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Determination of the structure and degree of polymerisation of fructans from *Echinacea purpurea* roots

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Abstract—Highly water soluble fructans have been isolated from *Echinacea purpurea* (L.) Moench. roots by hot water extraction and precipitation at three different ethanol concentrations (80% v/v, 60% v/v and 40% v/v). The structure of the fructans has been characterised by three analytical methods: GC of silylated oxime derivatives and partially methylated alditol acetates, respectively, as well as ^{13}C NMR analysis. The mean degree of polymerisation (mean DP) of each fructan has been determined by the glucose/fructose ratio. *E. purpurea* fructans represent linear inulin-type fructans with almost exclusively β-(2→1)-linked fructosyl units, terminal glucose and terminal fructose. Small proportions of β-(2→1,2→6)-linked branch point residues were detected. The mean DP of the fructan fractions depends on the ethanol concentration used for precipitation: the lower the ethanol concentration the higher the mean DP. Corresponding results were found with all of the three analytical methods: 80% ethanol-insoluble fructan from *E. purpurea* shows an average mean DP of 35, 60% ethanol-insoluble fructan of 44 and 40% ethanol-insoluble fructan of 55. The applied methods provide sufficient sensitivity to determine not only the composition and structure but also the mean degree of polymerisation of fructans.

Keywords: *Echinacea purpurea*; Asteraceae; Polysaccharides; Fructan; Inulin; Degree of polymerisation, DP

1. Introduction

Fructans are important storage polymers in numerous plant species including several Asteraceae, Liliaceae and Poaceae of major importance such as cereals and forage grasses. The most common sources are underground organs of chicory, Jerusalem artichoke, asparagus and members of the onion family.[1–5] Fructan molecules are fructose-rich polymers biosynthetically derived from sucrose, and therefore originally contain one glucose residue. This property is a good supposition for the determination of the chain length or degree of polymerisation (DP), respectively, resulting from the fructose/glucose ratio. Asteraceae store an inulin that contains mainly, if not exclusively, β-(2→1)-glycosidic linkages.[6] Jerusalem artichoke and chicory are the most productive fructan-accumulating members of that family, the latter being the most commonly used source. The structure of chicory inulin is well documented.[7–9] Careful analysis showed that even inulins of chicory and dahlia may contain small amounts of unusual linkages like β-(2→6)-linkages and branch point residues.[10,11] In inulin, the vast majority of glucose-containing molecules is often accompanied by small amounts of fructo-oligosaccharides probably arising from an plant-own inulinase activity. Graminan and phlein, storage carbohydrates of the Poaceae and bacterial levans, however, consist predominantly of β-(2→6)-linked fructosyl units or even highly branched structures comprised of both β-(2→1)- and β-(2→6)-linked fructosyl residues.[12,13] Linear and branched polymers show a wide range of DP depending not only on the species

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and plant tissue but also on the physiological state of the plant and environmental conditions. Oligo-fructans with DP 5–10 are found in *Avena sativa* and with a DP up to 260 in *Phleum pratense*.[2] In contrast to plant inulin, bacterial levan has very high DPs ranging from 10,000 to 100,000. When characterising the mean degree of polymerisation (mean DP) of total plant fructans, the applied isolation steps are of importance. Native tissue in addition to inulin always contains glucose, fructose, sucrose and small oligosaccharides. During isolation procedures, smaller oligosaccharides and monomers that are naturally present are commonly eliminated. Commercially available inulin may therefore have a mean DP of 12 (native) or 25.[14] Consequently, the mean DP of isolated fructan fractions is always dependent on the isolation procedure.

Up to now, techniques used for structural fructan analysis and determination of the DP include different chromatographic methods as TLC, GC and HPLC as well as enzymatic methods following total hydrolysis of the polymers.[15] For GC, glucose and fructose monomers must be converted to volatile derivatives. The critical factor of all these analyses is the hydrolysis procedure because of the instability of fructans in acids, where fructose units might be degraded resulting in the determination of a too low DP.

Therefore, we optimised the derivatisation procedures for GC analyses and compared DP results from GC separation of glucose and fructose derivatives of fructans with ^{13}C NMR spectroscopic data. NMR spectroscopy has the advantage of no need for hydrolysis and further derivatisation of hydrolysis products. The DP is deduced from the ratio of the signal intensities of relevant carbons.

The occurrence of fructans in *Echinacea* species has been known for a long time. Only preliminary results for the size and structure of fructans from pressed juice of *Echinacea purpurea* (L.) Moench. have been reported.[16] The present work describes the isolation procedure and structural analysis including the determination of the mean DP of fructans from aqueous extracts of the roots of *E. purpurea*. Characterising isolated fructan fractions by the mean DP with simple and rapid methods might be an option to characterise the origin and age of plant materials.

2. Results and discussion

Total fructan from roots of *E. purpurea* was extracted with hot water to inhibit enzyme activity. After removal of phenolic compounds, fructans were precipitated from the water solutions by the addition of ethanol using three final ethanol concentrations, and were purified by repeated dissolution and precipitation. Known fructan standards as well as fructans from *Echinacea* were analysed by GC following hydrolysis and derivatisation by silylation and methylation analysis, and ^{13}C NMR spectra were recorded. Assuming that each fructan molecule contains a single glucose moiety, the DP was calculated from the fructose/glucose ratio. GC analyses of the trimethylsilyloxime derivatives and partially methylated alditol acetates showed that all the peaks were fructan, since only glucose and fructose were present and no (1→4)-linked glucose residue was demonstrated. Also by ^{13}C NMR, only signals for terminal glucose residues were found. Silylation analysis revealed a mean DP of 12 for chicory inulin and a mean DP 33, DP 42 and DP 50 for 80%, 60% and 40% ethanol-insoluble fructan from *Echinacea* roots, respectively.

Results of methylation analyses of the same fructans provided in addition information on the molecular structure. Chromatograms of inulin and 80% ethanol-insoluble *Echinacea* fructans are shown in Figure 1. Results indicated an overall linear structure of the *Echinacea* fructan with D-glucopyranosyl end groups (Table 1). The major derivative of fructose is 3,4,6-trimethylated, indicating a β-(2→1)-linked backbone. In addition, a 3,4-dimethylated derivative was also found in agreement with the presence of branched residues. The number of terminal non-reducing fructose residues indicated by 1,3,4,6-tetramethyl-2,5-diacetyl-mannitol and 1,3,4,6-tetramethyl-2,5-diacetyl-glucitol (two peaks) is in accordance with the number of terminal glucose residues. Although some branching exists according to methylation analysis, the amount of terminal fructose is comparable to the amount of terminal glucose. The reason may be higher instability of terminal fructose compared to terminal glucose during derivatisation. All unlabelled peaks in Figure 1 result from non-carbohydrate impurities. Linear β-(2→6)-linkages between fructose residues can be excluded. Although partially methylated alditol acetates of β-(2→1)-linked and β-(2→6)-linked fructofuranoses have the same elution time on the used GC column, they can be distinguished by their mass spectra. The basis for the differentiation is the asymmetry introduced by reduction of the partially methylated fructoses at C-2 with sodium borodeuteride. Derivatives from β-(2→1)-linkages yield ions of m/z 190 and m/z 161 as primary fragments. No significant amounts of m/z 189 and m/z 162 typical for derivatives of β-(2→6)-linked fructofuranoses were detected, suggesting that these linear linkages are not present. Results of the methylation analyses confirmed DP 3 for 1-kestose and DP 4 for nystose. The commercially available chicory inulin was native and showed a mean DP of 12 (Table 1). In *Echinacea* extracts, precipitation with 40% ethanol leads to a mean DP of 59, with 60% ethanol to a mean DP of 46 and with 80% ethanol to a mean DP of 36 by methylation analysis.

The ^{13}C NMR spectrum of 80% ethanol-insoluble *Echinacea* fructan dissolved in D_2O is presented in

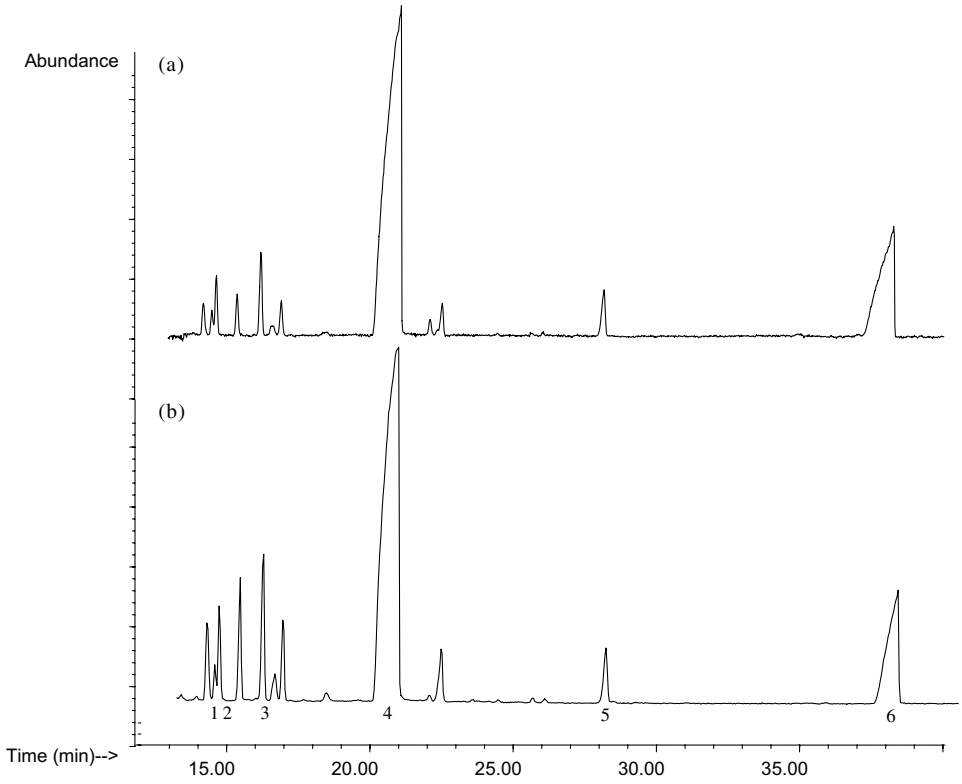

Figure 1. GC profiles of partially methylated alditol acetates of 80% ethanol-insoluble fructan from *Echinacea* roots (a) and chicory inulin (b). Peaks were: 1, t-Fru; 2, t-Fru; 3, t-Glc; 4, 1-Fru; 5, 1,6-Fru; 6 = internal standard myo-inositol.

Table 1. Percentage (%) and number (*n*) of the β-D-Fru and α-D-Glc residues in chicory inulin and ethanol-insoluble fructan fractions from *Echinacea* roots determined by methylation analysis

	Deduced linkage	Chicory inulin		*Echinacea* fructan					
				EtOH 80%		EtOH 60%		EtOH 40%	
		%	*n*	%	*n*	%	*n*	%	*n*
β-D-Fru	Terminal	6.3	0.8	2.3	0.9	1.4	0.7	1.4	0.9
	(2→1)-Linked	82.9	10.2	93.8	33.2	94.5	43.5	95.2	56.2
	(2→1, 2→6)-Linked	3.4	0.4	1.4	0.5	2.1	0.9	1.9	1.0
α-D-Glc	Terminal	7.5	1.0	2.6	1.0	2.0	1.0	1.6	1.0
DP			12		36		46		59

Figure 2. The spectra of 1-kestose, nystose and chicory inulin are shown in the same figure. These molecules were used as reference materials, all of which contain β-(2→1)-linked fructose residues. 1-kestose and nystose are supposed to be the structural building blocks of larger inulins from the Asteraceae. All resonances observed in the ^{13}C NMR spectra could be assigned to the carbons of the fructans (Table 2). The fructofuranosyl C-2 resonances for 1-kestose are clearly resolved and appear at 102.65 and 103.08 ppm. Three signals occur for the three anomeric fructosyl carbons in nystose at 102.65, 102.46 and 103.08 ppm. The signals of the ring carbons of the terminal glucose unit in the oligo-fructan are well separated from those of the fructosyl carbons, and on the basis of the intensities of the glucosyl carbon signals relative to those from the fructosyl carbons, the mean DP was estimated. Analysis confirmed the presence of glucose and fructose in the ratio 1:2 for 1-kestose, 1:3 for nystose and the simple structure of β-(2→1)-linked fructofuranan with DP 13 for commercially available inulin from chicory roots. The ^{13}C NMR data for the isolated fructan fractions from *Echinacea* roots clearly show the resonances of chicory inulin with only higher intensities for the β-(2→1)-linked fructofuranosyl residues relative to terminal fructosyl residues and glucosyl units. DP 37 is deduced from the relative signal intensities of glucose and fructose for 80% ethanol-insoluble material, DP 45 for 60% ethanol-insoluble material and DP 57 for 40% ethanol-insoluble material. These values are in accordance with those

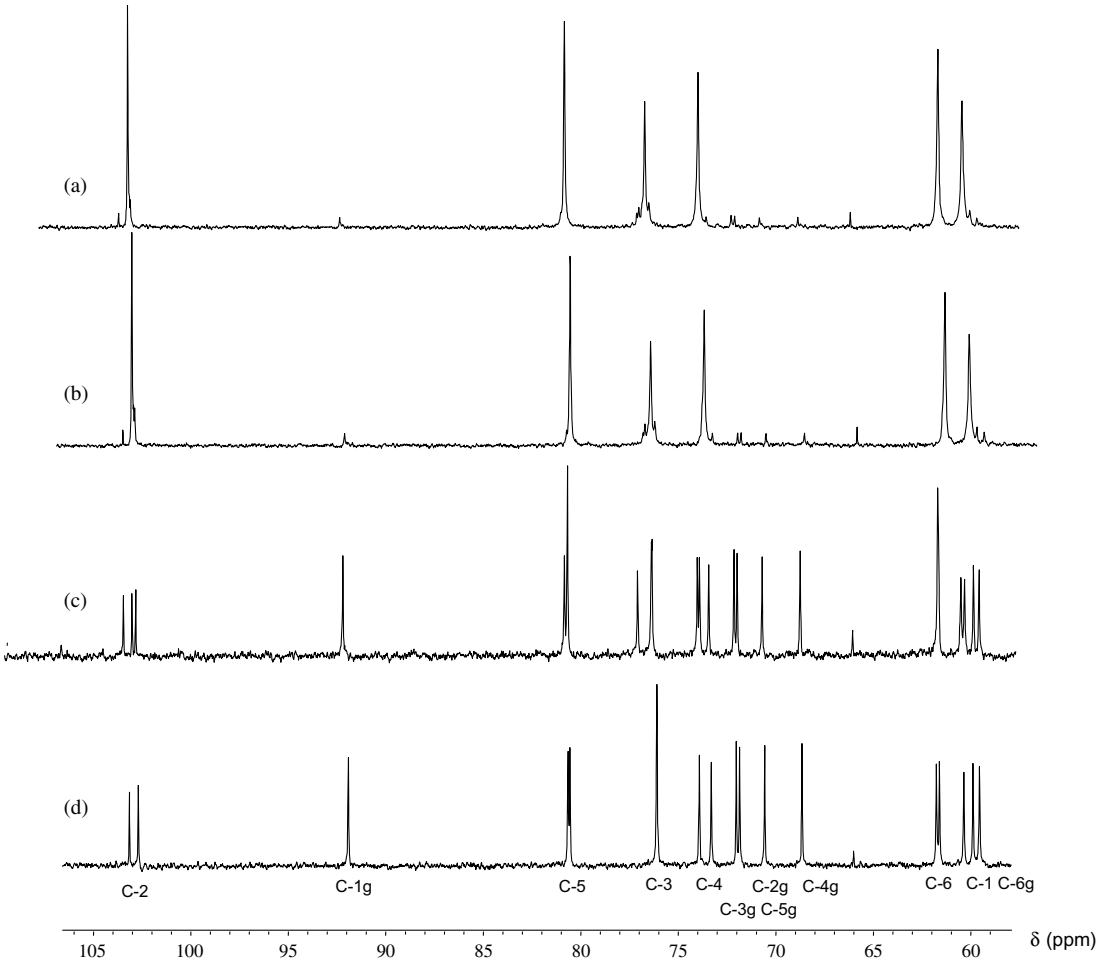

Figure 2. ^{13}C NMR spectra of 80% ethanol-insoluble fructan from *Echinacea purpurea* roots (a), chicory inulin (b), and the oligo-fructoses nystose (c) and 1-kestose (d) in D_2O. The letter 'g' denotes signals of the D-glucopyranosyl group of sucrose.

obtained for C-2 signal intensities of chain fructosyl residues (102.65 ppm) and the fructosyl moieties (103.1 ppm) of terminal sucrose in the spectra. Results correspond with the generally accepted role of sucrose-terminated β-D-fructosides in carbohydrate metabolism, in which sucrose is the primer onto which successive fructosyl groups are attached. Nevertheless, minor amounts of oligo-fructose without glucose end groups might occur (Ref. 11), which can be neglected in our analysed *Echinacea* fructan fractions (no signal for C-2 of uncombined fructose in the NMR spectra).

Fructan from *E. purpurea* has a chemical structure similar to that of the poly-fructosides generally occurring in roots or tubers of plants of the Asteraceae that is chicory inulin. It is interesting to note that our investigations revealed the occurrence of small amounts of branched residues in *Echinacea* fructan as well as in chicory inulin. Although branching is not typical for inulin-type fructans from the Asteraceae it has already been described for chicory inulin.

The mean DP of isolated *Echinacea* fructan is dependent on the isolation procedure that is the ethanol concentration used for the precipitation (Table 3). At low ethanol concentrations, only fructan molecules with high DP precipitate whereas high ethanol concentrations decrease the mean DP of fructan fractions because molecules with lower DP begin to precipitate. Preparation of an aqueous *Echinacea* extract including removal of phenolic compounds but no precipitation step for fructans lead to a mean DP of 7, which confirms the presence of appreciable amounts of monosaccharides and oligo-fructans (data not shown), which remained in solution during ethanol precipitation. These results are significant for the fructan isolation procedure because precipitability of fructans by ethanol defines the mean DP of resulting products. In other words, even the choice of the extracting agent has an influence on the mean DP of products. When using water/ethanol solutions for extraction, fructans with higher DPs might be excluded. Depending on the aspired preparation, the choice of the extraction medium as well as the isolation procedure may be variable.

Analysis by ^{13}C NMR spectroscopy is suitable not only for structural analysis of polysaccharides but also

Table 2. ^{13}C NMR chemical shifts in parts per million of the β-D-Fru*f* units and α-D-Glc*p* units of *Echinacea* fructan fractions and the model compounds 1-kestose, nystose and chicory inulin

Carbon		1-Kestose	Nystose	Chicory inulin	*Echinacea* fructan		
					80%	60%	40%
Fructose							
C-1	(2→1)-Linked	60.35	60.30	**60.33**[a]		**60.34**	
			60.49				
	Terminal	59.88	59.84	59.94		59.94	
C-2	(2→1)-Linked	102.68	102.65	**102.65**		**102.66**	
			102.46	102.51		102.51	
	Terminal	103.14	103.08	103.10		103.11	
C-3	(2→1)-Linked	76.10	76.23	**76.42**		**76.43**	
			76.93	76.72		76.72	
	Terminal	76.08	76.20	76.22		76.22	
C-4	(2→1)-Linked	73.91	73.78	**73.72**		**73.72**	
			73.89				
	Terminal	73.29	73.32	73.31			
C-5	(2→1)-Linked	80.51	**80.50**	**80.50**		**80.50**	
	Terminal	80.64	80.65				
C-6	(2→1)-Linked	61.75	**61.66**	**61.55**		**61.55**	
	Terminal	61.60	61.61				
Glucose							
C-1	Terminal	91.91	91.93	91.89		91.89	
C-2	Terminal	70.56	70.59	70.60		70.60	
C-3	Terminal	72.02	72.03	72.03		72.04	
C-4	Terminal	68.66	68.65	68.66		68.67	
C-5	Terminal	71.85	71.86	71.87		71.87	
C-6	Terminal	59.55	59.55	59.57		59.58	
DP		3	4	13	37	45	57

[a] Chemical shifts for the largest signals are in bold type and indicate the principal linkage group.

Table 3. Comparison of results for the mean DP of ethanol-insoluble fractions from *Echinacea* root extracts and chicory inulin determined by GC of trimethylsilyl oxime derivatives as well as partially methylated alditol acetates and ^{13}C NMR spectroscopy

Analytical method	Mean DP			
	Chicory inulin	*Echinacea* fructan		
		EtOH 80%	EtOH 60%	EtOH 40%
Silylation analysis	12	33	42	50
Methylation analysis	12	36	46	59
^{13}C NMR	13	37	45	57
Average mean DP	12	35	44	55

for the estimation of the mean DP of plant fructan fractions. Investigations might be limited by the signal intensities of glucose carbons relative to fructose carbons with increasing DP. The advantage of this method compared to GC is that the sample preparation procedure is easier and induces no loss or modification of the carbohydrates. However, for NMR analysis high amounts of the sample are needed. Small sample amounts in the range of 0.1–1 mg in turn can be well characterised by GC of silylated derivatives following hydrolysis. Within this work, we present hydrolysis procedures for fructans, which are ideal for oligomers as well as polymers. Corresponding results from silylation analysis, methylation analysis and NMR analysis indicate no loss of fructose residues. Thus, it is the size of the fructan molecule or fraction as well as the sample amount and the expected DP, which influence the choice of the analytical method.

3. Experimental

3.1. Material

Dried and cut roots from *E. purpurea* (L.) Moench. were purchased from Alfred Galke GmbH, Gittelde, Germany. Voucher specimens of the plant material are deposited at the Herbarium of the Department of Pharmaceutical Biology at the University of Kiel (identification number EPF1). 1-Kestose, nystose and inulin (from

chicory roots) were purchased from Sigma–Aldrich. All other reagents were of analytical grade.

3.2. Isolation of fructans

The dry roots were powdered and extracted with boiling water for 10 min (0.1 g mL^{-1}). The extract was stirred for another 3 h to cool down and centrifuged (20,000*g*; 30 min). Polyphenolic compounds were removed by precipitation with satd $Pb(CH_3COO)_2$ solution (0.5 mL mL^{-1} extract). Excess lead was precipitated with $(COOH)_2$ (2%; 20 mL). Pellets were removed by centrifugation (5000*g*; 10 min). Three fructan fractions were obtained from the supernatant by precipitation of three individual samples with different amounts of EtOH (final concentrations: 80% v/v, 60% v/v and 40% v/v) at 4 °C overnight. The fructan fractions were collected by centrifugation (5000*g*; 10 min), washed twice with the respective EtOH concentration and freeze-dried to give a white product.

3.3. Determination of sugar composition

Samples (0.1–1 mg) were hydrolysed in 500 μL 0.2 M TFA at 80 °C for 30 min and immediately cooled. The TFA was evaporated under a stream of N_2 within 2 h at ambient temperature and the residue was freeze-dried. Oxime derivatives were obtained with NH_2OH (1.25 g $NH_2OH{\cdot}HCl$ in 50 g pyridine) at 70–75 °C for 30 min. Trimethylsilylation was performed with a mixture of 780 μL pyridine and 220 μL *N*-(trimethylsilyl-)imidazole (TSIM) at 70–75 °C for 30 min. GC analysis of the trimethylsilyl oxime derivatives was performed using a Supelco SPB-1 fused silica column (0.25 μm, 25 m × 0.25 mm ID) on a HP 6890 Series gas chromatograph with FID detection (170 °C 1 min, 2 °C min^{-1} to 210 °C, 30 °C min^{-1} to 300 °C).

3.4. Linkage analysis

Dry samples of fructans (1–3 mg) were solubilised in dry DMSO (460 μL) and methylated by the Hakomori method, modified by Harris.[17] Methylated products were hydrolysed in 500 μL 2 M TFA for 30 min at 60 °C and dried after addition of 500 μL 2-methylpropan-2-ol to minimise losses through the decomposition of fructofuranosyl units prior to reduction and acetylation. GC–MS of partially methylated alditol acetate derivatives was performed on a HP 5890 Series gas chromatograph coupled with a HP MS Engine 5898A spectrometer. A Permabond OV-1701 fused silica column (25 m, i.d. 0.25 mm; 0.25 μm film thickness, Macherey & Nagel, Düren, Germany) was used with N_2 as carrier gas. The oven temperature was 170 °C for 2 min and then ramped at 1 °C min^{-1} to 210 °C and held for 10 min. The substitution patterns of the partially methylated alditol acetates were identified by comparison of their mass spectra with fragmentation patterns of the fructo-oligosaccharides 1-kestose and nystose.

3.5. ^{13}C NMR spectroscopy

Samples were prepared by dissolving 5–12 mg oligosaccharide or fructan in 0.6 mL D_2O at 60 °C for 10 min. The spectra were recorded at 300 K on a Bruker ARX 300 instrument at an operating frequency of 75.47 MHz for 17 h. Carbon signals were assigned by comparison with the chemical shifts deduced for 1-kestose, nystose and chicory inulin as well as literature data.[18] Dioxane was used as internal standard.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.03.034.

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