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Isolation and characterisation of cell wall polymers from the heavily lignified tissues of olive (*Olea europaea*) seed hull

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Cell wall material (CWM) was prepared from olive seed hull, which is heavily lignified and very tough. The material was cryomilled and delignified with chlorite/acetic acid for 9 h to give the holocellulose. Polymers were solubilised from the holocellulose by sequential extraction with cyclohexane-*trans*-1,2-diamine-*NN'N'*-tetra-acetate (CDTA, Na salt), DMSO, 0.5, 1 and 4 M KOH and 4 M KOH + borate to leave the α-cellulose residue. The suspension of α-cellulose on neutralisation released a small amount of pectic material virtually free of xylan to give α'-cellulose. The polymers from the various extracts were fractionated by graded precipitation with ethanol prior to anion-exchange chromatography, and selected fractions were subjected to methylation analysis. During delignification, glucuronoxylans with relatively low degrees of polymerisation (DP) and xylan–pectic polysaccharide complexes linked to degraded lignin were solubilised. A proportion of the xylan–pectic polysaccharide complexes were solubilised by 0.5 M KOH. The major hemicellulosic polysaccharides of the olive seed hulls are glucuronoxylans, which occur as highly branched short chains, with DP of 30–60; or slightly branched chains with DP of 90–110. Partial acid hydrolysis of the major acidic xylan, gel-filtration chromatography and methylation analysis allowed us to propose a tentative structure for the major glucuronoxylan in which one residue of Glc*p*A occurs in each 14 continuously linked Xyl*p* residues in a regular structure.

INTRODUCTION

The seed hull of the olive fruit is composed of different types of heavily lignified cells which form a hard structure that encloses the cotyledon (Winton & Winton, 1932). The hulls of drupe fruits such as olive become very hard on maturation and are very difficult to break open. There is very little, if any, definitive information on the cell walls of the seed hulls of the mature olive fruit and this study was undertaken to complement our work on the cell wall polymers of the olive pulp (Coimbra *et al.*, 1994). In contrast to olive seed hulls, a considerable amount of work has been done on the lignified hulls of cereal grains: the major polysaccharides of wheat and oat hulls are cellulose, glucuronoxylans and slightly branched arabinoxylans (Aspinall, 1959; Aspinall & Carpenter, 1984; Selvendran, 1985). In the case of dicotyledonous seeds such as peas and soya beans, the hulls are only slightly lignified. They are softer, and become pliable after imbibition of water. The major polysaccharides of these hulls are cellulose, pectic polysaccharides, acidic xylans and xyloglucans (Selvendran, 1984; Aspinall *et al.*, 1967). This paper reports the composition and structural features of the cell wall polymers of the seed hulls of olive fruit.

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MATERIALS AND METHODS

Plant material and experimental methods such as anion-exchange chromatography and pH fractionation were as described previously (Coimbra *et al.*, 1994).

Preparation of cell wall material (CWM)

Stones (olive seeds) were obtained from mature olives (*Olea europaea* L. cv Douro) and were frozen in liquid

nitrogen. The stones (135 g) were disrupted in distilled water with a Waring blender in order to remove any loosely adhering pulp tissue. After boiling in 95% EtOH, the stones were then broken into small pieces with a hammer, depleted of the cotyledon, and homogenised in a coffee grinder. The material was then freeze-milled (SPEX 6700) to a particle size of approximately 0.05 mm. It was subsequently washed with cold water, cold 95% EtOH, ether and then air-dried to a constant weight at 40°C. The yield of the recovered cell wall material was 75% that of the seeds.

Delignification of CWM

Prior to delignification, the CWM was extracted in DMSO for 4 days, which solubilised 0.3% of the CWM. The residue (12 g) was delignified according to the procedure of Jermyn and Isherwood (1964). The material was suspended in 300 ml of water and 200 μl of AcOH and 3 g sodium chlorite (80%) were added. The mixture was allowed to react at 70°C with continuous stirring. The round-bottom flask was closed with a loose-fitting glass stopper. Every hour, 200 μl of AcOH and 3 g of sodium chlorite were added. After 6 h the yellow supernatant solution was filtered through a sintered glass funnel. Since the residue remained pale yellow, 150 ml of water was added and the extract process was repeated with additions of AcOH and chlorite every hour. The reaction was stopped after 3 h by which time the insoluble residue had become light yellow. The solution was filtered and washed thoroughly with cold distilled water, a small amount of the residue was freeze-dried to determine the yield and for sugar composition. The filtrates, including the aqueous washings, were combined and flushed with argon until a pale yellow solution was obtained. The solution was then concentrated, dialysed and freeze-dried. Twelve grams of residue gave 8.5 g of holocellulose (71%) and 576 mg of chlorite/HOAc. soluble fraction (4.8%).

Sequential extraction of holocellulose

The holocellulose (8.5 g, dry weight) was extracted sequentially with (i) 50 mM cyclohexane-*trans*-1,2-diamine-*NNN'N'*-tetra-acetate (CDTA, Na salt), pH 6.5 at 20°C for 6 h; (ii) DMSO at 20°C for 24 h; (iii) 0.5 M KOH at 1°C for 2 h; (iv) 1 M KOH at 1°C for 2 h; (v) 1 M KOH at 20°C for 2 h; 4 M KOH at 20°C for 2 h; and 4 M KOH + 3.5% H_3BO_3 at 20°C for 2 h, to leave the α-cellulose residue. The KOH extractions were carried out under argon with O_2-free solutions containing 20 mM $NaBH_4$ to prevent β-elimination and peeling reactions. After each extraction, solubilised polymers were separated from the insoluble residue by filtration through a G1 sintered glass funnel. The alkali extracts were acidified to pH 5 with AcOH and dialysed. The precipitates formed during the dialysis were collected separately. The α-cellulose residue was suspended in water and the solution was acidified to pH 5, dialysed, and centrifuged to separate the final residue. All the dialysed extracts were concentrated and freeze-dried.

Graded precipitation with ethanol

The solubilised materials from above were dissolved in water or dilute KOH, and subjected to precipitation in AnalaR grade ethanol (Coimbra *et al.*, 1994). The precipitates obtained were dispersed in water, adjusted to pH 5 with AcOH, dialysed and freeze-dried. The alkali solutions were prepared with O_2-free water containing 20 mM $NaBH_4$.

Partial acid hydrolysis

Fraction Hp25 (30 mg) (aqueous-insoluble, but alkali-soluble precipitate from the 0.5 M KOH extract which had been reprecipitated with 25% EtOH), was hydrolysed in 1 ml 50 mM trifluoroacetic acid (TFA) at 100°C for 45 min and the supernatant was rendered free of TFA by repeated evaporation with water under vacuum.

Gel-filtration chromatography

The oligosaccharides released by TFA hydrolysis were dissolved in 0.5 ml HCO_2H (pH 3.6) and fractionated on a column (100 × 1.6 cm) of Bio-Gel P4 (−400 mesh) with HCO_2H (0.1 M, 7 ml/h) at 55°C. The elution sequence was monitored by measuring the refractive index of 2 ml fractions. Appropriate fractions were combined and the formic acid was removed by co-evaporation with water under reduced pressure.

Carbohydrate and linkage analysis

Neutral sugars were released by Saeman hydrolysis and analysed as their alditol acetates by gas chromatography (GC) (Blakeney *et al.*, 1983). Uronic acid was determined colorimetrically by a modification (Selvendran *et al.*, 1979, 1989) of the method of Blumenkrantz and Asboe-Hansen (1973). Polysaccharides were methylated by a modification of the method of Isogai *et al.* (1985). The sample (2–3 mg) was dispersed in 2 ml of dried DMSO and sonicated occasionally until dissolved. NaOH pellets were powdered under argon and 100 mg was added to the solution. The samples were sonicated for 90 min and left to stand for the same time. After cooling, 1 ml of CH_3I was added and allowed to react for 30 min in an ultrasound bath and 30 min without the bath. $CHCl_3$/MeOH (1:1, 3 ml) was added and the solutions were dialysed against three changes of 50%

EtOH. The methylated material was hydrolysed with 0.3 ml of TFA at 121°C for 1 h (Harris *et al.*, 1984), cooled and then evaporated to dryness. The partially methylated sugars were then suspended in 0.3 ml of NH_4OH (2 M) to which 20 mg of $NaBD_4$ were added. The mixture was allowed to react at 30°C for 1 h, and then treated with 0.1 ml AcOH. Acetylation was performed by adding 0.45 ml of methylimidazole and 3 ml acetic anhydride. The partially methylated alditol acetates were separated by GC on an OV-225 column and characterised by gas chromatography–mass spectrometry (GC/MS), using the molar response factors of Sweet *et al.* (1975). The chlorite-soluble fractions were first de-esterified according to Aspinall *et al.* (1970) as described in Coimbra *et al.* (1994). Selected methylated fractions (2–3 mg) were carboxyl reduced by a modification of the method described by Lindberg & Lönngren (1978) as described in Coimbra *et al.* (1994).

^{13}C-NMR spectroscopy

Spectra (75 MHz) were recorded under conditions of broad-band proton decoupling with a Brücker AMS 300 spectrometer in a solution of Me_2SO-d_6 at 20°C. Chemical shifts were compared with other ^{13}C spectral data for glucuronoxylans (Coimbra *et al.*, 1994; Kovak & Hirsch, 1982; Excoffier *et al.*, 1986).

RESULTS AND DISCUSSION

Delignification of CWM

The seed hull was very difficult to subdivide by ordinary milling and the material had to be cryomilled several times. The difficulty in finely subdividing the material could be due to its high lignin content as shown by phloroglucinol–HCl test (Siegel, 1953). The CWM of olive seed is rich in xylose and glucose and the total carbohydrate content accounted for only 62% of the dry weight of the CWM (Table 1). Initial attempts to delignify the CWM by treatment with chlorite/acetic acid at 70° for 4 h, as described by Jermyn and Isherwood (1964), were only partially successful, and a significant amount of residual (degraded) lignin was present in the residue. In this respect this material was unlike CWM from heavily lignified parchment fibres of mature runner bean pods (Selvendran & King, 1989). In order to 'completely' remove the lignin, the residue had to be treated for an additional 5 h with chlorite/acetic acid added at hourly intervals. Argon was passed through the delignification liquor to remove chlorine dioxide, and the solution was concentrated and dialysed. Chlorine dioxide can inhibit the free radical chain reaction between chlorine and the polysaccharides during delignification by acting as a free radical

Table 1. Sugar composition of fractions of cell wall material of olive seed hulls obtained by sequential extractions

Fraction	Recovery (%)	Cell wall sugars (mol %)								Total sugars[a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
CWM		1	t	2	48	t	1	41	8	619
Water	0.8	8	1	11	15	19	5	28	14	171
DMSO-1	0.3	2	t	7	40	2	5	31	13	328
Chlorite	4.8	4	2	16	51	1	4	2	19	210
Holocellulose	70.8	1	–	2	44	t	1	38	8	718
CDTA	0.4	3	1	10	61	1	4	3	16	313
DMSO-2	1.5	1	–	3	81	1	1	6	8	506
0.5 M KOH										
soluble[b]	11.5	1	–	3	77	–	2	t	17	616
ppt.[c]	13.6	1	–	1	86	–	t	t	12	773
1 M KOH 1°C										
soluble[b]	2.4	1	–	3	81	–	2	1	14	770
ppt.[c]	2.7	1	–	1	89	–	t	1	9	868
1 M KOH 20°C										
soluble[b]	0.8	1	–	3	77	t	2	2	15	836
ppt.[c]	1.0	1	–	t	91	–	t	t	8	889
4 M KOH	0.4	2	–	6	71	1	3	4	15	667
4 M KOH + borate	0.2	3	–	12	35	2	5	9	35	340
α-cellulose										
soluble	0.4	3	–	23	1	–	9	2	63	565
residue[d]	30.6	t	–	1	2	t	1	92	3	925

t, trace; UA, uronic acid.
[a]Values are expressed as μg anhydrosugar per mg.
[b]Material which remained soluble on dialysis.
[c]Material which precipitated on dialysis.
[d]α′-cellulose.

scavenger. It does this without affecting the chlorination of lignin, which is not a free radical reaction. Chlorine dioxide, by itself, reacts only very slowly with polysaccharides (Sjöstrom, 1981). Whilst it is probable that the delignification medium was acidic enough to partially hydrolyse arabinofuranosidic linkages (Ford, 1986), it is unlikely that this would have affected the integrity of the xylans.

Sequential extraction of holocellulose

In order to solubilise acetylated xylans, the CDTA-extracted holocellulose was treated with DMSO. The material solubilised was rich in acidic xylans but accounted for only 1.5% of the dry weight of the CWM. The DMSO-insoluble residue was extracted sequentially with increasing strengths of KOH as shown in Table 1 to give the α-cellulose residue, which accounted for 31% of the dry weight of the CWM. A suspension of the α-cellulose residue was adjusted to pH 5 with glacial acetic acid and dialysed against distilled water for 3 days. The resulting suspension was centrifuged and both the supernatant and the residue (α′-cellulose), were freeze-dried. The supernatant, which accounted for 0.4% of the dry weight of the CWM, was rich in uronic acid, arabinose and galactose and probably arose from the pectic polysaccharides. The α′-cellulose was rich in glucose derived from cellulose and had negligible amounts of other polysaccharides associated with it. This compares well with α-cellulose from parchment layers of runner bean pods (Selvendran & King, 1989). However, this is in contrast to the significant amounts of pectic polysaccharides and glycoproteins associated with the α-cellulose residue of parenchymatous tissues of runner beans (Ryden & Selvendran, 1990) and olive pulp (Coimbra *et al.*, 1994) and reflects the abundance of secondary cell walls in olive seed hull tissue. The alkali-soluble extracts were neutralised and, on dialysis, gave precipitates which were removed by centrifugation. It can be seen that, in general, the precipitate fractions had relatively higher levels of xylose and lower levels of uronic acid when compared with the polymeric material from the supernatant fractions. The presence of small, but significant, amounts of pectic polysaccharides could be inferred from the low levels of Rha*p*, Ara*f*, Gal*p* and uronic acids.

Chlorite-soluble polymers

As little is known about the polysaccharides solubilised during the delignification procedure, the chlorite-soluble polymers were subjected to graded precipitation with alcohol (Table 2). Interestingly, none of the polymers precipitated until the alcohol concentration reached 75% (v/v); this may be due to the hydrophobic properties of the associated degraded lignin. The material that precipitated with 85, 90 and 95% alcohol had relatively low levels of polysaccharides and the bulk of the material that remained in the 95% alcohol supernatant was mostly degraded lignin. The major fraction, which precipitated with 75% ethanol, was further resolved by anion-exchange chromatography using DEAE–Trisacryl M to give four main fractions (Fig. 1a and Table 2). All these fractions exhibited strong UV absorption due to the presence of degraded lignin and this is reflected on the low recovery of carbohydrate in all of them, particularly the fraction eluted with buffer containing 0.5 M NaCl. It is likely that the polysaccharides and phenolics are covalently linked since the carbohydrate and UV absorbing material co-eluted. Two of these fractions, ChA and ChB were subjected to methylation analysis.

Table 2. Sugar composition of fractions from chlorite extracts after graded precipitation with ethanol and anion-exchange chromatography of fraction EtOH 75%

Fraction		Recovery (%)	Cell wall sugars (mol %)								Total sugars[a] (μg/mg)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Insol. residue[b]		5.6	2	1	7	41	2	2	39	6	82
EtOH 75%		24.1	2	1	11	49	1	5	1	30	473
Insol. residue[b]		2.0	8		18	31	3	4	7	30	151
Buffer	(ChA)	25.7	2		3	74	1	3	2	16	693
0.125 M NaCl	(ChB)	14.6	5	t	18	30	1	5	2	41	546
0.25 M NaCl	(ChC)	13.1	5		21	19	1	6	3	46	359
0.5 M NaCl	(ChD)	13.6	5		24	16	2	8	6	39	174
EtOH 85%		10.6	2	1	22	54	3	4	4	11	225
EtOH 90%		18.5	2	1	28	56	2	3	3	8	133
EtOH 95%		9.8	4	1	25	47	2	3	3	16	73
EtOH 95% sol.		29.6	7		7	29	3	2	2	43	38

t, trace; UA, uronic acid.
[a]Values are expressed as μg anhydrosugar per mg.
[b]Material which became insoluble.

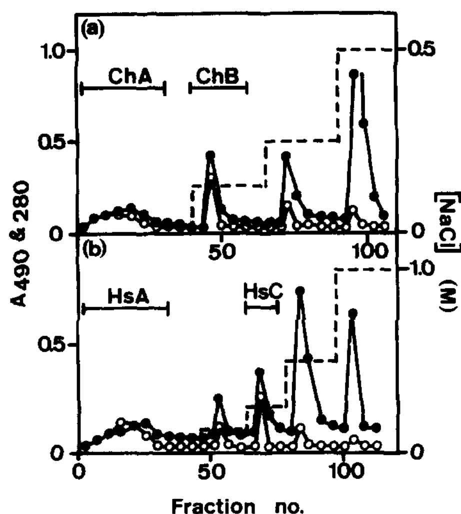

Fig. 1. Chromatography on DEAE–Trisacryl M of the extracts (a) chlorite, EtOH 75%; and (b) 0.5 M KOH, EtOH 30%, ○–○, total carbohydrate (A_{490}); ●–●, total phenolic (A_{280}); - - - -, NaCl gradient. A_{280} values are decreased 4-fold in (a) and 2-fold in (b); for experimental details see text.

Methylation analysis of fractions ChA and ChB, from the chlorite extract (Table 3, columns 2 and 3) showed that they contained xylans having a relatively low degree of polymerisation (DP) (~30–40 xylosyl residues) as calculated from the ratio of total xylose residues to terminally linked xylose residues. These xylans were shown to contain terminally linked glucuronic acid residues by reducing the methylated xylans with $LiAlD_4$, when 6,6′-dideutero terminally linked Glc residues were detected (data not shown). Whilst fraction ChA had very small amounts of non-hemicellulosic polysaccharides associated with it, fraction ChB contained significant amounts of pectic polysaccharides as inferred from the variously linked rhamnose residues, especially (1→2)- and (1→2,4)-linked Rha*p*, and variously linked arabinose residues and/or galactose residues. ChB also contained small quantities of wall glycoproteins, as inferred from the occurrence of 2- and 3-linked Ara*f* residues (Akiyama *et al.*, 1980).

0.5 M KOH: soluble fraction

Nearly all of this fraction dissolved readily in distilled water and was subsequently subjected to graded precipitation with alcohol (Table 4). At low alcohol concentration, the precipitated material was predominantly composed of xylose-rich polysaccharides. However, polymers that precipitated in alcohol at 80% or more, or which remained soluble in 90% alcohol, contained much non-carbohydrate material, probably of phenolic origin. The bulk (75%) of the polymeric material was precipitated with 50 and 60% ethanol and was rich in acidic xylans. The material which precipitated in EtOH at 30 and at 80% and above also contained significant quantities of pectic polysaccharides, suggesting the presence of associated xylans and pectic polysaccharides.

Methylation analysis of fraction Hs30 showed that it contained mainly acidic xylans and a small amount of pectic polysaccharides. The figure of 4.6 mol% for glucitol found in that fraction is not readily explicable but may be a contaminant. The xylans in fractions precipitated with higher concentrations of alcohol (Hs60 and Hs80) were more highly branched (Table 3).

The material precipitated with 30% ethanol was further resolved by anion-exchange chromatography on DEAE–Trisacryl M to give five main fractions (Fig. 1b). As before, the carbohydrate and UV-absorbing materials coeluted. The low recovery of carbohydrates in fractions 4 and 5 (HsD and HsE) is in accord with their high content of (degraded) phenolics. Fraction HsA, which was rich in acidic xylans, and fraction HsC, which had significant amounts of pectic polysaccharide, were subjected to methylation analysis. HsA consisted of a glucuronoxylan-type of polysaccharide whilst HsC was found to contain xylans and pectic polysaccharides. The fact that this fraction was retained on the anion-exchange column and eluted with 0.25 M NaCl suggests the occurrence of xylan–pectic polysaccharide complexes similar to those present in cell walls of olive pulp (Coimbra *et al.*, 1994). The overlap between UV absorption and the sugar profiles showed that degraded lignin components are probably associated with these complexes. Recently, both ester (Watanabe & Koshijima, 1988) and ether (Watanabe *et al.*, 1989) linkages between lignin and carbohydrate have been identified in the wood of *Pinus densiflora*.

0.5 and 1 M KOH: precipitate fractions

The precipitate from 0.5 M KOH extract was dissolved in 0.5 M NaOH and subjected to graded precipitation with ethanol. The polysaccharides that precipitated at different stages of alcohol concentration were dispersed in water and the suspension adjusted to pH 5 with acetic acid, dialysed and analysed for carbohydrate composition (Table 5). The major fraction (70%) was precipitated with 25% EtOH (Hp25) and was found to be an acidic xylan. This xylan had a molar ratio of xylose to uronic acid 9:1. A comparable xylan was obtained with 50% EtOH. The subsequent fractions had pectic polysaccharides and significant amounts of phenolics associated with the xylans.

The precipitate from 1 M KOH 1° was dispersed in 1 M NaOH and the insoluble residue was removed by centrifugation. The pH of the supernatant was lowered by gradual addition of HCl when a precipitate was obtained at pH 12 and another at pH 3. The sugar compositions of these fractions and of the material soluble at pH 3 are given in Table 5. All these fractions were rich in xylose and contained uronic acid, and it is probable that they are virtually pure acidic xylans.

Table 3. Glycosyl linkage composition of selected cell wall polysaccharides from chlorite and 0.5 M KOH (soluble) extracts (values expressed as relative mol%)

Linkage	Chlorite fraction		0.5 M KOH fraction					
	ChA	ChB	Hs30	HsA	HsC	Hs50	Hs60	Hs80
T-Rha*p*	0.2	1.4			1.7			
2-Rha*p*		1.4	0.3		1.1	0.1	0.1	0.2
3-Rha*p*	0.3	0.2	0.6	0.3	0.2	0.2	0.6	0.5
2,4-Rha*p*		0.7	0.5		1.0	0.1	0.3	0.6
3,4-Rha*p*						0.2		
T-Fuc*p*	0.3	0.5			0.3	0.1		0.3
T-Ara*p*					2.7			
T-Ara*f*	1.2	6.5	1.5	0.7	9.4	1.2	1.0	5.1
2-Ara*f*		1.2						
3-Ara*f*	0.3	2.7	0.3		2.5	0.1	0.1	0.9
5-Ara*f*	0.9	9.9	1.0		10.4	0.8	0.3	4.3
2,5-Ara*f*		0.5	0.2	0.2	0.4	0.2		0.5
3,5-Ara*f*	0.9	6.8	1.2	0.1	4.1	0.5	0.3	4.1
Arabinitol		0.5			0.1	0.1		
T-Xyl*p*	2.3	1.8	1.4	2.5	1.9	1.6	1.7	2.8
4-Xyl*p*	83.9	51.4	71.3	87.5	35.3	86.9	78.1	66.9
2,3-Xyl*p*		0.9						
2,4-Xyl*p*	3.6	3.9	6.8	8.0	2.0	5.7	10.1	8.5
3,4-Xyl*p*								1.1
Xylitol	0.2	0.2	0.4	0.1	0.7	0.1	0.1	0.3
T-Man*p*					0.1			
4-Man*p*	0.2				0.8			
3-Gal*p*	0.1	0.6	0.2		0.5	0.1		
6-Gal*p*	0.5	1.2	0.5		4.7	0.4		1.8
2,6-Gal*p*			0.3					
3,4-Gal*p*		0.3			0.8			
3,6-Gal*p*		1.4	0.2		5.5			1.3
Galactitol	0.4				0.2			
2-Gal*p*A				0.2				
4-Gal*p*A			0.8		7.0			
3,4-Gal*p*A					0.3			
T-Glc*p*					0.3			
2-Glc*p*				0.1	0.2			
4-Glc*p*	2.0	2.9	1.1		1.6	1.1	0.3	
4,6-Glc*p*	0.8	0.7						
Glucitol	1.9	2.4	4.6	0.1	1.1	0.5	1.2	0.8
T-Glc*p*A			6.4	0.2	2.0		5.8	
2-Glc*p*A			0.2		0.6			
3-Glc*p*A			0.2					
4-Glc*p*A					0.6			
DP[a]	39	32	56	39	21	60	53	29
BP[b]	4	7	8	8	5	6	11	12

[a]Degree of polymerisation of acidic xylans.
[b]Percentage of branch points of the acidic xylans.

Fractions Hp25, from 0.5 M KOH, K3, from 1 M KOH 1° and K20, from 1 M KOH 20° were subjected to methylation analysis (Table 6) and were found to be relatively pure glucuronoxylans. Fraction Hp25, containing the major acidic xylans from the seed hulls, was analysed by ^{13}C-NMR spectroscopy. From ^{13}C data it was inferred that the xylose residues are β-(1→4)-linked and the terminal glucuronic acid is α-linked and, from the relatively large signal for 4-*O*-Me at 59.1 ppm, it was inferred that most of the glucuronic acid carries a methyl substituent on C-4, as was reported for the glucuronoxylan isolated from olive pulp (Coimbra *et al.*, 1994). A comparison of the xylans from soluble fractions with those from the precipitate showed

Table 4. Sugar composition of fractions from 0.5 M KOH-soluble extracts after graded precipitation with ethanol and anion-exchange chromatography of fraction EtOH 30%

Fraction		Recovery (%)	Cell wall sugars (mol %)								Total sugars[a] (μg/mg)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Insol. residue[b]		3.9	t	1	3	80	t	1	t	15	654
EtOH 30%	(Hs30)	10.0	1	–	3	69	t	2	1	24	664
Insol. residue[b]		3.4	1	–	1	82	t	t	1	15	713
Buffer	(HsA)	42.1	2	–	2	80	t	t	1	15	755
0.125 M NaCl	(HsB)	8.8	2	–	6	68	1	4	3	16	556
0.25 M NaCl	(HsC)	22.3	3	–	12	12	1	7	2	64	572
0.5 M NaCl	(HsD)	14.4	4	t	23	10	1	11	13	49	414
1 M NaCl	(HsE)	4.6	4	–	25	26	3	13	7	23	199
EtOH 50%	(Hs50)	57.2	1	–	3	84	t	2	t	10	641
EtOH 60%	(Hs60)	18.1	1	t	2	82	t	1	t	13	792
EtOH 80%	(Hs80)	3.7	3	t	13	58	t	7	1	18	493
EtOH 90%	(Hs90)	2.1	3	t	14	47	1	t	9	25	314
EtOH 90% sol.		7.5	3	1	16	35	1	9	2	33	103

t, trace; UA, uronic acid.
[a]Values are expressed as μg anhydrosugar per mg.
[b]Material which became insoluble.

Table 5. Sugar composition of fractions from 0.5 M and 1 M KOH 1° extracts precipitated after neutralisation

Fraction	Recovery (%)	Cell wall sugars (mol %)								Total sugars[a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
0.5 M KOH ppt.										
NaOH insol. residue	2.7	1	–	1	75	t	1	2	20	611
NaOH soluble										
EtOH 25% (Hp25)	69.8	1	t	1	88	t	t	t	10	745
EtOH 50%	6.2	1	t	1	85	t	1	t	12	815
EtOH 70%	2.4	2	t	9	67	t	5	1	16	641
EtOH 80%	1.3	7	2	10	40	5	3	3	32	92
EtOH 80% sol.	2.4	1	t	3	76	t	1	1	17	272
1 M KOH 1°C ppt.										
NaOH insol. residue	5.7	1	–	1	86	t	t	6	5	327
NaOH soluble										
pH 12 ppt.	68.6	1	–	1	88	–	t	t	11	821
pH 3 ppt. (K3)	9.2	1	–	1	85	t	t	1	12	811
pH 3 sol.	4.4	1	t	2	82	t	1	1	13	805

t, trace; UA, uronic acid.
[a]Values are expressed as μg anhydrosugar per mg.

that the former have a higher DP and lower percentage of branch points.

In order to obtain information on the distribution of glucuronic acid on the xylan backbone, the fraction Hp25 was submitted to partial acid hydrolysis with TFA. The oligomers released were separated on a Bio-Gel P4 column. Selected fractions inferred to have DP of 7, 10, 14 and 17 were submitted to methylation analysis and the results are shown in Table 7. It can be seen that there is a very good correspondence between the DP inferred by methylation analysis with those obtained by gel-filtration chromatography. This observation thus lends support to the DP values we have reported for other acidic xylans in this study. The percentages of branch points of the oligosaccharides examined ranged from 5.3 to 7.3. This is consistent with a regular distribution of one glucuronic acid residue for every 15 xylose residues of the xylan backbone.

Fractions from α-cellulose

The small amount of pectic polysaccharide associated with the α-cellulose residue was released on neutralisation. Methylation analysis (Table 6) revealed that this

Table 6. Glycosyl linkage composition of selected cell wall polysaccharides from 0.5 M KOH (ppt) and subsequent extracts (values expressed as relative mol%)

Linkage		0.5 M KOH	1 M KOH		α-cellulose	
	Fraction:	Hp25	K3	K20	Sol.[a]	Res.[b]
T-Rha*p*					0.6	
2-Rha*p*					3.6	
3-Rha*p*					0.1	
2,4-Rha*p*					2.8	
T-Fuc*p*					0.3	0.2
T-Ara*p*					0.6	
T-Ara*f*		0.7	0.5	0.3	15.5	0.5
2-Ara*f*					0.4	
3-Ara*f*					4.8	0.2
5-Ara*f*					22.2	0.7
2,5-Ara*f*					0.3	
3,5-Ara*f*					19.4	5.3
Arabinitol			0.2		0.4	1.0
T-Xyl*p*		1.1	1.0	0.9	1.0	0.4
2-Xyl*p*					0.2	
4-Xyl*p*		91.4	91.0	92.4	1.4	4.2
2,4-Xyl*p*		6.8	5.3	5.3	1.0	
Xylitol			0.2		0.1	0.7
4-Man*p*						0.4
T-Gal*p*			0.1		7.8	
3-Gal*p*					0.9	
4-Gal*p*					2.2	
6-Gal*p*			0.1		3.0	
3,4-Gal*p*					3.1	
Galactitol					0.2	
4-Gal*p*A					4.7	
3,4-Gal*p*A					0.1	
T-Glc*p*					0.3	0.2
3-Glc*p*			1.2			
4-Glc*p*			0.5	0.5	1.1	54.6
2,4-Glc*p*					0.3	1.3
3,4-Glc*p*						2.8
4,6-Glc*p*					0.4	5.7
2,3,4-Glc*p*						1.9
2,4,6-Glc*p*						2.6
3,4,6-Glc*p*						6.0
Glucitol			0.9	0.6	0.6	11.3
T-Glc*p*A					0.6	
DP[c]		89	98	110		
BP[d]		7	5	5		

[a]Supernatant from α-cellulose (Table 1).
[b]Insoluble residue from α-cellulose (α′-cellulose).
[c]Degree of polymerisation of acidic xylans.
[d]Percentage of branch points of the acidic xylans.

exhibited a glycosidic linkage composition similar to the pectic polysaccharides isolated from olive pulp and also to the pectic polysaccharide that was released from the cellulosic residue of olive pulp on neutralisation (Coimbra *et al.*, 1994). The freeze-dried material from α′-cellulose residue did not disperse readily in DMSO prior to methylation and a proportion of it remained insoluble. Such insoluble material will have been difficult to methylate, and this probably accounts for the relatively high value for glucitol and variously linked glucose residues which would be consistent with under-methylation. Nevertheless, glucose was the predominant sugar as only small amounts of variously linked Ara*f* and Xyl*p* residues were detected.

Table 7. Glycosyl linkage composition of selected fractions from partial acid hydrolysis followed by gel filtration chromatography of Hp25 (values expressed as relative mol%)

Linkage	Fraction			
	7	10	14	17
T-Xyl*p*	13.9	9.7	7.1	5.7
4-Xyl*p*	78.9	80.7	87.0	88.1
2,4-Xyl*p*	5.8	7.3	5.3	5.6
Xylitol	1.4	2.3	0.6	0.6
DP[a]	7.2	10.3	14.1	17.5
PB[b]	5.8	7.3	5.3	5.6

[a]Degree of polymerisation.
[b]Percentage of branch points.

DISCUSSION

The extraction of polysaccharides from the cell walls of the lignified tissues of olive seed hulls was only possible after exhaustive delignification treatment. The major non-cellulosic polysaccharides of the seed hulls in decreasing amounts were: (a) slightly branched glucuronoxylans with DP ranging from 90 to 100; (b) highly branched glucuronoxylans with DP ranging from 30 to 60; (c) xylan–pectic polysaccharide complexes associated with phenolic material (degraded lignin); (d) small but significant amounts of pectic polysaccharides which were virtually free of xylans.

The occurrence of two major types of glucuronoxylans is in accord with the results of parchment fibres of runner bean pods and sunflower seed hulls (Bazus *et al.*, 1992). Unfortunately, the lack of information prevents generalisation of these to other lignified tissues of dicotyledonous plants.

The occurrence of 4-*O*-methylglucuronic acid in olive seed hull is in accord with the presence of this sugar residue in other types of lignified seed hulls such as sunflower (Bazus *et al.*, 1992; Düsterhöft *et al.*, 1991), palm kernel (Düsterhöft *et al.*, 1992) and cotton (Matsuo *et al.*, 1991). Early results on xylans present in the husk portion of seeds suggested that this type of tissue only contains glucuronoxylans without the 4-*O*-methylglucuron derivative (Swamy & Salimath, 1990). It is possible that in the same lignified seed tissues, glucuronic acid residues, 4-*O*-methyl substituted or not, can co-exist as was shown to occur in parchment fibres of mature runner bean pods (Selvendran & King, 1989).

By delignification and treatment with KOH solutions, it was possible to extract almost all of the non-cellulosic polymers. This result contrasts with the relatively high amounts of pectic material that remain in the residue of cellulosic material from the parenchymatous tissues of olive pulp (Coimbra *et al.*, 1994). The DP of the major glucuronoxylans of olive seed hulls are generally less than those of the parchment fibres of mature runner bean pods (Selvendran & King, 1989), which have a DP of approximately 190. This may be related to the functional differences between the tissues.

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REFERENCES

Akiyama, Y., Mori, M. & Kato, K. (1980). *Agric. Biol. Chem.*, **44**, 2487–2489.
Aspinall, G.O. (1959). *Adv. Carbohydr. Chem.*, **14**, 429–468.
Aspinall, G.O. & Carpenter, R.C. (1984). *Carbohydr. Polym.*, **4**, 271–282.
Aspinall, G.O., Cottrell, J.A., Molloy, J.A. & Uddin, M. (1970). *Can. J. Chem.*, **48**, 1290–1295.
Aspinall, G.O., Hunt, K. & Morrison, I.M. (1967). *J. Chem. Soc.* **C**, 1080–1086.
Bazus, A., Rigal, L., Fontaine, T., Fournet, B., Gosselin, M. & Debeire, P. (1992). *Biosci. Biotech. Biochem.*, **56**, 508–509.
Blakeney, A.B., Harris, P.J., Henry, R.J. & Stone, B.A. (1983). *Carbohydr. Res.*, **113**, 291–299.
Blumenkrantz, N. & Asboe-Hansen, G. (1973). *Analyt. Biochem.*, **96**, 484–489.
Brillouet, J.M., Joselau, J.P., Utile, J.P. & Lelievre, D. (1982). *J. Agric. Food Chem.*, **30**, 488–495.
Coimbra, M.A., Waldron, K.W. & Selvendran, R.R. (1994). *Carbohydr. Res.*, **252**, 245–262.
DuPont, M.S. & Selvendran, R.R. (1987). *Carbohydr. Res.*, **163**, 99–113.
Düsterhöft, E.M., Voragen, A.G.J. & Engles, F.M. (1991). *J. Sci. Food Agric.*, **55**, 411–422.
Düsterhöft, E.M., Posthmus, M.A. & Voragen, A.G.J. (1992). *J. Sci. Food Agric.*, **59**, 151–160.
Excoffier, G., Nardin, R. & Vignon, M.R. (1986). *Carbohydr. Res.*, **149**, 319–328.
Ford, C.W. (1986). *Carbohydr. Res.*, **147**, 101–117.
Harris, P.J., Henry, R.J., Blakeney, A.B. & Stone, B.A. (1984). *Carbohydr. Res.*, **127**, 59–73.
Isogai, A., Ishizu, A. & Nakoan, J. (1985). *Carbohydr. Res.*, **138**, 99–108.
Jermyn, M.A. & Isherwood, F.A. (1964). *Biochem. J.*, **64**, 123–132.
Kovak, P. & Hirsch, J. (1982). *Carbohydr. Res.*, **100**, 177–193.
Lindberg, B. & Lönngren, J. (1978). *Meth. Enzymol.*, **50**, 3–33.
Matsuo, N., Yoshida, S., Kusakabe, I. & Murakami, K. (1991). *Agric. Biol. Chem.*, **55**, 2905–2907.
Ring, S.G. & Selvendran, R.R. (1980). *Phytochemistry*, **19**, 1723–1730.
Ryden, S. & Selvendran, R.R. (1990). *Biochem. J.*, **269**, 393–402.
Selvendran, R.R. (1984). *Am. J. Clin. Nutr.*, **39**, 320–337.
Selvendran, R.R. (1985). *J. Cell Sci., Suppl.* **2**, 51–88.
Selvendran, R.R. & King, S.E. (1989). *Carbohydr. Res.*, **195**, 87–99.

Selvendran, R.R., March, J.F. & Ring, S.G. (1979). *Analyt. Biochem.*, **96**, 292–292.
Selvendran, R.R., Verne, A.V.F.V. & Faulks, R.M. (1989). *Mod. Methods Plant Anal.*, **10**, 234–259.
Siegel, S.M. (1953). *Physiol. Plantarum*, **6**, 134–139.
Sjöstrom, E. (1981). *Wood Chemistry: Fundamentals and Applications*. Academic Press, London.
Swamy, N.R. & Salimath, P.V. (1990). *Carbohydr. Res.*, **197**, 327–337.
Sweet, D.P., Shapiro, R.H. & Albersheim, P. (1975). *Carbohydr. Res.*, **40**, 217–225.
Watanabe, T. & Koshijima, T. (1988). *Agric. Biol. Chem.*, **52**, 2953–2955.
Watanabe, T., Ohnishi, J., Yamasaki, Y., Kaizu, S. & Koshijima, T. (1989). *Agric. Biol. Chem.*, **53**, 2233–2252.
Winton, A.L. & Winton, K.B. (1932). *Structure and Composition of Foods*, Vol. 1. John Wiley, Chichester.