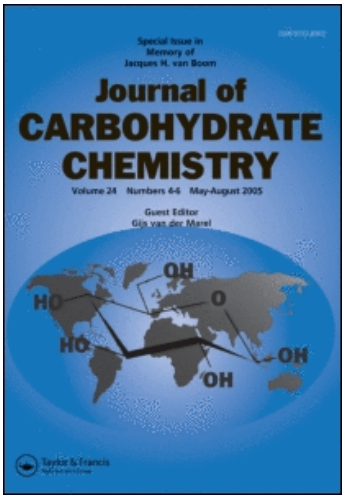

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QUANTITATIVE ANALYSIS OF THE MOLECULAR WEIGHT DISTRIBUTION OF INULIN BY MEANS OF ANION EXCHANGE HPLC WITH PULSED AMPEROMETRIC DETECTION

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ABSTRACT

A method, using anion exchange chromatography and pulsed amperometric detection, is described for quantitative analysis of the oligosaccharides present in inulin. The analysis of a number averaged and molecular weight averaged degree of polymerisation and the dispersion of inulin and inulin fractions is given.

INTRODUCTION

Inulin (Fig. 1) is a storage carbohydrate found in many plant species and several crops. Worldwide, many researchers are examining its structure and properties in order to facilitate the development of new applications. However, methods for the quantitative analysis of the composition of inulin are not available. Inulin oligomers with a degree of polymerisation (DP) up to 8 have been quantitatively analyzed[1] using High Performance Anion Exchange chromatography with Pulsed Amperometric Detection (HPAEC-PAD). This is not sufficient because inulin from, e.g., chicory, contains much larger oligomers. In this report a method is described, based on HPAEC-PAD, for quantitative analysis of inulin oligomers with DP up to 17. Furthermore, the number average DP ($\overline{DP_n}$) and weight average DP ($\overline{DP_w}$) of inulin from chicory have been determined.

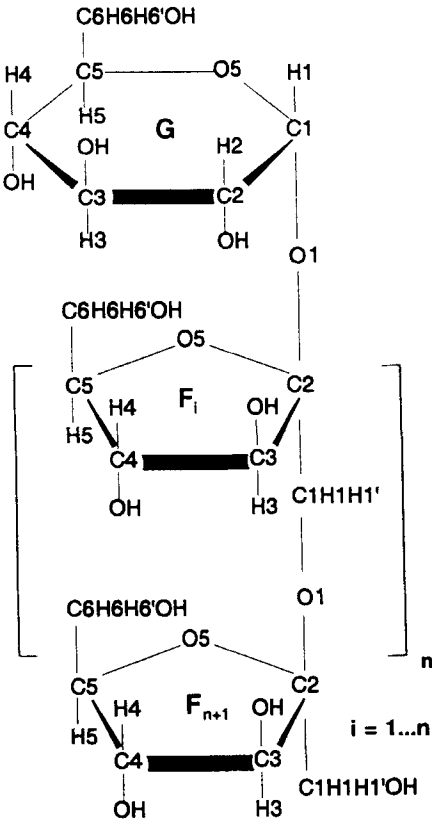

Fig. 1. Primary structure of oligosaccharides present in inulin.

RESULTS AND DISCUSSION

In the isolated inulin oligomers with DP 2-5 no other sugars could be detected using HPAEC-PAD. The oligomers with DP 11-17 were 95-98% pure according to HPAEC-PAD (Fig. 2). The retention time on the RP-18 HPLC column increases strongly when the DP increases from 2 to 6. Also for DP 9-17 the retention time increases with DP, however, this effect is significantly smaller. A transition between these series is formed by DP 7 which co-elutes with DP 6 and DP 10, and DP 8 which co-elutes with DP 9. It has been suggested[2] that this chromatographic behaviour is due to a difference in complex formation with metal ions, but this is not very likely because pure inulin and eluents have been used. A possible explanation is that GF_1-GF_5 are unfolded flexible molecules, which can easily interact with the

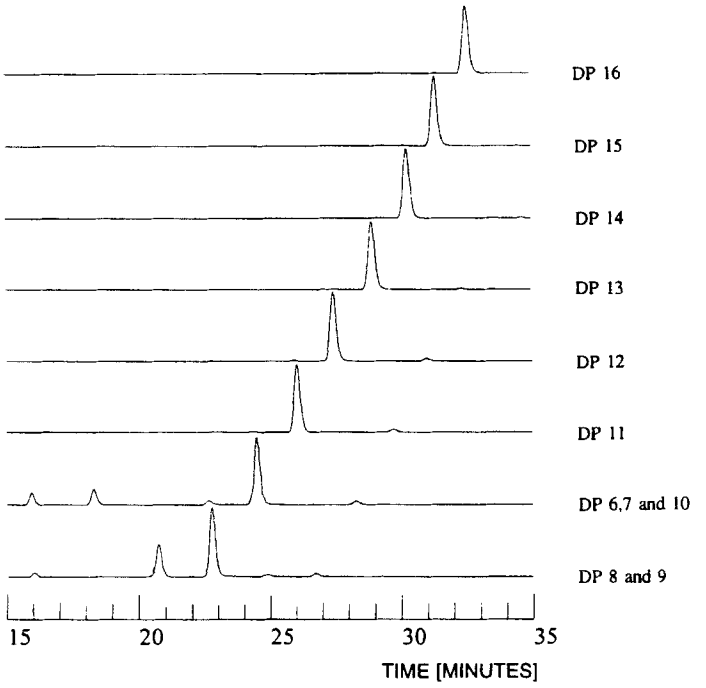

Fig. 2. HPAEC-PAD analysis of the inulin fractions obtained with RP 18 HPLC, using the water/methanol system.

acyl chains of the stationary phase, whereas inulin oligosaccharides larger than GF_7 are compact molecules with, e.g., a helical structure.

For analysis on HPAEC-PAD, all samples were injected two or three times for 24 different concentrations (Table 1). The measurements were carried out on three different days which will be referred to as series A, B and C. Concentrations of the individual compounds varied from 1.4 to 198 mg/L. In Fig. 3, the peak area is depicted as a function of the mass concentration. The curves obtained deviated slightly from a straight line up to concentrations of ca. 25 mg/L (Fig. 3a). For concentrations from 25 up to 200 mg/L (Fig. 3b) this deviation was very large. All peak areas (PA) have been fitted to the mass concentrations (w) using linear (equation 1) and nonlinear (equation 2) regression:

$$PA = a1 \times w \quad [1].$$

$$PA = a1 \times w + a2 \times w^2 \quad [2].$$

The difference between the calculated and measured peak areas is much larger for the first order fit than for the second order fit (Table 1). Considering this non-linearity, the detector

Table 1. PAD responses for inulin oligosaccharides and selected monosaccharides.

				PA = a1 x w		PA = a1 x w + a2 x w^2						
	Npoints	Cmin	Cmax	a1	av-dev	a1	a2	av-dev	Amax	sdm	ppp	series
DP 2	5	13.3	107.0	390	2259	506	-1.3	184	38963	0.63	2	A
DP 3	5	12.8	102.9	347	2840	498	-1.8	313	32307	1.1	2	A
DP 4	5	12.8	102.9	313	2596	451	-1.6	329	29136	1.2	2	A
DP 5	5	12.3	98.8	290	2076	404	-1.4	202	26114	1.2	2	A
DP 11	5	12.7	102.5	218	1546	300	-0.98	203	20505	1.4	2	A
DP 12	5	12.0	96.4	228	1505	312	-1.1	190	20170	1.4	2	A
DP 13	5	12.2	98.2	213	1428	292	-0.99	169	19175	0.97	2	A
DP 14	5	15.6	101.4	210	1387	285	-0.92	212	19535	1.5	2	A
DP 15	5	11.4	91.9	223	1273	298	-1.0	147	18915	1.5	2	A
DP 16	5	8.01	64.6	224	691	283	-1.1	67	13659	2.2	2	A
DP 17	5	4.55	36.7	248	275	289	-1.4	26	8765	2.6	2	A
Rha	9	3.39	198.4	606	1509	678	-0.44	141	117224	2.6	3	B
DP 2	9	3.11	182.1	311	2663	438	-0.84	602	52418	3.0	3	B
DP 3	9	3.27	191.7	240	3428	392	-0.95	985	41027	3.1	3	B
DP 4	9	2.98	174.7	223	2767	358	-0.93	712	34719	3.2	3	B
DP 11	9	1.89	110.7	185	982	263	-0.85	193	18883	2.8	3	B
DP 12	9	2.07	121.1	172	959	241	-0.69	226	19297	2.5	3	B
DP 14	9	2.21	129.7	159	1044	229	-0.65	218	19016	2.9	3	B
DP 15	9	1.47	86.2	185	536	239	-0.74	119	15119	5.8	3	B
Rha	10	3.12	24.8	562	134	529	1.7	52	14100	1.4	3	C
Glc	10	11.3	11.5	1051	80	-[a]	-[a]	-[a]	12154	2.6	3	C
Fru	10	11.6	11.9	725	127	-[a]	-[a]	-[a]	8777	2.1	3	C
DP 2	10	2.86	22.7	344	93	372	-1.6	5.7	7633	1.7	3	C
DP 3	10	3.01	24.0	325	135	362	-2.0	16	7551	1.2	3	C
DP 4	10	2.75	21.8	295	95	324	-1.7	8.7	6247	1.1	3	C
DP 11	10	1.74	13.8	197	18	206	-0.82	4.8	2681	1.0	3	C
DP 12	10	1.90	15.1	182	20	191	-0.73	4.5	2724	0.9	3	C
DP 14	10	2.04	16.2	175	18	183	-0.62	5.3	2785	1.0	3	C
DP 15	10	1.36	10.8	181	13	182	-0.20	12	1926	1.0	3	C

In a series (A, B or C) Npoints different concentrations (w), varying from cmin to cmax mg/L, have been used. The peak areas (PA) of ppp experiments, using the same solution, have been averaged (the largest standard deviation in one series is referred to as sdm). The difference between the calculated and measured peak area is averaged (av-dev) for each component in a series. The largest peak area in one series is referred to as Amax. a, Concentration range is too small to perform a second order fit.

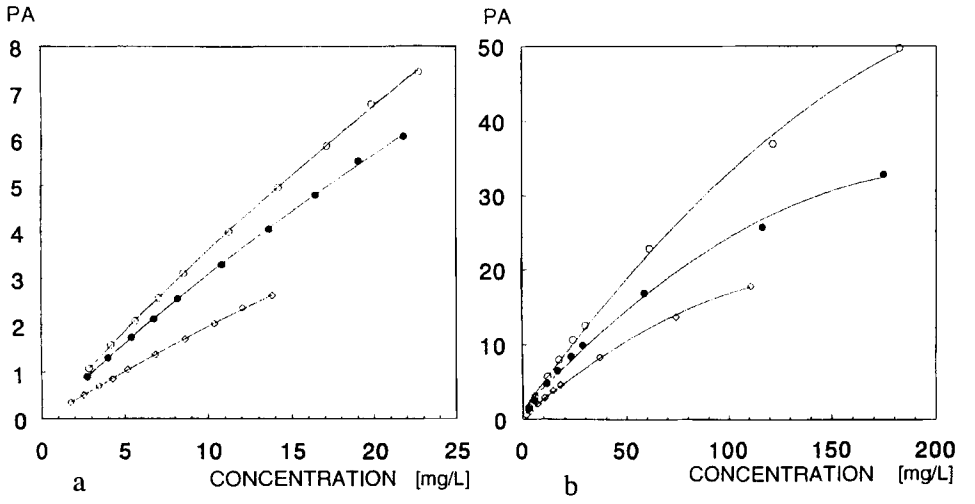

Fig. 3. PAD response for inulin oligosaccharides with DP 2 (○), 4 (●) and 11 (◊).

response for a certain compound is best described using the first order regression coefficient a1 of a second order fit (equation 2). This value varied only slightly within a series measured in one day. Although, on comparison with the other series, large deviations have been found, the a1 values of the different compounds related to the a1 value of sucrose ($a1_s$) or, e.g., rhamnose varied slightly (Table 2). This means that the relative responses can be used for quantitative analysis of the individual compounds using an internal standard. For calculation of $\overline{DP_n}$ and $\overline{DP_w}$ the use of an internal standard can be omitted, because relative concentrations are used.

The relative responses ($a1/a1_s$) are depicted (Fig. 4) as a function of the degree of polymerisation (DP). The sensitivity of the PAD detector decreases clearly from DP 2 to DP 5. This has also been found[1] for DP 2 - DP 8 using a PAD detector. Surprisingly, for longer oligomers (DP 11 - DP 17) the sensitivity of the detector decreases only slightly. A model for calculating the relative sensitivities has been obtained by linear regression of both series (DP 2-5 and DP 10-17), followed by interpolation for DP 6-9 and extrapolation for DP larger than 17. This model has been applied to calculate the $\overline{DP_n}$ and $\overline{DP_w}$ using equation 3-5, and dispersion ($D = \overline{DP_w} / \overline{DP_n}$) of inulin from chicory and inulin fractions I and II:

$$w_i = PA_i / (a1_i/a1_s) \quad [3].$$

$$\overline{DP_w} = \Sigma_i DP_i \times w_i / \Sigma_i w_i \quad [4].$$

$$\overline{DP_n} = \Sigma_i DP_i \times (w_i/M_i) / \Sigma_i (w_i/M_i) \quad [5].$$

Table 2. Relative PAD responses.

DP	series	a1/a1(Sucrose)
3	A	0.98
3	B	0.90
3	C	0.97
4	A	0.89
4	B	0.82
4	C	0.87
5	A	0.80
11	A	0.59
11	B	0.60
11	C	0.55
12	A	0.62
12	B	0.55
12	C	0.51
13	A	0.58
14	A	0.56
14	B	0.52
14	C	0.49
15	A	0.59
15	B	0.54
15	C	0.49
16	A	0.56
17	A	0.57

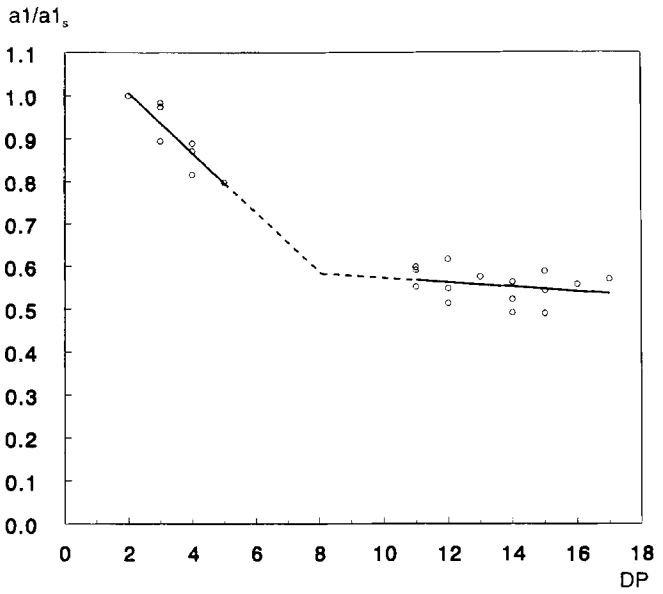

Fig 4. PAD response of inulin oligosaccharides with DP 2-17 relative to the PAD response of sucrose.

Table 3. Comparison of enzymatic and HPAE-PAD methods for determination of average DP values of inulin.

Fraction	Enzymatically Determination	HPAE-PAD			
	F/G + 1	F/G+1	$\overline{DP_n}$	$\overline{DP_w}$	D
Inulin I	5.3	4.9	4.4	6.5	1.5
chicory inulin	8.1	7.4	6.8	13	1.9
Inulin II	15	15	14	20	1.4

The applicability of the model has been examined by calculation of the fructose/glucose ratio and comparison with the enzymatically determined ratio after hydrolysis. This ratio, incremented by 1 (F/G + 1) is often used as an approximation for the $\overline{DP_n}$, because determination of the concentrations of glucose and fructose present before hydrolysis is omitted. The results are summarized in Table 3. A rather good agreement is obtained for the F/G ratio determined with HPAEC-PAD and the enzymatically determined F/G ratio. This result means that the model for the relative responses is valid, and, therefore, also the values for $\overline{DP_n}$, $\overline{DP_w}$ and the dispersion. It should be noted that there is a significant difference in the values for F/G + 1 and $\overline{DP_n}$. Therefore, for the determination of $\overline{DP_n}$ after hydrolysis also the concentrations of glucose and fructose before hydrolysis should be taken into account.

CONCLUSION

The quantitative analysis of oligosaccharides present in inulin is possible using HPAEC chromatography combined with pulsed amperometric detection. This method allows, besides the determination of a number average DP, also that of a molecular weight average DP and the dispersion. This is not possible with the currently used methods based on hydrolysis of inulin.

EXPERIMENTAL

Inulin oligomers with DP 2-5 have been isolated as described previously.[3,4] Larger oligomers have been isolated using preparative RP-18 HPLC chromatography.[2] The flow of the eluent (Milli-Q water with 0.5-2 % methanol) was 20-50 mL/min and samples of 50-200 mg inulin in 5 mL water were injected on a column of 50 x 300 mm. Lyophilisation of the

fractions with volumes of 50-500 mL yielded 5-20 mg of separated oligomers, which were 95 to 98 % pure according to HPAEC-PAD.

Inulin from chicory was fractionated by addition of ethanol to a solution in water. The precipitate will be referred to as fraction II and concentration of the filtrate yielded fraction I.

For separation on HPAEC-PAD, samples were eluted with a 60 min linear gradient of sodium hydroxide and sodium acetate in Milli-Q water running from 0.10:0.025 mol/L to 0.10:0.40 mol/L. All samples have been prepared by making mixtures of known amounts of all oligomers, sucrose, glucose and fructose, together with rhamnose. These compounds were all dried *in vacuo* by the use of P_2O_5. A CarboPac PA1 (4x250 mm) column was used with a CarboPac PA (3x25 mm) guard column. The system (DIONEX) was equipped with a Pulsed Electrochemical Detector (PED). The applied potential of a pulse has been kept at 0.1, 0.6, and -0.6 V during 0.5, 0.1 and 0.05 seconds, respectively. The signal has been integrated between 0.3 and 0.5 seconds after the beginning of the pulse.

The fructose/glucose ratio of the inulin fractions after acid hydrolysis have been determined enzymically (Boehringer-Mannheim GmbH, Cat. No. 139106, food analyses).

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