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Analysis of the substituent distribution in the glucosyl units and along the polymer chain of hydroxypropylmethyl celluloses and statistical evaluation

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Abstract

Three hydroxypropylmethyl celluloses (HPMC 1, 2, 3; $DS_{Me} = 2.06$, 1.99, 2.04; $MS_{HP} = 0.21$, 0.19, 0.21) have been analyzed with respect to their methyl and hydroxypropyl pattern in the glucosyl units and along the polymer chain. The determination of the methyl pattern in the glycosyl unit was performed by GLC/MS after hydrolysis, reduction, and acetylation, while the distribution of hydroxypropyl residues in the monomers could be analyzed with higher sensitivity including a permethylation step prior to hydrolysis. To determine the distribution of the substituents along the polymer chain, a method developed for hydroxyethylmethyl cellulose (HEMC) was applied. This method comprises random partial acid hydrolysis after perdeuteromethylation and reductive amination with propylamine, followed by *N*- and *O*-alkylation, yielding completely alkylated and permanently charged oligosaccharide derivatives. These compounds could be quantitatively analyzed by means of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), since all discrimination effects related to the hydroxyalkyl groups are leveled off by the sample preparation procedure in combination with the choice of a MALDI-TOF instrument. Methyl data deviate to some extent from the random distribution calculated from the monomer composition, but in contrast to methyl cellulose (MC) or HEMC, it is not heterogeneous, but more regular. The distribution of HP groups is random within experimental error as has been found for HEMC as well.

Introduction

The amount and the distribution of substituents in a polysaccharide derivative strongly influence the physicochemical properties and biological functions. While location on certain positions in the glycosyl units might be essential in molecular recognition processes, the distribution on the structural level of the polymer molecules is of predominant importance for all properties influenced by cooperative effects as chain–chain interactions, e.g. in thermoreversible gelation. Methods to determine the substituent distribution in the glucosyl units of starch and cellulose derivatives are well established. NMR spectroscopy (Tezuka and Tsuchiya 1995), chromatographic (Erler et al. 1992; Heinrich and Mischnick 1997; Horner et al. 1999; Adden et al. 2005) and electrophoretic (Lazik et al. 2002) separation of appropriate monomer derivatives have been

applied successfully. Analysis of the substituent distribution along the polymer chain is very challenging. Only very recently, a method for hydroxyethylmethyl cellulose (HEMC) has been reported by our group (Adden et al. 2005). It combines a statistical approach, which has been applied to more simple polysaccharide derivatives before (Arisz et al. 1995; Mischnick and Kühn 1996; Mischnick and Hennig 2001) with an appropriate labeling procedure. This procedure allows quantitative evaluation of matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) of oligomeric features representing the mixed cellulose derivative. Another approach uses enzymes as selective tools in combination with glucose determination, size exclusion chromatography and mass spectrometry of oligomeric products obtained (Mischnick 2001; Altaner et al. 2003; Tüting et al. 2004a; Momcilovic et al. 2005). Progress in this field has been achieved by using sophisticated MS instruments in particular tandem MS methods (Tüting et al. 2004b; Adden and Mischnick 2005; Momcilovic et al. 2005).

Hydroxypropylmethyl cellulose (HPMC) is commercially produced by reaction of alkali cellulose with racemic methyl oxirane and methyl chloride usually present at the same time in a heterogeneous process. The resulting polymer consists of glycosyl units substituted in position 2, 3 or 6 either with methyl or 2-hydroxypropyl, the latter additionally occurring in an *O*-methylated or, which is more rarely, in an *O*-hydroxypropylated form. HPMC is widely used for building materials, as food additive, and in cosmetical or pharmaceutical products, because of its properties as a water-soluble thickener or as regulator for controlled drug-release. Unfortunately, cellulose ethers of the same specification but from various batches often show different properties e.g. in clouding point, or drug release profiles when used in tablet coatings. Improved understanding of the relation between production process, structural features, and properties will help to solve these problems and give ideas for new applications. The monomer composition of mixed cellulose ethers including hydroxyalkyl substituents as in HPMC is very complex. Without regarding tandem reaction already $4^3 = 64$ different patterns in the glucosyl units are possible. Ethylhydroxyethyl celluloses (EHEC) have been analyzed by Lindberg (Lindberg et al. 1987, 1988), analysis of HPMC by GLC-MS after preparation of glucitol derivatives has already been described briefly by our group (Mischnick 1998). Due to the hydroxyl function of the HP residue, each chemical transformation of remaining polysaccharide OH will also involve the substituent OH. Therefore, the mass difference of 58 Da/HP-group is retained and chemical uniformity as required for quantitative MS cannot be achieved. We therefore focused on leveling off the influence of substituents by labeling all analytes with an ion signal intensity-enhancing tag. It has been shown for HEMC that introduction of a permanent charge and analysis of the oligomeric mixture by MALDI-TOF-MS is sufficient to overcome all discriminating effects, thus allowing quantitative evaluation (Adden et al. 2005). Here we present our results obtained for HPMC.

Experimental

General

All reagents were of highest purity available and purchased from Fluka, Aldrich or Merck. MeI-d_3 was purchased from Deutero, Kastellaun, Germany, the HPMC were obtained from Wolff Cellulosics, Walsrode, Germany.

Instrumental

Electrospray ionization mass spectra (ESI-MS, positive mode) were recorded on an Esquire LC (Bruker Daltonics, Bremen, Germany). The partially degraded samples were dissolved in MeOH, and introduced directly via a syringe at a flow of 200 μl/h. Nitrogen was used as drying gas (4 l/min, 300 °C) and as nebulizer gas (10 psi). The following voltages were used: capillary 4500 V, end plate offset −500 V, capillary exit 120.0 V, skim 1 40.0 V, and skim 2 10.0 V.

Positive ion matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was carried out using an ULTRAFLEX time-of-flight (TOF/TOF) instrument (Bruker Daltonics, Bremen, Germany) equipped with delayed extraction and reflectron systems and a N_2 laser (337 nm) operating with 3 ns pulse width and 10^7–10^8 W/cm^2 irradiance at the surface of 0.2 mm^2 spots.

One μl of the samples containing equal volumes of oligosaccharide solution (~10 pmol/μl anhydro glucose units) and the ultraviolet-absorbing matrix (19 mg) α-cyano-4-hydroxycinnamic acid in 400 μl acetonitrile and 600 μl 0.1% (v/v) trifluoroacetic acid in H_2O were spotted onto the stainless steel target and dried at room temperature (r.t.). Spectra were recorded at an acceleration voltage of 25 kV using the delayed extraction facility and the reflectron for enhanced resolution.

All reactions were carried out in 1-ml V-Vials in a heating block with a stirring and a evaporation unit from Barkey, GmbH & Co. KG, Germany. IR spectra were recorded using a Bruker Tensor 27 ATR-IR.

Gaschromatographic analysis was carried out with a GC-FID instrument Carlo Erba GC 6000 Vega Series 2 with a CPSil 8 column (25 m), a retention gap (1.5 m), and H_2 as carrier gas. Injection was carried out on-column.

The temperature program starts at 60 °C for 1 min, heats with 20 °C/min to 130 °C, and heats again with 4 °C/min to 290 °C, remaining constant for 30 min. Data were recorded with a Merck Hitachi D 2500 Chromato-Integrator. For peak identification GC-MS with a Agilent 6890 GC and a JEOL GCmate II bench top double-focusing magnetic sector mass spectrometer was applied. The GC was equipped with a HP-5 column (30 m). A split injection port at 250 °C was used for sample application, and the split ratio was set at 5:1. Helium carrier gas was set to 1.5 ml/min flow rate (constant flow mode). Transfer line was kept at 250 °C.

The MS was operating in electron impact ionization (EI) mode at 70 eV with an ion source temperature of 180 °C. Low-resolution mass spectra were acquired at a resolving power of 650 (20% height definition) and scanning from m/z 39 to m/z 650 at 1.0 s/scan with a 0.2 s inter-scan delay.

Sample preparation

Deuteromethylation

All samples were alkylated with MeI-d_3 according to Ciucanu and Kerek (1984) with NaOH/MeI-d_3, or according to a modified Hakomori procedure (Hakomori 1964) with Li-dimsyl/MeI-d_3 in DMSO. Sample cleanup was performed by dialysis of the reaction solution in a dialysis tube (MWCO of 12,000–14,000) against water for several days. Yields were 95.4% (HPMC 1), 90.6% (HPMC 2) and 88.5% (HPMC 3). Completeness of the reaction was controlled by means of ATR-IR spectroscopy.

Monomer analysis

HPMCs (ca. 2 mg) were hydrolyzed in a 1-ml V-Vial with 1 ml of 2 M trifluoroacetic acid for 120 min at 120 °C. After cooling to r.t., the solvent was evaporated in a stream of nitrogen. Residual acid was removed by co-distillation with toluene. The hydrolyzed sample was reduced with a solution of 0.5 ml of 0.25 M $NaBD_4$ in 2 M NH_3 at 60 °C for 120 min. After cooling to r.t., the solution was co-evaporated at 40 °C with 15% methanolic acetic acid in a stream of nitrogen to remove borate as its methyl ester.

After reduction, the residue was dissolved in 50 μl of pyridine, and 200 μl of acetic anhydride was added to acetylate the sample at 90 °C for 3 h. Saturated $NaHCO_3$ solution was added to the mixture and stirred until CO_2-formation ceased. Then the products were extracted four times with dichloromethane. The combined organic layer was first washed two times with saturated $NaHCO_3$ solution, once with cold 0.1 M HCl, once with water, and then dried over $CaCl_2$. After filtration, the solvent was removed at a rotary evaporator. Solution of the residue in 4 ml of CH_2Cl_2 was used for GLC/FID and GLC/MS analysis.

For analysis of the hydroxypropyl pattern the same procedure was performed after deuteropermethylation of HPMCs.

Oligomer analysis

The deuteromethylated HPMCs (3 mg) were partially hydrolyzed in a 1-ml V-Vial with 1 ml 2 M trifluoroacetic acid for 15 min at 120 °C. After cooling to r.t., the solvent was evaporated in a stream of nitrogen. Residual acid was removed by co-distillation with toluene (five times).

The hydrolyzate was dissolved in 150 μl of MeOH and reductively aminated by adding 17 μl of acetic acid and 20 μl (0.24 mmol) of *n*-propylamine. The solution was stirred for 30 min at r.t., then 30 μl of H_2O and 16 mg (0.25 mmol) of $NaCNBH_3$ in 100 μl of MeOH were added. After stirring over night at r.t., products were isolated and purified using size-exclusion chromatography

(Sephadex LH-20). The 1-deoxy-1-(*N*-propyl)amino-*O*-methyl/methyl-d_3-D-glucitol-terminated oligosaccharides were eluted with MeOH. Salts are longer retained on the column. Product containing fractions were eluted first and combined, the solvent was removed on a rotary evaporator.

The reductively aminated samples were *O*- and *N*-alkylated in a 1-ml V-Vial with 12.5 mg (0.31 mmol) of NaOH and 20 μl (0.32 mmol) of MeI in 300 μl of DMSO. 1-Deoxy-1-(*N*-dimethylpropyl)ammonium-*O*-methyl/methyl-d_3-D-glucitol-labeled oligosaccharides were purified by size-exclusion chromatography (Sephadex LH-20, eluent CH_2Cl_2 : MEOH (2:1, v/v)). The analyte molecules elute first while salts and DMSO are longer retained on the column. Collected fractions were evaporated in a stream of nitrogen, re-dissolved in MeOH to a concentration of approx. 0.25 mg/ml and directly submitted to ESI-IT-MS and MALDI-TOF-MS.

Results and discussion

This work reports on the application of a novel method, very recently described for HEMC (Adden et al. 2005), to three commercial HPMC with DS_{Me} ~2.0 and MS_{HP} ~0.2. After appropriate sample preparation, MALDI-TOF-MS allowed quantitative determination of the distribution of methyl and hydroxypropyl substituents on an oligomeric level and thus insight in the substituent patterns along the polymer chain. Knowledge of the exact monomer composition is an essential requirement for this analytical approach. Therefore, the substituent distribution in the glucsosyl units was thoroughly reinvestigated to detect and assign as many constituents of these very complex mixtures as possible.

Monomer analysis of HPMC

For each glycosyl unit in a 1,4-linked glucan three different hydroxyl groups are available for functionalization. The number of patterns can be calculated by n^3, where n is the number of different substituents. Since synthesis of HPMC is performed in a one-pot procedure with methyl oxirane and methyl chloride, HPMC can bear OH, OMe, OHP and OHPMe (*O*-(2-methoxy)propyl) groups, leading to $4^3 = 64$ different patterns, without considering the rather unlikely tandem reaction of the HP groups and formation of diasteroisomers due to the chirality of the HP residue. Capillary GLC is the method of choice to analyze these very complex mixtures due to its highly efficient separation power and its combination with mass spectrometry. By this approach, important structure information can be obtained. Another advantage is the high linear range of flame ionization detection (FID) and that standard compounds are not required. To minimize the complexity of the constituents as far as possible, reduction to glucitols was performed after acid hydrolysis and prior to acetylation. Alditol acetates were submitted to GLC-FID and GLC-MS. For quantitative evaluation the effective-carbon-response concept (ECR) was applied (Sweet et al. 1975; Scanlon and Willis 1985; Jorgensen et al. 1990). To analyze the distribution with the highest accuracy a twofold approach was used. First, the dominating methyl pattern was analyzed after direct hydrolysis of HPMC, reduction and acetylation. Peaks were assigned based on the well-known fragmentation of partially alkylated alditol acetates (Mischnick-Lübbecke and Krebber 1989). In a second step, the samples were permethylated prior to hydrolysis to focus all glucosyl units with a certain HP-pattern, but different combinations of methyl substitution, in one peak and thus enhance sensitivity of detection and reliability of assignment. Gas chromatograms in Figure 1 illustrates this reduction of complexity and improved sensitivity for HP-glucitol detection. MS_{HP} values were enhanced by 7–13% by this additional step. Table 1 lists retention times, corresponding peak assignments, ECR-values, and the normalized molar composition of all constituents.

Results for three HPMCs are summarized in Tables 2 and 3. The methyl pattern (Table 2 and Figure 2) is differentiated with respect to sugar core substitution and the minor contribution by HP-methylation, which is 3% for HPMC 1 and 2, while 5.4% of methyl groups are located on hydroxypropyl residues in HPMC 3. It is estimated that 35% of the hydroxypropyl residues are *O*-methylated in HPMC 1 and 2, while 55% are capped with methyl in HPMC 3. DS_{Me} values are all close to 2.0 (HPMC 1: 2.06, HPMC 2: 1.99, HPMC 3: 2.04). Regioselectivity of methylation (see x_2, x_3, and x_6 for O-Me in Table 2) is similar

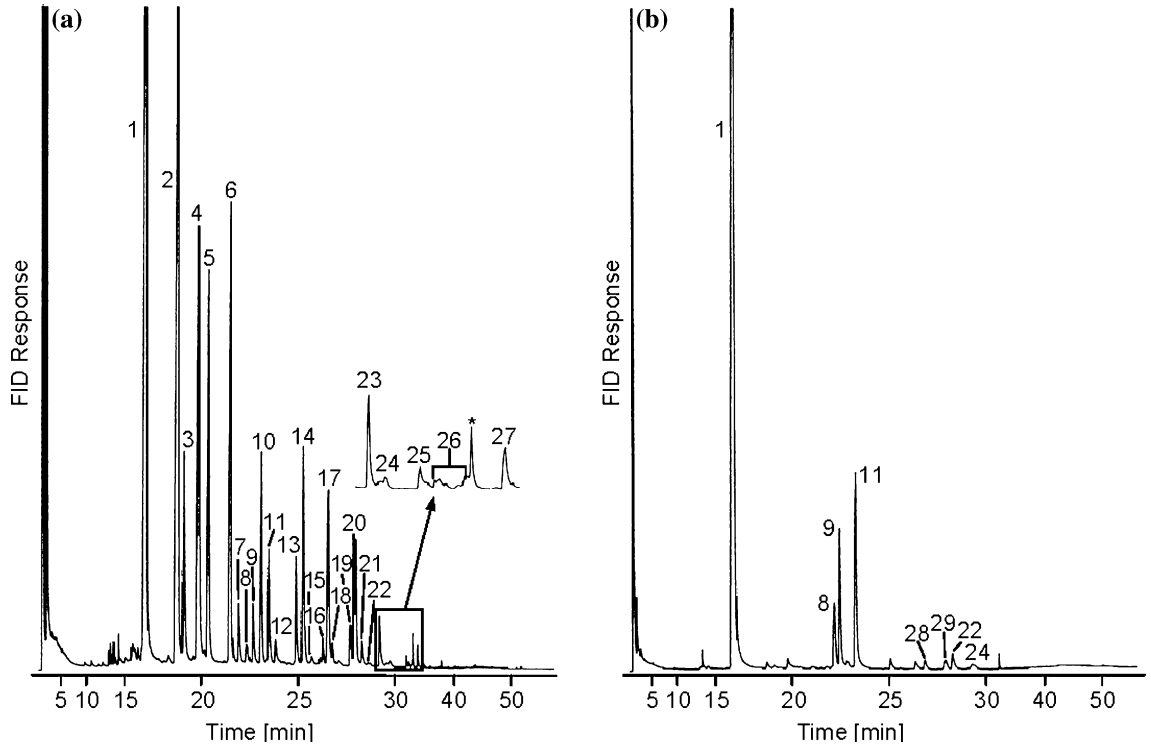


Figure 1. Gas chromatograms of *O*-methyl-*O*-(2-hydroxy)propyl-D-glucitol acetates obtained from HPMC 1 after direct hydrolysis, reduction, and acetylation (a), and after additional methylation prior to hydrolysis (b); peaks are assigned according to Table 1, *marks a plastiziser.

for all HPMC with 40% of the methyl groups at O-2. HPMC 3 shows a somewhat higher preference for O-3 (27.3%) compared to HPMC 1 and 2 (25.8 and 25.4%, respectively), on the expense of *O*-6-methylation (32.2% compared to 33.9 and 34.4%, respectively). Comparison with the statistical models of Spurlin (1939) and Reuben (1986) shows the latter being in better agreement with the experimental data than the random model of Spurlin. Deviations are always positive for un- and trisubstituted monomers (c_0 and c_3), negative for mono- and disubstituted fractions (c_1 and c_2). They are in agreement with a generally – not only specifically (Reuben) – enhanced reactivity of a glucosyl unit after primary substitution. Heterogeneity parameters (H_i) are given as indicator for the average deviation of experimental data from the model in Table 2. H_i-values are in the range of 2.6–3.8 for the Spurlin model, and even more modest for the Reuben model (HPMC 1:1.9, HPMC 2:2.9, HPMC 3:1.5). Table 3 and Figure 3 show the results for the HP-distribution in the glucosyl units. Minor products from tandem reaction could be determined even at this low amount of HP. Both, MS- and DS-values were calculated and are 0.22 and 0.21 for HPMC 1 and 3 and somewhat lower (0.19/0.19) for HPMC 2. Preferred oxirane addition was observed for the primary 6-OH, followed by O-2 > O-3 (x_6, Table 3). This regioselectivity was more pronounced for HPMC 3 (O-6: 56.0%, O-2: 33.3%) compared to approx. 49% (O-6) and 29% (O-2) for HPMC 1 and 2. Consequently, only 11% of HP residues were located at O-3 in HPMC 3, while 22% were found in this least reactive position for the other two HPMCs. Due to the higher 2,6-regioselectivity, one would expect HPMC 3 to have the lowest contribution of tandem products to the total MS, but with 5.3% it is significantly higher than for HPMC 1 and 2 (2.1% and 2.0%). It must also be considered that reactivity is not decoupled from the sugar core by the 'HP-spacer' as has been expected, but is highest for position 6 (see Table 2, compare x_i values for OMe and OHPMe). On average, 50% of HP-methyl groups are located at 6-*O*-HP, compared to only 32–34% of the methyl

Table 1. Peak assignment and relative molar composition of constituents for the GLC-analysis according to Figure 1a and 1b.

Pattern	Number	Retention time (min)	ECR[a]	HPMC 1 (Mol%)	HPMC 2 (Mol%)	HPMC 3 (Mol%)
Relative molar composition of constituents according to GC in Figure 1a						
236-Me	1	17.2	745	30.26	27.78	27.28
26-Me	2	18.9	790	22.95	22.65	20.24
36-Me	3	19.2	790	4.13	5.17	4.57
23-Me	4	19.9	800	8.06	7.43	9.66
6-Me	5	20.4	835	6.37	7.16	5.82
2-Me	6	21.5	845	7.71	9.44	9.70
3-Me	7	21.9	845	1.16	1.58	1.66
26-Me-3HPMe	8	22.4	945	0.44	0.39	0.55
36-Me-2-HPMe	9	22.6	945	0.82	0.73	1.56
un	10	23.0	890	2.71	3.56	3.51
23-Me-6-HPMe	11	23.4	945	1.44	1.23	2.93
6-Me-2-HPMe	12	23.8	990	0.56	0.50	0.68
2-Me-6-HPMe	13	24.8	990	1.24	1.10	2.03
26-Me-3-HP	14	25.1	990	1.43	1.33	0.94
3-Me-2HPMe	15	25.5	990	1.43	1.33	0.94
36-Me-2HP	15	25.5	990	0.18	0.20	0.31
6-HPMe	16	25.9	1035	0.30	0.30	0.51
23-Me-6-HP	17	26.0	990	3.05	2.77	2.08
6-Me-2-HP[b]/6-Me-3-HP	18	26.3	1035	0.46	0.43	0.19
2-Me-3-HP	19	27.4	1035	0.57	0.50	0.11
2-Me-6-HP	20	27.6	1035	2.34	2.33	1.18
3-Me-6-HP[b]/2-Me-36-HPMe	21	27.9	1035	0.50	0.58	0.63
3-Me-26-HPMe[b]/36-Me-22-HPMe	22	28.3	1145	0.07	0.04	0.37
23-HP	23	28.8	1280	0.56	0.70	0.42
23-Me-66-HPMe[b]/2-HPMe-3-HP-6-Me	24	29.3	1145	0.19	0.11	0.68
2-Me-3-HP-6-HPMe	25	30.4	1190	0.21	0.20	0.56
Di-HP[c]	26	30.5–31.7	1190	0.46	0.10	0.47
2-Me-66-HP	27	32.9	1235	0.41	0.35	0.43
Relative molar composition of constituents according to GC in Figure 1b						
236-Me	1	17.2	745	80.39	82.61	80.72
26-Me-3-HPMe	8	22.2	945	3.67	3.39	1.58
36-Me-2-HPMe	9	22.5	945	5.14	4.50	5.81
23-Me-6-HPMe	11	23.3	945	8.74	7.85	9.20
6-Me-23-HPMe	28	26.7	1145	0.42	0.26	0.23
2-Me-36-HPMe	29	27.8	1145	0.43	0.40	0.40
3-Me-26-HPMe[b]/36-Me-22-HPMe	22	28.1	1145	0.75	0.61	0.88
23-Me-66-HPMe	24	29.1	1145	0.46	0.38	1.17

[a]Effective carbon response.
[b]Peaks were assigned to more than one peak, the area was not distributed.
[c]Summarized signal intensities of pattern with two HP residues, some are HPMe.

Table 2. Distribution of methyl substituents of HPMC 1, 2 and 3.

Substituent pattern	HPMC 1					HPMC 2					HPMC 3				
	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ
s_0	4.03	3.11	0.92	3.70	0.33	4.66	3.85	0.82	4.29	0.37	4.91	3.87	1.03	4.39	0.52
s_2	12.47	12.67	−0.20	12.08	0.38	13.91	13.33	0.58	12.88	1.03	14.00	14.04	−0.04	13.53	0.48
s_3	3.16	3.28	−0.11	2.69	0.47	3.54	3.69	−0.15	3.24	0.30	3.59	4.31	−0.72	3.80	−0.21
s_6	7.38	6.49	0.90	7.71	−0.33	8.09	7.58	0.51	8.46	−0.37	6.69	6.36	0.33	7.21	−0.52
s_{23}	12.75	13.35	−0.60	13.93	−1.19	11.54	12.79	−1.25	13.23	−1.69	15.35	15.62	−0.27	16.14	−0.79
s_{26}	24.82	26.43	−1.61	25.20	−0.38	24.38	26.28	−1.91	25.41	−1.03	21.73	23.05	−1.32	22.21	−0.48
s_{36}	5.14	6.84	−1.70	5.61	−0.47	6.10	7.27	−1.17	6.39	−0.30	6.44	7.08	−0.64	6.23	0.21
s_{236}	30.26	27.84	2.41	29.07	1.19	27.78	25.21	2.57	26.09	1.69	27.28	25.65	1.63	26.50	0.79
c_0	4.03	3.11	0.92	3.70	0.33	4.66	3.85	0.82	4.29	0.37	4.91	3.87	1.03	4.39	0.52
c_1	23.01	22.43	0.58	22.48	0.53	25.54	24.60	0.94	24.59	0.95	24.28	24.71	−0.43	24.53	−0.25
c_2	42.70	46.61	−3.91	44.75	−2.05	42.01	46.34	−4.33	45.03	−3.02	43.53	45.76	−2.23	44.58	−1.06
c_3	30.26	27.84	2.41	29.07	1.19	27.78	25.21	2.57	26.09	1.69	27.28	25.65	1.63	26.50	0.79
H_1^a			3.7		1.9			3.8		2.9			2.6		1.5
O-Me															
x_2			0.803 (40.3%)				0.776 (40.2%)					0.784 (40.6%)			
x_3			0.513 (25.8%)				0.490 (25.4%)					0.527 (27.3%)			
x_6			0.676 (33.9%)				0.664 (34.4%)					0.621 (32.2%)			
DS_{Me}			1.99				1.93					1.93			
O-HPMe															
x_2			0.029 (43.6%)				0.026 (43.6%)					0.036 (43.6%)			
x_3			0.004 (6.6%)				0.004 (6.5%)					0.006 (6.5%)			
x_6			0.033 (49.8%)				0.030 (49.9%)					0.071 (49.9%)			
DS_{HPMe}			0.07				0.06					0.11			
O-Me total															
x_2			0.832 (40.4%)				0.802 (40.3%)					0.819 (40.1%)			
x_3			0.517 (25.1%)				0.493 (24.8%)					0.532 (26.0%)			
x_6			0.709 (34.4%)				0.693 (34.9%)					0.692 (33.9%)			
$DS_{\sum Me}$			2.06				1.99					2.04			

Monomer composition (Mol%) and comparison with models of Spurlin and Reuben.

[a]Heterogeneity parameter$(H_i) = \sqrt{\sum(\Delta^2)}$.

Table 3. Distribution of hydroxypropyl substituents of HPMC 1, 2 and 3.

Substituent pattern	HPMC 1						HPMC 2						HPMC 3					
	$GC_{(MS)}$	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ	$GC_{(MS)}$	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ	$GC_{(MS)}$	$GC_{(DS)}$	Spurlin	Δ	Reuben	Δ
s_0	80.39	80.39	80.16	0.23	80.28	0.10	82.61	82.61	82.41	0.21	82.41	0.17	80.72	80.72	80.41	0.31	80.48	0.24
s_2	5.14	5.14	5.40	0.26	5.28	−0.14	4.50	4.50	4.67	−0.18	4.64	−0.14	5.81	5.81	5.98	0.17	5.91	−0.10
s_3	3.67	3.67	3.80	−0.13	3.68	−0.01	3.39	3.39	3.48	−0.09	3.44	−0.05	1.58	1.58	1.83	−0.25	1.76	−0.17
s_6	8.74	9.20	9.29	−0.09	9.30	−0.10	7.85	8.23	8.39	−0.16	8.40	−0.17	9.20	10.37	10.60	−0.23	10.61	−0.24
s_{23}	0.42	0.42	0.26	0.16	0.38	0.04	0.26	0.26	0.20	0.06	0.23	0.02	0.23	0.23	0.14	0.10	0.21	0.03
s_{26}	0.75	0.75	0.63	0.12	0.61	0.14	0.61	0.61	0.48	0.14	0.47	0.14	0.88	0.88	0.79	−0.09	0.78	−0.10
s_{36}	0.43	0.43	0.44	−0.01	0.43	0.01	0.40	0.40	0.35	0.05	0.35	0.05	0.40	0.40	0.24	0.16	0.23	0.17
s_{22}	0.00	–	–	–	–	–	0.00	–	–	–	–	–	0.00	–	–	–	–	–
s_{33}	0.00	–	–	–	–	–	0.00	–	–	–	–	–	0.00	–	–	–	–	–
s_{66}	0.46	–	–	–	–	–	0.38	–	–	–	–	–	1.17	–	–	–	–	–
s_{236}	0.00	0.00	0.03	−0.03	0.04	−0.04	0.00	0.00	0.02	−0.02	0.02	−0.02	0.00	0.00	0.02	−0.02	0.03	−0.03
c_0	80.39	80.39	80.16	0.23	80.28	0.10	82.61	82.61	82.41	0.20	82.45	0.17	80.72	80.72	80.41	0.31	80.48	0.20
c_1	17.54	18.01	18.49	−0.48	18.26	−0.25	15.73	16.11	16.54	−0.43	16.47	−0.36	16.60	17.77	18.41	−0.64	18.28	−0.42
c_2	2.07	1.61	1.32	0.28	1.41	0.19	1.65	1.27	1.03	0.24	1.06	0.22	2.69	1.51	1.17	0.35	1.22	0.25
c_3	0.00	0.00	0.03	−0.03	0.04	−0.04	0.00	0.00	0.02	−0.02	0.02	−0.02	0.00	0.00	0.02	−0.02	0.03	−0.03
H_1^a				0.4		0.3				0.4		0.3				1.0		0.5
x_2	0.063 (29.7%)						0.054 (28.8%)						0.069 (33.3%)					
x_3	0.045 (21.3%)						0.040 (21.7%)						0.022 (10.7%)					
x_6	0.104 (48.9%)						0.092 (49.5%)						0.117 (56.0%)					
DS	0.21						0.19						0.21					
MS	0.22						0.19						0.22					

Monomer composition (Mol%) and comparison with models of Spurlin and Reuben.

[a]Heterogeneity parameter$(H_i) = \sqrt{\sum(\Delta^2)}$.

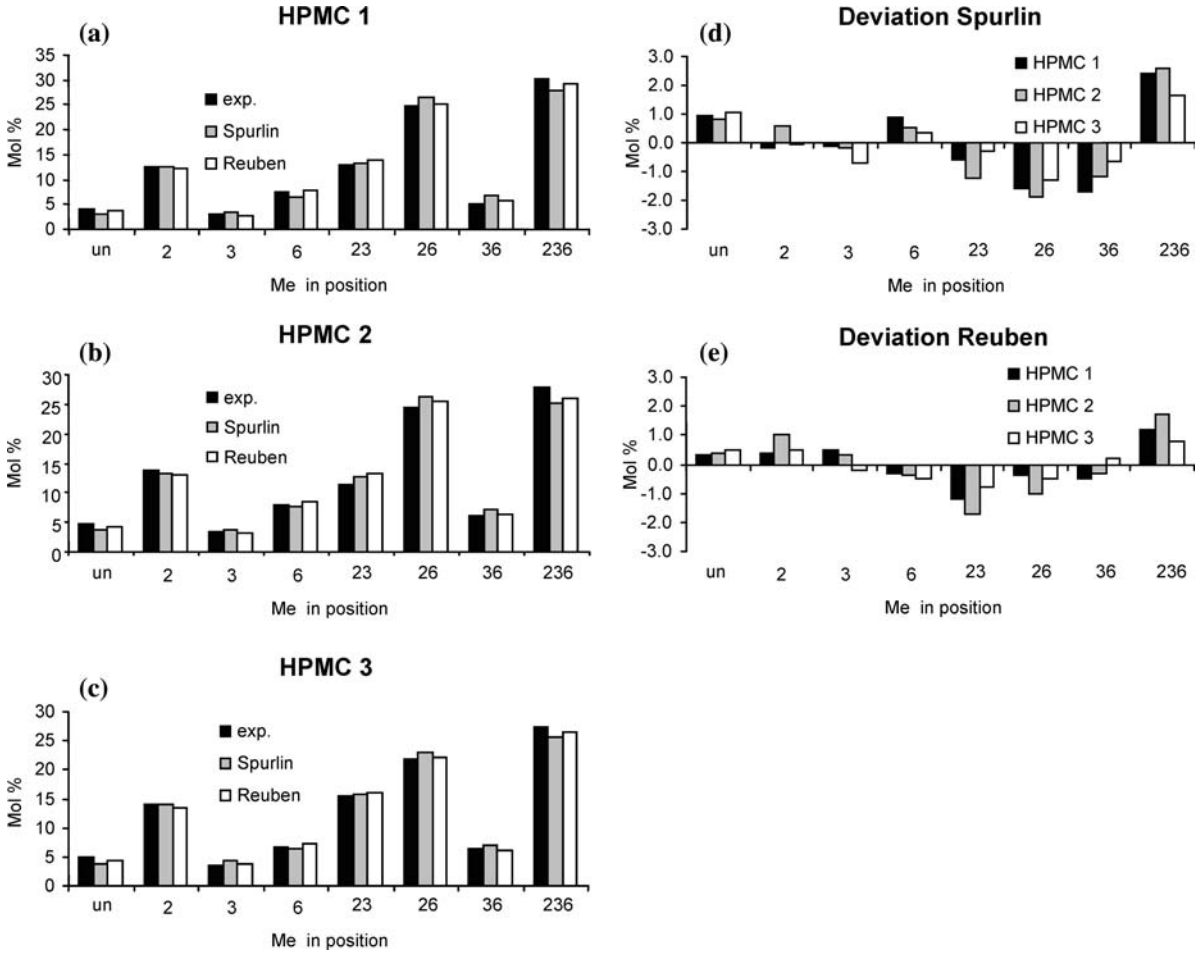


Figure 2. Distribution of methyl substituents in the glycosyl unit of HPMC 1–3 in comparison with the models of Spurlin and Reuben (a–c); deviation of the experimental data from the models of Spurlin (d) and Reuben (e).

groups, which are attached to *O*-6 of the sugar core. An increased DS_{HP} at *O*-6 will therefore also result in an increased chance of tandem reaction at this position (see Table 3, s_{66}). The special trends of regioselectivities cannot be explained at present. It cannot be excluded that hydroxypropylation started first and methyl chloride was added later. Thus, the methyl oxirane could react with the most reactive positions first, and consequently influence the methyl pattern.

For comparison of the data with the models of Spurlin and Reuben tandem reaction was neglected. All samples show very low heterogeneity values H_1 *Spurlin/Reuben)*: 0.4/0.3 (HPMC 1), 0.4/0.3 (HPMC 2), and 1.0/0.5 (HPMC 3) with a slightly better matching of the Reuben model.

Oligomer analysis of HPMC

As outlined in the introduction, it is not possible to obtain an isotopically labeled but apart from that chemically uniform polymer in case of hydroxyalkyl ethers, which would be the best prerequisite to guarantee representative quantitative data from MS. To develop an alternative approach one must consider the critical parameters that influence relative ion intensities of mixtures in MS, including the ionization/desorption process and ion transfer to the mass analysator. We focused during the method development on two different instrumental setups: ESI-MS connected to ion trap (ESI-IT-MS), and MALDI with TOF mass analysis, both in positive mode (Adden et al. 2005). Generally, ion yields of neutral carbohydrates depend on their

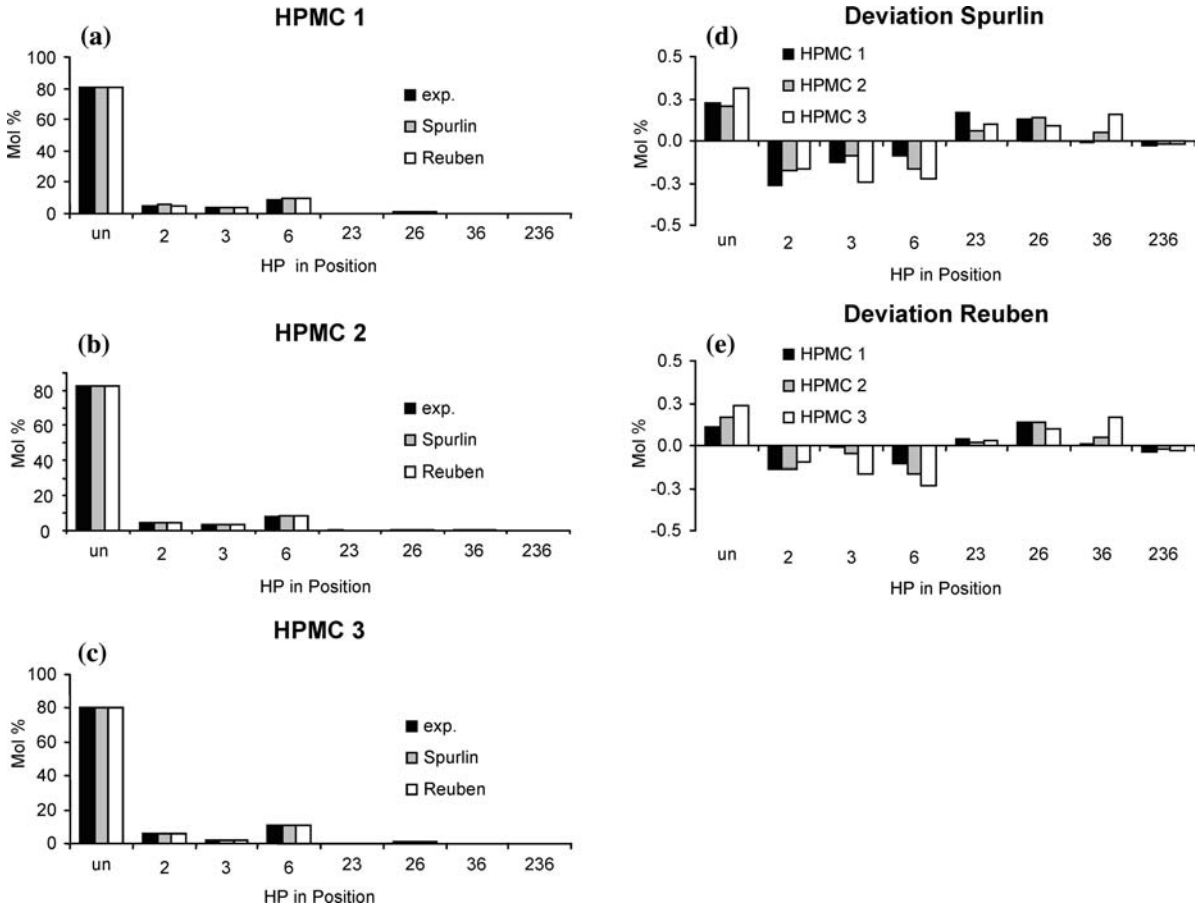


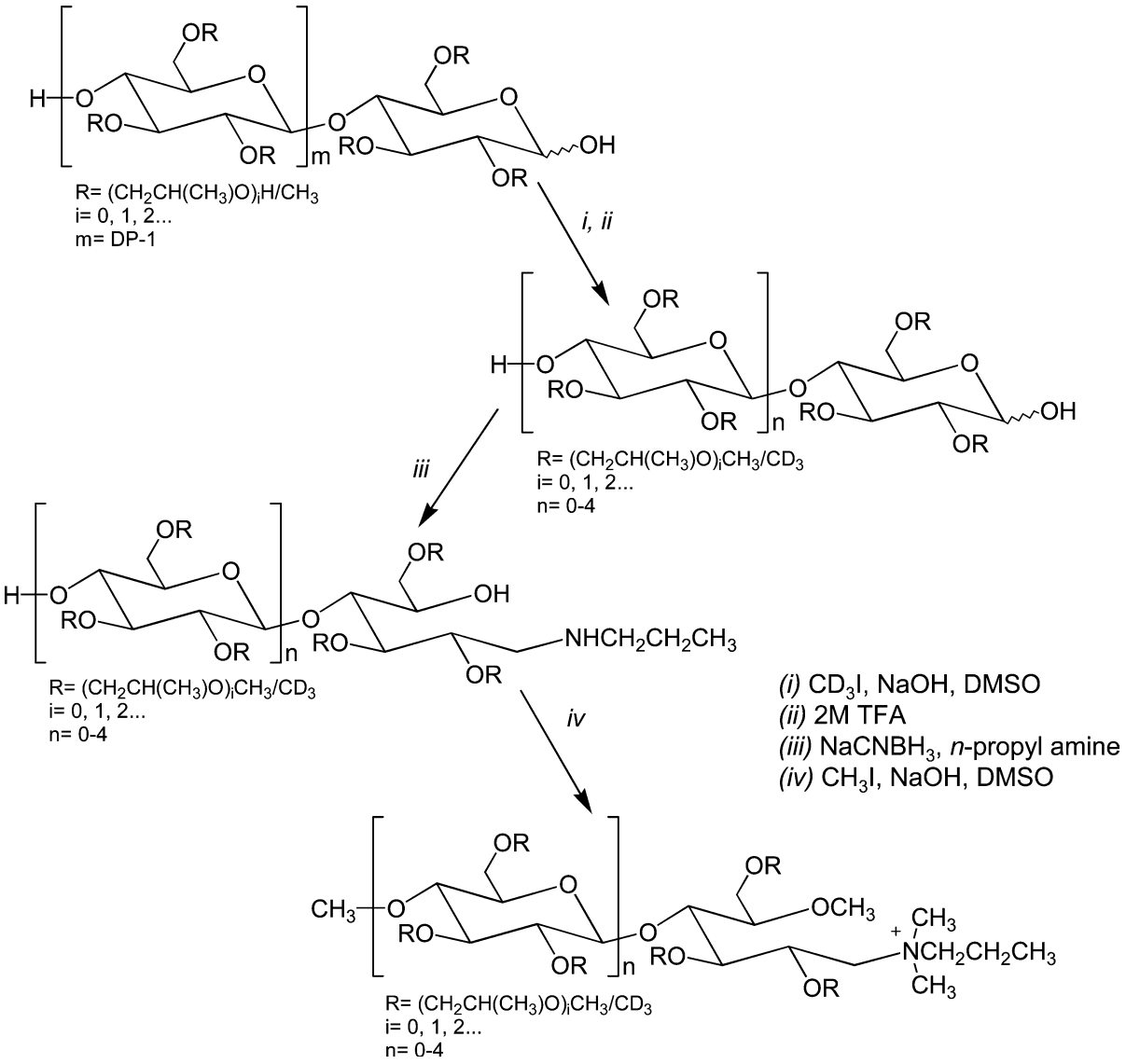
Figure 3. Distribution of hydroxypropyl substituents in the glycosyl unit of HPMC 1–3 in comparison with the models of Spurlin and Reuben (a–c); deviation of the experimental data from the models of Spurlin (d) and Reuben (e).

ability to form complexes with cations, usually ubiquitous sodium. This ability increases with the number of appropriate coordination sites, i.e. oxygen, and therefore strongly increases from DP 1 to 2, furthermore, also by reduction of the reducing end to a more flexible glucitol, and by appropriate substitution as well. Addition of the structural feature $CH_2CHOHCH_3$ or $CH_2CH(OCH_3)CH_3$ to a glucose is accompanied with a relatively large increase of coordination sites, and therefore causes huge intensity increase. By ESI-MS of HPMC hydrolysates even glucose residues with four HP residues could be detected (data not shown), and the MS_{HP} would be overestimated by a factor of about 7 from the relative intensities. In our studies on HEMC, we found that only MALDI-TOF-MS in combination with appropriate sample preparation allows to overcome all discrimination effects occurring in MS due to the chemical heterogeneity of the glucosyl units in hydroxyalkyl/methyl ether derivatives. For control, the average DS_{Me} and MS_{HP} are calculated for each DP from the signal intensity distribution. In case of a representative quantitative analysis these values must be constant and in agreement with the average reference value from monomer analysis. Applying the procedure in the way established for MC, including perdeuteromethylation and partial hydrolysis, by far too high MS_{HP} values are obtained for DP 1, decreasing with increasing DP, but not leveling off at a correct constant value as might have been expected, because the relative differences between the constituents become less significant with increasing complexity of the oligomeric mixtures. We therefore had to find appropriate sample preparation in connection with proper instrumental features to

enable quantitative evaluation of the MS experiments.

Sample preparation

All samples were permethylated with MeI-d_3 and submitted to partial acid hydrolysis as common for quantitative oligomer pattern analysis of methyl derivatives. Since this was not sufficient for quantitative analysis of HPMC, oligosaccharides were reductively aminated with *n*-propylamine to give still neutral, but basic nitrogen containing analytes. Reductive amination is very common in glycoconjugate analysis and various tags have been used (Harvey 2000). In the field of cellulose ether analysis, Momcilovic et al. (2005) recently reported on the successful use of dialkylamines, especially dimethylamine. For these compounds, always protonated and sodium co-ordinated analytes were observed in various ratios. To introduce a permanent charge, we subsequently alkylated the aminated compounds to obtain the quaternized ammonium compounds. The procedure is summarized in Scheme 1. An appropriate sample clean-up was very important to record mass



Scheme 1. HPMC sample preparation for MS analysis.

spectra with a high signal/noise ratio. We performed perdeuteromethylation as first step out of two reasons. First, we wanted to protect all free hydroxyl functions, because polarity-enhancing analytes are strongly discriminated with increasing number of free OH by a factor of 50–100. Second, peralkylation prevent side reactions during hydrolysis, which are favored by free OH, and levels off differences in relative stability of glucosidic linkages, which is important for the random course of the following partial hydrolysis. Reductive amination with *n*-propylamine was performed to introduce more basic nitrogen, one for each oligosaccharide. With the introduction of a permanent charge by subsequent peralkylation elimination of all problems related to ion formation in MS is addressed.

After quaternization of the amino group, very clean and well-resolved mass spectra with excellent signal/noise ratio were obtained (Harvey 2000; Adden et al. 2005). Enhancing the absolute intensity is a welcomed effect, but the main purpose of introducing a permanent charge into the analyte molecules is to minimize discrimination effects as outlined above. Permanently charged analytes do not require formation of sodium adducts, thus different complexation abilities have no longer to be considered. Therefore, all HPMCs were treated according to the procedure according to Scheme 1, and submitted to MALDI-TOF-MS. An example of a mass spectrum of HPMC 3 is given in Figure 4, the *m*/*z*-values up to DP 3 are listed in Table 4.

It has been shown that this sample preparation in combination with MALDI-TOF-MS is capable of analyzing both, the Me- and the HE-distribution along the polymer chain in HEMC (Adden et al. 2005). Therefore, this method was also applied to HPMCs.

Evaluation of data

HPMC 1–3 were submitted to the procedure outlined above and analyzed by MALDI-TOF-MS. If each glucosyl unit reacts with the same

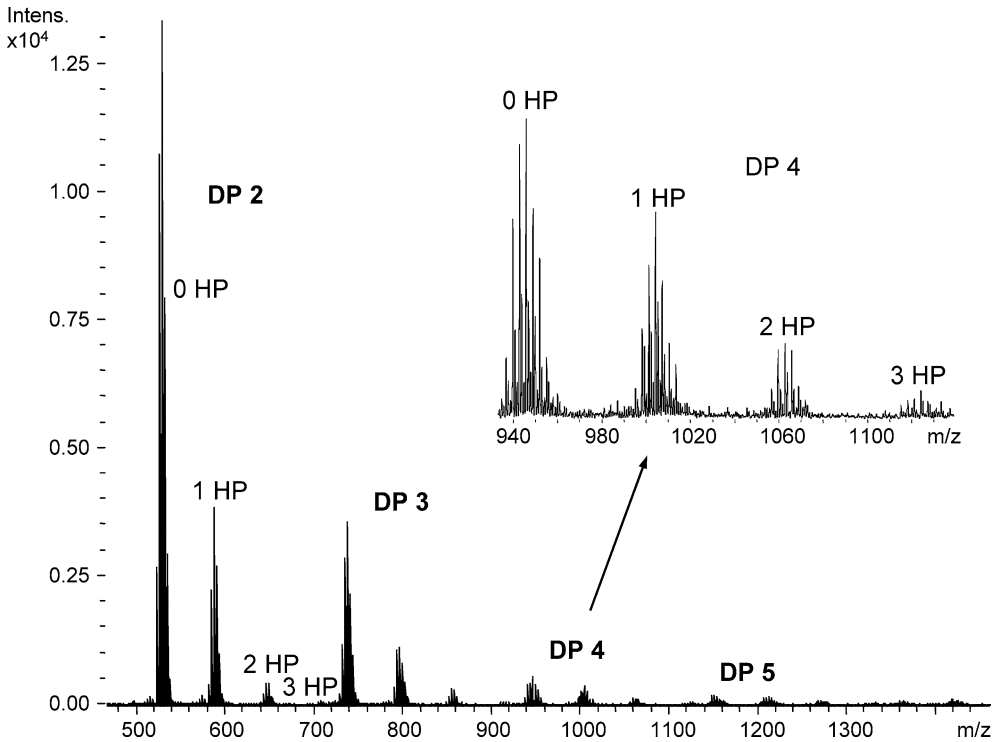


Figure 4. MALDI-TOF mass spectrum of HPMC 3 derived oligosaccharides prepared according to Scheme 1. The pattern of DP 4 is shown in detail. Signals are assigned according to DP and number of HP residues. Fine structure within a mixture of a certain number of HP-groups reflects the methyl distribution.

probability and primary alkylation does not influence the reactivity of neighbored units, a random distribution of the constituting monomer units is obtained. This random pattern can be calculated for oligomeric sequences from the molar fractions of un-, mono-, di-, and trisubstituted glucosyl units (c_0, c_1, c_2, c_3, Tables 2 and 3) as described (Arisz et al. 1995; Mischnick and Kühn 1996; Mischnick and Hennig 2001). Methyl groups linked to hydroxypropyl residues were referred to the corresponding HP position, regarding the $CH_2CH(CH_3)O$ element as a spacer. Methyl and hydroxypropyl patterns are calculated and evaluated separately. Comparison of experimental data with the random methyl distributions is shown in Figure 5. DS_{Me} values are in very good agreement with the average value from monomer analysis (see Table 5). For each HPMC, data of DP 2, 3, 4, and 5 have been evaluated. Methyl distribution of all HPMCs looked very similar.

Surprisingly, all samples showed a narrower profile compared to the random model. That means that methyl groups are distributed more regular on the expense of low and high substituted regions. The latter sequences, represented by the molar amount of low and high substituted oligosaccharides, occur with a lower probability as calculated for the random pattern. In contrast, commercial methyl celluloses show a more or less pronounced deviation in the opposite direction. Reactivity is improved in areas where methylation has started, presumably because of hydrogen bond interruption and improved local solubility of chain segments. The same tendency was principally observed for HEMCs (Adden et al. 2005). Since reaction conditions are comparable for HEMC and HPMC, this different behavior of HPMC must be related to the HP groups and will be discussed in context with the HP distribution. As a further difference to the HEMCs studied, we do not observe signals indicating unsubstituted sequences from unaffected crystalline domains according to the fringed fibril model (Schmidt et al. 1997), which are more difficult to activate with NaOH prior to the derivatization process. The heterogeneity parameters H_i are listed in Table 5 together with DS_{Me}. Since deviation of DS values from the reference DS of monomer analysis causes an apparent heterogeneity, H_i were only listed when $\Delta DS_{Me} \leq 0.03$ ($\pm 1.5\%$). Although the average heterogeneity parameters H_i are similar for monomer and oligomer analysis (in the range of 3–5, see Tables 2 and 5), deviation on the monomer level points in the opposite, but more common direction, i.e. increased unsubstituted and trisubstituted molar fractions compared to a random pattern. However, obviously this effect does not propagate along the chain. This is important to mention, since often monomer data in comparison to the Spurlin model are stressed with respect to the substituent distribution along the chain.

Table 4. m/z Values of oligosaccharides labeled according to Scheme 1 and detected in ESI and MALDI mass spectra.

	n(HP)	n(Me)	quat. $[M]^+$		n(HP)	n(Me)	quat. $[M]^+$
DP 1	0	0	331	DP 3	0	0	757
	0	1	328		0	1	754
	0	2	325		0	2	751
	0	3	322		0	3	748
	1	0	389		0	4	745
	1	1	386		0	5	742
	1	2	383		0	6	739
	1	3	380		0	7	736
	2	0	447		0	8	733
	2	1	444		0	9	730
	2	2	441		1	0	815
	2	3	438		1	1	812
					1	2	809
DP 2	0	0	544		1	3	806
	0	1	541		1	4	803
	0	2	538		1	5	800
	0	3	535		1	6	797
	0	4	532		1	7	794
	0	5	529		1	8	791
	0	6	526		1	9	788
	1	0	602		2	0	873
	1	1	599		2	1	870
	1	2	596		2	2	867
	1	3	593		2	3	864
	1	4	590		2	4	861
	1	5	587		2	5	858
	1	6	584		2	6	855
	2	0	660		2	7	852
	2	1	657		2	8	849
	2	2	654		2	9	846
	2	3	651		3	0	931
	2	4	648		3	1	928
	2	5	645		3	2	925
	2	6	642		3	3	922
	3	0	718		3	4	919
	3	1	715		3	5	916
	3	2	712		3	6	913
	3	3	709		3	7	910
	3	4	706		3	8	907
	3	5	703		3	9	904
	3	6	700				

Only values for DP 1–3 are given, the analysis was performed up to DP 5.

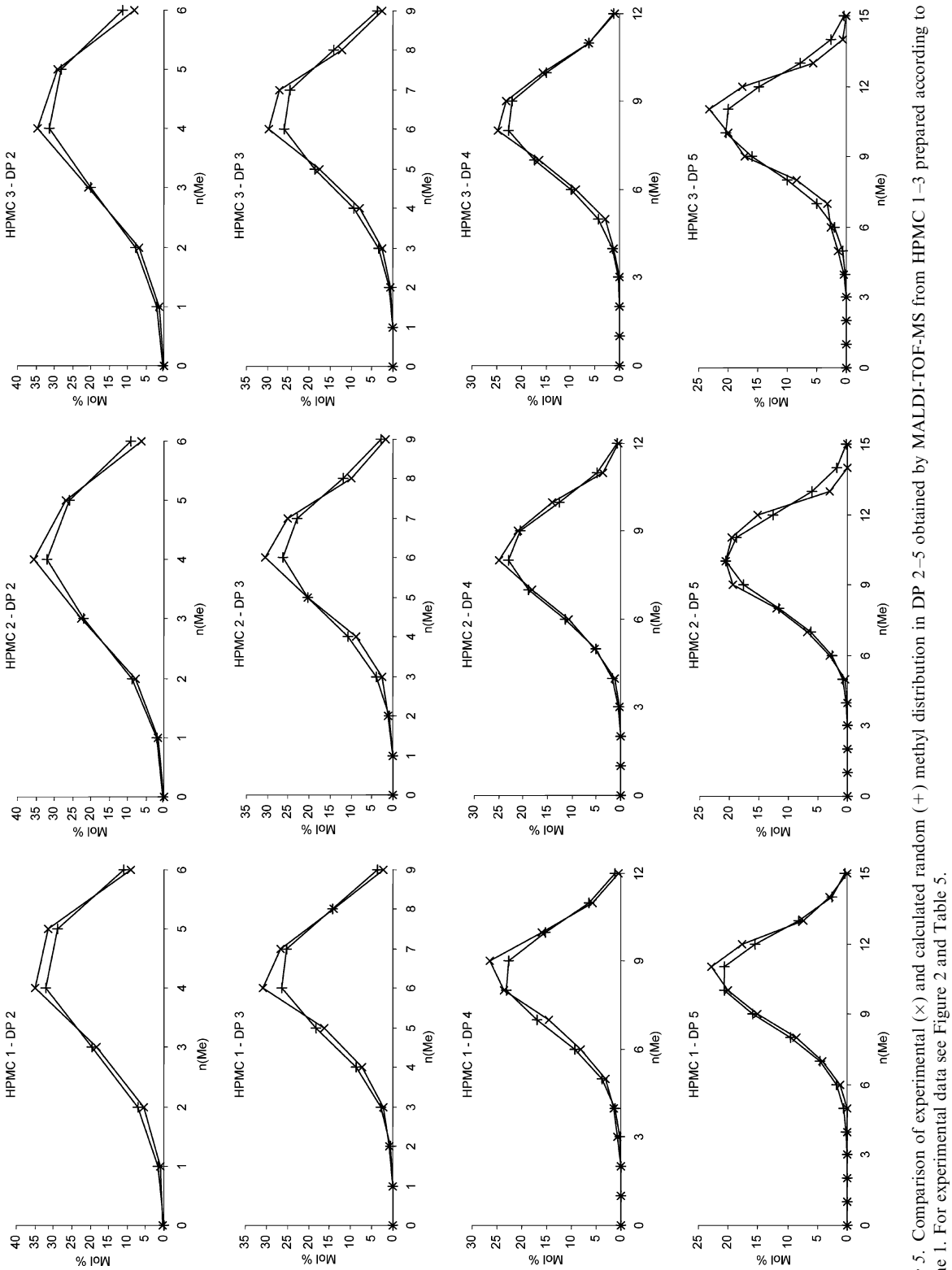


Figure 5. Comparison of experimental (×) and calculated random (+) methyl distribution in DP 2–5 obtained by MALDI-TOF-MS from HPMC 1–3 prepared according to Scheme 1. For experimental data see Figure 2 and Table 5.

Table 5. DS_{Me}-, MS_{HP}-values and heterogeneity parameters H_i of HPMC 1, 2, and 3.

	Pattern	DS/MS (MA)	DS_{Me} DP2	H_2	DS_{Me} DP3	H_3	DS_{Me} DP4	H_4	DS_{Me} DP5	H_5
HPMC 1	HP	0.22	0.15	n.e.	0.18	n.e.	0.20	2.3	0.19	5.1
	Me	2.06	2.08	5.0	2.07	5.5	2.07	4.9	2.09	3.5
HPMC 2	HP	0.19	0.14	n.e.	0.15	n.e.	0.17	7.1	0.18	3.0
	Me	1.99	1.98	5.0	2.00	5.7	2.00	3.1	1.96	4.9
HPMC 3	HP	0.22	0.18	n.e.	0.20	5.1	0.24	4.5	0.21	7.1
	Me	2.04	2.03	5.0	2.05	5.6	2.07	3.2	2.03	6.0

Labeling: reductive amination with propyl amine and quaternization with methyl iodide (Scheme 1). MA = monomer analysis. n.e.: not evaluated.

The results for the HP-pattern of HPMC 1 and 2 are in agreement with the random model within experimental error. Heterogeneity parameters H_i (2–3) are somewhat lower than for the methyl pattern. Since relative deviations are more pronounced due to the low MS_{HP}, H_i was only calculated when the deviation of MS_{HP} was $\leq \pm 0.03$ ($\pm 15\%$). To improve this analysis towards lower deviations is very difficult, if not impossible, because the deviations from DS_{Me} and MS_{HP} values are interrelated, but of course strongly differ in their relative meaning. At the same time, this is a control of reliability of this statistical approach. A systematic overestimation of HP would cause underestimation of DS_{Me}, because glycosyl units with HP groups carry less methyl groups. But this is not the case. As in the monomer composition, again HPMC 3 looks different. H_i values are in the range of 4–7 and type of deviation indicates a slight heterogeneity with enhanced contribution of sequences exhibiting higher and lower densities of HP groups than for a random pattern. Figure 6 shows the graphical comparison of experimental and calculated oligomer patterns.

The mass spectrometric data allow to compare how DS_{Me} develops in dependence on the number of HP groups in a certain DP. Figure 7 shows the graphical correlation. Again differences for HPMC 1 and 2 on one side and HPMC 3 on the other are found. While DS_{Me} decreases with increasing number of HP for HPMC 1 and more slightly for HPMC 2, it increases for HPMC 3 which corresponds to the higher degree of HP methylation for the latter.

Methylation and hydroxypropylation of cellulose are usually performed in a one-pot-procedure. Alkali-activated cellulose reacts with methyl oxirane and methyl chloride in a competing reaction. Differences in regioselectivity have been discussed above. While no interrelation between the two competing reagents was observed for HEMC (Adden et al. 2005), a fundamental change in the methylation pattern compared to simple MC is observed for HPMC. The more regular distribution of methyl groups means that methylation probability decreases in the near of methyl groups. MS cannot differentiate between methyl groups linked to the sugar core and methyl group at HP residues. The HP groups act as spacer between glucose and OH. The secondary hydroxyl group of HP is less reactive than the primary OH of HE residues. As already mentioned, only 35% of HP are *O*-methylated in HPMC 1 and 2 and 55% for HPMC 3. Thus, the decreased probability of methylation in a random manner might be a 'spacer effect' of HP residues, while HE groups with their higher reactivity do not exhibit such effect. Now the question arises, why there is no significant difference between the methyl patterns of HPMC 1 and 2 and HPMC 3. As already mentioned, regioselectivity was not leveled off by the spacer, but is shifted in favor of position 6, presumably due to sterical reasons. An increase in regioselectivity causes a more regular distribution, i.e. at a certain DS, the random pattern calculated for the monomer composition of higher regioselectivity is narrower than the random pattern of a mixture with a less selective pattern in the glucosyl units. The regioselectivity shift caused by the HP spacer is therefore assumed to explain the comparable methyl pattern of HPMC 3 in spite of higher HP methylation. The proposed spacer and regioselectivity effects will be the objective of further systematic studies.

Conclusion

A comprehensive study of the substituent distribution in HPMC has been performed. Three HPMCs with DS_{Me} of 2.0 and MS_{HP} of about

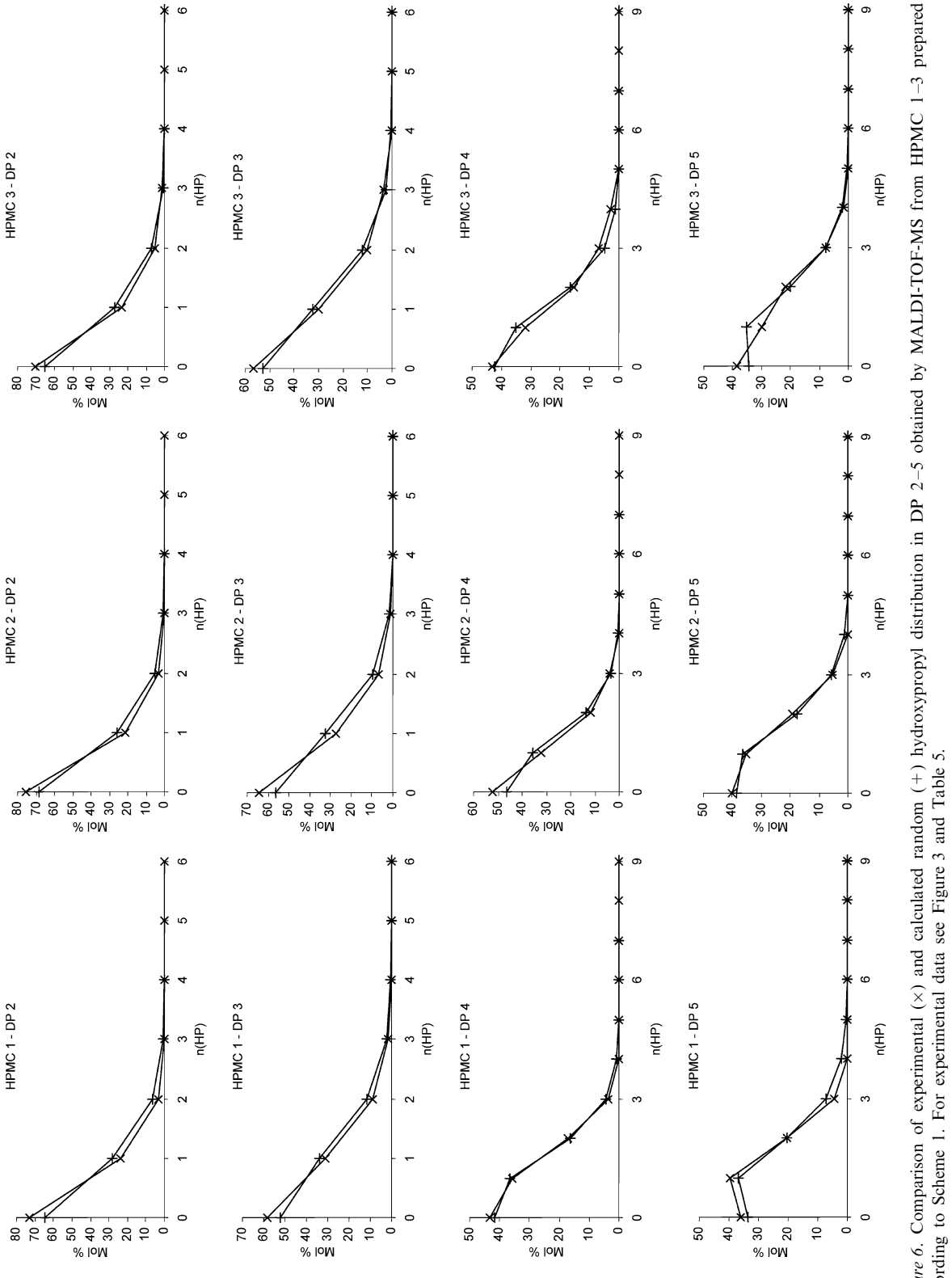


Figure 6. Comparison of experimental (×) and calculated random (+) hydroxypropyl distribution in DP 2–5 obtained by MALDI-TOF-MS from HPMC 1–3 prepared according to Scheme 1. For experimental data see Figure 3 and Table 5.

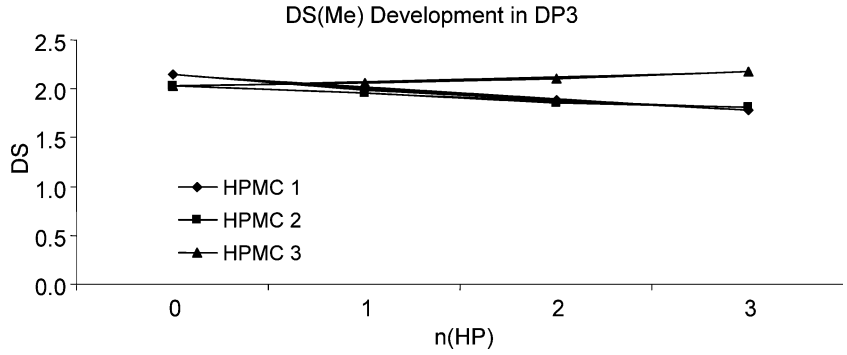


Figure 7. Dependence of the DS_{Me} from the number of hydroxypropyl groups in DP 3 for HPMC 1, 2 and 3.

0.2 have been investigated. Detailed analysis of the very complex monomer composition was a prerequisite for the statistical approach applied to the next structural level. Not only the distribution of methyl but also of hydroxypropyl groups along the polymer chain could be quantitatively analyzed for the first time by MALDI-TOF-MS of appropriate oligosaccharide derivatives. Sample preparation includes perdeuteromethylation, partial hydrolysis, reductive amination with *n*-propylamine and subsequent *O*- and *N*-alkylation. In contrast to MC and HEMC, methyl groups were found to be distributed in a more regular manner compared to the random model, while the hydroxypropyl pattern showed only slight deviations from this model. To further investigate this unusual effect, which is preliminary attributed to the lower reactivity of the secondary OH of HP for methylation compared to the primary OH-group of HE residues, hydroxyethyl and hydroxypropyl glucans of higher MS and mixed ethers produced in two steps shall be studied. Shift in regioselectivity of HP compared to the sugar core and steric requirements of the HP residue compared to the slim HE group must also be considered.

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