A New Approach for Studying Correlations between the Chemical Structure and the Rheological Properties in Carboxymethyl Cellulose

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Two model sodium carboxymethyl celluloses (CMC) with similar monomer composition but with significant differences in the viscoelastic properties, that could not be assigned to variations in the average molar mass or molar mass distribution, were investigated with respect to the fraction of nonsubstituted cellulose segments in the polymers. The CMCs were hydrolyzed by a purified highly selective endoglucanase. The average molar mass and molar mass distribution of the enzyme products, as measured by size-exclusion chromatography with online multi-angle light scattering and refractive index detection (SEC/MALS/RI), revealed that the enzyme-catalyzed hydrolysis was more effective on one of the CMCs. To investigate whether this was due to a higher fraction of nonsubstituted cellulose segments in the polymer, the concentrations of nonsubstituted enzyme products, e.g., cellotetraose and cellopentaose, were measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). It was concluded that the two CMCs displayed significant differences in the fraction of nonsubstituted cellulose segments. Furthermore, the CMC with the strongest attractive intermolecular interactions, according to rheometry, also contained the highest fraction of nonsubstituted cellulose segments.

Introduction

Carboxymethyl cellulose (CMC) is a commercial water-soluble product where carboxymethyl groups have been introduced along the cellulose backbone. CMC is biodegradable and nontoxic and can be found in an increasing number of applications, e.g., in food, health care and pharmaceutical products.[1] In order to improve the functionality in existing applications, or to develop new, it is essential to determine the physicochemical, e.g., viscoelastic and rheological, properties of CMC.[2] These properties are known to be affected by, not only the molar mass and molar mass distribution, but also the degree of substitution (DS) and the substituent distribution in the polymer.[2−4] For instance, it has been suggested that nonsubstituted cellulose segments cause attractive interactions between CMC molecules in solution, which may result in aggregation and therefore a change in the flow properties.[2,4,5]

There are several well-established characterization techniques for analyzing many of the parameters that affect the properties of CMC. The molar mass and molar mass distribution can be analyzed using size-exclusion chromatography (SEC)[6−9] while the DS can be analyzed by several techniques including nuclear magnetic resonance (NMR) spectroscopy, titrimetry, and chromatography.[10−15] In a recent publication, we showed that the DS in the intact CMC can be estimated by partial acidic hydrolysis in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS).[16] The determination of the substituent distribution in CMC is, however, still not a straightforward task even though several approaches have been presented in the literature.[17−22] Recently Adden et al.[23] presented a study on methyl cellulose where the substituent distribution at different levels was investigated. Fractionation, on basis of polarity, prior to chemical derivatization and chemical structure analysis, made it possible to differentiate between heterogeneities of the first (intermolecular) and second order (intramolecular).

By applying cellulose selective enzymes, such as endoglucanases, it is possible to selectively hydrolyze the glucosidic bonds in cellulose in regions containing few or no carboxymethyl groups.[24−26] The chemical structure, and concentration, of the enzyme products will depend on the selectivity of the enzyme and on the chemical composition (e.g., DS and substituent distribution) of the substrate. Therefore, selective hydrolysis may serve as an analytical tool for analysis of the substituent distribution in CMC.[4,9,17,19,21]

The objective of this study was to investigate correlations between the substituent distribution and the flow properties of CMC. In this new approach, highly selective endoglucanase hydrolysis of CMC was performed prior to SEC coupled to multi-angle light scattering and refractive index detection (MALS/RI) and MALDI-TOFMS. By using a highly selective endoglucanase, one benefits from a more restricted hydrolysis where longer nonsubstituted oligomers (e.g., cellotetraose and cellopentaose) are obtained as hydrolysis products. This made it possible to study the uniformity of the hydrolysis and also to quantify the nonsubstituted oligomers released by the enzyme. In addition, rheological oscillating experiments were performed on aqueous solutions of CMC in order to detect sensitive superstructures, originating from intermolecular interactions in the relaxed state.[2,27,28] The combined results from these analyses can be used as an indicator on how the detected variations in the flow properties are affected by the substituent distribution in the CMC.

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Experimental Section

Chemicals. Two model sodium carboxymethyl celluloses, CMC A (DS 0.98, 30.3% glucose) and CMC B (DS 0.99, 29.6% glucose), of purity >99.9%, according to the manufacturer, were provided by Akzo Nobel (Arnhem, Netherlands). The water contents in the CMCs were about 6% as measured by thermogravimetric analysis. The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich (Stockholm, Sweden). Ammonium sulfate ("for biochemistry" grade) and acetic acid were from Merck (Darmstadt, Germany). Anhydrous ammonium acetate was purchased from Acros Organics (Geel, Belgium). The cellotetraose (purity ≈ 95%) and cellopentaose (purity ≥85%) standards that were used for quantification were from Fluka (Buchs, Switzerland). MilliQ grade water from a Synergy 185 UV Ultrapure Water System (18.2 MΩcm, Millipore AB, Solna, Sweden) was used in all experiments. All chemicals were used without further purification.

Enzymatic Hydrolysis of CMC. The endoglucanase Cel45A of *Trichoderma reesei*, purified according to Karlsson et al.,[29] was used for hydrolyzing the CMCs. The endoglucanase showed no side activities. The CMCs were dissolved (10 g L^{-1}) in 50 mM ammonium acetate, pH 5.0, before addition of the enzyme (2 mg of enzyme/g CMC). Hydrolysis was performed for 24 h at 45 °C before termination, by boiling for 5 min, followed by centrifugation in order to remove enzyme precipitates. Substrate blanks, where buffer instead of enzyme was added and treated at identical conditions, indicated that no oligomers were formed without the addition of enzyme. Three separate hydrolysates were prepared from each CMC.

Quantification of Cellotetraose and Cellopentaose by Standard Addition. A small volume of the enzymatic hydrolysates were purified by filtration through a micro centrifuge filter with a molecular weight cutoff (MWCO) of 10 000 g/mol (Pall Norden AB, Lund, Sweden). When most of the solution had passed through the filter, additional water was added (three times) in order to recover as high fraction as possible of the low-mass analytes. The filtered solution was evaporated at 70 °C under nitrogen, and the residue was diluted to 0.50 g L^{-1} (calculated from the initial sample concentration) in water. These samples (filtrates) were used without further purification. Stock solutions of cellotetraose and cellopentaose were prepared by mixing 1.08 mg of cellotetraose and 1.16 mg cellopentaose with 1080 μL and 1160 μL H_2O, respectively. These solutions were further diluted to a final concentration of 0.0100 g L^{-1} and used as standard addition solutions. A 500 μL amount of the sample (filtrate) solution was used in each standard addition procedure where standard solution was added in 50 μL portions. After each addition, 10 μL of the mixed solution was extracted for analysis before continuing with the next of totally four additions.

Rheometry. The rheological measurements were made using a StressTech rheometer (Rheologica, Lund, Sweden) equipped with a 4 cm, 1° cone, and plate system. An external water bath, connected to the measuring geometry, ensured that the temperature was kept constant at 20.0 ± 0.1 °C. The measurements were performed by applying an oscillating stress sweep using an equilibrium time of 30 s, a frequency of 1 Hz, and a delay time of 1 s. Measurements were made using aqueous solutions (5 g L^{-1}) of CMC. Five separate measurements were made for each solution.

SEC/MALS/RI. The molar mass distributions in the intact and the enzymatically hydrolyzed CMCs were analyzed by SEC/MALS/RI. The equipment was comprised of an LC-10ADvp pump, a SIL-10ADvp autoinjector (Shimadzu Corporation, Tokyo, Japan) with DAWN EOS and Optilab rEX (Wyatt Technology Corp., Santa Barbara, CA) MALS and RI detectors. The effluent was degassed online with a Degasys Populaire (P4003) (Sanwa Tsusho Co., Ltd., Tokyo, Japan). The separation was carried out on a TSKgel $GMPW_{XL}$ mixed bed column 30 cm × 7.8 mm ID, particle size of 13 μm (TosoHaas Bioseparation Specialists, Stuttgart, Germany) at a flow rate of 0.5 mL min^{-1}. Ammonium acetate buffer (50 mM, pH 5) was used as mobile phase. The intact CMCs were dissolved to a concentration of 0.25 g L^{-1} while the hydrolysates were diluted to a concentration of 3 g L^{-1}. All samples were filtered through a 0.45 μm syringe filter (Millex-LCR, Millipore AB) before the analysis. The data from the detectors were processed using ASTRA software (ASTRA for Windows 4.90, Wyatt Technology Corp.). Refractive index increment $dn/dc = 0.136$ were used for the calculations.[30] Double injections of 100 μL were made for all samples.

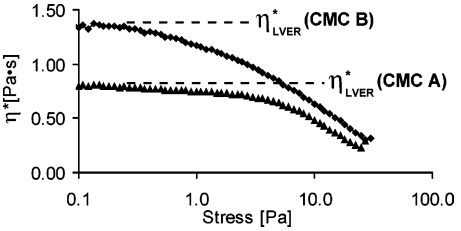

Figure 1. Complex viscosity [η^*] as a function of stress for aqueous solutions (5 g L^{-1}) of CMC A and B, measured by an oscillation stress sweep. It can be seen that the complex viscosity in the linear viscoelastic region (η^*_{LVER}) is significantly higher for the CMC B solution compared with CMC A. The plots are averages from five separate measurements (relative standard deviation (RSD) < 7%, $n = 5$).

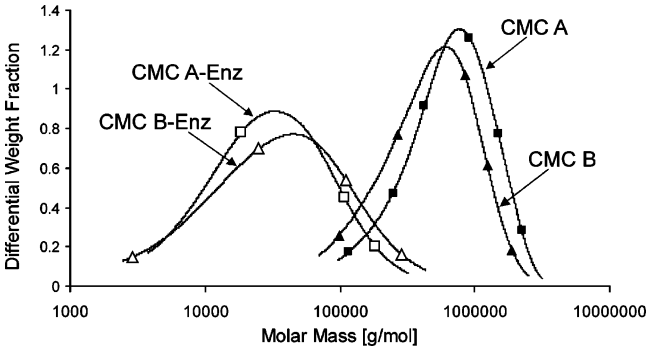

Figure 2. Differential molar masses for the intact (CMC A, CMC B) and enzymatically hydrolyzed (CMC A-Enz, CMC B-Enz) CMCs as measured by SEC/MALS/RI. It can be seen that the average molar mass of CMC A is higher compared to CMC B in the intact samples. For the hydrolysates, CMC B-Enz displays a more heterogeneous hydrolysis and a lower reduction in molar mass compared to CMC A-Enz. The symbols along the curves are presented in order to improve clarification.

MALDI-TOFMS. The MALDI-TOFMS experiments were performed using a Bruker UltraFlex MALDI-TOFMS (Bruker Daltonics, Bremen, Germany) according to Enebro et al.[16] with some modifications. The volume ratios used in the sample preparation were 20:20:1 (sample:matrix:$(NH_4)_2SO_4$). The detector mass range was set to m/z 650−1500 and spectra were gathered by irradiating 25−30 different positions at the center area on the sample spot with a total of 1250−1500 laser shots.

Results

Viscoelastic Properties. The viscoelastic properties of the aqueous CMC solutions were initially analyzed by rheometric measurements using an oscillation stress sweep. The results from the rheology measurements indicated large differences between the two CMCs. Figure 1 presents the complex viscosity as a function of stress. The complex viscosity in the linear viscoelastic region (η^*_{LVER}) was about 70% higher for CMC B than for CMC A. In addition, the phase angle was about 10° lower for CMC B, indicating a more elastic behavior of this solution.

SEC/MALS/RI. Molar mass determinations of the intact and enzymatically hydrolyzed CMCs were performed in order to investigate the extent of the hydrolysis. Figure 2 displays the differential molar mass plots of the intact and the hydrolyzed

Table 1. Calculated Weight Average Molar Mass (M_w), Number Average Molar Mass (M_n) and the Polydispersity Index (PDI) Values from the SEC/MALS/RI Data

sample	M_w [kg/mol]	M_n [kg/mol]	PDI (M_w/M_n)
CMC A[a]	800	490	1.64
	810	510	1.60
CMC B[a]	590	340	1.74
	580	330	1.78
CMC A-Enz[b]	48 (2%)	20 (3%)	2.46 (2%)
CMC B-Enz[b]	65 (5%)	22 (12%)	3.03 (7%)

[a] Individual values from two injections [b] Average values from six measurements (RSD in brackets)

CMCs, and the numerical results are presented in Table 1. Figure 2 and Table 1 show that CMC A had a slightly higher average molar mass than CMC B. After enzymatic hydrolysis, the average molar mass of CMC A (CMC A-Enz) was lower than that of CMC B (CMC B-Enz).

The results demonstrate that both CMCs were hydrolyzed by the endoglucanase. Both hydrolysates still contained rather high fractions of high mass CMC molecules. The reason for this is unknown but a possible explanation could be that the DS of the high mass fraction is too high for enzymatic hydrolysis. This limited enzyme efficiency for hydrolyzing CMC has previously been reported by Karlsson et al. where the catalytic core domain of the identical endoglucanase (Tr Cel45Acore) was used.[31]

The polydispersity index values (Table 1) showed that both the extent and the heterogeneity of the hydrolysis differed between the CMCs, where CMC B displayed a more heterogeneous hydrolysis, which resulted in a higher fraction of high mass products. In addition the amount of low mass products was also higher in CMC B (as presented in Quantification of Cellotetraose and Cellopentaose by Standard Addition below).

The low sensitivity of MALS for low molar masses makes SEC/MALS/RI a less appropriate analytical technique to measure the concentration of the low mass products. MALDI-TOFMS was, thus, employed for the chemical characterization and quantification of the low mass products.

MALDI-TOFMS. Figures 3a and 3b display mass spectra of hydrolyzed CMC A (Figure 3a) and CMC B (Figure 3b), covering the low-mass range where peaks corresponding to oligomers with a degree of polymerization (DP) of 4 and 5 have been labeled and connected by dashed lines.

Nonsubstituted cellotetraose and cellopentaose were readily observed in the spectra. It has previously been shown by Karlsson et al. that these oligosaccharides are enzyme products that cannot be further hydrolyzed by Tr Cel45A.[29] Furthermore, qualitative comparison of the two mass spectra (Figure 3a and Figure 3b) revealed that oligomers with similar chemical composition are present in the hydrolysates from both CMCs. This is expected because the chemical composition of the oligomers formed in the hydrolysis mainly depends on the endoglucanase selectivity on CMC.

The relative peak intensities of the detected oligomers differed between the two CMCs. CMC B contained a significantly higher fraction of cellotetraose and cellopentaose (in relation to the substituted oligomers) compared to CMC A. This alone does not prove that the CMC B hydrolysate contains a higher concentration of these compounds because MALDI-TOFMS is not an inherently quantitative method. It is therefore necessary to also determine the concentration of cellotetraose and cellopentaose in the hydrolysates in order to make the conclusions regarding the release of these compounds in the enzymatic hydrolysis.

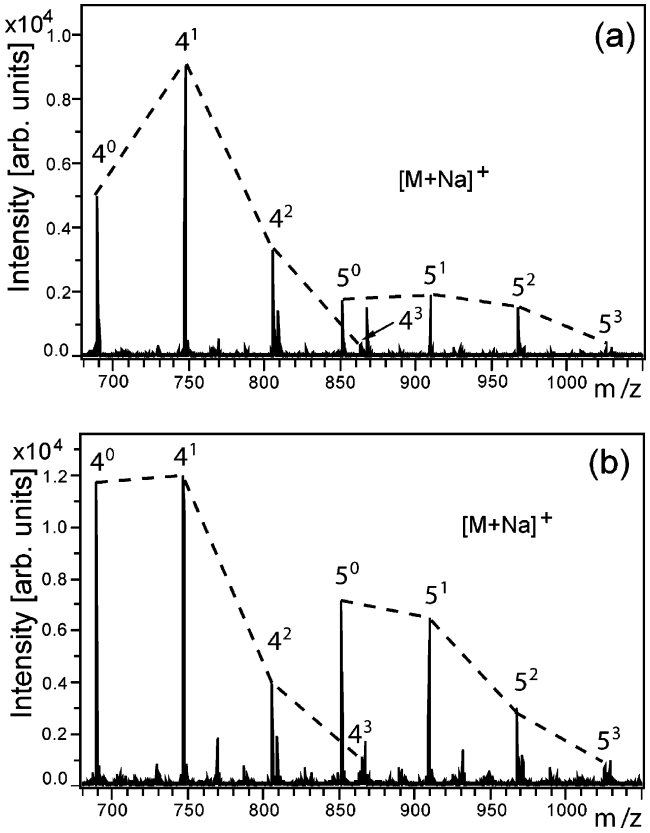

Figure 3. MALDI-TOF mass spectra of enzymatically hydrolyzed (a) CMC A and (b) CMC B. The labeling 4^1 indicates the ion corresponding to the oligomer with DP4 (large number) containing one carboxymethyl group (number in superscript). DHB (in H_2O) was used as matrix with $(NH_4)_2SO_4$ as additive,[16] and the spectra were collected in reflector positive ion mode.

Quantification of Cellotetraose and Cellopentaose by Standard Addition. The quantification of cellotetraose and cellopentaose in the hydrolysates were performed by consecutive standard additions of cellotetraose or cellopentaose before MALDI-TOFMS. Figure 4 presents the MALDI mass spectra before and after standard addition. The peak area ratios between peaks from nonsubstituted and monosubstituted oligomers increased for each addition.

Figure 5 displays standard addition plots where the peak area ratios for each addition have been plotted as a function of the concentration of added standard. A linear relationship was obtained between the added cellooligomer concentration and the detector response in the measured region. Therefore, it was possible to calculate the original cellooligomer concentration in the hydrolysates. Table 2 presents the concentration of cellotetraose and cellopentaose in the hydrolysates. The obtained data were reproducible as given by the standard deviation values.

The hydrolysate of CMC B contained twice as high concentrations of cellotetraose and cellopentaose as compared to the hydrolysate of CMC A. The accuracy in these values could be affected by the purity of the standards. Nevertheless, since the same standard solutions have been used for both samples, the relative differences in the cellotetraose and cellopentaose concentrations should not be affected.

Discussion

The rheometry measurements showed that CMC B had a significantly higher viscosity in the linear viscoelastic region,

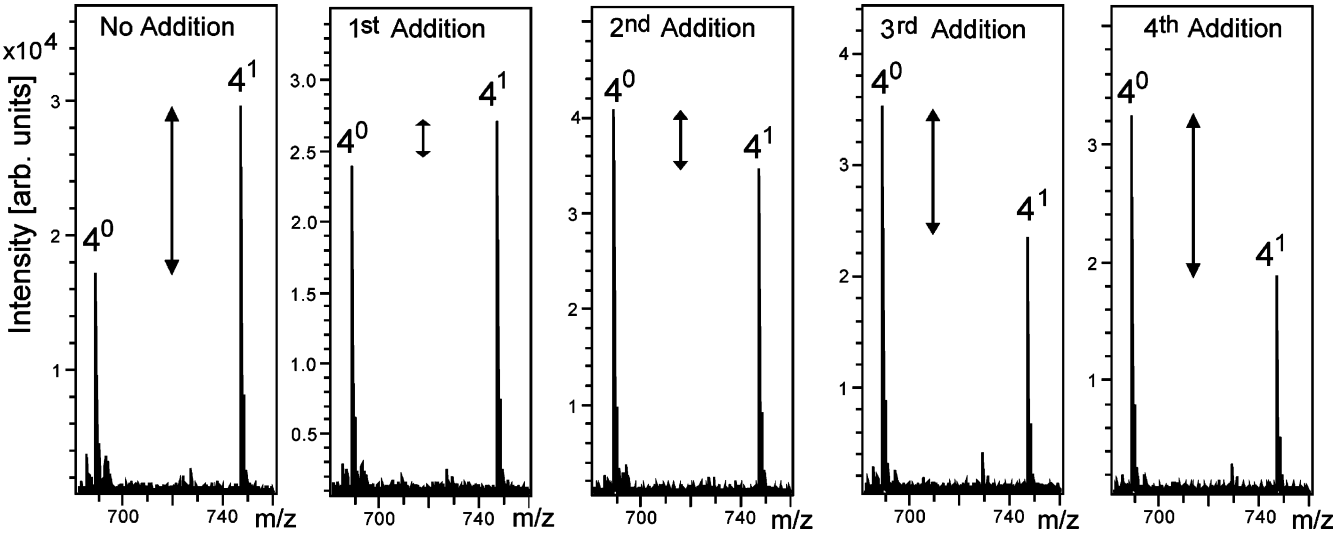

Figure 4. MALDI mass spectra from a standard addition series where cellotetraose standard has been added to the hydrolysate. It is seen that the peak ratio between the peaks corresponding to the nonsubstituted (4^0) and the monosubstituted (4^1) tetramers is increasing for each standard addition.

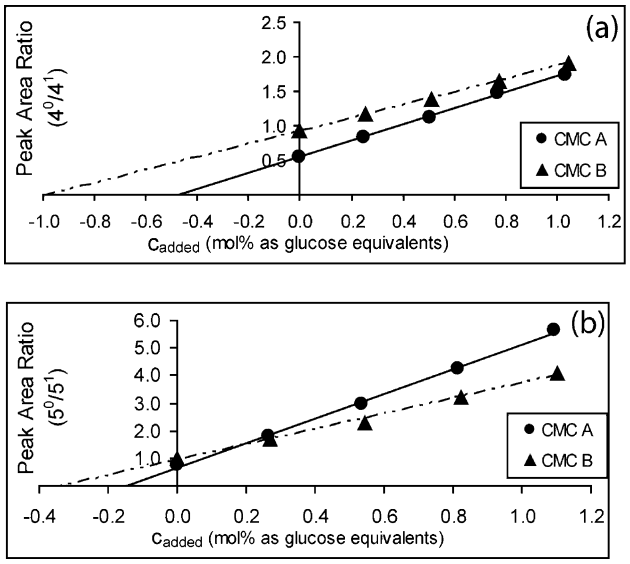

Figure 5. Standard addition plots for quantification of (a) cellotetraose and (b) cellopentaose concentrations in the hydrolysates from CMC A and CMC B. The plots are averages of three different hydrolysates for each sample.

Table 2. Concentration of Cellotetraose and Cellopentaose in the Hydrolysates of CMC A and CMC B as Measured by Standard Addition and MALDI-TOFMS

sample	cellotetraose [mol %][a]	cellopentaose [mol %][a]
CMC A	0.47 (±0.06)[b]	0.15 (±0.02)[b]
CMC B	0.98 (±0.03)[b]	0.34 (±0.01)[b]

[a] Calculated as glucose equivalents. [b] Standard deviation from three hydrolysates.

in comparison to CMC A (Figure 1). The molar mass of CMC B is lower than that of CMC A (Figure 2), which means that this effect cannot be explained by increased chain entanglements. It is known, however, that cellulose derivatives containing nonsubstituted segments can form associations by intermolecular interactions and thereby form superstructures in solution.[4,32] We therefore propose that the significant differences in the viscosity between CMC B and CMC A are due to a higher extent of intermolecular interactions caused by differences in the substituent distribution.

From the SEC/MALS/RI measurements it was seen that the fraction of high mass products was higher in CMC B than in CMC A. The low sensitivity of MALS in the low molar mass range ($<\sim$ 5 000 g/mol) does not allow for collection of information on the fraction of low mass products.

This was instead done by quantification of the release of cellotetraose and cellopentaose by MALDI-TOFMS. It was seen that the CMC B hydrolysate contained about twice as high concentration of these low mass products compared to CMC A. The higher fractions of low- and high-mass enzymatic products in CMC B indicate that the glucosidic linkages accessible to enzyme hydrolysis are more heterogeneously distributed in CMC B. This implies that the substituent distribution in CMC B is more heterogeneous compared to CMC A. The release of cellotetraose and cellopentaose is higher from CMC B but the glucose content is similar for both CMCs (≈30%). Therefore, it is proposed that the (nonsubstituted) glucose residues in CMC B are arranged into longer segments than in CMC A.

Conclusions

Use of enzymatic hydrolysis as pretreatment before MALDI-TOFMS and SEC/MALS/RI give results that can explain the variations in the viscoelastic properties of carboxymethyl cellulose. The CMC with the strongest intermolecular interactions in solution, as measured by rheometry, also contains the longest nonsubstituted segments according to our analytical method. SEC/MALS/RI and MALDI-TOFMS provide complementary information on molar mass and chemical structure of the enzymatic hydrolysis products. In addition, quantitative MALDI-TOFMS can be performed by standard addition methodology, similar as was earlier described for ESI-MS by Melander et al.[33]

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