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Structure characterization and carboxymethylation of arabinoxylan isolated from Ispaghula (*Plantago ovata*) seed husk

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ABSTRACT

As revealed by NMR spectroscopy (after ultrasonic degradation) and HPLC (after total hydrolysis) an arabinoxylan (AX) containing 74.8% Xyl*p* and 23.2% Ara*f* was isolated from Ispaghula (*Plantago ovata*) by soaking the seed husk with water, extraction with aqueous sodium hydroxide and coagulation with acetic acid. The AX with a molar mass of 364,470 g/mol shows high swelling ability in water. The carboxymethylation of AX was carried out heterogeneously with sodium monochloroacetate in the presence of aqueous sodium hydroxide. The reaction parameters were varied in terms of slurry medium, molar ratio, temperature, time, and sodium hydroxide concentration. For comparative studies, carboxymethylation of arabinan was carried out. In order to determine the total degree of substitution (DS) and mole fractions of the repeating units of carboxymethyl arabinoxylan (CMAX) and of carboxymethyl arabinan, HPLC and ^{1}H NMR spectroscopic investigations after total hydrolysis were carried out. DS values for CMAX as high as 1.81 were achieved. CMAX is water soluble starting at DS of 0.33.

1. Introduction

Ispaghula (*Plantago ovata*) is an indigenous product of south Asia and is a widely used herbal product both in traditional and modern medicines. Seed husk of Ispaghula has a long history of use as a dietary fiber supplement to promote the regulation of large bowel function (Cummings & Stephen, 1980). It has been shown to lower the blood cholesterol level in recent studies (Anderson et al., 2000). Moreover, it is used in folk medicines as demulcent, emollient, and laxative. It is used in the treatment of dysentery, constipation, catarrhal conditions of the genito-urinary tract, inflamed membranes of the intestinal canal etc. The succinic acid- and tartaric acid-treated Ispaghula seed husk powder has been studied for the preparation of modified release tablets of diltiazem hydrochloride (Gohel, Amin, Chhabaria, Panchal, & Lalwani, 2000; Gohel, Patel, & Amin, 2003).

Xylans are the most common hemicelluloses present in plants such as grasses, herbs and cereals. Heteroxylans of higher plants possess β-(1 → 4) linked Xyl*p* units as the backbone, usually substituted with sugar units and *O*-acetyl groups (Stephen, 1983). Structures of naturally occurring xylans, their isolation procedures and properties have been reviewed (Ebringerova & Heinze, 2000). Sulfated xylans are used as a biologically active component in drugs (Anees, 1996; Doctor et al., 1991). Biological and physiological effects of xylans including extraction methods, chemical modification and applications are discussed by Ebringerova and Hromadkova (1999) and Ebringerova (1992). Functionalization methods of xylan along with structural analysis of xylan derivatives are reviewed (Heinze, Koschella, & Ebringerova, 2004). Despite the large number of possible applications, xylan is not yet available in industrially required quantities for large scale processing. The seed husk of Ispaghula (*Plantago ovata Forsk*) contains a high amount of xylan (arabinoxylan) along with Rha*p* and Gal*p* as minor sugar components and may be a commercial source for this important biopolymer obtained by simple extraction. A recent study revealed the presence of a xylan-type polysaccharide in Ispaghula seed husk (Fischer et al., 2004).

Plantago ovata seed husk gum was carboxymethylated with sodium monochloroacetate and characterized by means of FTIR (Khasgiwal & Mithal, 1975). Recently, carboxymethylation of xylans from various sources like birch-, beech-, and eucalyptus wood

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and from oat husk, rye bran, and corn cob had been studied in detail to get xylans bearing anionic functions (Petzold, Schwikal, & Heinze, 2006; Petzold, Schwikal, & Günther, et al., 2006).

The paper describes structure characterization and carboxymethylation of arabinoxylan under different reaction conditions and detailed structure characterization of carboxymethylated arabinoxylan isolated from Ispaghula seed husk. A main objective of this research is to synthesize water soluble and water swellable products by carboxymethylation of arabinoxylan, which will be studied later on for pharmaceutical applications.

2. Experimental

2.1. Materials

Ispaghula seed husk was purchased from a local market in Pakistan. The sugar-beet arabinan **2** was purchased from Megazyme, Lot 80901 (Wicklow, Ireland). Sodium monochloroacetate (SMCA) was received from Fluka (Taufkirchen, Germany). Acetic acid was obtained from Merck (Darmstadt, Germany). Sodium hydroxide, ethanol, methanol and 2-propanol are reagent grade chemicals.

2.2. Measurements

FTIR spectra were recorded on a Nicolet AVATAR 370 DTGS spectrometer using the KBr-technique.

The determination of sugar composition by HPLC after acidic hydrolysis was carried out according to Puls (1993). For the one step hydrolysis, 100 mg of the sample was mixed with 8 ml of water and homogenized. It was autoclaved for 40 min at 120 °C after addition of 2.04 ml 0.5 M H_2SO_4. For the two step hydrolysis, a mixture of 200 mg of the sample with 2 ml H_2SO_4 (72%) was kept in a water bath for 1 h at 30 °C. The hydrolysis was quenched by addition of 6 ml of water. The mixture was transferred in a volumetric flask applying 50 ml of water and autoclaved for 40 min at 120 °C. The chromatographic separation of the sugars was carried out applying a strong anion exchange resin MCI Gel CA08F (Mitsubishi) packed in an Omnifit column (7 × 11.5 mm) at 60 °C using gradient elution (flow: 0.7 ml/min) with (A) 0.3 M potassium tetraborate and (B) 0.9 M potassium tetraborate. The gradient was set as follows: 0 min 90% A, 10% B; 35 min 10% A, 90%; 47 min end of elution. An after-column derivatization was carried out by injecting 0.35 ml/min copper bicinchoniate and subsequent heating of the mixture at 105 °C in a Teflon coil (inner diameter 0.3 mm, volume 30 ml) prior to the UV detection at λ = 560 nm.

The degree of substitution (DS) of the carboxymethyl groups was determined after total hydrolysis by HPLC (Heinze, Erler, Nehls, & Klemm, 1994; Heinze, Pfeiffer, Liebert, & Heinze, 1999) and ^{1}H NMR spectroscopy (Baar, Kulicke, Szablikowski, & Kiesewetter, 1994; Bach Tuyet, Iiyama, & Nakano, 1985). Both methods developed for carboxymethyl cellulose were adapted.

To hydrolyze the sample for the HPLC analysis, 2 ml $HClO_4$ (70%) were added to 100 mg of the sample and kept at room temperature for 10 min. After addition of 18 ml water, the mixture was treated for 16 h at 100 °C under shaking. After cooling, the solution was carefully neutralized with 2 M KOH, kept for 1 h at 4 °C to complete the precipitation of $KClO_4$, and filtered. The solution was concentrated on a rotary evaporator to a volume of approximately 5 ml and subjected to the HPLC analysis. A sample amount of 20 μl was injected in a HPLC system (KNAUER): column 1 Phenomenex Rezex ROA, column 2 Bio-Rad Aminex HPX-87H, 0.5 ml min^{-1}, 0.05 M H_2SO_4, intelligent pump (KNAUER HPLC pump 64), differential refractometer (KNAUER), operated with the BOR-WIN software package.

For ^{1}H NMR spectroscopy, 100 mg of the sample was mixed with 1 ml D_2SO_4 (25% in D_2O) and stirred for 5 h at 90 °C. After cooling to room temperature, the solution was centrifuged and transferred to the NMR tube containing few crystals of 3-(trimethylsilyl)-1-propanesulfonic acid (sodium salt) as internal standard. NMR spectra were measured on a Bruker AVANCE 250 and AVANCE 400 spectrometer at room temperature with Bruker standard pulse programs and processed with software package MestReC.

The molar mass was determined by GPC on a JASCO system with refractive index detector (RI-930), calibrated with pullulan- and dextran standards. The system was equipped with two columns (Novema 3000 rf and Novema 300 k) from Polymer Standards Service (PSS), using dimethyl sulfoxide (DMSO) containing 0.25% LiBr as eluent with a flow rate of 0.5 ml min^{-1} at 70 °C. In addition, water containing 0.1% $NaNO_3$ was used as eluent with a flow rate of 1.0 ml min^{-1} at 30 °C with two Suprema columns (1000$^+$ and 100).

Elemental analysis was performed on a CHNS analyzer Vario EL III (Elementaranalysensysteme, Hanau, Germany).

Ultrasonic degradation was carried out by means of Branson digital sonifier, model 450 equipped with a Haake PHOENIX II cryostat. The samples were treated at a temperature of 24–27 °C with a pulse amplitude of 75% at 450 W.

2.3. Methods

2.3.1. Isolation of arabinoxylan *1*

Ispaghula seed husk (50.0 g) was soaked in distilled water overnight (seed husk:water 1:50, w/v). Aqueous NaOH solution (2.5%) was added to the mixture to adjust the pH at ~12 and the husk was separated from the gel by vacuum filtration after stirring for 2–3 min. Concentrated acetic acid was added to coagulate the sample at pH ~ 3. The gel obtained was washed several times over a period of 3–4 days with distilled water until the pH remained constant and freeze dried for 1 week.

The yield was 22.5 g (45% related to the weight of seed husk) of arabinoxylan (AX) containing a very small amount of Rha*p* and Gal*p*.

IR (KBr): 3414 (ν OH), 2925 (ν CH), 1630 (absorbed H_2O), 1462 (in-plane δ OH), 1417 (δ CH_2), 1375 (δ CH), 1249, 1162 (antisym. bridge oxygen δ), 1043 (δ C–O), 896 (antisym. out-of-plan δ) 617, 534 (polymer backbone) cm^{-1}.

2.3.2. Solubility of arabinoxylan

Following methods were applied to study the solubility of AX (**1**):

(i) About 0.5 g of arabinoxylan was stirred in a beaker with 10 ml of 95% ethanol for few minutes. Ninety-milliliter water was added and stirring was continued at 100 °C until a solution was obtained. The solution formed a gel after 2–3 days at room temperature.
(ii) Arabinoxylan (0.5 g) was stirred with 25 ml aqueous NaOH (2.5%) for few minutes at room temperature.
(iii) Arabinoxylan (0.5 g) was stirred with 25 ml DMSO at 80 °C for 1 h.

The solubility was evaluated by naked eye. The gel became soluble after the above mentioned treatments (i–iii).

2.3.3. Ultrasonic degradation of arabinoxylan

About 250 mg of AX (**1**) in 225 ml water were treated with ultrasonic irradiation. The swollen AX became soluble after few minutes ultrasonic treatment. Samples were taken repeatedly, cen-

trifuged for 40 min at 4000 rpm to remove titanium particles, filtrated and freeze dried. The dried samples were subjected to GPC.

2.3.4. Carboxymethylation of arabinoxylan

In a typical synthesis, 2.5 g (18.9 mmole) of AX (**1**) was suspended in 180 ml of ethanol. The reaction mixture was vigorously stirred at room temperature for 1 h after addition of 9.0 ml of 25% aqueous NaOH solution (2.26 g, 56.7 mmole). 6.6 g (56.7 mmole, 3 mol/mol anhydrosugar unit) of SMCA was added and the temperature of the reaction bath was raised to 55 °C. The etherification was performed for 5 h. The product was filtered off, suspended in 80% (v/v) aqueous methanol, neutralized with diluted acetic acid, and washed five times with 50 ml of ethanol. The product was dried at 60 °C in vacuum (sample **15**).

Yield: 3.85 g (90%).

DS_{HPLC} 1.18.

IR (KBr): 3431 (ν OH), 2925 (CH), 1608 (ν_{as} CH_2COO^-), 1425 (ν_s CH_2COO^-), 1328, 1258, 1103, 1045, 898, 714, 601, 533 (polymer backbone) cm^{-1}.

^1H NMR: (D_2SO_4) δ: 5.47 (Ara*f* $H^1\alpha$-*O*-2s), 5.39 (Xyl*p* $H^1\alpha$-*O*-2s), 5.21 (Xyl*p* $H^1\alpha$-*O*-2u), 4.73–4.69 (Xyl*p* H^1 β-*O*-2s), 4.64–4.60 (Xyl*p* H^1 β-*O*-2u), 4.57–4.55 (Ara*f* H^1 β-*O*-2u), 4.51–4.47 (2α-*O*-CH_2, 3-*O*-CH_2), 4.39–4.36 (2β-*O*-CH_2) ppm (s stands for substituted and u for unsubstituted). The peaks were assigned according to Petzold, Schwikal, and Heinze (2006) and Petzold, Schwikal, and Günther, et al. (2006) and by comparison with the NMR spectra of CM arabinans (samples **22**–**24**).

2.3.5. Carboxymethylation of arabinan

For a typical synthesis, 2.0 g (15.15 mmole based on 100% arabinan) of arabinan **2** was suspended in 60.0 ml of 2-propanol. The reaction mixture was vigorously stirred at room temperature for 1 h after addition of 7.2 ml of 25% aqueous NaOH solution (1.8 g, 45.45 mmole). 5.29 g (45.45 mmole, 3 mol SMCA/mol anhydroarabinose unit) of SMCA was added and the temperature of the reaction bath was raised to 55 °C. The etherification was performed for 5 h. The product was filtered off, suspended in 80% (v/v) aqueous methanol, neutralized with diluted acetic acid, and washed two times with 50 ml ethanol, disintegrated into small particles and then washed three times with 100 ml ethanol. For purification, the product was dissolved in water and re-precipitated with ethanol, collected and dried at 60 °C in vacuum (sample **23**).

Yield: 4.35 g.

DS_{HPLC} 1.65.

IR (KBr): 3434 (ν_s OH), 2933 (CH), 1603 (ν_{as} CH_2COO^-), 1421 (ν_s CH_2COO^-), 1318, 1119, 1055, 723, 605 (polymer backbone) cm^{-1}.

^1H NMR: (D_2SO_4) δ: 5.49 (Ara*f* $H^1\alpha$-*O*-2s), 5.44 (b $H^1\alpha$-*O*-2s), 5.34–5.27 (Ara*f* $H^1\alpha$-*O*-2u b $H^1\alpha$-*O*-2u), 4.75–4.72 (b $H^1\beta$-*O*-2s) 4.68–4.66 (Ara*f* $H^1\beta$-*O*-2s), 4.60–4.53 (Ara*f* $H^1\beta$-*O*-2u, b $H^1\beta$-*O*-2u), 4.50–4.47 (2α-*O*-CH_2, 3-*O*-CH_2), 4.40–4.36 (2β-*O*-CH_2) ppm (b stands for other sugars, s for substituted and u for unsubstituted).

3. Results and discussion

3.1. Isolation and characterization of arabinoxylan *1*

A white gel was isolated from Ispaghula seed husk by alkali extraction and freeze drying that showed a pH depending water solubility and had high swelling ability in water. It was difficult to determine the water retention value of the polymer by means of the centrifugation method because the gel blocked the sintered pores, hence, excess water could not be removed completely. However, water was decanted off the gel after centrifugation and the average water retention value of 8100% was determined according to Jayme and Roffael (1970). Freeze drying of the gel yielded product **1**. Elemental analysis of **1** gave 40.38% C and 6.5% H. The absence of nitrogen indicates that the sample is free of protein. The molar mass of arabinoxylan (AX) was determined by means of GPC yielding a weight average molar mass (M_w) of 364,470 g/mol and a number average molar mass (M_n) of 12,800 g/mol.

The monosaccharide composition of the polysaccharide was determined by different HPLC methods (Puls, 1993; Heinze et al., 1994). The polymer contained 74.8% Xyl*p* and 23.3% Ara*f* relative to the total sugar content (Table 1). Overall, the sugar composition found by HPLC was almost comparable with the literature values (Fischer et al., 2004).

The ^1H NMR spectrum of AX (**1**) was badly resolved due to the high M_w of the sample and hence difficult to interpret. However, in

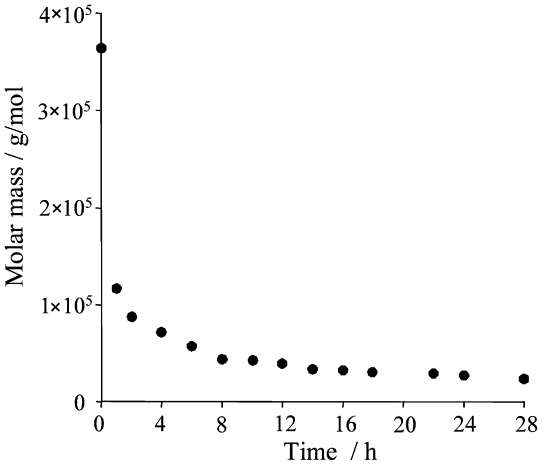


Fig. 1. Decrease of molar mass of arabinoxylan (**1**) during sonification for 28 h in water at 24–27 °C with pulse amplitude of 75% at 450 W.

Table 1
Comparison of the sugar composition of arabinoxylan (**1**) determined by different HPLC methods with reported values (Fischer et al., 2004, in italics)

Hydrolysis method[a]	Hydrolysis residue (%)	Sugar content (%)							
		∑sugar	Xyl*p*	Ara*f*	Rha*p*	Gal*p*	Glc*p*	Uronic acid	Cellobiose
One step (absolute)[b]	0.8	100.4	75.1	23.3	0.8	1.2	n.d.	n.d.	n.d.
Two step (absolute)[b]	1.0	90.8	66.0	21.3	1.7	1.4	0.2	n.d.	0.2
One step (relative)[c]	n.d.	–	74.8	23.2	0.8	1.2	n.d.	n.d.	n.d.
Two step (relative)[c]	n.d.	–	72.7	23.4	1.8	1.6	0.3	n.d.	0.3
One step[d]	n.d.	–	72.5	21.8	2.3	n.d.	n.d.	1.4	n.d.
		–	*74.6*	*22.6*	*0.4*	*1.5*	*0.3*	*0.7*	

[a] For details see Section 2.
[b] The sugar content is the mass percentage related to the starting material without consideration of ash, acetyl groups, extractives.
[c] The relative sugar content based on 100% total sugar content without consideration of different molar masses.
[d] Method adapted from Heinze et al. (1994).

Table 2
Comparison of ^{13}C NMR chemical shifts for glycosyl residues of a polysaccharide isolated from Ispaghula (*Plantago ovata*) after 16 h ultrasonic degradation with values (in italic) published by Fischer et al. (2004)

Glycosyl residue	Assignment				
	C-1	C-2	C-3	C-4	C-5
L-Ara*f*	108.6	81.9	77.1	84.6	61.9
	109.0	*82.0*	*77.4*	*85.0*	*61.8*
→3)-D-Xyl*p*-β-(1→	104.1	74.2	83.4	68.4	65.7
	104.5	*74.0*	*84.2*	*68.5*	*65.7*
T-Xyl*p*	103.4	73.9	76.2	69.8	65.7
	103.8	*73.7*	*76.4*	*70.0*	*66.0*
→2[→4]-D-Xyl*p*-β-(1→	101.0	81.2	73.5	76.0	63.1
	101.3	*81.4*	*74.3*	*77.3*	*63.5*

the ^{13}C NMR spectrum the signals corresponding to different major sugar moieties could be detected. The signal with very low intensity at 17.1 ppm and 168.8 ppm were assigned to the methyl group of the Rha*p* and COOH of GalA, present in very small amounts. A signal at 109.6 ppm is characteristic for C-1 of the Ara*f* units. Arabinoxylan (**1**) was swollen in water and subjected to ultrasonic degradation to decrease the molar mass. The sample dissolved after a treatment of few minutes. The degradation was studied over 28 h. It was observed that the molar mass of AX (**1**) was rapidly reduced during the first and second hour and after that the decrease of molar mass was significantly less (Fig. 1). In fact, the molar mass of AX (**1**) was reduced from 364,470 g/mol to 116,200 g/mol in first hour, which was about three times less then the initial molar mass. After 28 h the molar mass was 23,390 g/mol. This phenomenon has been described by Kulicke, Otto, and Baar (1993) both for coiled synthetic polymers, such as polyacrylamide or poly(acrylamide-co-sodium acrylate), and semi-rigid biopolymers, such as xanthan or schizophyllan.

The DEPT 135 NMR spectra were acquired using the samples after 1 h ultrasonic degradation. The signals at 61.6 ppm and 65.5 ppm were assigned to C-5 of Ara*f* and Xyl*p*, respectively. Some new signals appeared in the range of chemical shift values for C-5 after 16 h ultrasonic degradation. These signals may be assigned to Ara*f* and Xyl*p* moieties with a different linkage type (Table 2 and Fig. 2). The peak assignment to the different glycosyl residues was supported by literature values (Fischer et al., 2004).

According to our measurements and published data, a highly branched structure of the polysaccharide must be concluded. It was established that the main chain of AX (**1**) consists of β-(1 → 4)-linked D-Xyl*p* residues (Fischer et al., 2004). Some Xyl*p* residues of the main chain carry a single Xyl*p* moiety at position 2 with hydroxyl groups at position 2, 3, and 4, which are available for chemical modification. Other Xyl*p* residues of the main chain bear trisaccharide branches at position 3 with the sequence L-Ara*f*-α-(1 → 3)-D-Xyl*p*-β-(1 → 3)-L-Ara*f* (Fig. 2). As a consequence of the terminal branched Xyl*p* and Ara*f*, there was a possibility to obtain tri-*O*-functionalized products along with di- and mono-*O*-functionalized units in the main chain.

3.2. Carboxymethylation of arabinoxylan

The carboxymethylation of **1** was carried out heterogeneously under varying the molar ratio of anhydrosugar unit (ASU):sodium monochloroacetate (SMCA):NaOH, concentration of aqueous NaOH, slurry medium, reaction time, and temperature (Scheme 1).

An increase of the molar ratio of SMCA to ASU leads to an increase of the degree os substitution (DS) independent of the slurry media (Table 3). Compare samples **3**–**6** (methanol as slurry medium) and samples **16**, **19**, and **20** (ethanol as slurry medium) that were prepared with 15% aqueous NaOH as base. Increase of DS with increasing molar ratio of SMCA to ASU was also observed in the presence of 25% aqueous NaOH (samples **10** and **11**) and applying 45% aqueous NaOH (samples **8** and **9**).

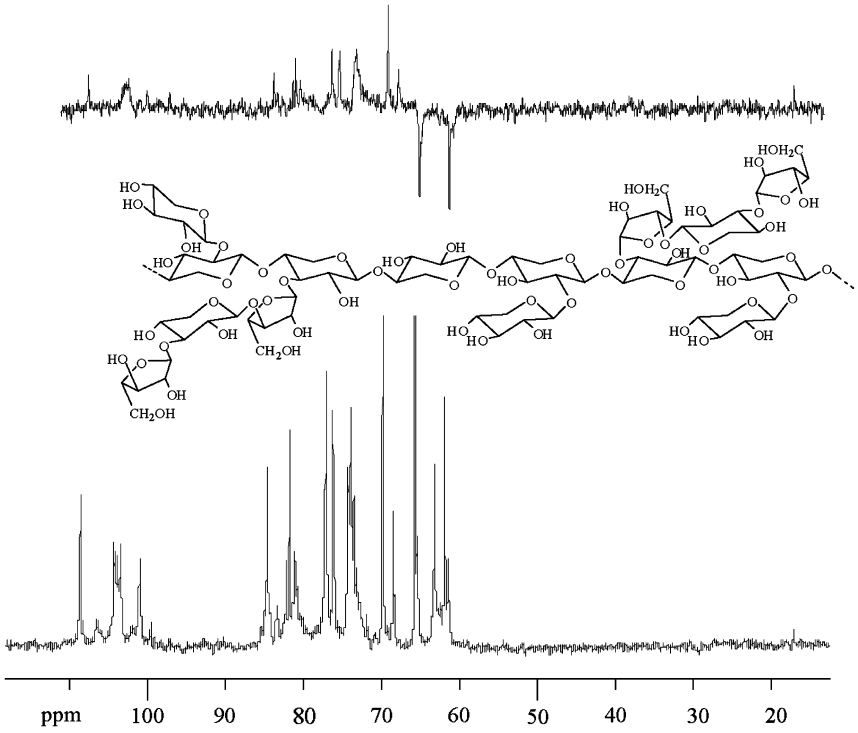
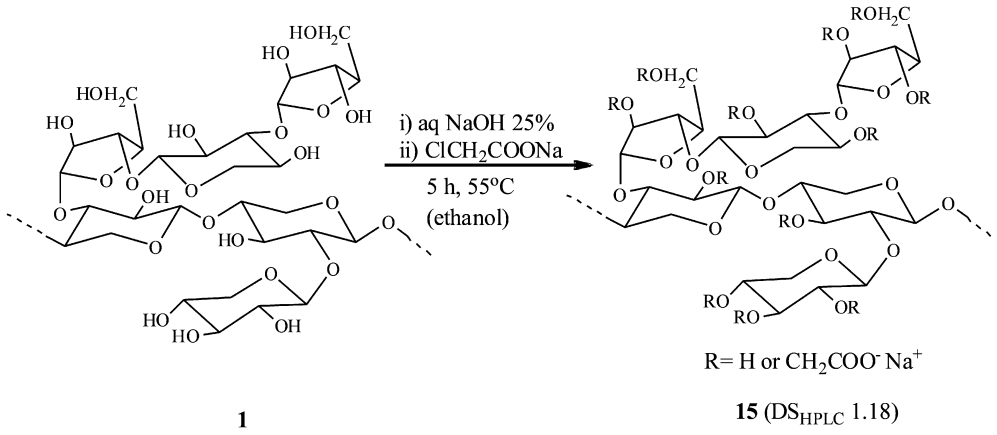


Fig. 2. DEPT 135 NMR spectrum of arabinoxylan (AX, **1**) after ultrasonic degradation for 1 h (top) and ^{13}C NMR spectrum of AX after ultrasonic degradation for 16 h (bottom). The structure of AX (**1**) is based on NMR characterization and according to (Fischer et al., 2004).



Scheme 1. Schematic representation of the synthesis of carboxymethyl arabinoxylan.

Table 3
Conditions for and results of the carboxymethylation of arabinoxylan (**1**) with sodium monochloroacetate (SMCA) and aqueous sodium hydroxide under heterogeneous conditions at 55 °C

Molar ratio ASU:SMCA:NaOH	Slurry medium	Time (h)	NaOH aq. (%)	Sample number	DS_{HPLC}	$DS_{^1H\ NMR}$		
						ΣDS	*O*-2	*O*-3[c]
1:5:5	Methanol	5	15	**3**	0.14	0.13	0.10	0.03
1:7:7	Methanol	5	15	**4**	0.33	0.31	0.22	0.09
1:10:10	Methanol	5	15	**5**	0.43	0.37	0.24	0.13
1:20:20	Methanol	5	15	**6**	0.48	0.38	0.20	0.18
1:20:20	Methanol	5	25	**7**	0.61	0.50	0.31	0.19
1:3:3	Methanol	5	45	**8**	0.08	0.07	0.05	0.02
1:5:5	Methanol	5	45	**9**	0.17	0.15	0.09	0.06
1:5:5	Methanol	5	25	**10**	0.27	0.23	0.14	0.09
1:3:3	Methanol	5	25	**11**	0.12	0.10	0.06	0.04
1:3:3	Methanol	10	25	**12**	0.16	0.14	0.11	0.03
1:3:3	Ethanol	10	25	**13**	1.72	1.12	0.52	0.60
1:3:3	Ethanol	20	25	**14**	1.81	1.33	0.59	0.74
1:3:3	Ethanol	5	25	**15**	1.18	0.91	0.42	0.49
1:3:3	Ethanol	5	15	**16**	1.07	0.74	0.35	0.39
1:3:3	Ethanol	5	15	**17**[a]	0.73	0.51	0.25	0.26
1:3:3	Ethanol	5	15	**18**[b]	0.26	0.20	0.12	0.08
1:5:5	Ethanol	5	15	**19**	1.37	1.14	0.45	0.69
1:10:10	Ethanol	5	15	**20**	1.47	1.34	0.54	0.80
1:3:3	2-Propanol	5	25	**21**	1.33	1.23	0.44	0.79

[a] At 45 °C.
[b] At 30 °C.
[c] *O*-3 representing substitution at all other expected positions except position.

Three different concentrations of aqueous NaOH solution, namely 15%, 25%, and 45%, were used. The highest DS value was obtained in the presence of 25% aqueous NaOH solution (sample **10**, DS_{CM} 0.27), while the lowest DS value was achieved applying 15% NaOH solution (**3**, DS_{CM} 0.14). Aqueous NaOH of 45% yielded product **9** with DS_{CM} 0.17. This low DS obtained in the presence of 45% aqueous NaOH (compared to 25% aqueous NaOH) may be caused by the formation of sodium glycolate as a side reaction.

The variation of the reaction time (5 h, 10 h, and 20 h) led to products with increasing DS with increasing reaction time. Carboxymethyl arabinoxylan (CMAX) **14** with maximum DS 1.81 was obtained after 20 h in ethanol as slurry medium at 55 °C applying a molar ratio of ASU:SCMA:NaOH of 1:3:3. The influence of the reaction temperature (30 °C, 45 °C, and 55 °C) was studied as well. Higher DS values were realized at 55° C (**16**, DS_{CM} 1.07) as compared to conversions at 45 °C (**17**, DS_{CM} 0.73) and 30 °C (**18**, DS_{CM} 0.26).

2-Propanol is an efficient slurry medium for carboxymethylation of cellulose or starch. However, AX (**1**) aggregates in 2-propanol after addition of aqueous NaOH to a lump, which prevented a proper mixing. Contrary, carboxymethylation of AX (**1**) in ethanol and methanol results a better handling. Applying ethanol leads to higher DS values as compared to the use of methanol as slurry medium. The highest DS values were obtained in ethanol combined with an easy work up (**13**, DS_{CM} 1.72; **14**, DS_{CM} 1.81; **15**, DS_{CM} 1.18; **16**, DS_{CM} 1.07; **17**, DS_{CM} 0.73; **19**, DS_{CM} 1.37; **20**, DS_{CM} 1.47).

HPLC investigations were applied in order to determine the mole fraction of differently carboxymethylated sugar moieties of CMAX. Arabinan (**2**) was carboxymethylated as a reference to underpin the peak assignment of carboxymethyl Ara*f*. After total hydrolysis of CMAX with $HClO_4$, the mixture of Xyl*p*, Ara*f* and their carboxymethylated products was separated on a cation exchange column (H^+ form). It was observed that mono-*O*-carboxymethyl Xyl*p* (mono-CMX) and mono-*O*-carboxymethyl Ara*f* (mono-CMA) appeared separately in the chromatogram. In contrast, the peaks for di-*O*-carboxymethyl sugars (di-*O*-CMX and di-*O*-CMA) as well as the tri-*O*-carboxymethyl sugars (tri-*O*-CMX and tri-*O*-CMA) could not be separated with the column applied. The peaks of di-*O*-CMX and di-*O*-CMA as well as of tri-*O*-CM sugars overlapped in the chromatogram (Fig. 3).

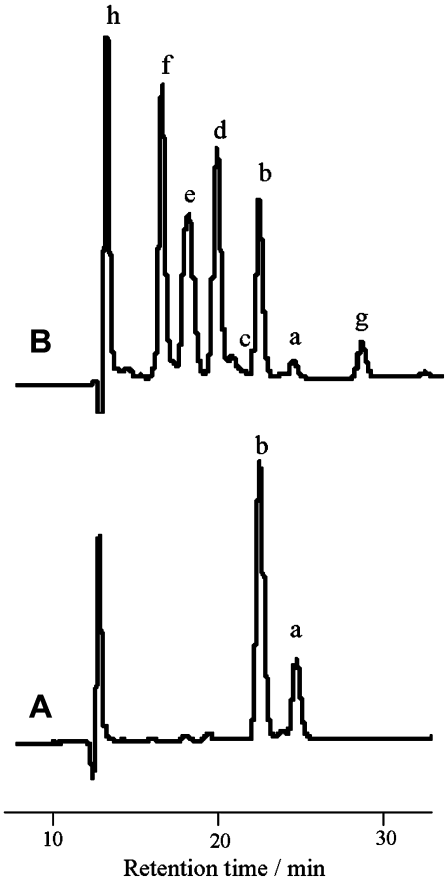


Fig. 3. HPL chromatograms of (A) arabinoxylan (**1**) and (B) carboxymethyl arabinoxylan (**21**) after total hydrolysis with $HClO_4$. (a) Ara*f*, (b) Xyl*p*, (c) mono-*O*-CMA, (d) mono-*O*-CMX, (e) di-*O*-CM, (f) tri-*O*-CM, (g) glycolic acid, (h) inorganic salt.

Not only the DS but also the functionalization pattern depends on the slurry medium. For example, carboxymethylation in methanol yields sample **7** with a maximum DS of 0.61, which contains only mono- and di-*O*-CM repeating units. In case of ethanol and 2-propanol as slurry media, a small amount of tri-*O*-CM units in addition to mono- and di-*O*-CM moieties could be detected in almost all samples (Table 4).

The ^{1}H NMR analysis of CMAX after hydrolysis with D_2SO_4 yielded lower DS_{NMR} values as compared to DS_{HPLC}. Chemical shifts were assigned with the help of published data on carboxymethyl xylan (Petzold, Schwikal, & Heinze, 2006) and by comparing the spectra with a spectrum of carboxymethylated arabinan from sugar-beet that was synthesized in this work as well (see below, Fig. 4). The peaks for carboxymethylated *O*-5 of Ara*f* and glycolic acid appeared with almost same chemical shift value at 4.3 ppm in ^{1}H NMR spectrum. That means this moiety can not be included in DS calculation that may be a reason for lower DS_{NMR} calculated.

It was found by ^{1}H NMR spectroscopic analysis of the D_2SO_4 hydrolyzates that the products **3**–**12** and **18** with low DS value had a higher partial DS at position 2 as compared to other positions. Products **13**–**16**, **19**–**21** with higher DS value had a comparable partial DS at position 2 due to carboxymethylation of position 3, 4 or 5 of branching sugars at higher DS values. These results are in accordance with the HPLC analysis.

3.3. Carboxymethylation of arabinan

Arabinan (**2**) was a commercial product. It was extracted with calcium hydroxide solution at 90 °C from sugar-beet pulp possessing a sugar composition of Ara*f*:Gal*p*:Rha*p*:GalA 88:3:2:7. Arabinan (**2**) was readily soluble in dimethyl sulfoxide (DMSO) and in water yielding a slightly acidic solution (pH 6). Before carboxymethylation, sample **2** was subjected to ^{13}C NMR studies and the peaks were assigned according to the literature (Habibi, Heyraud, Mahrouz, & Vignon, 2004; Vignon & Garcia-Jaldon, 1996). In the region from 110.1 to 109.1 ppm, the signals correspond to branched or terminal Ara*f* units (Table 5, Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007). In the region from 86.6 ppm to 79.2 ppm, the signals of C-2, C-3, and C-4 of the Ara*f* units appear. Peaks in the high field region from 63.8 to 69.0 ppm correspond to C-5 of the terminal Ara*f* units and a signal with low intensity at 63.4 ppm is assigned as C-6 of the Gal*p* moiety. A peak at 19.5 ppm corresponds to the CH_3 group of the Rha*p* moiety. Signals at 17.6 ppm, 21.6 ppm, and 56.9 ppm are attributed to the internal standard 3-(trimethylsilyl)-1-propanesulfonic acid (sodium salt).

Table 4
Degree of substitution (DS) and mole fractions of non-sub, stituted, mono-, di-, and tri-*O*-carboxymethylated repeating units of carboxymethyl (CM) arabinoxylans

Sample no.[a]	Slurry medium	DS_{HPLC}	Mole fraction							
			Non-substituted			Mono-*O*-CM			Di-*O*-CM	Tri-*O*-CM
			xyl	Ara	Total	CM_{Xyl}	CM_{Ara}	Total		
8	Methanol	0.08	0.688	0.241	0.929	0.053	0.012	0.064	0.007	–
11	Methanol	0.12	0.659	0.230	0.889	0.078	0.027	0.105	0.006	–
3	Methanol	0.14	0.649	0.213	0.862	0.096	0.036	0.132	0.009	–
12	Methanol	0.16	0.629	0.214	0.843	0.109	0.038	0.148	0.013	–
9	Methanol	0.17	0.639	0.190	0.829	0.115	0.034	0.149	–	–
18	Ethanol	0.26	0.576	0.162	0.738	0.146	0.064	0.209	0.052	–
10	Methanol	0.27	0.602	0.174	0.776	0.147	0.056	0.203	0.025	–
4	Methanol	0.33	0.563	0.135	0.698	0.196	0.07	0.269	0.064	–
5	Methanol	0.43	0.490	0.140	0.630	0.223	0.089	0.312	0.058	–
6	Methanol	0.48	0.469	0.120	0.593	0.240	0.092	0.335	0.073	–
7	Methanol	0.61	0.423	0.078	0.501	0.299	0.091	0.390	0.109	–
17	Ethanol	0.73	0.454	0.088	0.542	0.190	0.053	0.244	0.153	0.061
16	Ethanol	1.07	0.361	0.069	0.430	0.181	0.043	0.224	0.191	0.154
15	Ethanol	1.18	0.288	0.051	0.339	0.223	0.059	0.282	0.242	0.138
21	Isopropanol	1.33	0.174	0.025	0.199	0.243	0.028	0.271	0.251	0.279
19	Ethanol	1.37	0.245	0.024	0.260	0.227	0.045	0.271	0.286	0.176
20	Ethanol	1.47	0.188	–	0.188	0.275	0.045	0.320	0.321	0.171
13	Ethanol	1.72	0.170	0.016	0.186	0.197	0.025	0.222	0.281	0.311
14	Ethanol	1.81	0.144	0.033	0.177	0.179	0.016	0.195	0.262	0.365

[a] See Table 3.

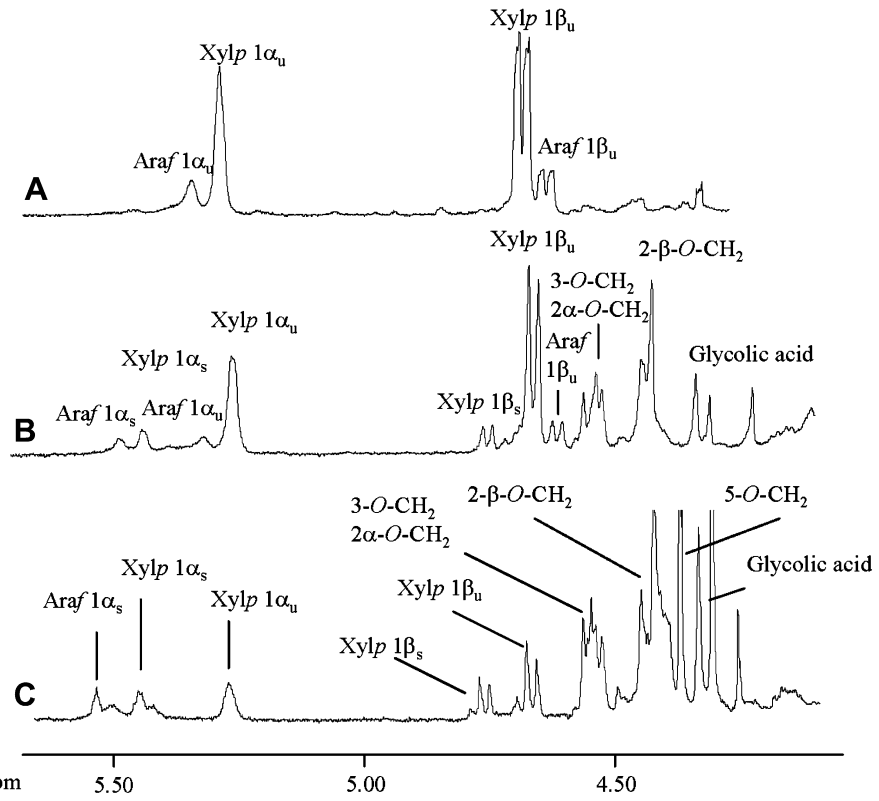


Fig. 4. ^{1}H NMR spectra of carboxymethyl arabinoxylans with different degree of substitution (DS) after hydrolysis in 25% D_2SO_4. s, substituted in neighbored position 2; u, unsubstituted in neighbored position 2, (A) sample **8** DS 0.07, (B) sample **6** DS 0.38, (C) sample **20** DS 1.34.

The main objective for the carboxymethylation of arabinan (**2**) was to confirm the assignment of peaks in HPLC and NMR spectra of CMAX. For carboxymethylation, arabinan (**2**) was slurried in 2-propanol followed by the addition of aqueous NaOH (25%) and SMCA. The reaction was carried out for 5 h at 55 °C (Scheme 2).

Carboxymethyl arabinan (CMA) samples **22**–**24** were soluble in water but insoluble in DMSO. The DS values of CMA were calculated by HPLC and ^{1}H NMR techniques. HPLC analysis showed higher amount of tri-*O*-CM units as compared to mono- and di-*O*-CM repeating units (Table 6). This may be caused by the presence of more branching of Ara*f* units with three available hydroxyl groups per repeating unit for carboxymethylation. Unexpectedly, the DS of **24** prepared with a molar ratio of anhydroarabinose unit (AAU):NaOH:SCMA of 1:6:6 was lower (DS 1.07) as compared to **22** (DS 1.60) and **23** (DS 1.65). Moreover, the mole fraction of non-functionalized sugar units of **24** determined by HPLC was higher as compared to those in **22** and **23**. It may be assumed that during workup of **24** a loss of highly functionalized CMA fraction with low molar mass occurred as indicated by GPC (Fig. 5). A M_w of 115,700 g/mol (**23**) and of 98,150 g/mol (**24**) was found. For **23**, two weak peaks in the range of low molar mass at ~150 g/mol and ~2700 g/mol and one intensive peak at a molar mass of ~160,000 g/mol were observed. It appears that **24** does not contain fractions around 150 g/mol. However, peaks at ~1000, ~2370 (of high intensity) and ~190,000 g/mol (of low intensity) were observed in **24**. Thus, applying a high molar ratio of SMCA to AAU, a large amount of highly functionalized CMA with comparatively low molar mass was obtained, which was lost during workup.

^{1}H NMR spectroscopy after total hydrolysis revealed a preferred carboxymethylation of position 2 compared to position 3. This was due to the well-known higher reactivity of *O*-2. Moreover, position 3 was more occupied by arabinofuranosyl branches and hence less available for carboxymethylation. The signals of the anomeric protons of carboxymethylated Ara*f* appear in the same chemical shift range compared with hydrolyzed CMAX (Fig. 6). This confirmed the peak assignments in ^{1}H NMR spectra of CMAX.

Table 5
^{13}C NMR chemical shift data for related glycosyl residues of arabinan (**2**)

Glycosyl residue[a]	Position					
	1	2	3	4	5	6
→4)-β-Gal*p*-(1→	105.8	73.0	74.5	79.4	75.4	63.4
→2)-α-L-Rha*p*(1→	101.0	77.7	70.6	71.3	69.2	19.5
→3,5)-α-Ara*f*-(1→	106.9	80.3	84.9	83.0	68.7	–
→5)-α-Ara*f*-(1→	110.1	81.8	76.0	83.5	69.0	–
T-α-Ara*f*-(1→3	109.8	83.9	79.3	84.9	63.8	–
T-α-Ara*f*-(1→2	109.1	83.9	79.3	84.9	63.8	–
→4)-α-GalpA-(1→	100.5	68.9	69.5	77.1	72.1	177.0

[a] T means terminal.

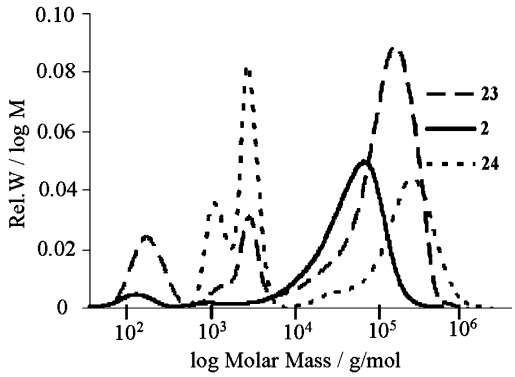
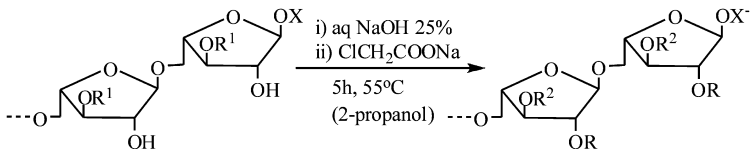


Fig. 5. GPC of arbinan (**2**) and carboxymethyl arabinans (**23** and **24**).



R= H or CH_2COO^- Na^+
R^1= H or Ara*f*
R^2= H or carboxymethyl Ara*f*
X= Pectic polysaccharide unit (with rhamnose, galactose and galacturonic acid)
X^-=Pectic polysaccharide unit after carboxymethylation

Scheme 2. Schematic representation of carboxymethylation of arabinan (**2**).

Table 6
Degree of substitution (DS) and mole fractions of carboxymethyl arabinans (**22–24**) determined by HPLC after total hydrolysis with $HClO_4$

Sample	Molar ratio AAU:SMCA:NaOH	DS_{HPLC}	Mole fraction			
			Non	Mono	Di	Tri
22	1:2:2	1.60	0.160	0.284	0.330	0.222
23	1:3:3	1.65	0.166	0.260	0.326	0.247
24	1:6:6	1.07	0.445	0.152	0.233	0.171

4. Conclusions

Ispaghula seed husk is a rich source of an arabinoxylan with high molar mass and high swelling ability in water. The carboxymethylation of arabinoxylan represents a useful way to get water soluble arabinoxylan derivatives with anionic functions. Carboxymethylation was carried out heterogeneously under different reaction conditions. Ethanol as slurry medium is most efficient to obtain the highest DS with easy workup. The maximum DS could be achieved applying 3 mol of sodium monochloroacetate and NaOH per mole of anhydroxylose unit, ethanol as slurry medium, 25% aqueous NaOH, a reaction temperature of 55 °C within 20 h. It should be emphasized that due to highly branched structure of the polymer, some sugar moieties bearing three hydroxyl groups were available, yielding tri-*O*-functionalized products along with di-*O*- and mono-*O*-carboxymethylated units. The degree of substitution can be calculated after total hydrolysis using ^{1}H NMR (25% D_2SO_4) and HPLC (70% $HClO_4$). Information about the partial degree of substitution at different positions can be determined by means of ^{1}H NMR spectroscopy. An arabinan containing 88% arabinose was included in these studies in order to confirm the assignment of the ^{1}H NMR spectra and HPLC after hydrolysis of the polymer. The mole fractions of unsubstituted and mono-, di-, and tri-*O*-carboxymethylated units can be quantified applying HPLC.

Further research will include investigations on the biological activity of carboxymethyl arabinoxylan (CMAX). The rheological characteristics of aqueous solution of CMAX regarding their applications as thickening agent will be studied.

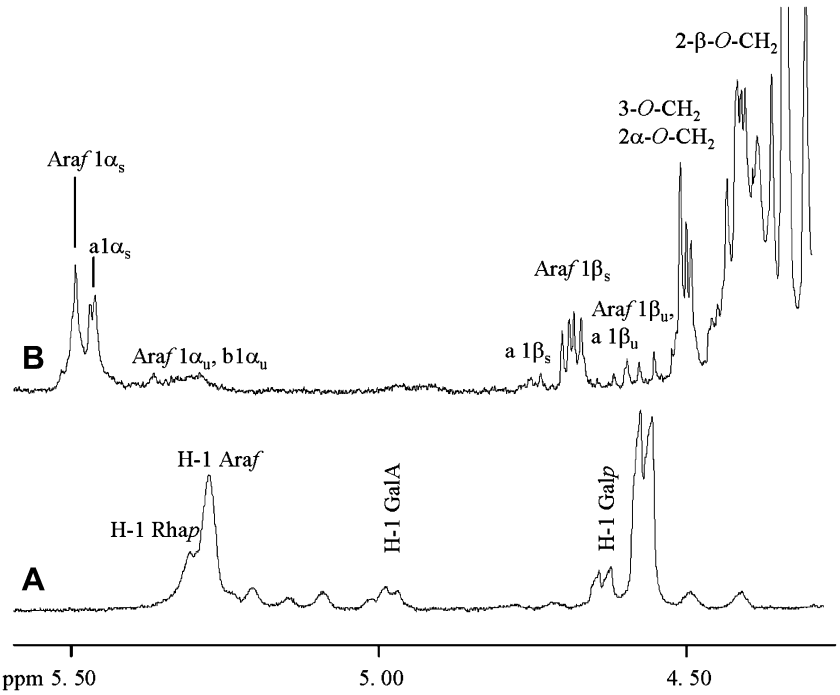


Fig. 6. Comparison of ^{1}H NMR spectra of (A) arabinan (**2**) and (B) carboxymethyl arabinan (**23**) after acidic hydrolysis in 25% aqueous D_2SO_4. (a, all sugar moieties in arabinan except Ara*f*; s, substituted; u, unsubstituted).

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