Carbohydrate Research, 252 (1994) 245–262
Elsevier Science B.V., Amsterdam

Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L.)

Manuel A. Coimbra [a], Keith W. Waldron [b,*] and Robert R. Selvendran [b,*]

[a] *Chemistry Department, University of Aveiro, 3800 Aveiro (Portugal)*
[b] *Department of Food Molecular Biochemistry, AFRC Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA (United Kingdom)*

(Received March 17th, 1993; accepted June 28th, 1993)

ABSTRACT

Cell wall material (CWM) was prepared from olive pulp, which is rich in oil. Polymers were solubilised from the CWM by sequential extraction with *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, sodium salt; Na_2CO_3; 1 M KOH; 4 M KOH; and 4 M KOH+borate to leave the cellulose-rich residue (CR). A suspension of this residue, on neutralisation, released a pectic material, to give residue CR1. The residue CR1 contained cross-linked pectic polysaccharides and some cell wall glycoproteins which were solubilised with chlorite–acetic acid. 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. Closely related pectic polysaccharides rich in arabinose were the major components of the cell walls and they differed in their ease of extraction from the wall matrix. Significant amounts of acidic xylans were isolated from the precipitates obtained on neutralisation of the 1 M KOH extracts, and a large proportion of these would have been derived from the lignified sclereids; the xyloglucans from the supernatant fractions had xylans associated with them. However, significant amounts of xyloglucans associated with glucomannans, but virtually free of xylans, were isolated from the 'neutral fractions' of the supernatant solutions from the 4 M KOH and 4 M KOH+borate extracts. These 'neutral fractions' also contained significant amounts of hydroxyproline-rich cell wall glycoproteins. The anomeric configurations of the sugars in the glucuronoxylans were determined by ^{13}C NMR spectroscopy. The structural features of the wall polymers are discussed in relation to the structure of the cell wall of the fruit pulp.

INTRODUCTION

The pulp of the olive fruit is composed of different tissue types[1,2] and there is a paucity of information on the cell walls of olives. The olive tree is very common in countries with a Mediterranean climate. The fruit is the raw material for a variety of products including olive oil and table olives. The preparation of table olives is a complex industrial process which involves fermentation and/or alkali treatment. The information on olive cell walls is crucial for a better understanding of the

* Corresponding authors.

SSDI 0008-6215(93)E0247-X

biochemical changes that occur in olives during growth, maturation, and processing. Previous work on olive cell walls has been restricted to the isolation and partial characterisation of a 4-*O*-methylglucuronoxylan from fresh olives[3] and a xyloglucan from processed olives[4]. This paper reports the composition and structural features of the cell wall polymers of olive pulp extracted and fractionated under conditions which cause minimum degradation of the polymers[5,6].

EXPERIMENTAL

Plant material.—Olive (*Olea europaea* L. cv Douro) fruits with an average length of 2.0 cm and a diameter of 1.2 cm were harvested at the mature green stage.

Preparation of the cell wall material (CWM).—Olive pulp CWM was prepared by adaptation of the method described in ref 6. Olives (800 g) were stoned and the pulp was frozen in liquid N_2. The tissue pieces of the pulp (620 g) were homogenised with aq 1.5% sodium dodecyl sulphate (SDS) (900 mL) containing 5 mM $Na_2S_2O_5$. The homogenate was filtered through a nylon cloth. The residue was washed with aq 0.5% SDS (2 × 700 mL) containing 3 mM $Na_2S_2O_5$, suspended in 500 mL of the same solution and, in order to break the clusters of cells, ball-milled (Pascall 1 L pot) for 10 h at 60 rpm and 1°C, and then centrifuged (20 000 rpm). The residue was washed with distilled water (twice, 1 L) and centrifuged. The residue was then stirred overnight in phenol–HOAc–H_2O (PAW, 2:1:1, w/v/v, 3 L) at 20°C, centrifuged, and stirred again for 2 h in PAW (1 L). The residue was collected, washed with distilled water (twice, 1 L), dispersed in water, dialysed at 1°C, and stored as a frozen suspension (1 L) in water at −20°C. A freeze-dried aliquot indicated the yield to be 23 g (3.7 g/100 g of fresh pulp tissue). At each stage of the wall preparation, aliquots (10%) of extraction media were dialysed. The dialyses of the SDS fractions were performed at 20°C to prevent SDS precipitation. Because a considerable amount of oil remained in the dialysed materials, SDS and PAW extracts were submitted to 2:1 $CHCl_3$–MeOH extractions and then freeze-dried.

Sequential extraction of CWM.—CWM was extracted by adaptation of the method proposed for the sequential extraction of CWM from parenchymatous tissues[6]. CWM (18.5 g) was sequentially extracted with (*i*) 50 mM *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetate (CDTA, Na salt; 1.5 L), pH 6.5 at 20°C for 6 h (CDTA-1); (*ii*) 50 mM CDTA (1.5 L), pH 6.5 at 20°C for 2 h (CDTA-2); (*iii*) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (1.5 L) at 1°C for 16 h (Na_2CO_3-1); (*iv*) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (1.5 L) at 20°C for 3 h (Na_2CO_3-2); (*v*) 1 M KOH + 20 mM $NaBH_4$ (1 L) at 1°C for 2 h; (*vi*) 1 M KOH + 20 mM $NaBH_4$ (1 L) at 20°C for 2 h; (*vii*) 4 M KOH + 20 mM $NaBH_4$ (1 L) at 20°C for 2 h; and (*viii*) 4 M KOH + 3.5% H_3BO_3 + 20 mM $NaBH_4$ (1 L) at 20°C for 2 h. The alkali extractions were carried out with O_2-free solutions under Ar. After each extraction, solubilised polymers were separated from the insoluble

residue by centrifugation (CDTA and Na_2CO_3 extracts) or by filtration through a G1 glass sinter (KOH extracts). All extracts were filtered through GF/C and dialysed exhaustively; Na_2CO_3 and KOH extracts were acidified to pH 5 with HOAc prior to dialysis. Precipitates formed during dialysis of alkali extracts were collected separately. The cellulose-rich residue (CR) remaining after the final alkali extraction (4 M KOH + borate) was suspended in water (300 mL), and the solution was acidified to pH 5 and dialysed. The supernatant solution of the CR dialysis was collected separately by centrifugation from the CR1 residue. All extracts collected after dialysis were concentrated and stored as frozen suspensions at −20°C. A small amount of each was freeze-dried.

Delignification of CR1.—CR1 (2 g) was stirred with $NaClO_2$ (0.3%, w/v) and HOAc (0.12%, v/v) in distilled water (200 mL) at 70°C for 15 min[7], during which the flask was flushed continuously with Ar. The residue was recovered on a G1 glass sinter and the treatment was repeated. The residue was washed thoroughly with distilled water and given one last extraction with 1 M KOH + 20 mM $NaBH_4$ (200 mL) at 20°C for 2 h. The combined chlorite supernatant solution and washing water was flushed with Ar until the yellow colour faded away. This and the alkali extract were filtered and neutralised as for the previous CWM extracts. The final residue (CR2) was suspended in water, neutralised, dialysed, and freeze-dried.

Graded precipitation with ethanol.—The polymers were dissolved in water and precipitated by the addition of EtOH, the concentration of which was increased in steps between 10 and 20%[8]. Each mixture was placed at 4°C for 1 h and the precipitate was collected by centrifugation. In order to remove the EtOH completely, each precipitate was dissolved in water and rotary-evaporated. The fractions were stored as frozen suspensions at −20°C. A small amount of each fraction was freeze-dried for sugar analysis.

Ion-exchange chromatography.—Some of the EtOH-precipitated fractions were suspended in water and stirred at 20°C. The insoluble residues were removed by centrifugation. To the supernatant solution was added K-Pi buffer (pH 6.5) to a final concentration of 50 mM and 1 mg/mL of material. Each solution was passed through a column of DEAE-Trisacryl M[5] (1 mL of ion-exchanger/7.5 μmol of uronic acid present), phosphate form, at 10 mL/h. The fractions were eluted sequentially with the same volume of buffer and buffer containing 0.125, 0.25, 0.5 and 1 M NaCl. Fractions (3 mL) were collected and aliquots (20 μL) were assayed for carbohydrate by the phenol–H_2SO_4 method[9]. The appropriate fractions were combined, dialysed, concentrated, and stored at −20°C. A small amount of each fraction was freeze-dried.

Copper precipitation of xylans.—Copper precipitation was carried out on the precipitate from the 1 M KOH 1°C fraction by adaptation of the method described[10]. The material (330 mg) was dispersed in 1 M NaOH and the insoluble residue (23 mg) was removed by centrifugation. To the solution (65 mL) was added copper acetate (7%, w/v; 10 mL), and the mixture was stirred for 3 h at room temperature, when a blue precipitate was obtained. This precipitate was isolated

by centrifugation and washed with distilled water. The precipitate was dissolved in a solution of 5% HCl in EtOH (50 mL) to give a pale-yellow solution. This solution, on addition of water (70 mL), gave a white precipitate which was collected by centrifugation, neutralised, and freeze-dried (145 mg).

pH Fractionation.—The foregoing purified xylan (80 mg) was dispersed in 5 mL of 1 M NaOH containing 20 mM $NaBH_4$, and the insoluble residue was removed by centrifugation (22 mg). The pH of the supernatant solution was lowered by gradual addition of 1 M HCl until a precipitate (68 mg) was obtained at pH 10 which was removed by centrifugation. No additional precipitate was obtained on further treatment of the supernatant solution with 1 M HCl to pH 6. The supernatant solution and precipitates were dialysed and freeze-dried.

Carbohydrate and linkage analysis.—Neutral sugars were released by Saeman hydrolysis and analysed[11] as their alditol acetates by GLC. Uronic acids were determined colorimetrically by a modification[11,12] of the method of Blumenkrantz and Asboe-Hansen[13]. Polysaccharides were methylated by a modification of the Hakomori method[14] as described[10,15], and then converted into partially methylated alditol acetates, which were separated by GLC on an OV-225 column and characterised by GLC–MS, using the molar response factors of Sweet et al.[16]. Prior to methylation, the CDTA-soluble fractions were de-esterified as described by Aspinall et al.[17]: the samples were stirred in 0.1 M NaOH + 20 mM $NaBH_4$ for 3 h at 1°C, neutralised, dialysed, and freeze-dried. Selected methylated fractions (2–5 mg) were carboxyl-reduced by a modification of the method described by Lindberg and Lönngren[18], as follows. The methylated fraction was heated in a sealed tube with a mixture of $LiAlD_4$ (20 mg) and THF (1 mL) at 65°C for 4 h. The excess of reagent was then destroyed with EtOH (2–3 drops) and water (2–3 drops), and the pH of the mixture was adjusted to neutrality with 2 M H_3PO_4. The reduced polymer was isolated by filtration, washed thoroughly with 2:1 $CHCl_3$–MeOH, evaporated to dryness, hydrolysed, reduced, and acetylated as described[10].

Hydroxyproline (Hyp) and protein analysis.—Total protein content was determined by Automatic Nitrogen Apparatus, Carlo Erba Instruments. Hyp was released by 6 M HCl at 110°C for 24 h and quantified by the method of Kivirikko and Liesmaa as described by Fry[19].

^{13}C NMR spectroscopy.—Spectra (100.4 MHz) were recorded under conditions of broad-band proton decoupling with a JEOL GX400 spectrometer in a solution of Me_2SO-d_6 at 27°C[20]. Chemical shifts were compared with other ^{13}C-spectral data for glucuronoxylans[21,22].

RESULTS AND DISCUSSION

Isolation of cell wall material (CWM).—Olive pulp was extracted with aq 1.5% sodium dodecyl sulphate (SDS) to remove the bulk of the intracellular compounds, including most of the olive oil. The residue was ball-milled in aq 0.5% SDS for 10 h at 1°C in order to disrupt the tissue structure more completely and thus render

TABLE I

Sugar composition of purified cell wall material of fresh olive pulp and of material solubilised during purification (the values within brackets given the percent carbohydrate content of the fractions)

Fraction	Yield [a] (g/kg)	Cell wall sugars (mol%)								Total sugar [b] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	
1.5% SDS	6.1 (2.9)	10	2	30	9	5	15	20	9	119
0.5% SDS										
soluble	0.8 (2.1)	1	t [c]	25	17	2	10	7	38	663
precipitate [d]	1.1 (1.4)	2	t	29	9	2	5	25	28	327
PAW										
soluble	0.4 (0.5)	1	t	19	3	1	3	6	67	338
precipitate [d]	1.0 (2.3)	2		27	15	2	3	30	21	581
Purified CWM	37.1 (90.8)	1	t	27	12	2	3	29	26	619

[a] Yield is expressed in g/kg fresh weight of olive pulp. The values in parentheses give the carbohydrate content (%) of the fractions. [b] Values are expressed as μg of "anhydrosugar"/mg dry polymer. [c] t, Trace. [d] Material which precipitated on dialysis.

the contents accessible to solvents: a significant amount of residual oil was removed at this stage. Phenol–HOAc–H_2O (PAW) treatment of the residue removed the remaining intracellular protein, adsorbed SDS, and the last traces of oil. Me_2SO treatment was omitted because the material was free of starch, as shown by negative reaction with I_2–KI. The purified CWM, which was pale green, was stored as a frozen suspension. The yield of CWM was 3.7% on a fresh weight basis. Table I shows the sugar composition of the various polymers solubilised and of the purified CWM. The material solubilised by 1.5% SDS was mainly of intracellular origin as shown by its very low content of carbohydrate. However, the material that was solubilised by 0.5% SDS during ball-milling contained significant amounts of uronic acid, arabinose, xylose, and galactose. Most of these sugars are likely to have arisen from intercellular polymers, which would include pectic polysaccharides. PAW treatment also solubilised small amounts of cell wall polymers. The total amount of cell wall polymers solubilised by the above treatments, based on carbohydrate recovery, was 6.3% of the dry weight of the purified CWM. The carbohydrate content of the purified CWM was only 62%, which is relatively small when compared with the CWM of onions[5], potatoes[23], runner beans[24], and carrots[25]. This, and the observation that the protein content was 8.6%, suggests that there may be lignin-like material in addition to polysccharides. The high content of glucose, arabinose, and uronic acid in the CWM indicated the presence of cellulose and large amounts of pectic polysaccharides rich in arabinose, and the presence of significant amounts of xylose indicated the presence of xylans in addition to xyloglucans.

Fractionation of CWM.—The CWM was sequentially extracted with aqueous inorganic solvents to leave a cellulose-rich residue CR (see Experimental). On neutralisation, a pectic polysaccharide was released into the supernatant solution,

TABLE II

Sugar composition of fractions of cell wall material of fresh olive pulp obtained by sequential extractions with aqueous solvents

Fraction	Recovery (%)	Cell wall sugars (mol%)								Total sugar [a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	
CDTA-1	10.8	1	t [b]	11	t	t	1	1	86	753
CDTA-2	6.6	1	t	21	2	1	2	2	71	318
Na_2CO_3-1	14.4	2		40	1	t	3	1	53	767
Na_2CO_3-2	4.8	1		52	1	t	2	1	44	882
1 M KOH 1°C										
supt. [c]	3.4	1	t	27	31	1	5	13	22	789
ppt. [d]	5.2	1	t	8	67	2	2	7	13	547
1 M KOH 20°C										
supt.	4.2	1	t	48	8	9	7	13	14	471
ppt.	6.3	1	t	10	53	2	2	14	18	368
4 M KOH										
supt.	1.6	t	1	15	25	11	10	30	8	936
ppt.	3.3	1	t	41	28	2	2	8	18	134
4 M KOH + borate										
supt.	1.9	1	t	64	5	5	5	8	13	588
ppt.	1.7	1	t	52	12	1	2	9	23	188
CR										
supt.	4.3	2		53	1		3	1	40	936
CR1	31.5	1		18	8	1	2	56	14	722
Chlorite–HOAc-sol.	2.6	3		56	1	t	4	1	35	732
1 M KOH	4.5	2	t	48	16	2	5	9	18	505
Final residue (CR2)	22.8	1	t	6	7	2	1	78	5	725

[a] Values are expressed as μg of "anhydrosugar"/mg dry polymer. [b] t, Trace. [c] Material isolated from the supernatant solution. [d] Material which precipitated on dialysis.

leaving a residue (CR1). The extraction procedures were designed to minimise degradation of pectic polysaccharides during the initial stages of extraction, and to solubilise the polysaccharides in as close to their native form as possible. As the CR1 residue contained significant amounts of pectic polysaccharides and possibly glycoproteins, it was given a short treatment with chlorite–HOAc, washed thoroughly, and then extracted with 1 M KOH. The amounts of polymers solubilised and their sugar composition are shown in Table II. The CDTA extractions solubilised those pectic substances held in the walls by Ca^{2+}, the bulk of which probably originated from the middle lamella. The second CDTA extract (CDTA-2) had a low content (32%) of carbohydrate and was shown by ^{13}C NMR data to be contaminated with CDTA. Recently, attention was drawn to the fact that CDTA was not completely removed from these extracts even after extensive dialysis[26]. In contrast, when citrus pectin was mixed with the sodium salt of CDTA and the solution was dialysed, > 95% of the CDTA was removed by dialysis (K.W. Waldron and R.R. Selvendran, unpublished results). The incomplete removal of CDTA in this study is probably due to the presence of calcium ions in the extract,

which complex with the CDTA and thus prevent its complete removal by dialysis. The CDTA-insoluble residue was washed with water to remove any CDTA held in the cell wall matrix prior to extraction with Na_2CO_3, which resulted in the high carbohydrate content of the Na_2CO_3 extracts. Successive Na_2CO_3 extractions (1 and 20°C) solubilised pectic material presumably by hydrolysis of weak ester cross-links. The Na_2CO_3-1 extraction was performed at 1°C in order to minimise β-elimination of pectic polysaccharides in all subsequent alkali extractions. The Na_2CO_3 extracts are rich in pectic polysaccharides as shown by the high levels of uronic (galacturonic) acid and arabinose, and the occurrence of rhamnose as the main deoxy sugar.

The alkali extracts, on neutralisation and dialysis, gave precipitates which were removed by centrifugation, and the polymers in the supernatant fractions and precipitates were analysed separately. The precipitates from the 1 M KOH (1 and 20°C) extracts contained predominantly acidic xylans, and the polysaccharides in the supernatant solution were mostly xyloglucans and pectic polysaccharides; these inferences are based on the results of methylation analysis described later. The precipitate from the 1 M KOH (20°C) had a relatively low content (37%) of carbohydrate and contained a significant amount of UV-absorbing material. The material solubilised by 4 M KOH (supernatant fraction) was rich in carbohydrate and had much xyloglucan(s), whereas the precipitate was relatively poor in carbohydrate and contained a significant amount of UV-absorbing material. The material solubilised by 4 M KOH + borate (supernatant fraction) was very rich in arabinose and had a relatively low amount of uronic acid, and a large proportion of the arabinose was shown to be derived from cell wall glycoproteins by methylation analysis (described later). Interestingly, 4 M KOH + borate was also shown to solubilise cell wall glycoproteins from potatoes[23] and runner beans[24]. The precipitate from the 4 M KOH + borate extract was dark brown, had a low content of carbohydrate, and also contained a significant amount of UV-absorbing material.

The residue remaining after 4 M KOH + borate extraction was neutralised and dialysed. The polymeric material isolated from the supernatant solution, which accounted for 4.3% of the CWM, was found to be rich in pectic polysaccharides, as shown by the high levels of arabinose and uronic acid. It is conceivable that, in the strong alkali, the ionisation of the $-CH_2OH$ groups on cellulose prevents the diffusion of negatively charged pectic polysaccharides enmeshed within the swollen cellulose matrix. On neutralisation of the cellulose-rich suspension, the loss of negative charges on cellulose facilitates diffusion of the entangled pectic polysaccharides.

The residue from CR (CR1) accounted for 31.5% of the dry weight of the cell wall and contained hydroxyproline (Hyp). CR1 was therefore treated with chlorite–HOAc to solubilise cell wall glycoproteins and associated pectic polysaccharides. The chlorite-insoluble residue was washed thoroughly and extracted with 1 M KOH to leave the final residue (CR2). The low recovery of carbohydrate in the final residue (73%) may be due to the presence of lignin-like material.

TABLE III

Sugar composition of fractions from CDTA-soluble and Na_2CO_3-soluble extracts after ethanol precipitation followed by anion-exchange chromatography

Fraction		Recovery (%)	Cell wall sugars (mol%)								Total sugar [a] (μg/mg)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	
CDTA-1											
EtOH 60%		35.6	2	t [b]	29	t		1	1	67	472
Buffer	(CIA)	64.0	2	1	26	t	1	1	2	67	554
0.125 M NaCl		12.0	3	t	34	1	t	2	2	58	489
0.25 M NaCl		12.0	2	t	31	1	1	2	2	61	531
0.5 M NaCl		6.7	2	1	17	1	t	2	6	72	428
EtOH 75%		20.0	3	t	25	t		2	1	69	615
EtOH 85%		5.3	2	t	29	1		1	2	65	586
EtOH 85% supt. [c]		37.7	32		48	7				13	12
Na_2CO_3 1°C											
Insol. res.		3.0	2	t	41	1	t	3	3	50	495
EtOH 70%		89.3	2	t	36	t	t	2	1	59	914
Buffer		3.1	2	t	50	8	1	3	15	21	164
0.125 M NaCl		4.9	3		71	1	t	4	2	19	581
0.25 M NaCl	(NC1)	38.2	3		68	t	t	3	1	25	804
	(NC2)	47.4	2		12	1	1	2	1	81	590
0.5 M NaCl		5.2	1	t	17	1	1	2	2	76	605

[a] Values are expressed as μg of "anhydrosugar"/mg dry polymer. [b] t, Trace. [c] Supernatant solution.

Fractionation of the soluble extracts.—The polymers present in CDTA-1, Na_2CO_3-1, and 1 M KOH 1°C (soluble fraction) were subjected to graded precipitation with ethanol, and the major fractions were further resolved by anion-exchange chromatography (Tables III and IV). We found that this resulted in much better recovery of the material applied to the anion column compared with unfractionated material. Ethanol precipitation of the CDTA-1 extract gave 4 fractions (Table III). The bulk of the pectic polysaccharides were precipitated with 60% ethanol. The material that remained soluble in 85% ethanol was shown to have very little carbohydrate, and the non-carbohydrate material was shown by ^{13}C NMR to be mainly CDTA. The 60% fraction was further resolved by anion-exchange chromatography to give 4 fractions (Fig. 1a). The recovery of the polymers from the column was > 95%. The major pectic polysaccharides were rich in arabinose and uronic acid. The ratio of neutral sugars to uronic acid decreased only slightly with increase in NaCl concentration, indicating that the degree of esterification of the pectic polysaccharides was mainly responsible for the separation.

The Na_2CO_3-1 extract was likewise subjected to graded precipitation with ethanol, and the material precipitated with 70% ethanol (major fraction) was fractionated by anion-exchange chromatography to give 4 fractions (Table III). The

TABLE IV

Sugar composition of fractions from 1 M KOH-soluble extract

Fraction	Recovery (%)	Cell wall sugars (mol%)								Total sugar [a] (μg/mg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	
1 M KOH 1°C supt. [b]										
Insol. res. [c]	41.5	1	1	54	16	1	4	6	17	158
EtOH 70%	2.0	1	1	17	45	1	6	20	9	861
EtOH 70% supt.	25.0	1	1	15	46	2	6	21	8	796
Buffer (K1A)	62.9	1	1	10	44	2	7	28	7	915
0.125 M NaCl (K1B)	17.1	1		15	64	t [d]	2	4	14	796
0.25 M NaCl	2.9	2		39	20	2	4	10	23	774
1 M KOH 1°C ppt.										
NaOH insol. res. (K1R)	6.7	1	t	11	11	t	1	74	2	212
NaOH soluble										
$Cu(OAc)_2$ ppt.	43.9	1		1	91		t	1	6	923
pH 10 ppt. (K10)	85.0	1		1	87	t	t	2	9	869
pH 10 sol. (K6)	10.0	2	t	3	78	1	1	4	11	821

[a] Values are expressed as μg of "anhydrosugar"/mg dry polymer. [b] Supernatant solution. [c] Insoluble material obtained on thawing the 1°C extract. [d] t, Trace.

recovery of the polysaccharides from the column was 99%. The third fraction (eluted with 0.25 M NaCl) had a shoulder and the faster running component of this fraction exhibited UV absorption (Fig. 1b). This fraction (NC1) was much richer in arabinose but relatively poorer in uronic acid when compared with NC2 (Table III).

The 1 M KOH 1°C supernatant fraction yielded a considerable amount of precipitate upon thawing. The soluble material was treated with ethanol and gave only a very small amount of material at 70% ethanol concentration. As no additional material precipitated on further addition of ethanol, the supernatant solution was concentrated and fractionated by anion-exchange chromatography (Fig. 1c). The material eluted with buffer (K1A) was very rich in xylose and glucose, and contained a relatively small amount of uronic acid. This fraction can be inferred to contain xyloglucans and xylans. The material eluted with 0.125 M NaCl (K1B) was rich in xylose, arabinose, and uronic acid, and is probably a xylan–pectic polysaccharide complex (Table IV).

The 1 M KOH 1°C precipitate was dispersed in NaOH and the insoluble residue was removed by centrifugation (K1R). The soluble fraction was treated with copper acetate and the blue precipitate formed was freed of copper by treatment with ethanolic HCl. The resulting white precipitate was dissolved in 1 M NaOH and the pH of the solution was lowered by gradual addition of HCl; a precipitate was obtained at pH 10 (K10). The pH of the supernatant solution was lowered to 6 and, as no other precipitate formed, the solution was dialysed and

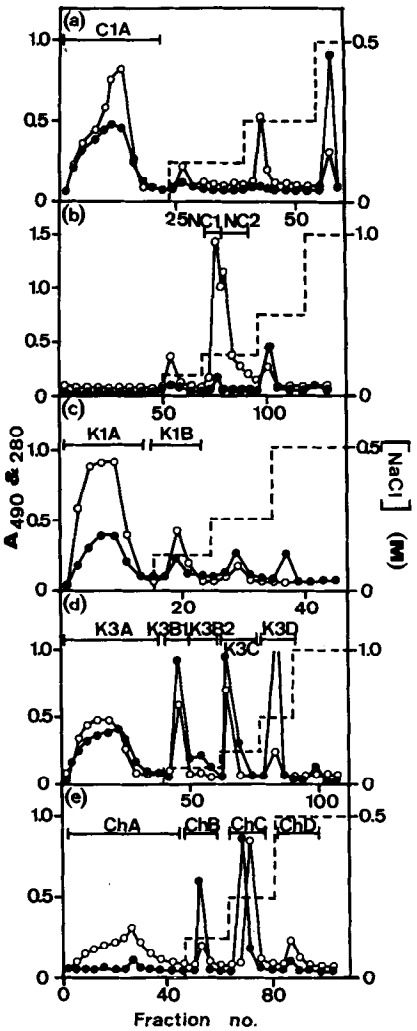

Fig. 1. Chromatography on DEAE-Trisacryl M of the extracts (a) CDTA-1, EtOH 60%; (b) Na_2CO_3-1, EtOH 70%; (c) 1 M KOH 1°C, EtOH soluble; (d) 4 M KOH; and (e) chlorite–HOAc. For experimental details, see the text. –○–, Total carbohydrate (A_{490}); –●–, total phenolic (A_{280}); - - - - - -, NaCl gradient.

freeze-dried to give K6. The sugar compositions of these fractions are given in Table IV. These fractions were rich in xylose, showing that they contain much xylan, and it is probable that fraction K10 is a pure acidic-xylan. This was verified by methylation analysis (Table V).

The 4 M KOH supernatant fraction was dispersed in water and the insoluble residue (10%) was removed by centrifugation. The supernatant solution was subjected to anion-exchange chromatography when 4 main fractions (K3A to K3D) were obtained (Fig. 1d). All these fractions exhibited UV absorption. With the

TABLE V

Glycosyl linkage composition of selected cell wall polysaccharides from CDTA, Na_2CO_3, and 1 M KOH extracts (values expressed as relative mol%)

Linkage	Fraction:	CDTA	Na_2CO_3		1 M KOH				
		C1A	NC1	NC2	K1A	K1B	K1R	K10	K6
T-Rha*p*		0.3		1.6					
2-Rha*p*		2.3	3.2	1.7		0.4			0.2
3-Rha*p*		0.2	0.1	0.8	0.2	0.4		0.2	0.3
2,4-Rha*p*		2.4	2.3	1.6		0.6			0.3
3,4-Rha*p*		0.3	0.1	1.0				0.2	
T-Fuc*p*		0.3	0.3	0.5	0.3				
T-Ara*f*		17.4	21.3	11.0	6.1	2.9	1.7	0.3	2.4
2-Ara*f*		0.2	0.3	0.3	0.4				
3-Ara*f*		7.2	9.6	5.2	0.7	1.5	1.1		0.6
5-Ara*f*		20.1	26.3	13.3	2.6	4.7	2.9		2.0
2,5-Ara*f*		0.9	1.3	0.4					
3,5-Ara*f*		20.6	25.4	14.2	2.6	5.6	3.9		2.2
Arabinitol		0.9	0.8	0.6	0.2	0.5	1.3		
T-Xyl*p*		0.4	0.4	0.8	3.4	0.5		0.4	0.7
2-Xyl*p*					7.6				
4-Xyl*p*		2.2	0.3	2.4	23.3	61.3	9.0	81.5	71.9
2,4-Xyl*p*					8.5	10.4	2.2	12.5	12.4
Xylitol			0.1	0.6	0.8	0.9	0.4	0.5	0.7
4-Man*p*					2.1				
4,6-Man*p*					0.5				
T-Gal*p*		1.0	1.0	1.2	4.0				
3-Gal*p*		0.7	0.9	0.3					
4-Gal*p*		1.1	0.5	3.5					
3,4-Gal*p*		0.4	0.4	0.4					
3,6-Gal*p*		0.3							
T-Gal*p*A		0.7	0.2	1.6					
4-Gal*p*A		15.3	4.8	33.3		1.1			0.6
3,4-Gal*p*A			0.2	0.7					
T-Glc*p*					0.3	0.3	0.8	0.4	
4-Glc*p*		3.0		3.0	9.0	0.8	61.0		
2,4-Glc*p*		0.7	0.2		0.5		2.4		
3,4-Glc*p*					0.5		2.8		
4,6-Glc*p*					21.2	1.5	3.9		0.6
Glucitol		1.1			1.7	1.8	6.6	0.2	0.3
T-Glc*p*A					3.5	4.8		3.8	4.8

possible exception of K3A, all the other fractions, particularly K3B2 and K3D, had low levels of sugars. This may be due to the fact that these polysaccharides are associated with phenolics as can be seen in Fig. 1d. Fraction K3B had a shoulder and the slower component (K3B2) was shown to be very rich in phenolics by UV absorption. The fraction eluted with buffer (K3A) contained significant amounts of

TABLE VI

Sugar composition of fractions from 4 M KOH-soluble, 4 M KOH + borate-soluble, and chlorite-soluble extracts after anion-exchange chromatography

Fraction		Recovery (%)	Cell wall sugars (mol%)								Total sugar [a] (μg/mg)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	
4 M KOh soluble											
Insol. residue		10.0	1	t [b]	32	21	9	6	20	11	590
Buffer	(K3A)	34.0	1	1	41	5	18	9	21	4	509
0.125 M NaCl	(K3B1)	12.0	2	t	69	3	2	4	7	13	324
	(K3B2)	6.0	2	1	46	7	10	3	31		63
0.25 M NaCl	(K3C)	18.0	3	1	48	2	2	4	8	32	212
0.5 M NaCl	(K3D)	4.0	2	2	23	2	12	2	29	28	88
4 M KOH + borate soluble											
Buffer	(K4A)	21.3	1	t	66	3	10	6	10	42	679
0.125 M NaCl	(K4B)	9.3	2	–	76	2	1	3	4	12	635
0.25 M NaCl	(K4C)	4.7	3	t	62	2	t	4	5	24	561
0.5 M NaCl	(K4D)	1.3	3	1	28	2	38	3	18	7	355
Chlorite											
Insol. residue		25.8	2		71	1		4	3	19	712
Buffer	(ChA)	10.0	1		80	2		3	2	12	858
0.125 M NaCl	(ChB)	14.2	2	t	72	1		5	2	18	861
0.25 M NaCl	(ChC)	30.0	2		74	1		3		20	838
0.5 M NaCl	(ChD)	2.5	2	t	65	1		4	3	25	520

[a] Values are expressed as μg of "anhydrosugar"/mg dry polymer. [b] t, Trace.

arabinose, glucose, and mannose (Table VI) and the bulk of the arabinose was shown by methylation analysis to be derived from cell wall glycoproteins. The 4 M KOH + borate fraction dissolved completely in water and on anion-exchange chromatography gave 4 main fractions (Table VI). It can be seen that the first 3 fractions were very rich in arabinose, and the bulk of the arabinose present in the K4A was shown to be derived from cell wall glycoproteins by methylation analysis. The chlorite-soluble fraction was dispersed in water and the insoluble residue (26%) was removed by centrifugation. The soluble material was fractionated by anion-exchange chromatography when 3 main fractions rich in arabinose and uronic acid were obtained (Table VI and Fig. 1e). A significant proportion of the arabinose in ChA was shown to be derived from the wall glycoproteins, and the remainder from pectic polysaccharides.

Methylation analysis.—Selected fractions were methylated and carboxyl-reduced with $LiAlD_4$. Fractions K1R, K3A, and K4A were not carboxyl-reduced because of the low content of uronic acid, and the residues CR1 and CR2 were also not carboxyl-reduced. The results are shown in Tables V and VII. Fraction C1A, from CDTA-1, was de-esterified with cold dilute alkali prior to methylation. The results of the carboxyl-reduced methylation analyses were not quantitative, as shown by

the low recoveries of galacturonic acid (16–54%) and glucuronic acid (~ 40%). The 40% recovery of glucuronic acid is based on the assumption that the T-Glc*p*A is linked to C-2 of the glucuronoxylan. The low recovery of carboxyl-reduced galacturonic acid cannot be explained readily. The de-esterified pectic polysaccharides dissolved completely in Me_2SO to give clear, colourless solutions, and methylation analysis was quantitative for all the neutral sugars present in the fractions — this could have been due to the low galactose content of the fractions, and contrasts with the low recovery of galactose on methylation analysis of galactose-rich pectic polysaccharides from a number of plant organs[23,24,27,28]. The efficiency of carboxyl reduction appears to depend on the pectic polysaccharide. Using similar pectic polysaccharide preparative methods, we have obtained, at times, 90–100% carboxyl reduction. However, with onion pectic polysaccharides[5], the efficiency of carboxyl reduction ranged from 30–100%.

Glycosidic-linkage analysis of CDTA and Na_2CO_3 fractions.—Methylation analysis of C1A (Table V) showed the presence of small amounts of terminal galacturonic acid residues in addition to (1 → 4)-linked Gal*p*A. Variously linked rhamnose residues were detected, the preponderant ones being (1 → 2)- and (1 → 2,4)-linked Rha*p*. The relative proportions of the variously linked major arabinose residues were as follows: T-Ara*f*–3-Ara*f*–5-Ara*f*–3,5-Ara*f* = 2.4 : 1.0 : 2.8 : 2.9. Interestingly, this overall structural feature of the "arabinan" moiety is present in the other fractions rich in pectic polysaccharides such as NC1, NC2, and the soluble fraction from CR (Tables V and VII). In addition, all these pectic fractions have small amounts of (1 → 4)-linked Xyl*p* and terminally linked Xyl. A small amount of (1 → 3,4)-linked Gal*p*A was detected in NC1 and NC2, showing that a small proportion of the (1 → 4)-linked Gal*p*A residues were substituted in position 3, as has been observed with pectic polysaccharides from other tissues[24,28].

Glycosidic-linkage analysis of 1 M KOH fractions.—The occurrence of xyloglucans can be inferred from the presence of (1 → 4)- and (1 → 4,6)-linked Glc*p*, terminally and (1 → 2)-linked Xyl*p*, and terminally linked Gal*p*. The presence of acidic xylans can be inferred from (1 → 4)-linked, (1 → 2,4)-linked Xyl*p*, and terminally linked Glc*p*A. In most of the hemicellulosic fractions (K1A, K1B, K10, and K6), unambiguous evidence was obtained for the occurrence of terminally linked Glc*p*A. Fraction K1A can be inferred to contain both xyloglucans and glucuronoxylans. As the degree of polymerisation of the xylan moiety is apparently low, it is possible that K1A is a complex containing mainly glucuronoxylan and xyloglucan. K1B was retained on the anion-exchange column and was eluted with buffer containing 0.125 M NaCl. It contained glycosidic linkages usually associated with glucuronoxylans and pectic polysaccharides, and it is most probably a complex of these two polysaccharides. K1R was the insoluble fraction obtained from the 1 M KOH extract. This material was not easily dispersible in Me_2SO and a proportion of it remained insoluble, which probably accounts for the relatively high value for glucitol. Nevertheless, it can be inferred to have significant amounts of a glucan and a relatively small proportion of xylan. The major component (> 95%)

TABLE VII

Glycosyl linkage composition of selected cell wall polysaccharides from 4 M KOH and subsequent fractions (values expressed as relative mol%)

Linkage	4 M KOH	1 M KOH + borate			CR					
Fraction:	K3A	K4A	K4B	K4C	Supt. [a]	Res. [b]	Chlorite–HOAc			CR2
							ChA	ChB	ChC	
2-Rha*p*		0.4	2.7	3.0	3.7	1.0	1.9	2.9	3.9	0.9
3-Rha*p*				0.3	0.2				0.1	
2,4-Rha*p*		0.3	1.8	2.1	2.2	0.8	1.5	2.1	2.4	0.3
3,4-Rha*p*				0.3					0.2	
T-Fuc*p*				0.3	0.2	0.4			0.2	
T-Ara*p*									0.1	
T-Ara*f*	10.9	16.1	22.1	19.2	21.5	6.9	15.3	21.3	19.8	4.5
2-Ara*f*	13.0	20.3	3.4	3.1	0.4	0.4	2.4	0.7	0.4	0.3
3-Ara*f*	7.1	12.2	10.0	8.1	9.5	2.6	11.8	8.9	8.7	1.7
5-Ara*f*	4.0	7.3	24.6	19.8	23.8	8.2	24.4	25.1	24.1	5.2
2,5-Ara*f*					0.6					
3,5-Ara*f*	3.4	6.9	21.2	19.7	24.3	8.0	26.7	23.8	22.1	5.2
Arabinitol	0.3	0.5	0.8	0.9	0.7	0.6		0.8	0.7	0.8
T-Xyl*p*	0.6	0.4	0.5	1.1	0.4	0.6		0.4	0.4	0.7
2-Xyl*p*	0.9	0.7		0.1					0.1	1.4
4-Xyl*p*	2.1	1.3		0.5	0.8	6.0	2.9		0.2	6.8
2,3-Xyl*p*									0.2	
2,4-Xyl*p*			0.5	0.4			1.3	0.7	0.5	0.8
Xylitol				0.3	0.4	1.4				0.5
T-Man*p*	0.6	0.3								
4-Man*p*	17.5	10.4	0.7	0.8						2.4
6-Man*p*	0.5									
4,6-Man*p*	5.3	3.1								0.7
T-Gal*p*	6.7	3.7	2.5	3.4	1.2	0.6	0.9	2.2	1.3	
2-Gal*p*	1.4	0.7								
3-Gal*p*	0.4	0.3			0.8				0.6	
4-Gal*p*	0.5	1.4	1.0	1.2	1.3		3.2	1.1	1.4	
3,4-Gal*p*			0.4	0.4	0.4			0.4		0.1
3,6-Gal*p*	1.0	0.7	0.4					0.5		
4,6-Gal*p*										0.2
T-Gal*p*A			0.3	0.7	0.3		0.4	0.3	0.6	
4-Gal*p*A			3.9	6.0	5.9		5.0	4.5	9.6	
2,4-Gal*p*A				0.4				0.1	0.2	
3,4-Gal*p*A			0.2	0.5	0.2			0.3	0.3	
T-Glc*p*	0.4	0.2	0.1	0.2				0.1		0.6
4-Glc*p*	18.3	10.0	1.2	3.9	0.5	56.2	1.2	2.6	0.5	62.4
2,4-Glc*p*				0.3	0.1				0.2	0.3
3,4-Glc*p*						1.2			0.4	0.4
4,6-Glc*p*	4.9	2.8	1.0	2.1	0.4	3.3	0.6	0.7	0.6	2.3
Glucitol	0.2		0.4	0.9	0.2	1.8	0.5	0.5	0.3	1.5
T-Glc*p*A			0.3							

[a] Supernatant solution from CR (Table II). [b] Insoluble residue from CR (CR1).

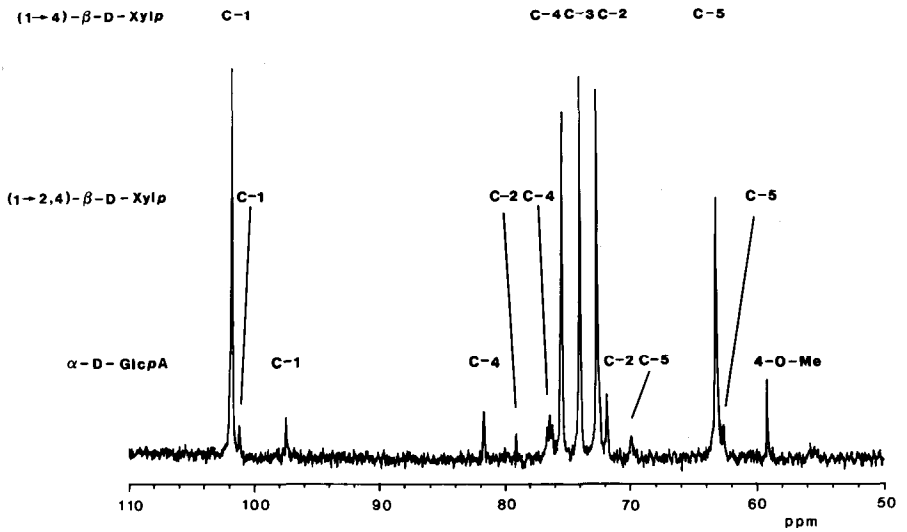

Fig. 2. ^{13}C NMR spectrum of glucuronoxylan from 1 M KOH 1°C extract (K10).

of fraction K10 was a xylan which was 13% branched and had an average degree of polymerisation of 200, as inferred from the ratio of total xylose residues to terminally linked xylose. ^{13}C NMR spectroscopy (Fig. 2) was used to determine the anomeric configuration of the sugar residues and also to compare the relative proportions of the variously linked sugar residues. From ^{13}C NMR data, it can be 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 can be inferred that most of the glucuronic acid carries a methyl substituent on C-4. From the ratio of the areas for C-1 of (1 → 4)-linked + (1 → 2,4)-linked Xyl*p* residues to (1 → 2,4)-linked Xyl*p* residues, it can be inferred that 1 in 11 of the xylose residues are branched. This value is at variance with the value for branching obtained by direct methylation analysis which suggests that 1 in 8 of the xylose residues are branched. The major component of fraction K6 was an acidic xylan and it was contaminated with a small amount of pectic polysaccharide.

Glycosidic-linkage analysis of 4 M KOH and 4 M KOH + borate fractions.—The major polysaccharide moieties in fractions K3A and K4A can be inferred to be a slightly branched xyloglucomannan (or a mixture of xyloglucan and glucomannan) from the occurrence of (1 → 4)- and (1 → 4,6)-linked glucose and mannose residues. These fractions also contained significant amounts of (1 → 2)-, (1 → 3)-, and terminally linked Ara*f* residues, which are characteristic of hydroxyproline-rich cell wall glycoproteins[29]. It should, however, be noted that the pectic polysaccharides of olive (C1A, NC1, and NC2) contained significant amounts of (1 → 3)-linked Ara*f* residues. The hydroxyproline (Hyp) contents of K3A and K4A were 2.0 and 3.0%, respectively, on a dry weight basis. The (1 → 2)- and (1 → 3)-linked Ara*f* residues in K3A and K4A are in the ratios 1.8:1 and 1.7:1, respectively, and it is probable that most of these residues are derived from Hyp tetra-arabinosides in

which the proportions of T-Ara*f* to (1 → 2)-Ara*f* to (1 → 3)-Ara*f* are 1:2:1[30]. The small amounts of (1 → 5)- and (1 → 3,5)-linked Ara*f* residues and some of the terminally linked Ara*f* residues in both fractions would have arisen from the associated pectic polysaccharides. From the glycosidic composition of the fractions K4B and K4C, it can be seen that the major components of these fractions are pectic polysaccharides and Hyp-rich glycoproteins. The fact that these fractions were retained on the anion-exchange column and eluted as homogeneous peaks suggests that they were similar to the complexes isolated from *Vicia faba*[31] and runner-bean parenchyma[24].

Glycosidic-linkage analysis of fractions from CR.—The water-soluble fraction from CR (Table VII, column 5) can be inferred to be rich in pectic polysaccharides and is comparable to the pectic polysaccharides present in fractions C1A, NC1, and NC2. The insoluble residue (CR1) can be inferred to contain much cellulose and small, but significant, amounts of pectic polysaccharides and glycoproteins. Most of these polymers were solubilised with chlorite–HOAc, and the neutral fraction from the anion-exchange column can be inferred to contain significant amounts of pectic polysaccharides and some Hyp-rich glycoproteins. The fact that these polymers had not been released by chaotropic agents such as alkali or borate and alkali, but were subsequently released by a short treatment with chlorite–HOAc, which is a relatively mild extraction (longer and more vigorous treatment is used in the delignification of secondary cell walls), suggests that they were probably cross-linked within the cellulose matrix by phenolics. The occurrence of polysaccharide–protein–phenolic complexes in the cell walls of parenchymatous tissue has been reported in our earlier work on runner beans[32]; also, the occurrence of pectic polysaccharides in covalent association with glycoproteins has been demonstrated by Pusztai et al.[33]. The occurrence of small but significant amounts of variously branched arabinose, xylose, and mannose residues in the final residue CR2, in addition to (1 → 4)-linked glucose, shows that cross-linked polymers within the cellulose complex cannot be completely removed.

GENERAL DISCUSSION

The major problem in studying the structural aspects of cell walls is the isolation of relatively pure walls and the solubilisation of the constituent polymers with minimum degradation. The procedures used in this study removed a large proportion of the intracellular components, including the oil which was removed completely; a small proportion of the phenolics was associated with the cell wall material (CWM), could not be readily removed by further washing, and gave a slight green colour to the CWM. The CWM was kept hydrated and stored as a frozen suspension rather than as a freeze-dried powder. The cellulose-rich residue (CR) obtained after sequential extraction with various aqueous inorganic solvents contained significant amounts of pectic polysaccharides and small amounts of cell wall glycoproteins. A proportion of these (apparently) cross-linked pectic polysac-

charides was solubilised on allowing CR to stand in distilled water. This observation is of interest because, in our work with runner-bean parenchyma, we have treated the cellulose-rich residue directly with chlorite–HOAc to solubilise cross-linked polymers[24]. The occurrence of pectic polysaccharides and some hydroxyproline-rich glycoproteins in CR1 and its solubilisation by chlorite–HOAc clearly suggest that these polymers are very closely enmeshed within the cellulose microfibrils, and this property may have to be taken into account in any realistic model of the parenchyma cell wall. The pectic polysaccharides of olive pulp consisted of a range of structurally related polymers that differ widely in their ease of extraction from the cell wall matrix. Interestingly, the relative proportions of the variously linked arabinose residues in all the pectic polysaccharides examined are highly comparable. In this connection, it should be remembered that, in those pectic fractions that have significant amounts of cell wall glycoproteins, an allowance has to be made for the occurrence of arabinosyl residues found in wall glycoproteins. Some of the pectic polymers (e.g., those in fractions K4B, K4C, and ChA) may be cross-linked to Hyp-rich glycoproteins by phenolics. The occurrence of pectic polysaccharide–glycoprotein complexes has been reported in other tissues[24,31]. The 1 M KOH-soluble fractions contained significant amounts of acidic xylans in addition to pectic polysaccharides. Relatively pure acidic xylans free of associated pectic polysaccharides have been isolated from the precipitates obtained on neutralisation of these extracts. The hemicelluloses in the supernatant fractions of the alkali extracts are largely xyloglucans some of which appear to be associated with xylans. It is very likely that a large proportion of the glucuronoxylans was derived from the lignified sclereids of the olive pulp[1], as is the case in stone cells of pear (Martin, Waldron, and Selvendran, unpublished results) and guava fruit[34]. However, a small proportion may have originated from the parenchyma tissues. Recently, xylans have been detected in stone cell-free cell wall preparations from guava fruit[34]. Furthermore, we have previously identified xylans in the primary cell walls of non-lignified parenchyma cells in asparagus stems[28].

ACKNOWLEDGMENTS

We thank Macaricos, olive industry from Aveiro, for providing the olives and introducing us to olive processing; Peter Ryden and Neil Rigby for helpful discussions; John Eagles for the mass spectrometry; Dr. Ian Colquhoun for the NMR spectroscopy; and Dr. Ivonne Delgadillo for her interest in the work. This work was partially funded by the Calouste Gulbenkian Foundation, Instituto Nacional de Investigacao Cientifica (INIC), and the British Council (Treaty of Windsor).

REFERENCES

1 A.L. Winton and K.B. Winton, *Structure and Composition of Foods*, Vol. 1, Wiley, 1932.
2 A. Ouguerram, *Anal. Sci. Nat. (Botanique) (Paris)*, 13 (1989) 77–96.

3 A. Gil-Serrano, M.I. Mateos-Matos, and M.P. Tejero-Mateo, *Phytochemistry,* 25 (1986) 2653–2654.
4 A. Gil-Serrano and M.P. Tejero-Mateo, *Carbohydr. Res.,* 181 (1988) 278–281.
5 R.J. Redgwell and R.R. Selvendran, *Carbohydr. Res.,* 157 (1986) 183–199.
6 R.R. Selvendran and M.A. O'Neill, *Methods Biochem. Anal.,* 32 (1987) 25–153.
7 M.A. O'Neill and R.R. Selvendran, *Biochem. J.,* 187 (1980) 53–63.
8 R.R. Selvendran and S.E. King, *Carbohydr. Res.,* 195 (1989) 87–99.
9 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.,* 28 (1956) 350–356.
10 S.G. Ring and R.R. Selvendran, *Phytochemistry,* 17 (1978) 745–752.
11 R.R. Selvendran, J.F. March, and S.G. Ring, *Anal. Biochem.,* 96 (1979) 282–292.
12 R.R. Selvendran, A.V.F.V. Verne, and R.M. Faulks, *Mod. Methods Plant Anal.,* 10 (1989) 234–259.
13 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.,* 96 (1973) 484–489.
14 S. Hakomori, *J. Biochem. (Tokyo),* 55 (1964) 205–208.
15 K.W. Waldron and R.R. Selvendran, *Physiol. Plant.,* 80 (1990) 568–575.
16 D.P. Sweet, R.H. Shapiro, and P. Albersheim, *Carbohydr. Res.,* 40 (1975) 217–225.
17 G.O. Aspinall, J.W. Cottrell, J.A. Molloy, and M. Uddin, *Can. J. Chem.,* 48 (1970) 1290–1295.
18 B. Lindberg and J. Lönngren, *Methods Enzymol.,* 50 (1978) 3–13.
19 S.C. Fry, *The Growing Plant Cell Wall: Chemical and Metabolic Analysis,* Longman, Harlow, UK, 1988.
20 P. Ryden, I.J. Colquhoun, and R.R. Selvendran, *Carbohydr. Res.,* 185 (1989) 233–237.
21 P. Kovak and J. Hirsch, *Carbohydr. Res.,* 100 (1982) 177–193.
22 G. Excoffier, R. Nardin, and M.R. Vignon, *Carbohydr. Res.,* 149 (1986) 319–328.
23 P. Ryden and R.R. Selvendran, *Carbohydr. Res.,* 195 (1990) 257–272.
24 P. Ryden and R.R. Selvendran, *Biochem. J.,* 269 (1990) 393–402.
25 P. Massiot, X. Rouau, and J.F. Thibault, *Carbohydr. Res.,* 172 (1988) 217–227.
26 A.J. Mort, B.M. Moerschbacher, M.L. Pierce, and N.O. Maness, *Carbohydr. Res.,* 215 (1991) 219–227.
27 G.B. Seymour, I.J. Colquhoun, M.S. DuPont, K.R. Parsley, and R.R. Selvendran, *Phytochemistry,* 29 (1990) 725–731.
28 K.W. Waldron and R.R. Selvendran, *Phytochemistry,* 31 (1992) 1931–1940.
29 D.T.A. Lamport and L. Epstein, *Proc. Annu. Plant Biochem. Physiol. Symp., 2nd,* Columbia, 1983, pp 73–83.
30 Y. Akiyama, M. Mori, and K. Kato, *Agric. Biol. Chem.,* 44 (1980) 2487–2489.
31 A. Pusztai and W.B. Watt, *Eur. J. Biochem.,* 10 (1969) 523–532.
32 M.A. O'Neill and R.R. Selvendran, *Biochem. J.,* 227 (1985) 475–481.
33 A. Pusztai, R. Begbie, and I. Duncan, *J. Sci. Food Agric.,* 22 (1971) 514–519.
34 O. Marcelin, P. Williams, and J.-M. Brillouet, *Carbohydr. Res.,* 240 (1993) 233–243.