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# Structural characterization of polysaccharides isolated from grape stalks of *Vitis vinifera* L.

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ABSTRACT

The main structural polysaccharides of grape stalks are cellulose, heteroxylan, and glucan. Cellulose contributes 30.3% of grape stalk matter and has an unusually high degree of the crystallinity (75.4%). Among hemicelluloses, xylan was the most abundant one, contributing ~12% to the weight. The heteroxylan was isolated from the corresponding peracetic holocellulose by DMSO extraction followed by precipitation in ethanol. The $M_w$ of heteroxylan (19.0 kDa) was assessed by size exclusion chromatography (SEC) and the structure was inferred by methanolysis and methylation linkage analysis, as well as 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. The heteroxylan is a partially acetylated (DS = 0.49) glucuronoxylan possessing the main backbone composed by β-(1→4)-linked D-xylopyranosyl units ramified with α-(1→2)-linked 4-*O*-methyl-α-D-glucuronosyl residues (MeGlc*p*A) at a molar ratio 25:1. The isolated heteroxylan contained concomitant β-glucan (ca. 15%), whose structure was elucidated by methylation linkage analysis and by NMR spectroscopy. The results obtained revealed mixed β-(1→3; 1→4)-D-glucan with a molar ratio of β-(1→3)- to β-(1→4)-linked glucopyranosyl units of 1:2.



## 1. Introduction

Grape stalks are a large byproduct of the wine sector and are mainly composed of cellulose, hemicelluloses, tannins, and lignin.[1–3] Previous studies have shown that polysaccharides constitute more than 50% of grape stalk components representing an economically attractive source of fiber material for energy crops (renewable fuels) or papermaking/biocomposites.[1–3] However, only limited knowledge is available concerning structural features of polysaccharides from grape stalks.

Hemicelluloses are non-cellulosic polysaccharides surrounding the cellulose fibers and can be divided into four groups of structurally different polysaccharide types: the xylans, the mannans, the xyloglucans, and the mixed linkage β-glucans. The hemicelluloses together with cellulose have a support function in the cell wall.[4] The hemicelluloses are mainly heteropolysaccharides that are composed by repeating units of pentoses (D-xylose and L-arabinose), hexoses (mainly D-galactose, D-glucose and D-mannose), and uronic acids.[5] In particular, glucuronoxylans are the major hemicelluloses class in angiosperms, representing 15–30% of total dry weight and they are widely found in plant species, especially in woods.[5,6] *O*-Acetyl-4-*O*-methylglucurono-β-D-xylan, usually designated as glucuronoxylan, consists of the main backbone composed by β-(1→4)-linked β-D-xylopyranose units (β-D-Xyl*p*) partially substituted at *O*-2 by the 4-*O*-methyl-α-D-glucuronic acid (MeGlc*p*A). The glucuronoxylans are also substituted at *O*-2 and/or at *O*-3 by acetyl groups. The degree of acetylation ranges from 10% to 20%, corresponding to approximately from 3 to 7 acetyl groups per 10 units of D-xylose.[5,6] In some plants, the glucuronoxylans can be structurally associated with other cell wall polysaccharides. Thus, in *Eucalyptus globulus*, the glucuronoxylan backbone is substituted at *O*-2 with MeGlc*p*A substituted in turn with galactosyl and glucosyl structural units.[7–9]

Among structural glucans, xyloglucans and the mixed β-(1→3;1→4)-D-glucans are the most abundant. The xyloglucans have a β-(1→4)-glucopyranose backbone branched with α-D-Xyl*p* residues at position 6 of the Glc*p* residues, when 30–65% of Glc*p* units are xylosylated.[5,9] The mixed β-(1→3;1→4)-D-glucans possess a usually unbranched backbone composed of β-D-Glc*p* units with a diverse number of molar ratios between β-(1→3) and β-(1→4)-linked units.[5,9] As a rule, the structural blocks constituted by several β-(1→4)-linked D-Glc*p* units (cellobiose, cellotriose, cellotetraose, etc.) are irregularly spaced by β-(1→3)-linked D-Glc*p* residues.

Further research and development trials on the conversion of grape stalks into value-added products (fuels, chemicals and materials) run into the difficulties related to the lack of knowledge

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about the structure of its carbohydrates. Hence this study aims to fill this gap by clarifying the structural features of the carbohydrate polymers in grape stalks.

The present work deals primarily with the study on the xylan isolated from peracetic holocellulose of grape stalks by extraction with dimethyl sulfoxide (DMSO). The xylans and concomitant β-glucan were characterized by methanolysis and methylation linkage analysis, SEC, 1D, and 2D NMR spectroscopy and mass spectrometry techniques.

## 2. Experimental

### 2.1. Materials

The grape stalks sample of the variety *Vitis vinifera* L. (Touriga Nacional) was supplied by Tavfer Group (Quinta do Serrado in Penalva do Castelo in Dão Region of Portugal). The grape stalks were separated from the grape clusters by a mechanical destemmer operation. The material was dried at room temperature, milled on a Retsch cross-beater mill SKl, and sieved to 1–2 mm particles. The grape stalks were characterized regarding the ash content (Tappi T 211 om-91), extractives content in acetone (Tappi T 204 om-88), cellulose *Kürschner* and *Höffer* content,[10] proteins and tannins content,[1] and lignin content according to Tappi T 222 om-88.

### 2.2. Preparation of holocellulose and isolation of xylan

Holocellulose in∼41% yield was obtained by delignification of grape stalks with peracetic acid (14% AcOOH) at 85 °C during 30 min.[8] The holocellulose (ca. 2 g) was milled in a vibratory zirconium ball mill for 40 min and extracted twice by dimethylsulfoxide (DMSO) at 60 °C for 24 h under a nitrogen atmosphere while being stirred (solid-to-liquid ratio 50). The resulting filtrates were precipitated in 800 mL of ethanol, acidified with formic acid to pH 2–3 and kept for two days in the fridge at +4 °C to coagulate the precipitated xylan. After centrifugation, the residue was washed five times in absolute methanol and dried under vacuum. A part of crude xylan was re-dissolved in DMSO (ca. 1:50 w/w) and purified by graded precipitation with ethanol thus obtaining the purified xylan.

### 2.3. Neutral sugars analysis

The holocellulose and xylan were submitted to the neutral sugar analysis after Saeman hydrolysis.[11] The neutral sugars were analyzed by GC as alditol acetates (Varian 3350 gas chromatograph equipped with a FID detector (260 °C) and with a DB-225J&W column), under the following conditions: initial temperature −220 °C (5 min); temperature gradient of 10 °C/min; final temperature −240 °C (6 min).

### 2.4. Acid methanolysis for analysis of sugars and uronic acids

The xylan was subjected to acid methanolysis and subsequent silylation according to methodology described previously.[12] The samples were analyzed by GC (Trace Gas Chromatograph 2000 series), equipped with mass detector (Thermo Scientific DSQII), using helium as carrier gas (35 cm/s). The chromatographic conditions were as follows: column capillary DB-1J&W (30 m × 0.32 mm i.d. 0.25 μm); initial temperature of the column −100 to 4 °C/min −175 °C following by 175–12 °C/min; final temperature −290 °C; detector temperature −290 °C.

### 2.5. Methylation linkage analysis

The xylan was activated with powdered NaOH and methylated with $CH_3I$[13,14] followed by a remethylation to ensure complete methylation of the polysaccharides.[15] The methylated xylan was hydrolyzed by treatment with 2 M trifluoroacetic acid for 1.5 h at 120 °C, and the partially methylated sugars were reduced with sodium borohydrate and acetylated.[15,16] Partially methylated alditol acetates (PMAA) were dissolved in dichloromethane and analyzed by GC/MS (Trace GC 2000 series coupled with Thermo Scientific DSQII mass spectrometer) using a column capillary DB-1J&W (30 m × 0.32 mm i.d. 0.25 μm). The chromatographic conditions were as follows: injector and detector operating at 220 and 280 °C, respectively. Temperature program used was as follows: 10 min at 45 °C with a linear increase of 10 °C/min until 140 °C and standby 5 min at 140 °C; a linear increase from 140 to 170 °C by 0.5 °C/min and standby 1 min at 170 °C; a linear increase from 170 to 280 °C by of 15 °C/min and standby 5 min at 280 °C.

### 2.6. Size exclusion chromatography (SEC)

The xylan solution was prepared immediately before the analysis by dissolution of ca 5 mg of xylan in 50 μL of 10% LiCl solution in *N*,*N*-dimethylacetamide (DMAC) at 100 °C for 15 min and further dilution with DMAC to a xylan concentration of about 1% (w/w). The xylan was analyzed by GPC in a PL-GPC 110 system, equipped with a 10 μm Plgel pre-column and two 10 μm Plgel columns MIXED D 300 × 7.5 mm in a series, and a refraction index detector. The pre-column, SEC columns, and the injection system were maintained at temperature of 70 °C during the analysis. The eluent flow (0.1 M LiCl in DMAC) was 0.9 mL/min. The SEC columns were calibrated using pullulan reference materials (Polymer Laboratories, UK) in a range of 1–100 kDa.

### 2.7. NMR spectroscopy

Holocellulose was analyzed by Cross Polarization/Magic Angle Spinning (CP/MAS) $^{13}C$ NMR. Solid state $^{13}C$ NMR spectra were recorded on a NMR BRUKER AVANCE 400, with a magnetic field of 9.4 T. The sample was spun in a zirconium rotor sealed with Kel-FTM caps at 9 kHz. The acquisition parameters used were as follows: proton pulse of 4 μs, contact time of 2 ms, recovery delay of 4 s and 7000 scans were accumulated.

The isolated and extracted xylan with DMSO was analyzed by $^1H$ NMR and $^{13}C$ NMR on a Bruker AVANCE 300 spectrometer operating at 300.13 MHz for proton and at 75.2 MHz for carbon, respectively, at 303 or 323 K. The xylan was dissolved in $D_2O$ (ca. 2% w/w) and the sodium 3-(trimethylsilyl)propionate-$d_4$ (TMSP, $\delta$ 0.00) was used as the internal standard. The acquisition parameters for the proton spectra were as follows: 12.2 μs pulse width (90°), 18 s relaxation delay, and 300 scans were collected. The acquisition parameters for the carbon spectra were as follows: 60° pulse, 8 s relaxation delay, and 5000 scans were collected. All 2D NMR spectra were recorded on a Bruker AVANCE 300 spectrometer. 2D $^1H$–$^1H$ (absolute-mode COSY spectrum) spectrum was recorded at 323 K using a standard COSY sequence (relaxation delay 2 s) acquiring 2 K × 512 increments transformed to a 2 K × 1 K data matrix after zero-filling, FT, and squared sine-bell apodization applied to both dimensions. COSY spectrum was acquired over a 7.0 ppm window in both F2 and F1 directions. For each t1 value 600 scans were accumulated. 2D $^1H$–$^1H$ (TOCSY) spectroscopy was recorded at 323 K and the spectra were acquired at a spectral width of 2185 Hz in both dimensions ($\tau_{mix}$ = 0.050 s).The relaxation delay was 2.0 s. For each FID, 128 transients were acquired; the data size was 1024 in $t_1$ × 512 in $t_2$. The phase sensitive $^1H$–$^{13}C$(HSQC) spectrum was acquired at 50 °C over a F1 spectral weight of 12,000 Hz and a F2 width of 2000 Hz with a 2048 × 1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarization transfer was optimized for $^1J_{CH}$ = 148 Hz.

### 2.8. Enzymatic hydrolysis and fractionation of XOS

The isolated xylan (10 mg) was treated with *endo*-β-(1→4)-xylanase (EC# 3.2.1.8) from *Thermomyces lanuginosus* (Pentopan Mono BG→X2753) (ca. 1 U/mg) in 2 mL of 0.05 M sodium acetate buffer (pH 5.0) at 40 °C for 2 h. The xylo-oligosaccharides (XOS) were then separated by semi-preparative LEX/SEC (pump Knauer K-1001, RI detector Knauer K-2401) using a Shodex sugar KS 2002 (Showa Denko K.K.) column (300 mm × 20 mm) at 30 °C and ultra-pure water (pH 6.5) as eluent, at a flow rate of 2.80 mL/min. The injected sample volume was 500 μL.

### 2.9. ESI–MS/MS analysis

Electrospray ionization mass spectra (ESI-MS) and tandem mass spectra (ESI-MS/MS) analyses, acquired in a positive mode, were carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. Samples were introduced at a flow rate of 10 μL/min into the ESI source. In the MS and MS/MS experiments the time-of-flight (TOF) mass resolution was set to approximately 9000. For ESI analysis, oligosaccharides were diluted in methanol–water–formic acid (50:50:0.1 v/v/v). The cone voltage was 35 V, and the capillary voltage was 3 kV. The source temperature was 150 °C. The data were processed using MassLynx software (version 4.0). MS/MS spectra were obtained using argon as the collision gas with the collision energy set between 35 and 65 V.

## 3. Results and discussion

### 3.1. General chemical composition of grape stalks

The chemical composition of grape stalks has been recently assessed and is briefly reviewed in Table 1.[1] Overall, grape stalks represent a lignocellulosic material composed essentially of cellulose, lignin, tannins, and hemicelluloses. According to these results, the contribution of polysaccharides in the composition of grape stalks exceeds 50%. In our previous work, cellulose isolated by the *Kürschner* and *Höffer* method was characterized by X-ray scattering analysis (WAXS), which revealed a degree of crystallinity (DC) of 75.4%, a value much superior to that normally found in the wood (55–65%) and comparable with DC of cellulose in cotton.[1] However, the detailed structure of hemicelluloses was not assessed, and was the main topic of the present study.

The polysaccharides were isolated as peracetic holocellulose in ca. 42% yield. The solid state $^{13}C$ NMR spectrum of holocellulose (Fig. 1) clearly confirmed cellulose as the major component, which carbon resonances were unambiguously assigned based on known data.[17–19] Notable shoulders at ca. 64 and 77 ppm were assigned to C5 and C4, respectively, in xylopyranoside moieties of xylan, which must be partially acetylated due to the presence of characteristic signals from acetyl groups at 21 and 173 ppm.[20] The high xylan content in holocellulose was confirmed by the analysis of neutral sugars (Table 1). Taken into account the xylan content in holocellulose, the yield of holocellulose and eventual contribution of acetyl and uronic moieties (15–25%), the total xylan content in grape stalks must be about 12%. This is in agreement with the recently published xylan content in grape stalks.[1]

### 3.2. Isolation and chemical analysis of xylan from grape stalks

The xylan was isolated from peracetic holocellulose by two consecutive extractions with DMSO followed by precipitation of united extracts in ethanol. The approximate yield of xylan was 70% (w/w) from its total content in holocellulose. The extraction with DMSO allows obtaining almost intact xylan and to assess additional structural information concerning the relative abundance and the location of the acetyl groups in the backbone.[8,21] The isolated xylan was analyzed for sugar composition (Table 2). The analysis of neutral sugars revealed a reasonable purity of isolated crude xylan (xylose content almost 84%) though being contaminated by glucan (glucose content almost 15%). The presence of non-cellulosic glucan in grape stalks (about 6–7%) was proposed previously based on polysaccharide balance.[1] The complementary sugar analysis by methanolysis confirmed the presence of non-cellulosic glucan in crude heteroxylan and indicated the probable substitution of the heteroxylan backbone by terminal MeGlc*p*A residues (Table 3). The small amounts of detected rhamnose (Rha) and galacturonic acid (GalA) residues agree with the presence of unique structural fragment [→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p*] at the reducing end in xylan.[9]

The concomitant non-cellulosic glucan was impossible to separate completely from heteroxylan by graded precipitation with ethanol due to their strong bounding or structural association. The purified heteroxylan still contained about 5% of glucan (Table 2). This purified heteroxylan was used for the structural studies, whereas crude heteroxylan was availed for the evaluation of glucan structure.

The molecular weight ($M_w$) of purified heteroxylan was 19 kDa as assessed by SEC. This is a rather small value when compared to the molecular weights of xylan isolated by the same method from wood (25–40 kDa),[22] but very similar to molecular weights normally reported for xylan from monocotyledons.[12,23]

### 3.3. Structural analysis by NMR

The purified heteroxylan from grape stalks was structurally analyzed employing single-bond (COSY) and multiple-bond (TOCSY) proton–proton and single-bond proton–carbon (HSQC) correlation NMR spectroscopy. The correlations obtained from COSY spectrum (Fig. 2) were combined with correlations in TOCSY (Appendix A. Supplementary data) and in HSQC (Fig. 3) spectra. The obtained assignments were identical to those previously reported for *O*-acetyl-(4-*O*-methyl-α-D-glucurono)-β-D-xylan.[8,12,20,24,25] The chemical shifts to the corresponding protons and carbons are summarized in Table 4. The *O*-2 and *O*-3 acetylated Xyl*p* units were unambiguously identified together with non-substituted terminal α-(1→2)-linked MeGlc*p*A residue.

The contribution of structural units with different substitution patterns in the heteroxylan backbone was determined by quantitative $^1H$ NMR spectroscopy (Fig. 4). The expanded anomeric region (4.3–5.4 ppm) of the spectrum shows the designations for the specified groups of protons in particular substructures used in the integration trials. Non-acetylated internal xylose residues and glucopyranosyluronic residues were assessed based on their anomeric proton resonances, whereas the amounts of *O*-acetylated were estimated based on H-3 resonances in

**Table 1**
Chemical composition of grape stalks[1]

| Grape stalks components | Ash | Extractives in acetone | Cellulose | Proteins | Tannins | Klason lignin | Hemicelluloses |
|---|---|---|---|---|---|---|---|
| %, w/w | 7.0 | 2.3 | 30.3 | 6.1 | 15.9 | 17.4 | 21.0 |

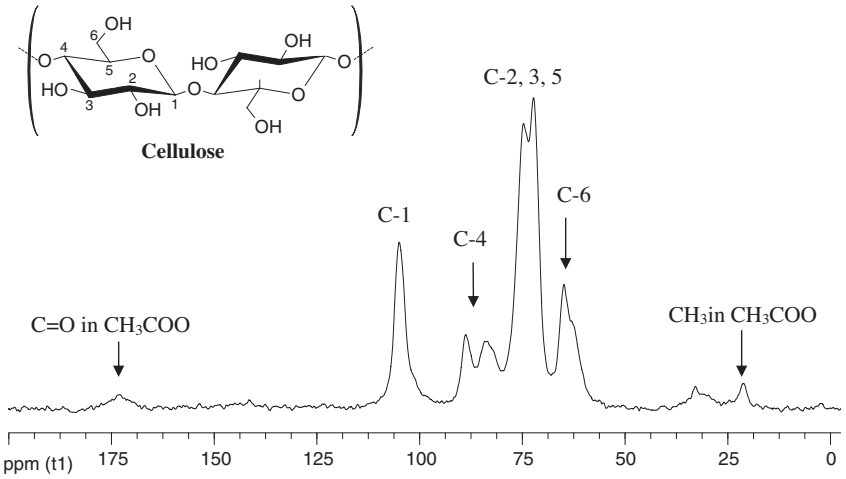



**Figure 1.** CP/MAS $^{13}$C NMR spectrum of peracetic holocellulose from grape stalks.

**Table 2**
Composition of neutral sugars (% wt) in peracetic holocellulose and in isolated xylans

| Monosaccharides | Holocellulose | Xylan[a] |
|---|---|---|
| Rhamnose | 0.5 | 0.2 (0.4) |
| Arabinose | – | 0.4 (0.1) |
| Xylose | 22.6 | 83.5 (93.6) |
| Mannose | 1.1 | 0.6 (0.2) |
| Galactose | 0.9 | 0.6 (0.2) |
| Glucose | 74.9 | 14.7 (5.5) |

[a] Data in parentheses correspond to sugars analysis of purified xylan, obtained by graded precipitation of crude xylan with ethanol.

**Table 3**
Composition of monosaccharides and uronic acids (% wt) determined by methanolysis of crude xylan from grape stalks

| Monosaccharides | Xylan |
|---|---|
| Ara | 0.3 |
| Rha | 0.8 |
| Xyl | 77.2 |
| GalA | 1.0 |
| 4-*O*-Me-GlcA | 2.7 |
| Gal | 1.1 |
| Man | 0.4 |
| Glc | 16.5 |

corresponding structures (Fig. 4). The total balance of the acetyls groups was controlled by the integration of proton resonances in acetyl groups at 2.05–2.30 ppm and the integrals of characteristic protons in acetylated xylopyranosyl residues.

The distribution of acetyl groups per 100 of xylopyranose units is presented in Table 5. According to the obtained results, 43% mol of the Xyl*p* residues was acetylated, mostly 3-*O*-acetylated (29 mol %). The total degree of acetylation (DS) was found to be 0.49. Practically all β-D-Xyl*p* units ramified at *O*-2 with MeGlc*p*A residues were 3-*O*-acetylated, as follows from the equal amounts of [4-*O*-methyl-α-D-Glc*p*A-(1→] and [→4)[4-*O*-methyl-α-D-Glc*p*A-(1→2)][3-*O*-Ac]-β-D-Xyl*p*-(1→] residues detected in the heteroxylan. The estimation of molar proportion of MeGlc*p*A and internal Xyl*p* units, assessed by integrals of anomeric protons in corresponding structures, was around 1:25. This is coherent with data obtained by sugars analysis after methanolysis (Table 3). Overall, the heteroxylan from grape stalks may be defined as *O*-acetyl-glucuronoxylan of relatively low molecular weight and low degree of substitution with uronosyl moieties.

### 3.4. Linkage analysis

The methylation analysis was carried out using crude heteroxylan to assess the major substitution patterns in the heteroxylan backbone and in the concomitant glucan. The relative abundance of the identified partially methylated products normalized to one non-reducing xylopyranose unit is presented in Table 6. The (1→4)-linked D-xylopyranosyl units represented the relative molar ratio of 80. The molar ratio of →4)-Xyl*p*-(1→ to →2,4)-Xyl*p*-(1→ in methylation analysis was practically the same as found for the ratio of Xyl*p* to MeGlc*p*A residues in the sugars analysis by methanolysis (Table 3). Hence practically all *O*-2 substitutes in the heteroxylan backbone are MeGlc*p*A residues. A small amount of *O*-3 substituted Xyl*p* units (→3,4)-Xyl*p*-(1→) may be tentatively explained by the presence of other substitutes in the heteroxylan backbone. For example, a structural association between glucan and heteroxylan may be proposed. However, the incomplete permethylation of *O*-3 linked acetyl groups in heteroxylan backbone also cannot be completely excluded.

The concomitant glucan in crude heteroxylan was composed of structural units →3)-Glc*p*-(1→ and →4)-Glc*p*-(1→ at a molar ratio 1:2. This glucan was assigned to the mixed linear β-(1→3;1→4)-D-glucan, because α-(1→3;1→4)-D-glucopyranosides were not detected in proton (no strong resonance of anomeric protons at around 5.40 ppm in Fig. 4) and in carbon spectra (no resonance of anomeric carbon in α-D-Glc*p* residue at ca. 100.6 ppm in Fig. B, Supplementary data) of purified and of crude (Fig. C, Supplementary data) heteroxylans. The $^{13}$C NMR spectrum showed resonances of anomeric carbons in internal β-D-Glc*p* residues at 103.5–103.7 ppm (Fig. B, Supplementary data). In addition, the characteristic proton correlations for β-D-glucans have been detected in the TOCSY spectrum of the crude heteroxylan (Fig. D, Supplementary data). The hypothetic structure of mixed linear β-(1→3;1→4)-D-glucan from grape stalks is presented in Figure 5. Nevertheless, an additional study is required for the more comprehensive structural elucidation of this β-D-glucan to assess the detailed information about its building blocks. The possible structural association of heteroxylan and β-D-glucan in the isolated crude heteroxylan was clarified by structural analysis of xylo-oligosaccharides (XOS) released upon enzymatic hydrolysis by xylanase as discussed below.

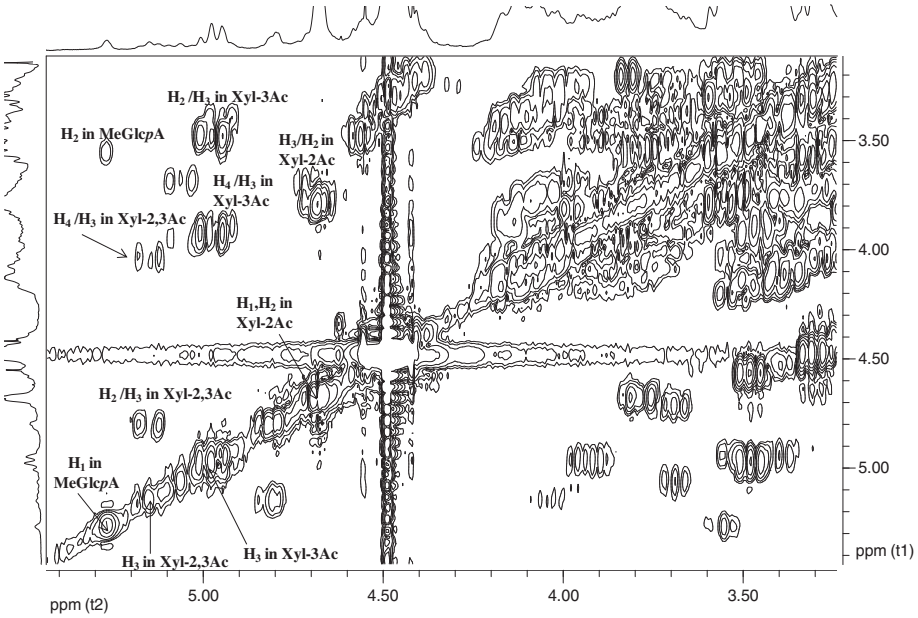



**Figure 2.** Expanded anomeric protons region of $^{1}H–^{1}H$ COSY spectrum ($D_2O$, 50 °C) of purified xylan from grape stalks. Designations are as follows: Xyl*p*-2Ac, 2-*O*-acetylated Xyl*p*; Xyl*p*-3Ac, 3-*O*-acetylated Xyl*p*; Xyl*p*-2,3Ac, 2,3-di-*O*-acetylated Xyl*p*; MeGlc*p*A, 2-*O*-linked MeGlc*p*A.

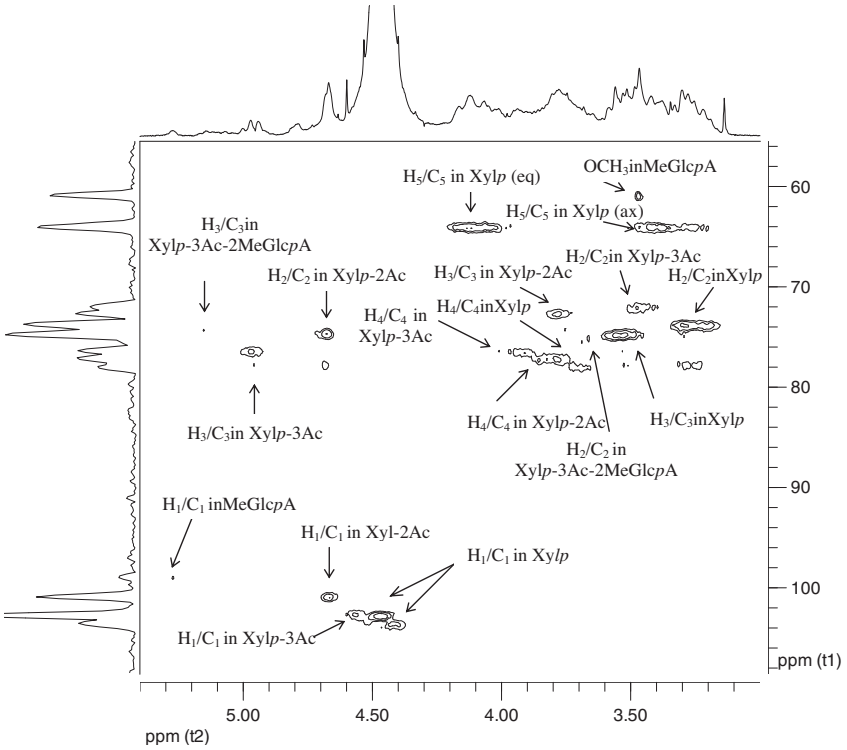



**Figure 3.** HSQC spectrum ($D_2O$, 50 °C) of purified xylan from grape stalks.

### 3.5. Fractionation and analysis of XOS

The heteroxylan was hydrolyzed using a commercial xylanase to produce a series of XOS that were fractionated by semi-preparative ligand-exchange/size exclusion chromatography (LEX/SEC).[7] All XOS were separated into five fractions (F1–F5) as it is depicted in Figure 6. Fraction F1 contained acidic XOS (XOS containing uronic moieties). Fraction F2 represented high molecular neutral XOS (>2000 Da) and was contaminated by enzymatic complex. The fractions F3 and F4 were

**Table 4**
Proton/carbon assignments of xylan from grape stalks ($D_2O$, 50 °C).

| Structural fragment and short designation | Assignments | | | | | |
|---|---|---|---|---|---|---|
| | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | |
| | | | | | ax | eq |
| Xyl*p* (isol) | 4.46/102.9 | 3.30/73.8 | 3.55/74.8 | 3.79/77.3 | 3.40/64.0 | 4.11/64.0 |
| Xyl*p*-3Ac | 4.56/102.6 | 3.46/72.0 | 4.97/76.1 | 3.91/76.4 | 3.46/63.9 | 4.14/63.9 |
| Xyl*p*-2Ac | 4.66/100.9 | 4.67/74.6 | 3.78/72.5 | 3.85/77.2 | 3.44/63.9 | n.d./63.9 |
| Xyl*p*-2,3Ac | 4.79/100.4 | 4.80/74.6 | 5.14/75.9 | 4.05/78.1 | 3.51/63.9 | 4.20/63.9 |
| Xyl*p*-3Ac-2MeGlc*p*A | 4.72/102.0 | 3.66/75.5 | 5.07/75.0 | 3.95/77.5 | n.d./n.d. | n.d./n.d. |
| MeGlc*p*A | 5.27/98.8 | 3.56/72.4 | 3.80/73.8 | 3.24/83.1 | n.d./n.d. | Non relevant |

Designations are as follows: Xyl (isol.), non-acetylated Xyl*p* in the backbone isolated from other acetylated Xyl*p* units; Xyl*p*-3Ac, 3-*O*-acetylated Xyl*p*; Xyl*p*-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2,3-di-*O*-acetylated Xylp; Xyl*p*-3Ac-2MeGlc*p*A, MeGlc*p*A 2-*O*-linked and 3-*O*-acetylated Xyl*p*; MeGlc*p*A, 2-*O*-linked MeGlc*p*A.

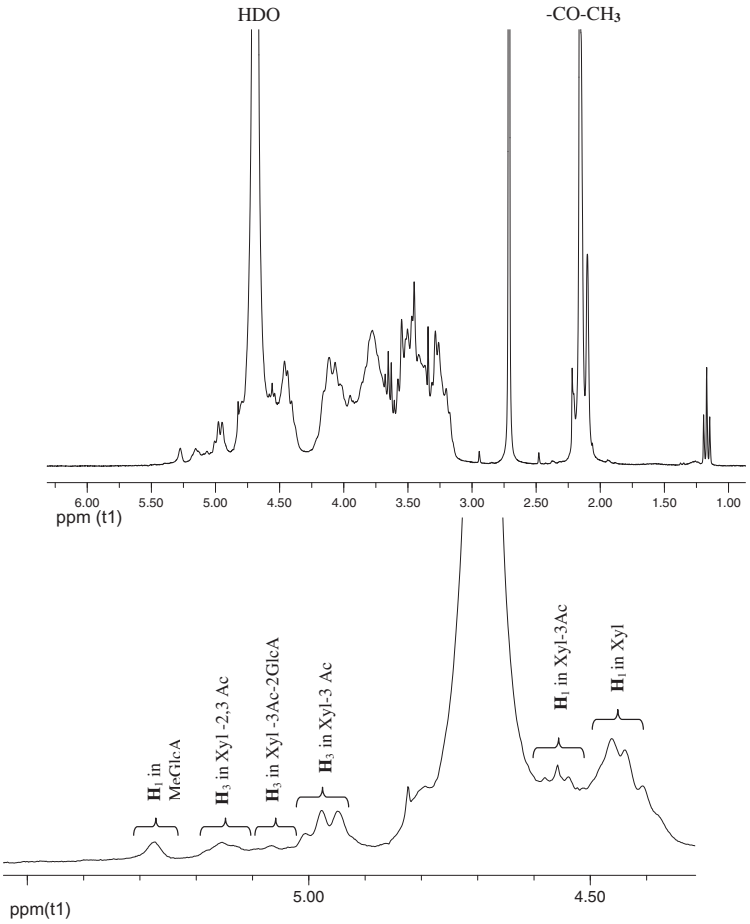



**Figure 4.** $^1H$ NMR spectrum ($D_2O$, 30 °C) of purified xylan (top image) and the expanded region of anomeric protons (bottom image). The designations are the same as presented in Table 4.

**Table 5**
Relative content in acetyl groups in structural units of purified xylan

| Structural fragment and short designation[a] | Relative abundance (per 100 Xyl*p* units) |
|---|---|
| Xyl*p* | 57 |
| Xyl*p*-2Ac | 14 |
| Xyl*p*-3Ac | 19 |
| Xyl*p*-2,3Ac | 6 |
| Xyl*p*-3Ac-2MeGlc*p*A | 4 |
| MeGlc*p*A | 4 |

[a] Designations for the structural fragments are the same as depicted in Table 4. The results were the same as presented in our previous work.[1]

**Table 6**
Results of methylation analysis of crude xylan from grape stalks

| Methylated residue[a] | Structural units deduced | Relative mole ratio[b] |
|---|---|---|
| Xyl-2,3,4 | Xyl*p*-(1→ | 1.0 |
| Xyl-2,3 | →4)-Xyl*p*-(1→ | 80.0 |
| Xyl-3 | →2,4)-Xyl*p*-(1→ | 2.7 |
| Xyl-2 | →3,4)-Xyl*p*-(1→ | 0.4 |
| Ara-2,3,5 | Ara*f*-(1→ | 0.2 |
| Rha-2,4 | →3)-Rha*p*-(1→ | 0.4 |
| Glc-2,3,4,6 | Glc*p*-(1→ | 0.1 |
| Glc-2,4,6 | →3)-Glc*p*-(1→ | 3 |
| Glc-2,3,6 | →4)-Glc*p*-(1→ | 6 |

[a] Only trace amounts of Man-2,3,6, Gal-2,3,6 and Gal-2,3 were detected.
[b] Normalized to one non-reducing xylopyranose unit.

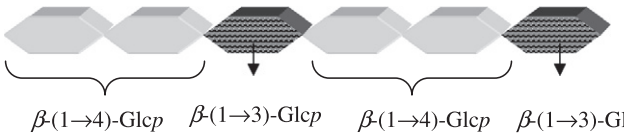



**Figure 5.** Schematic representation of β-(1→3;1→4)-glucan from grape stalks.

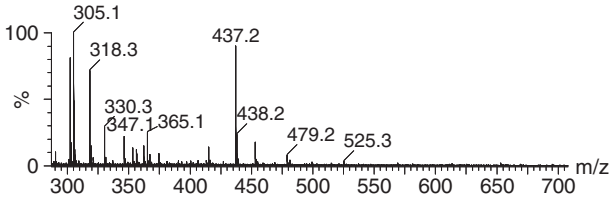



**Figure 7.** ESI-MS spectrum (positive mode) of XOS collected in fraction F3 (Fig. 6).

composed for neutral oligomers and finally, the fraction F5 corresponded to sugar monomers. The most prominent for the structural analysis XOS, in relation to their possible structural association with glucan, are fractions F1 (acidic XOS) and F3 (neutral dimers/trimers), which were assessed by ESI-MS/MS.

### 3.6. Mass spectrometry analysis

The mass spectrometry analysis of fractions F1 and F3 by ESI-MS was carried out in a positive mode according to previously described methodology of the analysis.[7,20] XOS were ionized preferentially as sodium adducts, $[M+Na]^+$, which were further analyzed by tandem mass spectroscopy (MS/MS). Electrospray tandem mass spectrometry (ESI-MS/MS) allows the assessment not only the distribution patterns of acetyl groups and MeGlc*p*A residues in XOS,[7,20] but also the structural elucidation of complex XOS.[26]

The analysis of acidic XOS by ESI-MS/MS revealed a series of non-acetylated ($Xyl_nMeGlcpA_m$, $n = 1–5$; $m = 1–2$) and acetylated ($Xyl_nAc_kMeGlcpA_m$, $n = 1–5$; $m = 1–2$; $k = 1–3$) oligosaccharides, just the same as detected in the fraction of acidic XOS from other xylans.[7,20] No acid XOS of eventual structure $Xyl_nAc_kHex_pMeGlcpA_m$ (Hex is hexose, $p \geqslant 1$) have been detected.

The analysis of neutral fraction F3 (Fig. 7) allowed the structural evaluation of dimers and trimers that could contain mixed pentose/hexose structures thus providing a proof for the eventual structural association between heteroxylan and β-glucan. The analysis of ESI-MS spectrum revealed expected non-acetylated ($Xyl_2$ at $m/z$ 305 and $Xyl_3$ at $m/z$ 437) and acetylated ($Xyl_2Ac$ at $m/z$ 347 and $Xyl_3Ac$ at $m/z$ 479) XOS. The eventually derived from β-glucan dimeric ($Hex_2$ at $m/z$ 365) and trimetric ($Hex_3$ at $m/z$ 527) gluco-oligosaccharides have been also detected. However, no signals from oligomers of series $Xyl_mHex_n$ ($m = 1–2$, $n = 1–2$) were registered. Hence ESI-MS analysis did not confirm covalent bonding between heteroxylan and β-glucan.

## 4. Conclusion

Results of this work revealed cellulose, heteroxylan, and mixed β-(1→3;1→4)-D-glucan as the main structural polysaccharides of the grape stalks from *Vitis vinifera* L. The heteroxylan was the second most abundant polysaccharide in grape stalks, after cellulose, and composed by partially acetylated β-(1→4)-linked xylopyranose units (Xyl*p*) ramified at *O*-2 with 4-*O*-methyl-α-D-glucuronosyl residues (Xyl*p* to MeGlc*p*A molar ratio 1:25). The acetyl groups are attached at *O*-2 (14 mol %), *O*-3 (19 mol %) and at *O*-2/*O*-3 (6 mol %) of Xyl*p* residues. The heteroxylan from grape stalks can be defined as an *O*-acetyl-(4-*O*-methyl-α-D-glucurono)-β-D-xylan with the empirical structure per 100 Xyl*p* units, as presented below:

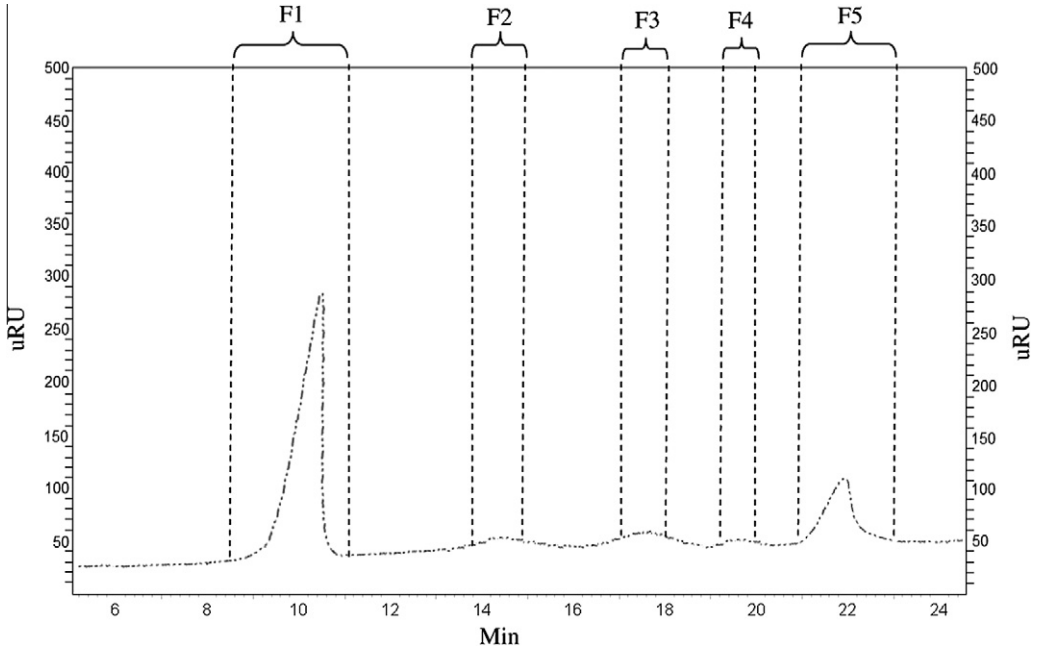
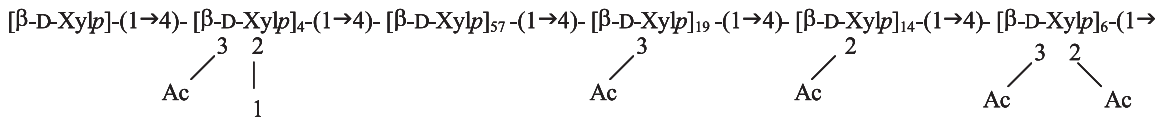



**Figure 6.** LEX/SEC chromatogram of xylo-oligosaccharides after enzymatic hydrolysis of heteroxylan. Collected fractions are depicted as F1–F5.




The glucan strongly interacting with heteroxylan was suggested to be a mixed β-(1→3;1→4)-D-glucan as revealed from methylation linkage analysis and NMR studies. This glucan is not linked covalently to heteroxylan, as confirmed by ESI-MS analysis of the XOS released after enzymatic hydrolysis of xylan by xylanase.

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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.2012.02.001.

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