49 531 391 7230.
E-mail address: p.mischnick@tu-braunschweig.de (P. Mischnick).

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amination[15–18] is a powerful tool for chromatographic, electrophoretic, and mass spectrometric applications and it is also used in this work for quantification of O-CH_3-cellooligomers gained by partial degradation.

2. Results and discussion

Methyl cellulose (MC, Methocel™ A4M of DowWolff Cellulosics, DS(GC) = 1.96, DS(Zeisel) = 1.82) was perdeuteromethylated and submitted to partial hydrolysis with trifluoroacetic acid according to the common procedure for oligomer analysis of cellulose derivatives.[4] To simultaneously control the degree of depolymerization, oligosaccharides were labeled with *m*-aminobenzoic acid (*m*ABA) and quantified by HPLC/UV. The determined DP distribution is compared with the model for a random (most probable) depolymerization.[19]

ESI-MS data of the oligomers were used for evaluation of the *O*-Me/*O*-Me-d_3 pattern which is representative for the substituent distribution in the polymer chains and thus can indicate heterogeneities over the polymer chains (heterogeneities of first order) and along the chains (heterogeneities of second order).

2.1. HPLC method development

HPLC with fluorescent or UV detection is widely applied for separation and characterization of carbohydrates.[17,18] Therefore a chromophore has to be introduced to the molecule which is usually performed by reductive amination at the reducing end.[18,20–22] This procedure facilitates quantification and detection sensitivity,[21] since for example refractive index (RI) detection is not very sensitive and not applicable for gradient elution.

For substituent pattern analysis by ESI-MS, labeling of the oligomers prior to HPLC separation is not necessary but provides additional information on DP distribution and thus the degree of depolymerization. The kinetics of hydrolysis can be assessed to gain understanding whether partial depolymerization proceeds randomly. Thus, for screening of the partial hydrolysis with respect to optimize yields of oligomers of interest, cellooligomers were labeled by reductive amination with *meta*-aminobenzoic acid and 2-picoline borane[23] under conditions elaborated for quantitative labeling with *o*-aminobenzoic acid.[24]

By pre-testing the retention behavior of the oligosaccharides by thin layer chromatography (TLC), a RP_{18} stationary phase turned out to be the most suitable, using acetonitrile and water as basic components of the mobile phase. Since *m*ABA bears a carboxyl group and an amine function, the pH is an important factor during chromatography on a reversed phase column. It is necessary to either add an acid to the mobile phase so the carboxyl group stays non-dissociated, or perform ion pair chromatography at higher pH.

Two different eluent additives were tested. First acetic acid (1 vol %) was added to both components of the mobile phase resulting in a pH of approx. 2.8 (pK_a of *m*ABA = 4.75)[25] to repress dissociation. For good separation the acetonitrile concentration was increased linearly from 20% (t = 0 min) to 100% (t = 60 min).

As a second additive for good chromatographic performance at a higher pH, triethylammonium acetate (TEAA) was added to the mobile phase at a concentration of 10 mM.[26] The pH of this buffer solution is nearly neutral, so the carboxyl group is deprotonated and paired with the triethylammonium cation. With the same gradient as mentioned before, good separation could be achieved, except for DP1 and the excess reagent used for reductive amination. The two peaks partially overlap unless the gradient is changed to lower rate of acetonitrile increase.

Figure 1 shows two UV chromatograms of an oligomer separation with both mobile phase systems. The TEAA additive gives narrower peaks at shorter retention times but also some non-assignable signals. Due to the pH-sensitive UV absorption of compounds like *m*ABA, wavelengths for detection have to be adapted to both mobile phase systems.

*m*ABA shows two absorption maxima for the $\pi\rightarrow\pi^*$ and the $n\rightarrow\pi^*$ excitation, 254 nm and 330 nm at pH 2.8 (HOAc), and with TEAA as mobile phase additive (pH 7) at 254 nm and 314 nm. For detection at $\lambda \leqslant 300$ nm a problem arises from the absorption of excess 2-picolin borane which elutes after approximately 30 min and can interfere with signals of the labeled carbohydrates. At wavelengths below 250 nm the increasing acetonitrile ratio during gradient elution caused a slope of the baseline but when using gradient grade solvents quantification is not influenced.

In conclusion, all following HPLC and LC–MS experiments shown here were carried out with ACN/H_2O with 1% acetic acid as mobile phase, due to the easier handling, cleaner spectra, and better compatibility with the mass spectrometer.

2.2. Calibration of HPLC-method

For the quantification of the *O*-Me/*O*-Me-d_3-cellooligomers the HPLC-method was calibrated with *N*-(2-carboxyphenyl)-1-amino-1-deoxy-2,3,6-tri-*O*-methyl-D-glucitol, which represents DP1 of the partially hydrolyzed permethylated and *m*ABA-labeled MC.

Application of this calibration to all homologs requires that the molar absorption of the oligomers does not change with their size, and that the changing ACN/H_2O ratio during gradient elution has no solvent-effect on the absorption. In order to control these potential effects, a simple experiment was performed using permethylated β-cyclodextrin (Me-β-CD) as a model compound. Two equal portions of Me-β-CD were submitted to hydrolysis, in parallel. One sample was partially hydrolyzed to give oligomers in the range of DP1–DP7, while the other one was completely depolymerized to 2,3,6-tri-*O*-methylglucose. The chromatograms are shown in Figure 2. If the molar absorption is independent of DP and the eluent composition, but caused by the chromophore (the label) only, evaluation on basis of the calibration with 2,3,6-tri-*O*-Me-glc should give the same total amount of anhydroglucose units (AGU) for both Me-β-CD samples.

This study is only performed with HOAc as mobile phase additive, because TEAA shows additional peaks even in this very neat sample (data not shown). Moreover using triethylamine for ESI-IT mass spectrometry is not advisable for different reasons mentioned in Section 2.4.

The peak area of the UV signals is converted into molar amounts and then multiplied by DP to obtain total molar amounts of AGU. As shown in Figure 3, the same amount of AGU is found for total and partial hydrolysis at λ = 330 nm.

The sample recovery of the labeled oligomers is 102% for the total hydrolysis and 104% for the partial hydrolysis. Since the number of oligomers is limited and no tetra-*O*-methylated end groups are present, the recovery is very good. But it has to be kept in mind that the relative error increases with increasing DP and decreasing intensity. In conclusion the molar UV response is independent of the DP and the eluent composition of permethylated *m*ABA labeled cellooligosaccharides.

2.3. Estimation of degree of depolymerization

To find the most suitable conditions of partial hydrolysis for oligomer analysis of MC, a time course study was performed with 1 M TFA at 120 °C. After labeling with *m*ABA the samples were analyzed by HPLC.

In all chromatograms obtained by HPLC/UV (ACN/H_2O/HOAc, λ = 330 nm) the peak for DP1 is the most abundant in all samples as expected for a most-probable distribution.[19,27] The molar

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