*Carbohydrate Research*, 243 (1993) 323–332
Elsevier Science Publishers B.V., Amsterdam



# Isolation and characterisation of hemicelluloses from sunflower hulls

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(Received December 23rd, 1991; accepted November 11th, 1992)

ABSTRACT

The hemicelluloses extracted from sunflower hulls by repeated alternating oxidative and alkaline treatments were purified by precipitation with Cetavlon and then ion-exchange chromatography on DEAE-trisacryl. The resulting fractions were examined by hydrolysis, methylation, GLC–MS, and NMR spectroscopy. The hemicelluloses are of the glucuronoxylan type with the following structure → 4)-β-D-Xyl*p*-(1 → 4)-[4-*O*-Me-α-D-Glc*p*A-(1 → 2)]-β-D-Xyl*p*-(1 →. The polysaccharides differed in the amount of branching; the ratio of the main fraction 4-*O*-MeGlcA : Xyl was 1 : 8-9.

## INTRODUCTION

The increasing growing of sunflowers and the improvement in the trituration processes through seed decortication[1,2] has resulted in large amounts of hulls remaining in the sunflower-oil mills.

Analysis of the main constituents from sunflower hulls showed a significant proportion of hemicelluloses[2–5] and various procedures for their characterisation have been attempted. After delignification, glucuronoxylan, glucomannan, and arabinogalactan have been identified, of which the first is similar to (4-*O*-methylglucurono)xylan[6].

Previous studies[7–22] have shown that the hemicelluloses of hardwoods and annual plants consist mainly of chains of (1 → 4)-linked β-D-xylopyranose residues, some of which are 2-substituted by 4-*O*-methyl-α-D-glucopyranosyluronic acid and others are acetylated. In contrast, these glucuronoxylans are seldom found in softwoods[10,23,24].

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We now report on the structure of the main glucuronoxylan isolated from sunflower hulls.

## EXPERIMENTAL

*Preparation of the polysaccharides.*—The hemicelluloses were isolated at room temperature as described by Darling[25]. Sunflower hulls (300 g) were shredded to a fine powder (80–100 mesh) then treated with hot water (80°C, 15 min, 1.5 L), collected, and stirred with aq NaOCl (1 L, 2% of chlorine) for 1 h. The insoluble material was collected, then washed with water, and a suspension in aq 0.2% NaOH (2 L) was stirred for 4 h. The mixture was filtered, the insoluble material was washed with water, and the whole process was repeated twice except for the concentration of hypochlorite (1% of chlorine). The hemicelluloses were then stirred with aq 5.5% NaOH (2 L) for 2 h. The insoluble material was removed by filtration and the pH of the filtrate was adjusted to 4 by adding acetic acid. The crude hemicelluloses were precipitated with EtOH (3 vol), collected, washed with 19:1 EtOH–$H_2O$, and dried under vacuum at room temperature.

*Cetavlon precipitation*[26].—To a solution of the above polysaccharides (10 g) in water (700 mL) was added aq 3% Cetavlon (200 mL) with stirring. After storage overnight at 4°C, the precipitate was collected by centrifugation (20 min, 3000 rpm) and dissolved in 300 mM $Na_2SO_4$ (180 mL). The acidic polysaccharides were precipitated with EtOH (3 vol), collected by centrifugation, and dissolved in water, and the solution was dialysed against distilled water and freeze-dried.

*Anion-exchange chromatography.*—The glucuronoxylans were isolated by anion-exchange chromatography of the foregoing acidic polysaccharides (1.8 g) on a column (20 × 2 cm) of DEAE-trisacryl (IBF-France) equilibrated with 0.05 M Tris/HCl (pH 8.6) by elution with the same buffer followed by a step-wise gradient of NaCl (0.05, 0.1, 0.2, and 0.5 M) in the same buffer at 20 mL/h with detection by UV absorption (280 nm). The fractions collected were tested for sugar content with the phenol–$H_2SO_4$ reagent[27], combined as appropriate, dialysed, and lyophilised.

*Sugar analysis.*—Qualitative and quantitative sugar analyses were carried out by using the orcinol reagent[28,29] for neutral sugars, the *m*-hydroxybiphenyl reagent[30] for uronic acids, and GLC–MS of the trimethylsilylated methyl glycosides obtained[31] after methanolysis (0.5 M HCl in MeOH, 24 h, 80°C) and trimethylsilylation.

*Mass spectrometry.*—The foregoing trimethylsilylated methyl glycosides and the oligosaccharide-alditol derivatives obtained after partial acid hydrolysis, reduction, and ethylation were subjected to GLC–MS using a Girdel 300 or Delsi gas chromatograph with a capillary column (25 m × 0.2 mm) coated with DB 1, a He pressure of $4 \times 10^4$ Pa, and temperature programs 120 → 240°C at 2°C/min for the trimethylsilylated methyl glycosides and 150 → 270°C at 5°C/min for the ethylated disaccharide-alditol. The EI-mass spectra were recorded with a Nermag

R 10-10 S mass spectrometer (Rueil-Malmaison, France) at 70 eV with an ionising current of $2 \times 10^{-4}$ A. Positive-ion CI-mass spectra were obtained at 50 eV using ammonia as the reagent gas.

FABMS was preformed with a Kratos concept II HH mass spectrometer equipped with a DS 90 (DGDG/30) data system and an accelerating potential of 8 keV with Xe as the bombarding atom (7.3 keV, $1.2 \times 10^{-3}$ A). A solution of the oligosaccharide (1–5 $\mu$g) in distilled water (1 $\mu$L) was loaded onto the copper tip with 5% triethylamine in glycerol as the matrix.

*NMR spectroscopy.*—The 400-MHz $^1$H NMR experiments were performed at 25 or 80°C with a Bruker AM-400 WB spectrometer equipped with a 5-mm $^1$H–$^{13}$C mixed probe head, operating in the pulsed FT mode, and controlled by an Aspect 3000 computer. After one exchange with $D_2O$ (99.96 atom% D, Aldrich) and lyophilisation, the products were analysed with a spectral width of 3000 Hz for 16 K frequency-domain and time-domain data points, giving a final digital resolution of 0.365 Hz/point[32]. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation. The 100-MHz $^{13}$C NMR experiments were carried out at 25 or 80°C with the standard Bruker pulse program POWGATE with $^1$H broad-band composite-pulse decoupling. A 90° pulse (4.8 $\mu$s) and a recycle delay ($D_1$ + ACQ = 2 s) were used. The chemical shifts ($\delta$) were expressed in ppm relative to those for internal acetone ($^1$H, 2.225 ppm; $^{13}$C, 31.55 ppm for $CH_3$) or external sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-$d_4$ (0.0 ppm for $^1$H and $^{13}$C). The 2D-homonuclear experiments were performed with the standard Bruker pulse programs COSY and COSYRCT. The 2D-heteronuclear correlated experiments were performed with simultaneous $^1$H-decoupling, using the standard Bruker pulse program XHCORRD.

*Methylation analysis.*—Polysaccharide fractions were methylated as described[33]. The methylated polysaccharides were hydrolysed (4 M $CF_3CO_2H$, 4 h, 100°C), and the products were reduced with $NaBD_4$, acetylated, and analysed by GLC–MS.

*Partial acid hydrolysis.*—The aldobiouronic acids were isolated after partial acid hydrolysis of the polysaccharide fraction II (2 M $CF_3CO_2H$, 2.5 h, 100°C) by GPC on a column (90 × 1.2 cm) of Sephadex G-10 (Pharmacia) by elution with water. The disaccharide obtained was analysed by $^1$H and $^{13}$C NMR spectroscopy and GLC–MS of the ethylated alditol acetate derivative.

## RESULTS AND DISCUSSION

*Isolation and fractionation of the hemicelluloses.*—In order to decolorise and eliminate most of the polyphenolic compounds, the powdered hulls were subjected to alternate oxidative and alkaline treatments. The yields of crude polysaccharides were 6–15%. Treatment of the crude polysaccharide with Cetavlon yielded the acidic polysaccharides, ion-exchange chromatography of which on DEAE-trisacryl gave five fractions. The polysaccharide-containing fractions II-V were eluted by 0.05, 0.1, 0.2, and 0.5 M NaCl, respectively (Table I).

TABLE I

Molar compositions and weights of the fractions obtained from 1.8 g of Cetavlon precipitate on DEAE-trisacryl

| Fractions | Xyl | Monosaccharides | | | | | Weights (mg) |
|---|---|---|---|---|---|---|---|
| | | 4-*O*-MeGlcA [a] | GlcA | Glc | Gal | Man | |
| I | | | | | | | 61.2 |
| II | 9.1 | 1 | | | | | 351.6 |
| III | 7.2 | 1 | | | | | 34.4 |
| IV | 3.4 | 1 | | | | | 59.4 |
| V [b] | 1 | | + | 0.3 | 0.07 | 0.17 | 10.2 |

[a] Values are given relative to one unit of 4-*O*-MeGlcA. [b] Molar ratio calculated by GLC of trimethylsilylated methyl glycosides.

*Sugar analysis.*—GLC–MS of the derived trimethylsilylated methyl glycosides revealed xylose and 4-*O*-methylglucuronic acid in fractions II–IV ($[M + NH_4]^+$ at $m/z$ 398, $[M + H]^+$ at $m/z$ 381, $[M - 32 + NH_4]^+$ at $m/z$ 366, $A_1$ ion at $m/z$ 349 in CIMS, and ions at $m/z$ 204, 159, and 146 with no ion at $m/z$ 217 in EIMS[34]).

The composition of fractions II–IV, determined by colorimetric methods, are given in Table I. The molar ratio Xyl:4-*O*-MeGlcA decreased from 9.1:1 for fraction II to 3.4:1 for fraction IV in accord with the order of elution from DEAE-trisacryl. The more complex fraction V did not contain 4-*O*-MeGlcA, but glucuronic acid together with xylose and hexoses.

*Methylation analysis of fractions II–IV.*—After hydrolysis of the methylated polysaccharide, reduction of the products, and acetylation, the partially methylated and acetylated alditols were analysed by GLC–MS. Two products were identified (Table II) as 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol and 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylxylitol. These results suggests that fractions II–IV comprised a backbone of (1 → 4)-linked xylose residues and side chains of a 4-*O*-MeGlcA linked to position 2. The molar ratio of the partially methylated xylitol acetates was in accord with the molar composition of native polysaccharide fractions.

Previous methylation analyses[10,35] showed that a typical 4-*O*-methylglucuronoxylan contains one 4-*O*-methyl-D-glucuronic acid residue per 5–20 (1 →

TABLE II

Molar ratios of partially methylated and acetylated alditols obtained after methylation, hydrolysis, reduction, and acetylation of DEAE-trisacryl fractions II–IV

| Fractions | Partially methylated and acetylated alditols | |
|---|---|---|
| | 2,3-Xyl [a] | 3-Xyl [b] |
| II | 11.4 | 1 |
| III | 7.1 | 1 |
| IV | 5.1 | 1 |

[a] 1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methylxylitol. [b] 1,2,4,5-Tetra-*O*-acetyl-3-*O*-methylxylitol.

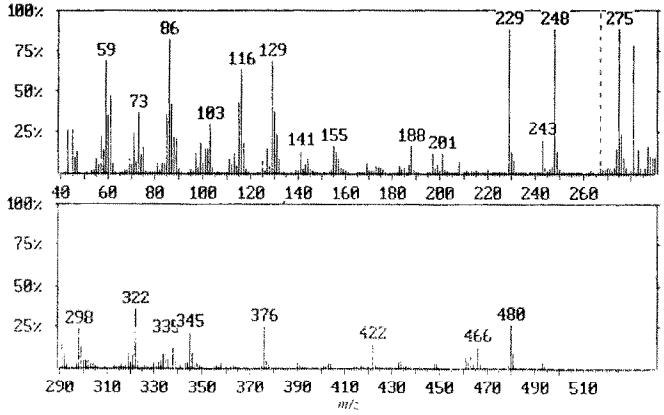
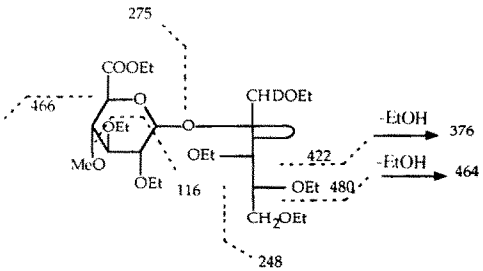



Fig. 1. EI-mass spectrum of the reduced ($NaBD_4$) and ethylated disaccharide obtained by partial acid hydrolysis of polysaccharide fraction II.

4)-linked β-D-xylose residues. Generally, 4-*O*-methyl-D-glucuronic acid side chains are more numerous in softwood than in hardwood xylans, with 5–6 xylose residues being present per acid group in the former, and 10 in the latter[10,22].

*Partial acid hydrolysis.*—Partial acid hydrolysis (2 M $CF_3CO_2H$, 2.5 h, 100°C) of fraction II, followed by GPC on Sephadex G-10, yielded a disaccharide as indicated by FABMS ($[M - H]^-$ at $m/z$ 339). GLC–MS of the borodeuteride-reduced and ethylated disaccharide (Fig. 1) gave a fragmentation pattern (Fig. 2) consistent with structure 4-*O*-MeGlc*p*A-(1 → 2)-Xyl-ol. The $^1H$ and $^{13}C$ NMR data for the disaccharide are listed in Tables III and IV. The resonances in the region for anomeric protons (Fig. 3) confirmed the disaccharide structure with the xylose as the reducing moiety (δ 5.372 for H-1α and 4.709 for H-1β). That the non-reducing residue was α, i.e., 4-*O*-Me-α-D-Glc*p*A, is indicated by the H-1′ resonances (δ 5.066, $J_{1,2}$ 4.02 Hz). That this residue was 2-linked to the xylose residue is confirmed by the chemical shifts of the C-2 resonances (C-2α at 77.10 ppm and




$J_1 = 248+74 = 322$

Fig. 2. Fragmentation pattern of the disaccharide derivative in Fig. 1.

C-2$\beta$ at 79.64 ppm) (see Fig. 4) and by the downfield position of the C-2 resonance by 4.26 ppm in comparison with the C-2 resonances of D-xylose residues that lacked a side chain. This observation was noted by Shibuya et al.[35] for rice endosperm cell-wall polysaccharide and by Cavagna et al.[36] for the aldotriouronic acid obtained from birchwood. On the basis of the NMR and MS data, the structure 4-*O*-Me-$\alpha$-D-Glc*p*A-(1 → 2)-$\alpha$,$\beta$-D-Xyl*p* can be assigned to the disaccharide.

*NMR spectroscopy of the polysaccharide fraction II.*—The $^{1}$H and $^{13}$C chemical shift values (determined at 80°C and assigned on the basis of homonuclear and

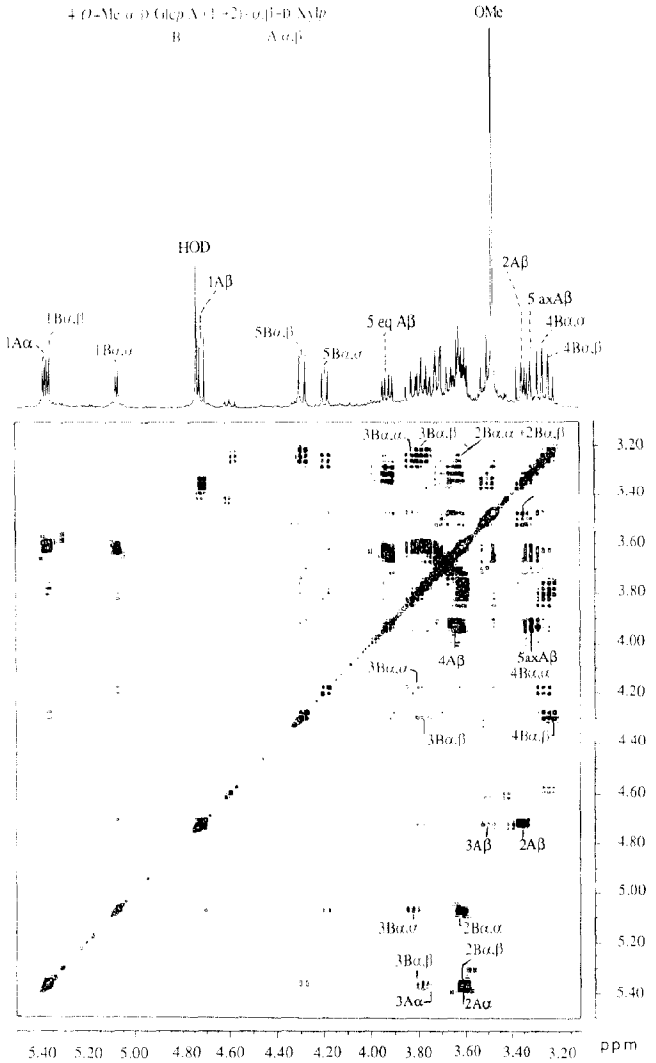



Fig. 3. 2D-COSY spectrum of 4-*O*-Me-$\alpha$-D-Glc*p*A-(1 → 2)-$\alpha$,$\beta$-D-Xyl*p*.

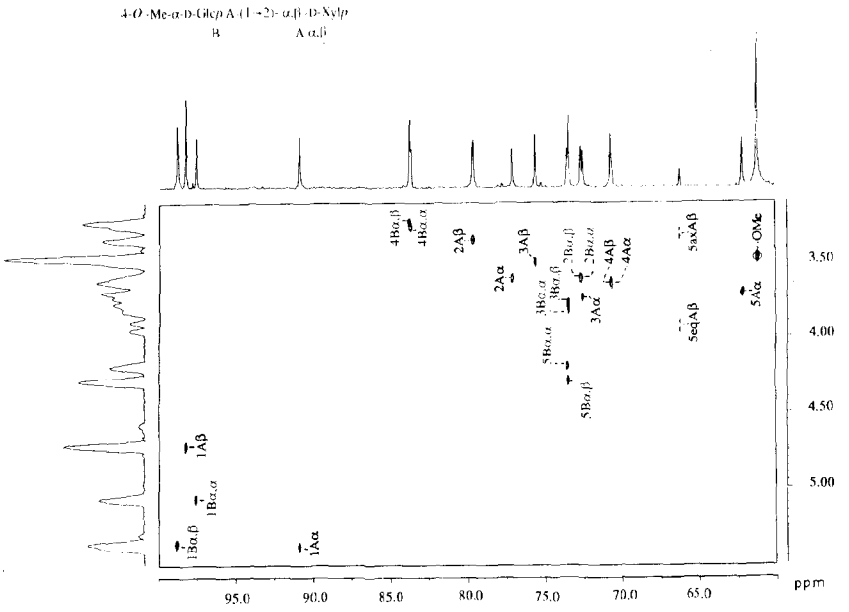



Fig. 4. 2D-Heteronuclear COSY experiment of 4-*O*-Me-α-D-Glc*p*A-(1 → 2)-α,β-D-Xyl*p*.

heteronuclear experiments) are reported in Tables III and IV, and the spectra are shown in Figs. 5 and 6. The intensities of the C-1 resonances confirm the 1 : 9 ratio of the glucuronic acid and xylose residues. The $^1$H NMR spectrum confirms that the 4-*O*-MeGlc*p*A is α and the xylose residues are β ($J_{1,2}$ 7.5 Hz). The mono- (δ 4.483 for H-1) and di-substituted (δ 4.639 for H-1) xylose residues can be discriminated. That the disubstituted xylose residues are linked through C-2 and

TABLE III

$^1$H NMR chemical shifts (δ in ppm) for polysaccharide fraction II isolated from sunflower hulls and for 4-*O*-Me-α-D-Glc*p*A-(1 → 2)-α,β-D-Xyl*p*

| Unit | H-1α,β | H-2α,β | H-3α,β | H-4α,β | H-5α*x*, 5*eq* α,β | OMe |
|---|---|---|---|---|---|---|
| Polysaccharide fraction II [a] | | | | | | |
| → 4)-β-D-Xyl*p*-(1 → | 4.483 | 3.327 | 3.578 | 3.787 | 3.400/4.115 | |
| → 4)-β-D-Xyl*p*-(1 →<br>2<br>↑ | 4.639 | 3.482 | 3.672 | 3.811 | 3.331/3.984 | |
| 4-*O*-Me-α-D-Glc*p*A-(1 → | 5.247 | 3.589 | 3.780 | 3.295 | 4.274 | 3.481 |
| Disaccharide [b] | | | | | | |
| 4-*O*-Me-α-D-Glc*p*A-(1 → | 5.066/<br>5.358 | 3.609/<br>3.602 | 3.821/<br>3.779 | 3.258/<br>3.235 | 4.186/4.284 | 3.474 |
| → 2)-α,β-D-Xyl*p*-(1 → | 5.372/<br>4.709 | 3.602/<br>3.348 | 3.740/<br>3.497 | 3.646/<br>3.633 | 3.697/3.309/<br>3.922 | |

[a] Values obtained at 80°C. [b] Values obtained at 25°C.

TABLE IV

$^{13}$C NMR chemical shifts ($\delta$ in ppm) for polysaccharide fraction II isolated from sunflower hulls and 4-*O*-Me-α-D-Glc*p*A-(1 → 2)-α,β-D-Xyl*p*

| Unit | C-1α,β | C-2α,β | C-3α,β | C-4α,β | C-5α,β | OMe | COOHα,β |
|---|---|---|---|---|---|---|---|
| Polysaccharide fraction II [a] | | | | | | | |
| → 4)-β-D-Xyl*p*-(1 → | 104.33 | 75.43 | 76.47 | 79.16 | 65.74 | | |
| → 4)-β-D-Xyl*p*-(1 → | 103.82 | 79.69 | 75.08 | 79.52 | 65.49 | | |
| 2 ↑ | | | | | | | |
| 4-*O*-Me-α-D-Glc*p*A-(1 → | 100.36 | 74.13 | 74.96 | 84.80 | 74.88 | 62.14 | 178.80 |
| Disaccharide [b] | | | | | | | |
| 4-*O*-Me-α-D-Glc*p*A-(1 → | 97.55/ 98.80 | 72.62/ 72.66 | 73.45 | 83.64/ 83.73 | 73.45/ 73.52 | 61.18 | 178.08/ 177.84 |
| → 2)-α,β-D-Xyl*p*-(1 → | 90.89/ 98.22 | 77.10/ 79.64 | 72.50/ 75.63 | 70.65/ 70.71 | 62.15/ 66.27 | | |

[a] Values obtained at 80°C. [b] Values obtained at 25°C.

C-4 is shown by the chemical shifts for C-2 (79.69 ppm) and C-4 (79.52 ppm). The simplicity of the $^{13}$C spectrum of fraction II indicates that this polysaccharide comprises a regular decasaccharidic unit with the structure: → 4)-β-D-Xyl*p*-(1 → 4)-[β-D-Xyl*p*-(1 → 4)]$_7$-[4-*O*-Me-α-D-Glc*p*A-(1 → 2)]-β-D-Xyl*p*-(1 → .

These results are in good agreement with those obtained for the hydrolysis of glucuronoxylans from sunflower hulls by endoxylanases[37]. Fraction II was hydrolysed completely by the enzyme, suggesting a regular pattern of uronic acid groups.

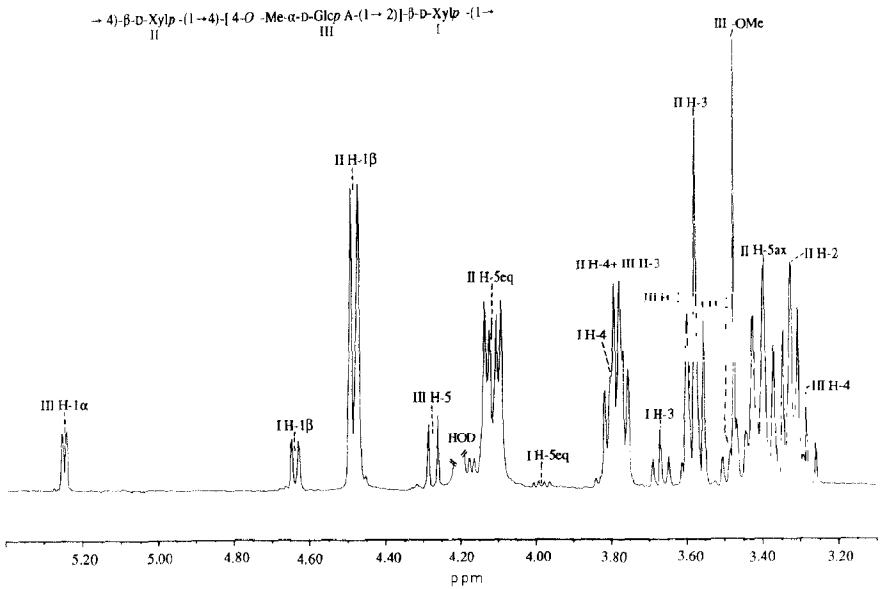



Fig. 5. $^1$H NMR spectrum of polysaccharide fraction II.

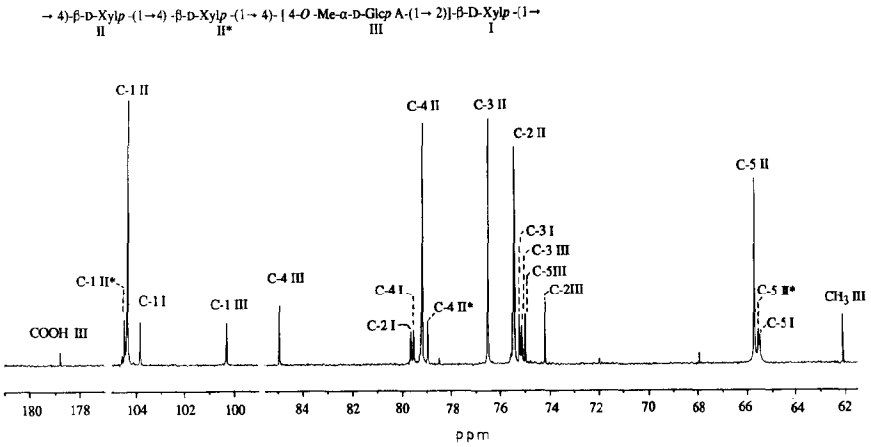



Fig. 6. $^{13}C$ NMR spectrum of polysaccharide fraction II.

From the above results, it is concluded that the distribution of uronic acid side chains in glucuronoxylans from sunflower hulls, for the main fraction, is similar to those of the xylans of hardwoods and some annual plants, but different from those of softwood xylans which have an irregular distribution[9,38,39].

AKNOWLEDGMENTS

This work was supported by ONIDOL (Organisation Nationale Interprofessionnelle Des Oléagineux), the Centre National de la Recherche Scientifique Unité Mixte de Recherche No. 111 (Directeur Professeur A. Verbert), and the Université des Sciences et Technologies de Lille. The authors thank Yves Leroy and Guy Ricart for frequent help with the MS analysis.

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