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Distribution of substituents in *O*-carboxymethyl and *O*-cyanoethyl ethers of inulin

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Abstract

The distribution of substituents in *O*-carboxymethyl and *O*-cyanoethyl ethers of inulin was studied using ^{13}C NMR spectroscopy and HPLC analysis. For both types of inulin derivatives, the distribution of substituents can be described by the statistical model of Spurlin, showing that the substituents are uniformly distributed along the inulin chains and that the reactivities of the hydroxyl groups in the sugar units are independent upon substitution of a neighbouring hydroxyl group. The 4-position of the D-fructofuranosyl units was found to be the most reactive in the etherifications. © 1997 Elsevier Science Ltd.

Keywords: Inulin; Etherification; Carboxymethylation; Cyanoethylation; ^{13}C NMR spectroscopy

1. Introduction

Etherification is a widely used industrial method to prepare useful derivatives of polysaccharides. We have studied two etherification methods of inulin, a β-(2 → 1)-fructan with a D-glucose unit at the reducing end (Fig. 1). *O*-(Carboxymethyl)inulin was obtained by reaction with monochloroacetate in aqueous alkaline medium [1] and a neutral inulin derivative was prepared by the *Michael*-type addition of acrylonitrile, yielding *O*-(cyanoethyl)inulin [2]. The reactions are usually not aimed at complete substitution of the hydroxyl groups. The inulin ethers may, therefore, be considered as copolymers of unsubstituted,

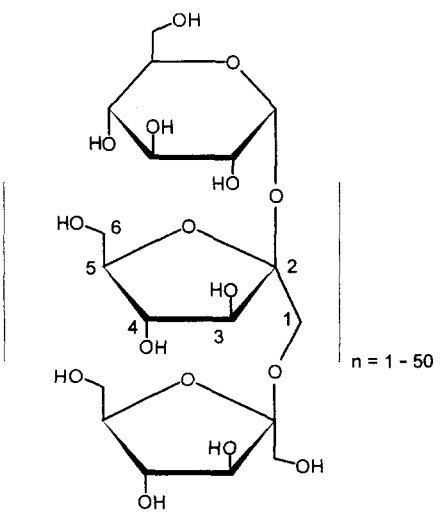

Fig. 1. Inulin.

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monosubstituted (3 regioisomers), disubstituted (3 regioisomers) and trisubstituted D-fructofuranose units, with a D-glucopyranose and a D-fructofuranose end group which have theoretically up to four substituents.

The properties of the inulin derivatives obtained depend, amongst others, on the average degree of polymerization (dp), on the degree of substitution (ds) and on the distribution of the substituents along the inulin backbone (uniformity of the product) and within the D-fructofuranose units (the relative reactivity of the three available hydroxyl groups). The first two parameters have been described in previous papers [1,2]. In the present paper, the attention is focussed on the distribution of the substituents in the inulin ethers.

The distribution of substituents has been extensively studied for derivatives of cellulose [3–9], a polysaccharide which consists of rigid chains stabilized by intramolecular hydrogen bonds [10]. Inulin, by contrast, is assumed to be very flexible [11]: the polysaccharide backbone is a polyethylene glycol chain onto which the furanose rings are attached. There is no evidence for the existence of intramolecular hydrogen bonds in solution. Electron diffraction studies show that inulin crystals are stabilized only by interchain hydrogen bonds and hydrogen bonds involving water molecules [12].

In 1939, Spurlin developed a statistical model [3,4] to evaluate the distribution of substituents along the cellulose backbone [5,6] and in the D-glucopyranose units [7–9] of cellulose ethers. In this paper, it will be shown that this model can also be applied to inulin ethers, allowing an estimation of the relative reactivities of the three hydroxyl groups in the D-furanose units.

2. Results and discussion

Reactivity of the hydroxyl groups of the D-*fructose units in inulin during etherification.*—For the study of the distribution of substituents in inulin ethers, materials with an average dp 30 were used. Upon hydrolysis of an inulin ether, a complex mixture of unsubstituted, monosubstituted (3-, 4- and 6-), disubstituted (3,4-, 4,6- and 3,6-) and trisubstituted (3,4,6-) D-fructose units is obtained. The amounts of (substituted) D-glucoses and 1-substituted D-fructoses (originating from the end groups of the inulin chains) are very low ($< 5\%$) and, therefore, these were not taken into consideration. The complexity of the mixture is enhanced by mutarotation of the monosaccharides in solution (equilibrium for D-fructose in D_2O at 27 °C: 75% β pyranose; 21% β furanose; 4% α furanose; trace of α pyranose and of open form) [13].

In a first approach to analyze the relative reactivities of the hydroxyl groups during etherification, *O*-(carboxymethyl)inulin with a low degree of substitution (ds 0.68) was hydrolyzed in acidic medium. HPLC analysis showed that the products obtained are D-fructose (48%), monosubstituted D-fructose (41%) and disubstituted D-fructose (10%). Only traces of trisubstituted D-fructose were present ($< 1\%$) at this low degree of substitution. D-Fructose was removed by incubation with *Saccharomyces cerevisiae*. Glycerol was the only organic fermentation product formed. Although the resulting mixture still is very complex (3 monosubstituted regioisomers and 3 disubstituted regioisomers, each in several anomeric forms), the ^{13}C NMR spectrum revealed one predominant monosubstituted product ($> 50\%$), showing that one of the hydroxyl groups of the D-fructose unit is much more reactive than the others during carboxymethylation.

The structure of this product was elucidated by an NMR study on the hydrolysate described above after fermentation of D-fructose. The chemical shift of the anomeric carbon C-2 (99.4 ppm) indicated a β pyranose structure [14], which implies that C-6 is not substituted since this carbon is part of the pyranose ring. The ^{13}C chemical shifts of C-1, C-6 and C-2 were assigned by comparison with unsubstituted β-D-fructopyranose (Table 1). A HETCOR (^{13}C–{^1H}) experiment (Fig. 2) identified the protons attached to C-1 and C-6. The H-6 atoms were then used as a starting point in the identification of the other protons of the pyranose ring (H-3, H-4 and H-5) using a ^1H COSY spectrum. Subsequently, C-3, C-4 and C-5 were assigned with the use of the HETCOR spectrum. The large upfield shift of H-4 and downfield shift of C-4, and the smaller opposite shifts of the neighbouring atoms (H-3, H-5, C-3, C-5) with respect to the corresponding nuclei in β-D-fructopyranose demonstrate that the substituent is located at the 4-position (Table 1). Similar substituent effects have been observed in *O*-carboxymethyl derivatives of D-glucose [5,15]. As shown by the HETCOR spectrum, the methylene ^1H resonances of the substituent (CH_2COONa) coincide at 4.05 ppm. This is supported by a triplet for the carbon at 69.8 ppm (CH_2COONa) in an ^1H-coupled ^{13}C spectrum, which collapses into a singlet upon selective irradiation of the ^1H resonance at 4.05 ppm. In conclusion, the

Table 1
^{13}C and ^{1}H chemical shifts of β-D-fructopyranose (**a**) and 4-*O*-carboxymethyl-β-D-fructopyranose (**b**), and ^{13}C chemical shifts of 4-*O*-cyanoethyl-β-D-fructopyranose (**c**). The spectra were recorded in D_2O (sample: 100 mg/mL) with *tert*-BuOH as internal standard at 25 °C and at pH 7

Compound	δ (ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	CH_2R [a]	
a [b]	65.4	99.6	69.1	71.2	70.7	64.8		
b	65.4	99.4	68.1	80.7	67.4	64.3	69.8	
c	65.3	99.6	67.2	79.9	67.7	64.7	65.7	
Δδ (**b**)	0	−0.2	−1.0	+9.5	−3.3	−0.5		
Δδ (**c**)	−0.1	0	−1.9	+8.7	−3.0	−0.1		
	δ (ppm)							
	H-1a	H-1b	H-3	H-4	H-5	H-6a	H-6b	CH_2COO^-
a [c]	3.57	3.71	3.80	3.89	3.99	3.71	4.03	
b	3.52	3.67	3.86	3.66	4.11	3.71	3.96	4.05
Δδ (**b**)	−0.05	−0.04	+0.06	−0.23	+0.12	0	−0.07	

[a] R = COONa for (**b**) and CH_2CN for (**c**).
[b] Taken from Ref. [14].
[c] Taken from Ref. [22].

main product formed by hydrolysis of *O*-(carboxymethyl)inulin with a relatively low ds (ds 0.68) is 4-*O*-carboxymethyl-β-D-fructopyranose (Fig. 3) showing that the hydroxyl group at the 4-position is the most reactive during carboxymethylation.

Similarly, 4-*O*-cyanoethyl-β-D-fructopyranose was found to be the main product (about 50%) upon similar treatment of *O*-(cyanoethyl)inulin with a ds 0.70. The ^{13}C chemical shifts are included in Table 1. These data unequivocally show that also in cyanoethylation the hydroxyl group at the 4-position is the most reactive.

One of the possible explanations for the high preference for the 4-position might be the relatively high acidity of secondary hydroxyl groups as compared to primary ones. This higher acidity was also found for D-glucose units in starch, cellulose, β-maltose, and cyclodextrins based on molecular calculations [16], and was suggested to be responsible for the higher reactivity of the 2-positions during etherification of cellulose [5,17]. However, during etherification, the concentration of NaOH used is high (0.6 M for cyanoethylation; > 1 M for carboxymethylation; pH > 13). It may, therefore, be assumed that

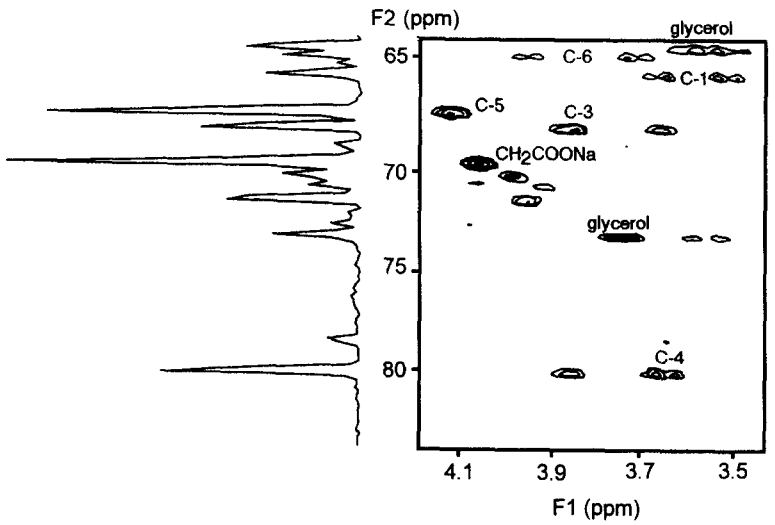

Fig. 2. HETCOR spectrum of 4-*O*-carboxymethyl-β-D-fructopyranose in D_2O, pH 7 (100 MHz, 25 °C).

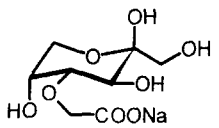

Fig. 3. 4-*O*-carboxymethyl-β-D-fructopyranose.

under these conditions secondary as well as primary hydroxyl functions are at least partially dissociated. This was supported by the considerable displacements of the ^{13}C chemical shifts (0.6–1.2 ppm) of the primary and secondary alcohol carbon atoms of a sample of inulin dissolved in D_2O upon addition of NaOH (2 equiv with respect to hydroxyl groups). The results of these measurements should, however, be regarded with care, because the displacements are probably not only caused by deprotonation but also by conformational changes upon deprotonation.

Methyl α-D-fructofuranoside, a model compound representing one D-fructofuranoside moiety of inulin, was carboxymethylated and cyanoethylated under the same reaction conditions as inulin. ^{13}C NMR spectra of the products showed that there was no main product formed as in the case of inulin, but that the four regioisomers (1-, 3-, 4- and 6-substituted methyl α-D-fructofuranosides) were formed in about equal amounts, which points to comparable reactivities of all available hydroxyl groups of the monomeric D-fructofuranoside. These experiments confirm that the concentration of NaOH used is high enough to deprotonate (at least partially) secondary as well as primary hydroxyl groups. The selectivity for the 4-position of the D-fructofuranoside units in inulin during etherification reactions must thus be ascribed to structural features of the inulin chain in solution, rather than to differences in acidity of the hydroxyl groups. The lower reactivity of the 3-position may be explained by its lower accessibility for the reagents as this position is located close to the inulin backbone. Furthermore, the hydroxyl function at the 3-position is oriented cis with respect to the hydroxyl group at the 6-position. Steric effects will thus favour the 4-position. The ^{13}C NMR spectrum of the hydrolyzed product of cyanoethylated nystose revealed also 4-*O*-cyanoethyl-β-D-fructopyranose as the main product. However, the selectivity for the 4-position was lower than in the case of inulin (dp 30), which, once again, confirms that steric factors play an important role. Obviously, inulin has a conformation in solution wherein the hydroxyl groups at the 4-positions are in particular exposed.

Analysis of hydrolyzed O-*(carboxymethyl)inulin and* O-*(cyanoethyl)inulin: evaluation of substitution patterns.*—In order to obtain information on the distribution of substituents along the inulin chains (uniformity of the products), the hydrolyzed ethers were analyzed using HPLC and ^{13}C NMR spectroscopy. The results were evaluated using the statistical model developed by Spurlin [3].

^{13}C NMR spectra of O-cyanoethyl-D-*fructoses.*—Mono-, di- and trisubstituted *O*-cyanoethyl-D-fructoses, obtained by acidic hydrolysis of *O*-(cyanoethyl)inulin, were separated by preparative reversed phase HPLC and then identified using ^{13}C NMR spectroscopy. The substituent effects observed for 4-*O*-cyanoethyl-β-D-fructopyranose (see above), and the peak intensities were used for the assignment. Only the analytically interesting region of the NMR spectra (70–90 ppm) was taken into account. In this region the signals of C-3, C-4 and C-5 of the D-fructoses are found. The differences in chemical shifts of the signals of the other carbon atoms (C-1, C-2, C-6 and CH_2COONa) are too small to make assignments.

In order to identify the monosubstituted D-fructoses, the model compound methyl α-D-fructofuranoside was cyanoethylated to a ds of about 1, and the products were separated using preparative reversed phase HPLC. Subsequently, the fractions were analyzed using ^{13}C NMR spectroscopy. Fractions containing pure methyl 3-*O*-cyanoethyl-α-D-fructofuranoside (fraction I) and a mixture of 1-, 4- and 6-substituted methyl α-D-fructofuranosides (fraction II) were obtained. Fraction I was hydrolyzed in acidic medium and then the peaks of the 3-substituted D-fructoses (β pyranose and β furanose) in the ^{13}C NMR spectrum were assigned. From a similar NMR analysis of hydrolyzed fraction II, the ^{13}C NMR resonances of the 4- and the 6-substituted β furanoses were assigned (Table 2). The α anomers were only present in minor amounts and were not further analyzed.

For the assignment of the signals of the disubstituted D-fructoses, a sample of *O*-(cyanoethyl)inulin (ds 1.0) was hydrolyzed and partly separated with preparative HPLC. NMR analysis revealed that one fraction contained pure 3,4-disubstituted D-fructose while another consisted of 3,4- and 4,6-disubstituted D-fructose. The peaks were assigned as shown in Table 2. The NMR spectrum of highly cyanoethylated inulin (ds 2.0), in which the trisubstituted D-fructoses are predominant, revealed the signals of 3,4,6-trisubstituted D-fructose. Only in the case of 4,6-di-*O*-cyanoethyl-D-fructose and 3,4,6-tri-*O*-

Table 2
^{13}C chemical shifts of *O*-cyanoethyl-D-fructoses. The spectra are recorded in D_2O (sample: 100 mg/mL) with *tert*-BuOH as internal standard at 25 °C and at pH 7

	β-D-fructopyranose			β-D-fructofuranose			α-D-fructofuranose		
	C-3	C-4	C-5	C-3	C-4	C-5	C-3	C-4	C-5
Unsubstituted	68.4	70.5	70.1	76.5	75.4	81.5	82.9 [a]	77.0 [a]	82.2 [a]
3-substituted	77.8	71.1	69.3	84.9	75.6	82.2	n.i. [b]		
4-substituted	67.2	79.9	67.7	73.1	84.9	81.2	n.i. [b]		
6-substituted				76.5	76.1	80.4	n.i. [b]		
3,4-substituted	76.2	80.0	69.2	84.6	84.4	80.6	n.i. [b]		
4,6-substituted				76.2	85.4	79.4	81.2	87.3	80.8
3,4,6-substituted				84.9	84.4	79.7	89.2	87.1	81.3

[a] Data taken from Ref. [14].
[b] n.i.: not identified.

cyanoethyl-D-fructose, the α furanose anomers were identified. For the other isomers, the amount of this anomer was too low to be detectable with NMR spectroscopy. Only traces of the α pyranoses of the various isomers were present in the samples. 3,6-Di-*O*-cyanoethyl-D-fructose was not found in any of the mixtures. Complete overlap of their ^{13}C NMR signals with those of other disubstituted D-fructoses is not likely, as its C-4 atom is expected to undergo an upfield shift due to the substituent on the 3-position, in contrast with the downfield shift of C-4 in the other disubstituted D-fructoses.

The relative amounts of each isomer in *O*-(cyanoethyl)inulin samples with varying ds were determined. Each sample was first hydrolyzed and subjected to HPLC analysis in order to quantify the fraction of unsubstituted D-fructoses. Then, D-fructose was removed from the mixture by incubation with *Saccharomyces cerevisiae* and the samples were analyzed using quantitative ^{13}C NMR spectroscopy with signal deconvolution of the spectral region of interest. An example of a ^{13}C NMR spectrum is shown in Fig. 4. The monomer compositions of *O*-(cyanoethyl)inulin samples with varying ds are given in Table 3. The error in the relative amounts of each isomer is quite large (about 10%) due to the neglect of the α anomers and the 3,6-disubstituted isomers and the complexity of the spectra. During the calculation of the relative rate constants, these errors were taken into account (see below) and general trends in the distribution of substituents could be revealed.

^{13}C NMR spectra of O-carboxymethyl-D-fructoses. —A similar procedure was used for the analysis of the relative amounts of the (substituted) D-fructoses

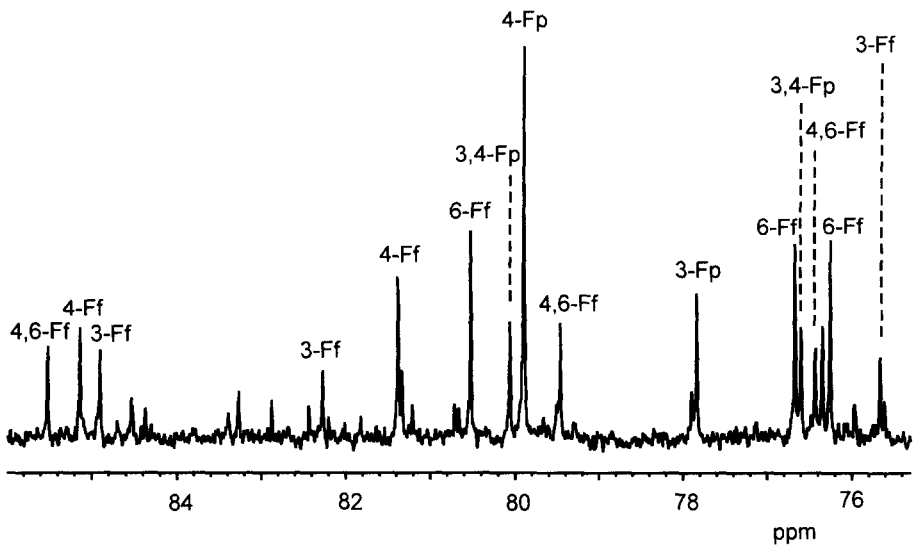

Fig. 4. ^{13}C NMR spectrum of *O*-(cyanoethyl)inulin after hydrolysis and removal of the unsubstituted monosaccharides (100 mg/mL D_2O, pH 7, 25 °C).

Table 3
Monomer composition of *O*-(cyanoethyl)inulin with varying ds. The data were obtained by HPLC and deconvolution of quantitative ^{13}C NMR spectra (see text)

Molar fraction	ds				
	0.45	0.70	1.10	1.33	1.98
Unsubstituted	0.60	0.43	0.26	0.19	0.02
3-substituted	0.08	0.10	0.09	0.09	0.04
4-substituted	0.22	0.27	0.22	0.25	0.15
6-substituted	0.06	0.08	0.09	0.10	0.03
3,4-substituted	0.04	0.06	0.16	0.19	0.26
4,6-substituted	0.02	0.05	0.12	0.17	0.20
3,6-substituted	0	0	0	0	0
3,4,6-substituted	0	0	0.05	0.05	0.31

obtained by hydrolysis of *O*-(carboxymethyl)inulin. The relative amounts of unsubstituted, mono-, di- and trisubstituted D-fructoses were determined by HPLC. Preparative separation of these groups of isomers was performed on an anion-exchange resin. By analyzing each fraction with ^{13}C NMR spectroscopy, the signals of each isomer were identified (Table 4). Because the methylene carbon of the substituent of *O*-carboxymethyl-D-fructose is located around 70 ppm, the useful spectral region is narrower than in the case of the *O*-cyanoethyl-D-fructoses and therefore not all C-3, C-4 and C-5 signals could be identified. The relative amounts of each isomer in the polymeric chain was obtained by deconvolution of the spectral region of interest (75–90 ppm) in quantitative ^{13}C NMR spectra of samples of hydrolyzed *O*-(carboxymethyl)inulin of varying ds, from which the unsubstituted D-fructose had been removed (Table 5).

Monomer composition of hydrolyzed O-*(cyanoethyl)-* *and* O-*(carboxymethyl)inulin.*—The statistical model which has been developed by Spurlin [3,4], and further elaborated by Reuben and coworkers [7–9] for cellulose ethers, is used here for the evaluation of the substituent distribution in inulin ethers. The model assumes that (1) the substitution pattern is governed exclusively by the relative reactivities of the hydroxyl groups; (2) the substituent distribution along the backbone is homogeneous; (3) the relative reactivities of the hydroxyl groups are independent of the ds and are not influenced by substitution of other positions in the unit; (4) the influence of the end groups of the polymer can be neglected. Two sets of equations have been derived, one for the case that the substitution is governed by an equilibrium, and another for first order kinetic processes. Carboxymethylation of polysaccharides with monochloroacetate is known to be a first order irreversible reaction [3,7], while the addition of acrylonitrile is in principle reversible [2]. However, when *O*-(cyanoethyl)inulin was hydrolyzed and the monosaccharides obtained were subjected to the reaction conditions used for cyanoethylation, no change in monomer composition (from preferentially 4-substituted D-fructoses to a thermodynamic equilibrium of about equal amounts of 3-, 4- and 6-substituted D-fructoses) was observed. It can be concluded that the cyanoethylation of inulin is kinetically controlled. The monomer compositions of both *O*-(carboxymethyl)inulin and *O*-(cyanoethyl)inulin were therefore calculated with the kinetic model. [1]

[1] Besides the kinetic model, also the equilibrium model was used to fit the data points obtained for *O*-(cyanoethyl)inulin. The resulting values for the relative reactivities and the curves obtained appeared to be similar for both models. It was thus not possible to conclude from the fitting results whether the cyanoethylation is kinetically or thermodynamically controlled.

Table 4
^{13}C chemical shifts of *O*-carboxymethyl-D-fructoses. The spectra were recorded in D_2O (sample: 100 mg/mL) with *tert*-BuOH as internal standard at 25 °C and at pH 7

	β-D-fructopyranose			β-D-fructofuranose			α-D-fructofuranose		
	C-3	C-4	C-5	C-3	C-4	C-5	C-3	C-4	C-5
Unsubstituted	68.4	70.5	70.1	76.5	75.4	81.5	82.9 [a]	77.0 [a]	82.2 [a]
3-substituted	78.8			85.5	75.7	82.8	n.i. [b]		
4-substituted	68.1	80.7	67.4		85.2	81.3	n.i. [b]		
6-substituted				76.6	76.4	81.2	n.i. [b]		
3,4-substituted	76.7	81.2		84.6	84.4	81.9	n.i. [b]		
4,6-substituted				76.4	85.3	79.9	n.i. [b]		
3,4,6-substituted				85.0	84.7	80.3	87.1	84.5	80.5

[a] Data taken from Ref. [14].
[b] n.i.: not identified.

Table 5
Monomer composition of *O*-(carboxymethyl)inulin with varying ds. The data were obtained by HPLC and deconvolution of quantitative ^{13}C NMR spectra (see text)

Molar fraction	ds		
	0.68	1.04	1.76
Unsubstituted	0.42	0.25	0.05
3-substituted	0.11	0.11	0.04
4-substituted	0.33	0.36	0.27
6-substituted	0.06	0.07	0.03
3,4-substituted	0.04	0.12	0.24
4,6-substituted	0.03	0.08	0.19
3,6-substituted	0	0	0
3,4,6-substituted	0	0	0.25

The probability of having an unsubstituted hydroxyl group in position i can be described in terms of the relative rate constant (k_i) and a time parameter, B, related to the duration of the reaction [3,7].

$$p_i = e^{-Bk_i}, \quad i = 3, 4 \text{ or } 6. \tag{1}$$

The fraction of D-fructose moieties in which position i is substituted, x_i, is thus given by:

$$x_i = 1 - p_i. \tag{2}$$

The degree of substitution, ds, is calculated as:

$$\text{ds} = x_3 + x_4 + x_6 = 3 - e^{-Bk_3} - e^{-Bk_4} - e^{-Bk_6}. \tag{3}$$

The experimental values of x_i as a function of ds were fitted with Eq. (2) for each position using a least squares procedure. During the minimization, B was recalculated as a function of ds for each new set of k_i-values using Eq. (3). The values for k_i obtained are given in Table 6. In order to estimate uncertainties on k_i, random errors (up to 10%, corresponding to the experimental errors, see above) were imposed on the calculated data. For five independent sets of data obtained by this means, values of k_i were determined. Subsequently, uncertainties were calculated from the standard deviation. Curves of molar fractions of unsubstituted, mono-, di- and trisubstituted D-fructose moieties as a function of ds were

Table 6
Relative rate constants of 3-, 4-, and 6-position (k_3/k_4, k_6/k_4) for etherification of inulin with monochloroacetate (CMI) and with acrylonitrile (CEI) in aqueous alkaline medium. The uncertainties were calculated from the standard deviations as described in the text

	k_3/k_4	k_6/k_4
CMI	0.34 ± 0.06	0.25 ± 0.07
CEI	0.42 ± 0.06	0.36 ± 0.05

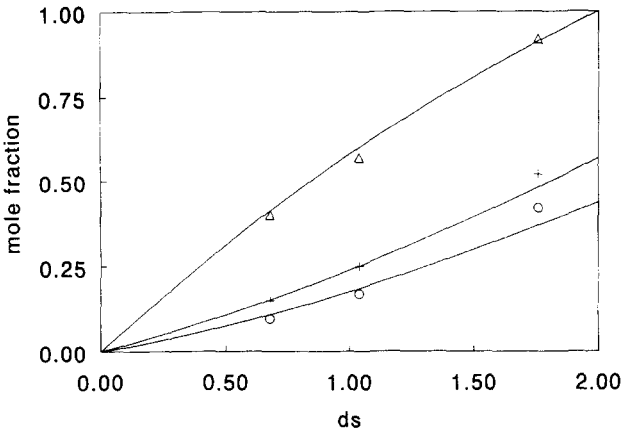

Fig. 5. Molar fractions of D-fructose units in which position i is carboxymethylated (x_i) in function of ds: +: x_3; △: x_4; ○: x_6. The curves are calculated using the statistical kinetical model: agreement factor $\Re = 0.08$ (x_3); 0.08 (x_4); 0.17 (x_6).

calculated using the values of k_i and B obtained. The close conformity of the experimental data to the calculated values (agreement factors [2] $\Re \simeq 0.1$, see Figs. 5–8) indicates that the assumptions made in the statistical model are justified: The inulin ethers have a homogeneous substituent distribution along their backbone. A heterogeneous distribution would be reflected in larger fractions of unsubstituted and highly substituted monomers [5,6]. No enhancement of reactivities of hydroxyl groups upon substitution

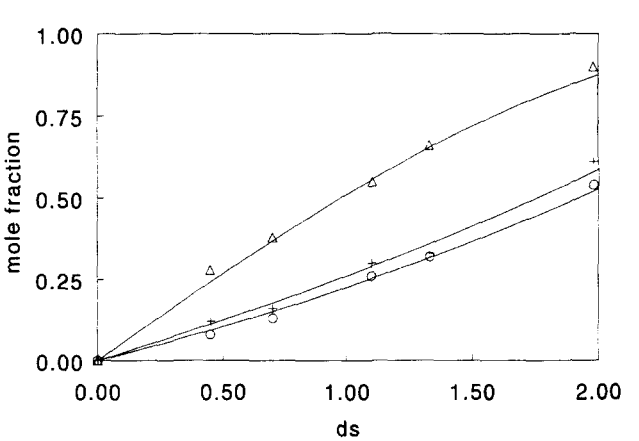

Fig. 6. Molar fractions of D-fructose units in which position i is cyanoethylated (x_i) in function of ds: +: x_3; △: x_4; ○: x_6. The curves are calculated using the statistical kinetical model: agreement factor $\Re = 0.06$ (x_3); 0.04 (x_4); 0.05 (x_6).

[2] $\Re = (\Sigma(\text{exp} - \text{fit})^2 / \Sigma(\text{exp})^2)^{1/2}$ with 'exp' is the experimental value and 'fit' the calculated value.

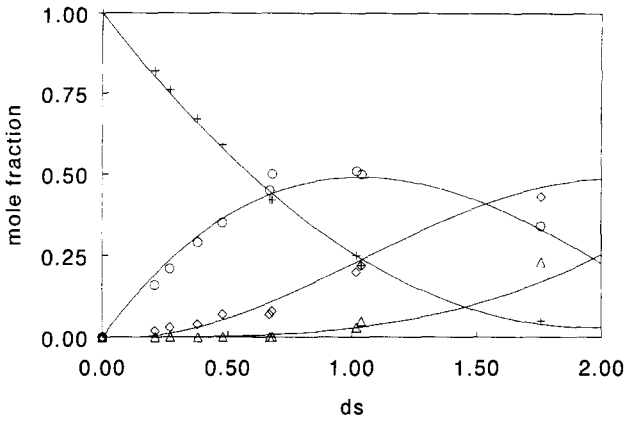

Fig. 7. Molar fractions of unsubstituted (c_0, +), mono- (c_1, ○), di- (c_2, ◇) and tricarboxymethylated (c_3, △) D-fructose units in function of ds. The datapoints were obtained from HPLC analysis and from ^{13}C NMR spectroscopy. The curves are calculated using the statistical kinetical model: agreement factor $\Re = 0.02$ (c_0); 0.06 (c_1); 0.10 (c_2); 0.20 (c_3).

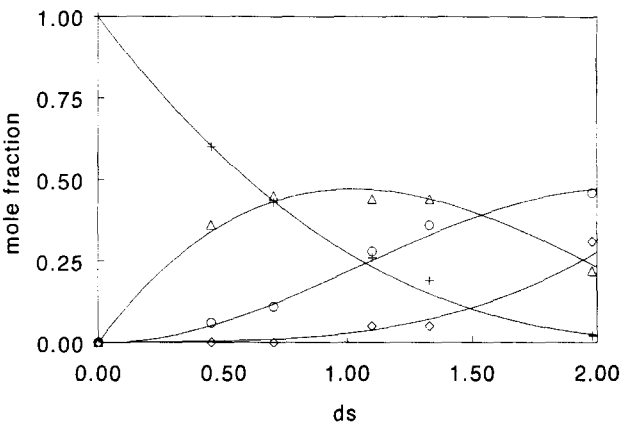

Fig. 8. Molar fractions of unsubstituted (c_0, +), mono- (c_1, △), di- (c_2, ○) and tricyanoethylated (c_3, ◇) D-fructose units in function of ds. The curves are calculated using the statistical kinetical model: agreement factor $\Re$ = 0.06 (c_0); 0.05 (c_1); 0.06 (c_2); 0.17 (c_3).

of neighbouring ones has been found, by contrast to studies of cellulose ethers [9,18]. The main factor responsible for the enhancement of reactivity of the 3-position in cellulose is its involvement in an intramolecular hydrogen-bond [10], which is disrupted upon 2-substitution. This is only observed with neutral reactants, which are not able to break intramolecular hydrogen-bonds (e.g. ethyl chloride) [7,9,19]. For the etherification of inulin with an ionized reactant (monochloroacetate), as well as with a neutral reactant (acrylonitrile), no such effects were observed. Apparently, the hydroxyl groups of inulin are not involved in strong hydrogen bonds. The absence of intramolecular hydrogen bonds has also been suggested based on molecular modeling [20] and electron diffraction data [12]. Intermolecular hydrogen bonds which exist in crystalline inulin [12] are presumably broken upon dissolution.

The monomer composition of *O*-(carboxymethyl)inulin prepared from commercially available inulin with a relatively low dp (ex chicory, average dp 10) was analyzed by HPLC. To this end, a set of samples with varying ds, obtained from an independent study on technical properties of *O*-(carboxymethyl)inulin, was used. The data for the

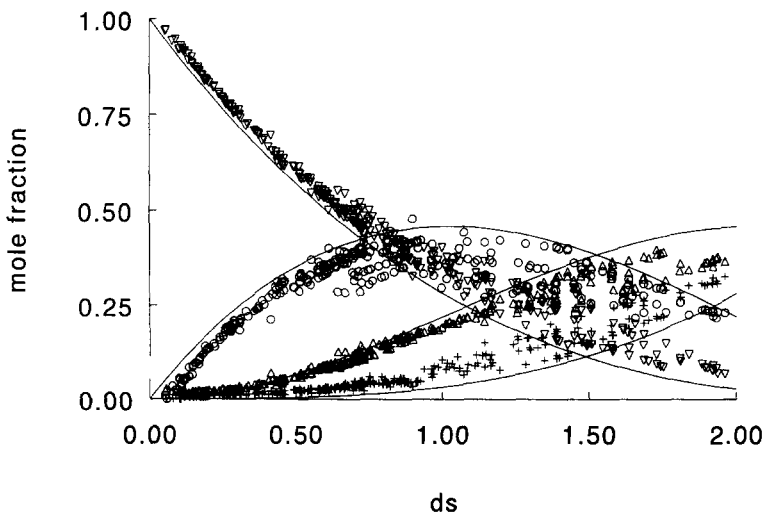

Fig. 9. Molar fractions of unsubstituted (c_0, ▽), mono- (c_1, ○), di- (c_2, △) and tricarboxymethylated (c_3, +) D-fructose units in function of ds. The datapoints were obtained from HPLC analysis. The curves are calculated using the statistical kinetical model.

molar fraction of unsubstituted, mono-, di- and trisubstituted monomers were compared to curves calculated with relative k_i-values: $k_3:k_4:k_6$ = 0.5:1:0.5 (Fig. 9). An accurate determination of the relative k_i values was not necessary because these curves are not much influenced by the relative k_i values [4]. The experimental monomer compositions agree reasonably well with the calculated values (Fig. 9). The deviations may be explained by the relatively large amount of monosaccharides (D-glucose and D-fructose) in the starting material (see Experimental), which may carry up to five substituents. Moreover, the end groups of the inulin chains are present in a higher amount than in the case of long chain inulin and the assumption that these end groups can be neglected is less justified here. We tentatively conclude that also in short chain inulin ethers, the substituents are homogeneously distributed along the polysaccharide chains.

3. Experimental

Materials.—Inulin with an average dp 30 was obtained from E. Merck (Darmstadt, Germany) and is essentially monosaccharide free. Inulin isolated from chicory root (Coöperatie Cosun U.A., Roosendaal, The Netherlands) with an average dp 10 contains 5.4% monosaccharides (D-glucose and D-fructose) and 4.5% sucrose (HPLC analysis on a CarboPac PA-1 column). Methyl α-D-fructofuranoside was synthesized and purified as previously described [21]. Methyl α-D-glucopyranoside was obtained from Acros Chimica (Geel, Belgium). Carboxymethylations and cyanoethylations were performed as described in detail elsewhere [1,2]. The inulin ethers were hydrolyzed to monosaccharides prior to analysis by dissolving 1 g of product in 50 mL H_2O, acidifying the solution to pH 1.5 by adding 2 M HCl and heating at 70 °C for 1 h. The unsubstituted units were removed by neutralisation of the solution, adding 0.5 g of *Saccharomyces cerevisiae* and incubation at room temperature for 24 h.

HPLC analysis.—HPLC of carboxymethylated monosaccharides on a Phenomenex column (Bester, Amstelveen, The Netherlands), Rezex Organic Acid (7.8 mm i.d. × 300 mm), using 0.01 M CF_3CO_2H (60 °C; flow rate: 0.6 mL/min; RI and UV_{215} detection) yielded a good separation of unsubstituted, mono-, di-, and trisubstituted monosaccharides (retention time 11.8 min, 10.5 min, 9.8 min, and 9.3 min, respectively). For the quantitative determination of the relative amounts of monosaccharides, the relative peak areas of the RI signals, corrected for the molecular mass of the compounds, were used. The reliability of this method was confirmed by checking the mass balance by analyzing samples during carboxymethylation. HPLC analysis of cyanoethylated monosaccharides was carried out on a Phenomenex column (Bester, Amstelveen, The Netherlands), Rezex Cal Monosaccharide (7.8 mm i.d. × 300 mm), using water (80 °C; flow rate: 0.6 mL/min; RI detection). Separation of unsubstituted and substituted monosaccharides was obtained (retention time 13.6 min and 11.0–12.5, min respectively). With this method, the fraction of unsubstituted D-fructose units was determined using D-ribose (retention time 21.8 min) as internal standard.

NMR spectroscopy.—^{1}H NMR spectra were recorded on a Varian VXR-400 S spectrometer and ^{13}C NMR spectra on a Nicolet NT-200 WB or a Varian VXR-400 S spectrometer. D_2O was used as solvent and *tert*-butanol as internal standard (^{1}H NMR, CH_3 at 1.2 ppm; ^{13}C NMR, CH_3 at 31.2 ppm). Quantitative ^{13}C NMR spectra were recorded with a pulse angle of 45°, a relaxation delay of 30 s and decoupling during acquisition only. Solutions containing 100 mg product in 1 mL D_2O and pH 7 were used.

Column chromatography.—The carboxymethylated monosaccharides (2 g) were separated on an anion-exchange resin (Dowex 1X8-200 in the carbonate form, column 20 × 130 mm). The unsubstituted monosaccharides were eluted with water (150 mL) and subsequently, the carboxymethylated monosaccharides were eluted with 0.5 M $(NH_4)_2CO_3$. Fractions of 50 mL were collected and analyzed with HPLC and NMR spectroscopy.

The cyanoethylated monosaccharides (0.5 g) were injected onto a preparative reversed phase HPLC column (Waters RCM-base, Novapack C_{18} 6 μm, 40 × 200 mm) using water as eluent at a flow rate of 20 mL/min and RI detection. Separation of cyanoethylated methyl α-D-fructofuranoside yielded four fractions which were analyzed with ^{13}C NMR spectroscopy. The first fraction (130 mL) contained no organic material and the second (60 mL) contained the unsubstituted starting material. The monosubstituted methyl *O*-cyanoethyl-D-fructosides eluted in the third (110 mL) and the fourth (250 mL) fraction. Higher substituted methyl *O*-cyanoethyl-D-fructosides were not recovered from the column using water as the eluent. These compounds were eluted with MeOH as the eluent. Separation of the cyanoeth-

ylated monosaccharides obtained by hydrolysis of *O*-(cyanoethyl)inulin yielded unsubstituted saccharides in the first fraction (10 mL) and monosubstituted D-fructoses in the second fraction (60 mL). Disubstituted D-fructoses eluted in the third (100 mL) and the fourth (500 mL) fraction. This is in contrast with the disubstituted methyl α-D-fructofuranosides, which were not eluted from the column due to their higher hydrophobicity. The trisubstituted D-fructoses were recovered from the column using MeOH as the eluent.

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