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Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv koroneiki) at different ripening stages

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Abstract

Olive fruit (*Olea europaea* cv koroneiki) were picked at the immature green, green, turning and purple ripening stages and cell wall material was isolated. During ripening the sugar composition changed and the level of methyl esters and acetyl groups in the cell wall decreased. The cell wall material of green and purple olives was fractionated by successive extractions with 0.05 M NaOAc buffer, 0.05 M cyclohexane-*trans*-1,2-diaminetetra-acetate (CDTA) and 0.05 M NH_4-oxalate in 0.05 M NaOAc buffer, 0.05 M NaOH, 1 M KOH, 4 M KOH and 6 M NaOH. The amount of pectic material extracted with NaOAc buffer increased considerably during ripening. The molecular weight distribution and the sugar composition of the pectic polymers hardly changed. The yields and the sugar composition of the hemicellulose-rich fractions showed little change throughout development. The molecular weight profiles of the hemicellulosic fractions were similar.

For further study, the hemicellulose-rich fractions were fractionated by anion-exchange chromatography. The fractions had similar elution behaviours and all contained a xyloglucan-rich pool and four xylan-rich pools. The yields of the subpopulations differed for the 1 and 4 M KOH fractions. During ripening no detectable changes in the sugar composition and the molecular weight distribution of the xyloglucan-rich and xylan-rich fractions were found.

Keywords: Olive fruit; Ripening; Pectin; Hemicellulose; Xyloglucan; Xylan

1. Introduction

Ripening associated changes of the cell wall have been studied for many fruits often in relation to textural changes. Changes to the pectic polymers are the most common and can involve an increased solubility, depolymerisation, de-esterification and a loss of neutral sugars associated side chains. In some cases a decrease in the molecular mass of hemicelluloses has also been reported (Seymour & Gross, 1996). Knowledge about the composition of the cell wall material from olive fruit and about changes in the cell wall material during ripening is limited. Sequential extraction of cell wall material shows that the major components of the cell wall from olive fruit are pectic polysaccharides rich in arabinose (Coimbra, Waldron & Selvendran, 1994; Huisman, Schols & Voragen, 1996). Besides pectins the cell wall also contains significant amounts of acidic xylans and xyloglucans (Gil-Serrano & Tejero-Mateo, 1988; Gil-Serrano, Mateos-Matos & Tejero-Mateo, 1986; Coimbra et al., 1994).

Ripening related changes in the composition of the olive fruit cell wall have been studied by Heredia, Guillén, Jimenez and Fernández-Bolaños (1993). They have studied the neutral sugar composition of the cell wall material during the development and ripening of the olive fruit but did not sequentially extract the material for a more detailed analysis. Huisman et al. (1996) have also studied the overall composition of the cell wall material. The ripening associated changes of the cell wall sugars they have described were less pronounced compared to the results of Heredia et al. (1993). This may be due to the use of a different variety but may also originate from the fact that Huisman et al. (1996) have used olive fruit harvested at one moment and re-divided this batch on the basis of their colour into mature green olives, turning olives and purple olives. For our research we will use, as Heredia et al. (1993), olive fruit harvested at specific moments during the season. The objective is to examine changes in the cell wall material and the pectin and hemicellulosic fractions during ripening. The fractions are compared on the basis of yield, sugar composition and molecular weight distribution. A more detailed characterisation of the hemicellulose-rich fractions isolated from different ripening stages will be described in the last

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part of the paper to complete previous work of our group described by Huisman et al. (1996) on the characterisation of pectin-rich fractions from different ripening stages.

2. Experimental

2.1. Materials

Olive (*Olea europaea* cv koroneiki) fruit of four stages of ripening were kindly supplied by Dr E. Stefanoudaki, Institute of Subtropical Plants and Olives, Chania, Greece. Fruit from each ripening stage was selected for uniformity of colour and size and the damaged fruit was discarded. Classes: immature green (small, underdeveloped olive fruit, F0), green (FI), turning (FII) and purple (FIII).

2.2. Isolation of alcohol-insoluble solids

Olives (2000 g) were boiled in water (10 min) and destoned. The pulp was freeze-dried and defatted by Soxhlet extraction with petroleum ether (40–60). Subsequently, the lipid free material was extracted with 70% (v/v) ethanol at 40°C for 1 h and centrifuged. The extraction with 70% (v/v) ethanol was repeated until the extracts were free of sugars as monitored by the phenol–sulphuric acid test (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The residue (AIS) was dried by solvent exchange (96% (v/v) ethanol and acetone) at room temperature and ground (particle size <1 mm).

2.3. Sequential extraction of AIS

The AIS prepared from olive pulp was sequentially extracted with various solvents as described by Huisman et al. (1996). The extraction procedure was extended with an extraction with 1.5% (w/v) sodium dodecylsulphate (SDS) in 10 mM 1,4-dithiothreitol (DTT) to remove proteins. AIS (20 g) was sequentially extracted with 0.05 M NaOAc buffer, pH 5.2 (three times, 600 ml) at 70°C for 30 min (hot buffer soluble solids, HBSS); 1.5% (w/v) SDS in 10 mM DTT (three times, 300 ml) at room temperature for 3 h (sodium dodecyl soluble solids, SDSS); 0.05 M CDTA and 0.05 M NH_4-oxalate in 0.05 M NaOAc-buffer, pH 5.2 (two times, 600 ml) at 70°C for 30 min (chelating agent soluble solids, ChSS); washed with distilled water; extracted with 0.05 M NaOH (two times, 600 ml) at 4°C for 30 min (dilute alkali soluble solids, 0.05 M NaOH); 1.0 M KOH + 20 mM $NaBH_4$ (two times, 600 ml) at room temperature for 2 h (1 M alkali soluble solids, 1 M KOH); 4.0 M KOH + 20 mM $NaBH_4$ (600 ml) at room temperature for 2 h (4 M alkali soluble solids, 4 M KOH); 6.0 M NaOH + 20 mM $NaBH_4$ (600 ml) at room temperature for 2 h (6 M alkali soluble solids, 6 M NaOH). After each extraction, solubilised polymers were separated from the insoluble residue by centrifugation. The supernatants were filtered through a G3 glass sinter (those containing alkali were neutralised by adding glacial acetic acid), ultrafiltrated (nominal molecular weight cut-off 30 kDa) and freeze-dried. The final residue was dialysed and freeze-dried.

2.4. Ion-exchange chromatography

Ion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column (50×2.6 cm) equilibrated with 0.005 M NaOAc (pH 5.0). After loading with sample the column was washed with the same buffer (380 ml) and eluted successively with a NaOAc buffer gradient and NaOH (0.005 M → 1 M NaOAc (1600 ml), 1 M NaOAc (300 ml), 1 M → 2 M NaOAc (300 ml), 2 M NaOAc (300 ml), 0.005 M NaOAc (380 ml), 0.25 M NaOH (1000 ml) and 0.5 M NaOH (1500 ml)). During the elution with NaOAc and NaOH fractions of 20 ml were collected and analysed for neutral sugar and uronic acid content as described. The alkali fractions were neutralised before analysis.

2.5. Analytical methods

2.5.1. Total neutral sugar content

Total neutral sugar content was determined colorimetrically by the automated orcinol/sulphuric acid assay (Tollier & Robin, 1979). Xylose was used as a standard. Corrections were made for the interference of uronic acids in the samples.

2.5.2. Total uronic acid content

Total uronic acid content was determined colorometrically by the automated *m*-hydroxydiphenyl assay (Thibault, 1979). Galacturonic acid was used as a standard. Corrections were made for the interference of neutral sugars in the samples.

2.5.3. The neutral sugar composition

The neutral sugar composition of the AISs and the fractions was determined by gas chromatography according to Englyst and Cummings (1984) using inositol as internal standard. The samples were treated with 72% (w/w) sulphuric acid for 1 h at 30°C prior to hydrolyses with 1 M sulphuric acid for 3 h at 100°C. The released constituent sugars were analysed as their alditol acetates. Cellulosic glucose in the residue was calculated as the difference between the glucose contents determined with and without pre-treatment with 72% (w/w) sulphuric acid. The sugar composition of the xyloglucan-rich and xylan-rich pools were determined by direct hydrolyses without a pre-treatment with 72% (w/w) sulphuric acid because no cellulose was expected in these soluble samples.

2.5.4. The uronic acid composition

The uronic acid composition of the xylan-rich pools was determined using methanolysis combined with trifluoroacetic acid hydrolysis as described by De Ruiter, Schols,

Table 1
Yield and sugar composition (mol%) of AIS isolated from olive fruit at four ripening stages

Sample	Yield[a]	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	OMe/OAc[b]	Carbohydrate content[c]	Protein content[c]
AIS F0	8.9	1 (5)[d]	20 (65)	18 (57)	2 (8)	4 (17)	31 (122)	23 (99)	68/72	37	18
AIS FI	6.8	1 (5)	18 (57)	20 (64)	2 (7)	4 (16)	32 (126)	23 (97)	67/72	37	20
AIS FII	5.1	2 (6)	18 (52)	19 (55)	2 (7)	5 (17)	32 (116)	22 (85)	69/72	34	23
AIS FIII	4.7	3 (9)	17 (47)	17 (46)	2 (7)	5 (16)	34 (113)	23 (83)	59/73	32	24

[a] Expressed as % of fresh weight of destoned olive pulp.
[b] Expressed as mol methyl esters or acetyl groups per 100 mol uronic acid.
[c] Expressed as % (w/w).
[d] Expressed as mg/g AIS.

Voragen and Rombouts (1992) followed by an enzymatic hydrolysis of incomplete hydrolysed aldobiuronic acids. The xylan-rich pools were heated for 16 h at 80°C with 1 ml anhydrous 2 M hydrochloric acid in absolute methanol. After cooling to room temperature the liquid was evaporated by a stream of air. The remaining carbohydrates were hydrolysed further with 2 M trifluoroacetic acid (1 h, 121°C), which was removed again by evaporation (air stream, at room temperature). Two times, 1 ml of methanol was added and evaporated to dryness to remove residual acetic acid from the samples. Subsequently, the samples were dissolved in distilled water and (4-*O*-methyl)-α-glucuronidase was added to enzymatically hydrolyse residual aldobiuronic acids (4-*O*-MeGlcA-Xyl and GlcA-Xyl). The incubation was performed at 40°C for 24 h. The (4-*O*-methyl)-α-glucuronidase was purified from a commercial enzyme preparation from *Trichoderma viride* (Kroef, Beldman & Voragen, 1992). A standard mixture of galacturonic acid, glucuronic acid and (4-*O*-methyl)-glucuronic acid-xylose was treated likewise. The response of glucuronic acid was used to calculate the (4-*O*-methyl)-glucuronic acid content because no standard for (4-*O*-methyl)-glucuronic acid was available. Standards and samples were analysed by HPAEC.

2.5.5. Protein content

Protein contents of the AISs were determined by a semi-automated Micro Kjeldahl assay (Roozen & Van Boxtel, 1979). All nitrogen (N) was assumed to be of protein origin and the protein content was calculated as 6.25 × N.

2.5.6. Degrees of methylation and acetylation

Degrees of methylation and acetylation of AISs were determined by HPLC after saponification with 0.4 M NaOH according to Voragen, Schols and Pilnik (1986) and expressed as mol methyl esters or acetyl groups per 100 mol uronic acid.

2.5.7. High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed using a Dionex BIO LC GMP-11 gradient module equipped with a CarboPac PA-1 column (250 × 4 mm) in combination with a CarboPac PA guard column (25 × 3 mm) of Dionex. Elution took place at 20°C at a flow rate of 1.0 ml/min. The gradient was obtained by mixing solutions of 0.1 M NaOH, 1 M NaOAc in 0.1 M NaOH and distilled water at 1.0 ml/min. The uronic acids were analysed with the following gradient: 0 → 26 min, isocratic with 15 mM NaOH; 26 → 33 min, linear gradient of 15 → 100 mM NaOH; 33 → 95.5 min, linear gradient of 0 → 100 mM NaOAc in 0.1 M NaOH. After each run the column was washed for 5 min with 100 mM NaOH containing 1 M NaOAc, for 5 min with 100 mM NaOH and subsequently equilibrated for 15 min with the starting eluent. Solvents were degassed and stored under helium using a Dionex eluent degassing module. The column effluent was monitored using a Dionex pulsed electrochemical detector (PED) in the pulsed amperometric detection (PAD) mode. A reference silver/silver chloride electrode was used containing a gold electrode using the following pulse potentials and durations: E_1 0.1 V and 0.4 s, E_2 0.7 V and 0.2 s, E_3 −0.1 V and 0.4 s.

2.5.8. High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8810 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 × 7.5 mm) in series (40XL, 30XL and 20XL; Bio-Rad Labs) in combination with a TSK XL guard column (40 × 6 mm). Elution took place at 30°C with 0.4 M NaOAc (pH 3.0) at a flow rate of 0.8 ml/min. The column effluent was monitored using a refractive index detector (Shodex RI SE-61). Calibration was performed using dextrans (M_w 4000–500 000 Da).

3. Results and discussion

3.1. Composition of the AIS

The AIS from olive fruit (*O. europaea* cv Koroneiki) contained glucose, arabinose and xylose as the major neutral sugars (Table 1). Rhamnose, mannose and galactose were present in relatively small amounts (a total maximum of 10 mol%). During ripening the yield of AIS expressed as % of fresh weight of destoned olive pulp decreased from 8.9 to

Table 2
Yield on sugar basis (%) and sugar composition (mol%) of the fractions and the residue of AIS isolated from olive fruit of the first ripening stage (FI) (tr = trace amount)

Sample	Yield	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	Carbohydrate content[a]
AIS FI	100	1 (5)[b]	18 (57)	20 (64)	2 (7)	4 (16)	32 (126)	23 (97)	37
HBSS FI	9.3	2 (0.6)	17 (4.7)	2 (0.5)	1 (0.3)	12 (3.9)	2 (0.8)	65 (24)	66
ChSS FI	2.6	2 (0.2)	25 (2.0)	1 (0.1)	tr (tr)	4 (0.4)	1 (0.1)	66 (7.0)	22
0.05 M NaOH FI	5.7	3 (0.7)	42 (7.7)	2 (0.4)	tr (0.2)	5 (1.1)	2 (0.4)	45 (11)	53
1 M KOH FI	7.3	1 (0.2)	8 (2.1)	66 (17)	1 (0.2)	5 (1.5)	9 (2.9)	10 (3.4)	30
4 M KOH FI	> 4.7	tr (0.1)	8 (1.3)	43 (6.7)	10 (2.0)	8 (1.5)	26 (5.0)	4 (0.8)	50
6 M NaOH FI	5.2	3 (0.6)	42 (7.2)	7 (1.1)	6 (1.2)	7 (1.5)	10 (2.0)	26 (5.8)	24
RES FI	42.3	1 (1.2)	15 (20)	20 (27)	tr (0.8)	2 (2.6)	54 (90)	8 (15)	47

[a] Expressed as % (w/w).
[b] Expressed as mg/g AIS.

4.7%. The carbohydrate content of the AIS was only 32–37%, which is relatively small but agrees well with the findings of other workers (Heredia et al., 1993; Huisman et al., 1996). In addition to polysaccharides the AIS contained 18–24% of proteins. The remaining part contains probably lignin-like material (Coimbra et al., 1994).

No distinct differences between the four ripening stages (F0 immature green, FI green, FII turning and FIII purple olives) could be found in the sugar composition but some trends could be noticed. Arabinose decreased from 20 to 17 mol%, glucose increased from 31 to 34 mol% and rhamnose increased from 1 to 3 mol% during ripening. The amount of xylose first increased from 18 (F0) to 20 mol% (FI) and then decreased till 17 mol% (FIII). The amount of uronic acid showed no consistent change from the unripe to ripe stage. The differences described were relatively small but they were reproducible and consistent.

The sugar composition of the AISs isolated from four different stages of ripeness show a good resemblance with the previous reported results of Coimbra et al. (1994) and Huisman et al. (1996) who have worked on the varieties Douro and Frantoio, respectively. Small differences in the sugar composition may be due to the different varieties used.

The amount of each monosaccharide per g AIS is also shown in Table 1. The results showed that during ripening a loss of arabinose per g AIS could be noticed of about 30%. Also, the amount of uronic acid per g AIS decreased. The amount of galactose did not change. In general, our results agree rather well with the neutral sugar composition of AIS as described by Heredia et al. (1993). However, in contrast to our findings and the findings of Huisman et al. (1996); Heredia et al. (1993) have found a considerable decrease of galactose per g AIS during ripening (about 60%). A loss of arabinose during ripening as found in the present study is mentioned in several other fruits like avocado, pear and apple but even more often in fruits a marked decrease in the galactose content is mentioned (references in Redgwell, Fischer, Kendal & Macrae, 1997; references in Voragen, Pilnik, Thibault, Axelos & Renard, 1995).

The degrees of methylation and acetylation were high for all ripening stages. The degree of methyl esterification was constant during the first three ripening stages and decreased from 68 to 59% when the olives became purple. The same trend was observed for the degree of acetylation which was also constant in the first three ripening stages and then decreased from 72 to 63% for purple olives.

3.2. Composition of the fractionated AIS

The AISs of green (FI) and purple (FIII) olives were sequentially extracted with different solvents. To solubilise the major part of the pectic material the AIS

Table 3
Yield on sugar basis (%) and sugar composition (mol%) of the fractions and the residue of AIS isolated from olive fruit of the third ripening stage (FIII) (tr = trace amount)

Sample	Yield	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	Carbohydrate content[a]
AIS FIII	100	3 (9)[b]	17 (47)	17 (46)	2 (7)	5 (16)	34 (113)	23 (83)	32
HBSS FIII	15.9	4 (1.7)	17 (7.1)	2 (1.0)	1 (0.5)	10 (4.9)	3 (1.5)	62 (34)	73
ChSS FIII	2.2	4 (0.3)	28 (1.6)	1 (0.1)	1 (0.1)	6 (0.4)	2 (0.2)	57 (4.4)	20
0.05 M NaOH FIII	4.3	5 (0.7)	39 (4.7)	3 (0.4)	1 (0.1)	7 (1.1)	5 (0.7)	40 (6.3)	49
1 M KOH FIII	8.2	2 (0.6)	12 (2.9)	52 (13)	2 (0.5)	6 (1.9)	15 (4.4)	11 (3.5)	25
4 M KOH FIII	6.0	2 (0.4)	10 (1.7)	39 (6.8)	8 (1.8)	7 (1.5)	27 (5.8)	6 (1.3)	25
6 M NaOH FIII	4.2	5 (0.7)	31 (3.6)	6 (0.8)	13 (1.9)	8 (1.2)	18 (2.6)	18 (2.8)	22
RES FIII	45.2	1 (1.6)	14 (18)	19 (23)	tr (0.7)	1 (2.3)	58 (89)	6 (9.9)	47

[a] Expressed as % (w/w).
[b] Expressed as mg/g AIS.

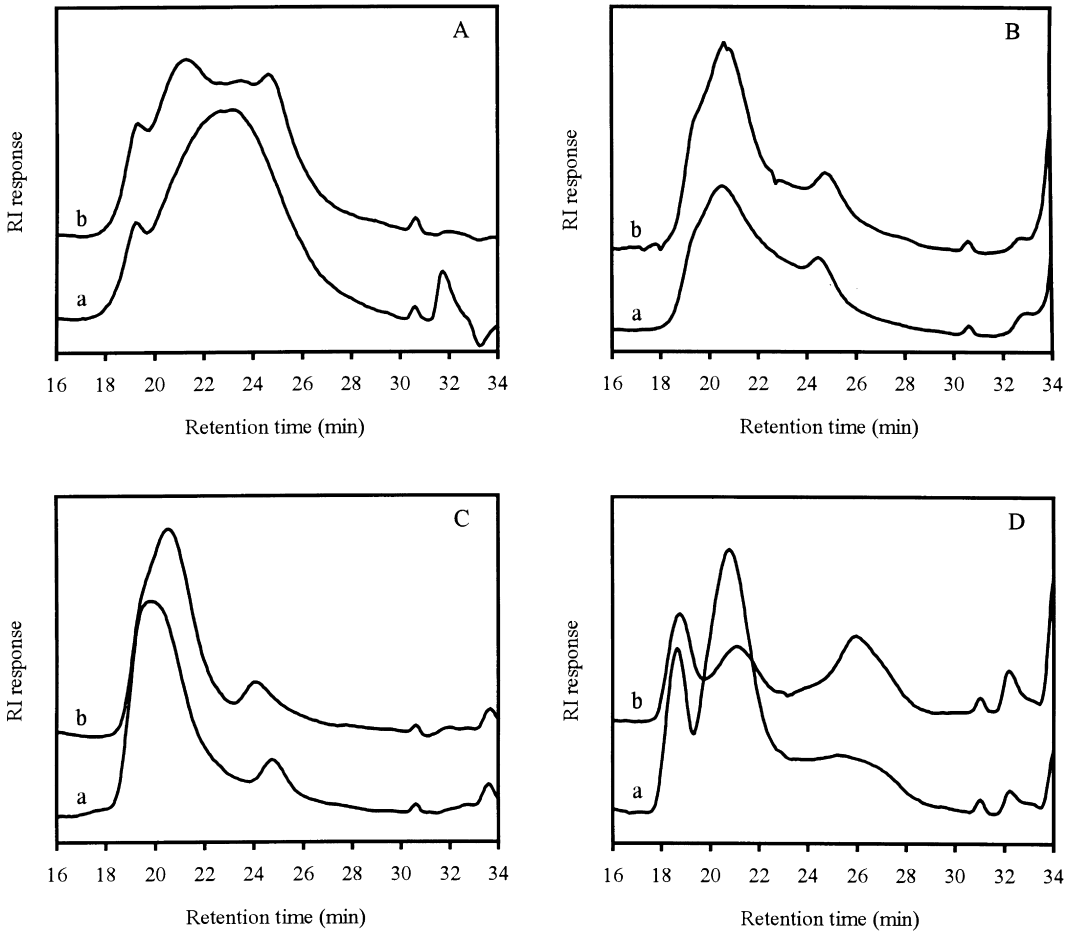

Fig. 1. HPSEC-patterns of the pectin-rich fractions from olive fruit. A: HBSS FI (a), HBSS FIII (b), B: ChSS FI (a), ChSS FIII (b), C: 0.05 M NaOH FI (a), 0.05 M NaOH FIII (b), D: 6 M NaOH FI (a), 6 M NaOH FIII (b).

was fractionated with hot buffer, chelating agent and cold dilute alkali. Subsequently, a fractionation with 1 and 4 M alkali was performed to solubilise the hemicellulosic material. A final fractionation with 6 M alkali was performed to solublilise the residual pectins. Besides pectic material this fraction may also contain small amounts of hemicelluloses (Huisman et al., 1996). An extraction with SDSS was performed after the extraction of the buffer soluble pectins to prevent contamination of the fractions with proteins as much as possible. In Tables 2 and 3 the yields and the sugar composition of the fractions are shown. The recoveries based on the total sugar contents were 78 and 87% for the fractions of AIS FI and FIII, respectively. Some of the material of the 4 M KOH FI fraction was lost during the extraction procedure and is the cause of a lower recovery for the total fractions of AIS FI.

Analysis of the residue fraction showed that the solvents used to extract the AIS were not able to solubilise all pectic and hemicellulosic substances. About 50% of the AIS were extracted leaving a residue, which consisted of about 58% of cellulose and about 42% of (highly branched) pectic polysaccharides, xylans and xyloglucans. The material which remains in the residue is probably tightly bound to the other cell wall components, but the possibility cannot be ruled out that the preparation of the AIS may cause some of the material to become insoluble (Massiot, Rouau & Thibault, 1988). In Sections 3.3 and 3.4 the pectin and hemicellulose-rich fractions are described in more detail.

3.3. Pectin-rich fractions

Total pectins as obtained in the HBSS, ChSS, 0.05 M NaOH and 6 M NaOH fractions hardly changed during ripening of the olive fruit. However, a shift in the relative amounts of the various pectic fractions could be noticed during ripening. The amount of pectic material extracted with hot buffer increased markedly from 40% of the total extractable pectin for green olive AIS to 60% of the total extractable pectin for purple olive AIS. A solubilisation of pectic polymers from the cell wall during ripening has also been described for other fruits (references in Redgwell et al., 1997; Seymour & Gross, 1996; references in Voragen et al., 1995). The amounts of pectic material extracted with CDTA, 0.05 M NaOH and 6 M NaOH all decreased during ripening.

Although the yield of the HBSS fraction increased, the

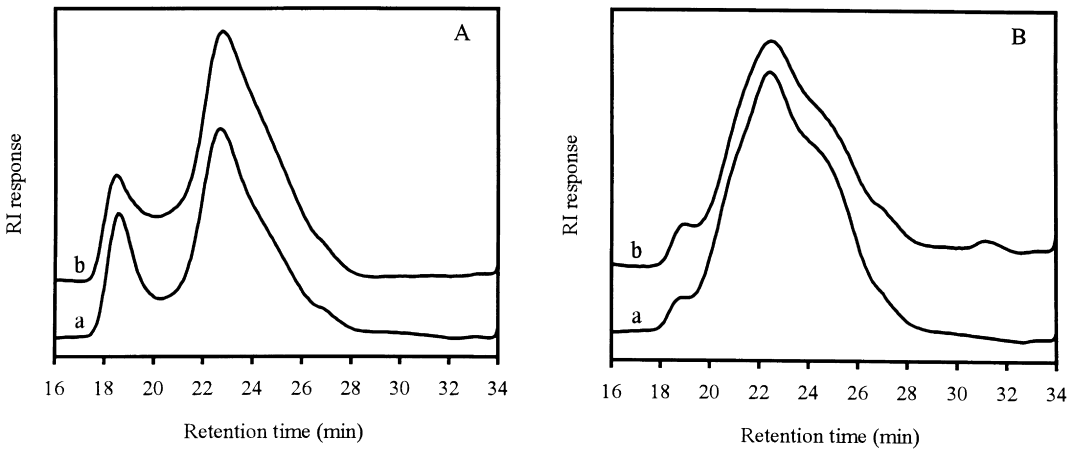

Fig. 2. HPSEC-patterns of the hemicellulose-rich fractions from olive fruit. A: 1 M KOH FI (a), 1 M KOH FIII (b), B: 4 M KOH FI (a), 4 M KOH FIII (b).

sugar composition did not change appreciably during ripening. The HBSS fractions contained 62–65 mol% of uronic acids and arabinose and galactose were the main neutral sugars in these fractions and represented 17 and 10–12 mol%, respectively. The ChSS fractions had an uronic acid content of 57–65 mol% and an arabinose content of 25–28 mol% which was substantially higher compared to the arabinose content of the HBSS fractions. The galactose contents were lower compared to the HBSS fractions. The carbohydrate contents of the samples are rather low because of residual CDTA and other salts (Mort, Moerschbacher, Pierce & Maness, 1991). The pectic polysaccharides solubilised by diluted alkali were relatively rich in arabinose (39–42 mol%) and had a uronic acid content of 40–45 mol%. Also, the 6 M NaOH fractions were relatively rich in arabinose. Besides the sugars characteristic for pectins, these fractions also contained significant amounts of glucose, mannose and xylose.

Calculation of the ratio of galactose and arabinose to uronic acid showed that the pectins extracted with stronger solvents had a higher ratio compared to the more easy extractable pectins. This implied that the degree of branching was higher for pectins extracted with stronger solvents or that the arabinose and galactose side chains of these pectins were longer. The ratios of galactose and arabinose to uronic acid showed only small differences for ripe and unripe olive fruit.

Calculation of the rhamnose to uronic acid ratios showed that pectins from ripe olives had a higher ratio compared to pectins extracted from unripe olives. This agreed with the relative increase in rhamnose and the loss of uronic acid in the cell wall material (AIS) during ripening (Table 1). Assuming that the rhamnose residues are partly substituted, a higher rhamnose to uronic acid ratio might indicate that the pectins in ripe olive fruit were more highly branched.

The HPSEC-elution patterns of the pectin fractions of green and purple olive fruit are shown in Fig. 1. The HBSS fraction showed a broad molecular weight distribution for both ripening stages. The profile of the HBSS fraction extracted from purple olive fruit showed two additional populations compared to the HBSS fraction from green olive fruit. No striking decrease in the molecular mass was found, even though the extractability of pectins with hot buffer increased during ripening. The HPSEC patterns of the CHSS and 0.05 M NaOH fractions mainly showed the presence of polymers with a high molecular mass eluting between 18 and 22 min. The CHSS fractions from both ripening stages had the same HPSEC elution behaviour. The HPSEC profiles of the 0.05 M NaOH fraction extracted from purple olives showed a slight shift to a lower molecular mass compared to the pectic material extracted from green olives with the same solvent. The elution profiles of the fractions extracted with 6 M NaOH showed three populations of polymers of which the relative amounts depended on the ripening stage. The molecular weight distribution shifted towards lower molecular weight ranges during ripening. However, it was not determined whether this shift was due to a degradation of pectic material or due to other polymers that were co-extracted with 6 M NaOH.

The increase in the yield of the hot buffer soluble pectins during ripening was accompanied by a decrease in the pectins extracted with stronger solvents. This may suggest that the additional pectic polymers extracted with hot buffer from ripe olive fruit cell wall material originated from pectic material which needed stronger solvents to be extracted from unripe olive fruit. The additional pectins in the HBSS fraction might originate from cleavage of cross-linkages or from hydrolysis of large pectin molecules during ripening. However, it should be kept in mind that the modification of cell wall material during ripening is a dynamic process in which not only degradation is involved but synthesis of polymers also occurs.

3.4. Hemicellulose-rich fractions

Total hemicellulose as obtained in the 1 and 4 M KOH fractions did not change appreciably during the ripening of

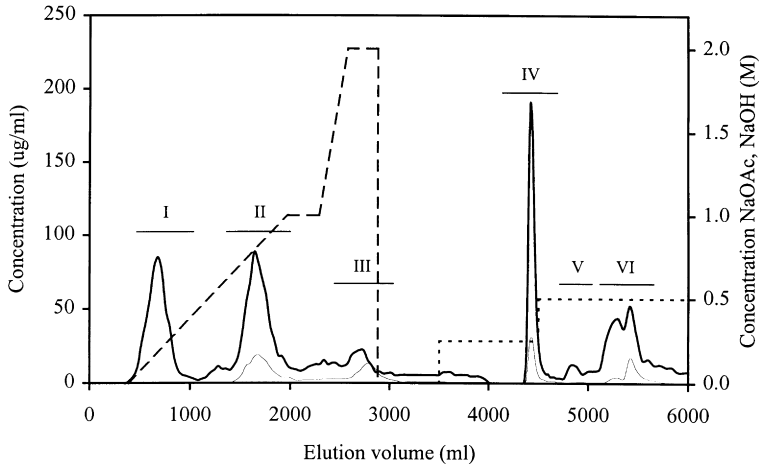

Fig. 3. Elution profile of 1 M KOH FIII on DEAE Sepharose Fast Flow. Elution with NaOAc (– –), elution with NaOH (– – –), neutral sugars (—), uronic acids (—).

the olive fruit (Tables 2 and 3). Also, the amounts of material extracted per fraction were about equal for both ripening stages, although because of some loss of material of the 4 M KOH FI fraction this cannot be completely certified. However, for the variety Frantoio the amounts of material extracted with 1 and 4 M KOH are also about equal for both ripening stages (Huisman et al., 1996).

The most abundant sugar of the 1 M KOH fraction was xylose, which accounted for 66 and 52 mol% of the neutral sugars for the green and purple ripening stage, respectively. Most of the xylose residues probably originated from the backbone of a xylan. The presence of glucose residues indicated that xyloglucans were also a part of this fraction (Coimbra et al., 1994; Gil-Serrano & Tejero-Mateo, 1988; Gil-Serrano et al., 1986).

The sugar composition of the 4 M KOH fraction hardly changed during ripening. This fraction had a low xylose content and a relatively high glucose content compared to the 1 M KOH fractions. The 4 M KOH fractions from both ripening stages also contained 8–10 mol% mannose, suggesting the presence of glucomannans or galactomannans.

The size-exclusion patterns of the 1 and 4 M KOH fractions of the two stages of fruit development are shown in Fig. 2. The elution pattern of the 1 M KOH fractions showed two distinct populations: a small population with a high molecular mass which eluted in the void of the column and a major population eluting around 23 min. No distinct differences could be noticed between the two ripening stages except for small differences in the proportions between the first and second population. The HPSEC-elution patterns of both 4 M KOH fractions indicated that the fractions were heterogeneous in molecular size. The fractions contained a major population which eluted at 23 min, preceded by a small population and a shoulder eluting at about 24–25 min. The molecular weight

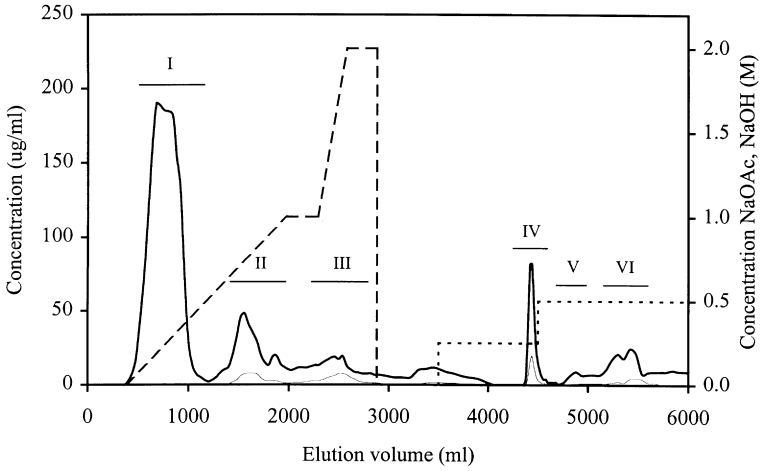

Fig. 4. Elution profile of 4 M KOH FIII on DEAE Sepharose Fast Flow. Elution with NaOAc (– –), elution with NaOH (– – –), neutral sugars (—), uronic acids (—).

Table 4
Yield on sugar basis (%) and sugar composition (mol%) of the DEAE pools of the 1 M KOH FIII fraction from olive fruit (tr = trace amount)

Pool	Yield[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	4-*O*-MeglcA	Carbohydrate content[b]
I (Xyloglucan)	20	1	1	11	31	1	11	43	1	0	0	69
II (Xylan 1)	27	3	0	7	70	tr	5	2	2	4	8	52
III (Pectin)	10	8	0	32	15	1	8	7	28	tr	tr	30
IV (Xylan 2)	16	2	0	1	83	tr	tr	1	2	1	10	78
V (Xylan 3)	3	5	0	1	79	1	tr	3	4	1	6	22
VI (Xylan 4)	24	4	0	9	65	1	3	6	10	tr	2	22

[a] Expressed as % of the sugars recovered in the 6 pools.
[b] Expressed as % (w/w).

distribution of the 4 M KOH soluble fractions did not change during ripening.

Although in olive fruit no ripening associated modifications of hemicellulosic fractions were found by size-exclusion analysis, modifications to lower molecular masses have been documented in several other fruits as tomato, pepper, strawberry and melons. The decrease of molecular mass may result from the modification of existing polymers, but a synthesis of small polymers can also be involved (Fischer & Bennett, 1991; Seymour & Gross, 1996).

Analysis of the fractions showed no marked differences in the sugar composition and molecular weight distribution of the fractions extracted from unripe and ripe olive fruit. Only a solubilisation of the pectic polymers from the cell wall during ripening was observed. Previous work performed by our group has been directed to the characterisation of the pectin-rich fractions isolated from unripe and ripe olive fruit (Huisman et al., 1996). We will continue our research with the characterisation of the hemicellulose-rich fractions isolated from olive fruit at two ripening stages.

3.5. Fractionation of the 1 and 4 M KOH fractions on DEAE Sepharose Fast Flow

The 1 and 4 M KOH fractions of the green and purple ripening stages were applied on a DEAE Sepharose Fast Flow column to be able to distinguish between the various polysaccharides. The elution patterns (sugar content) of the 1 and 4 M KOH fractions isolated from ripe olive fruit are shown in Figs. 3 and 4. The material bound to the column was eluted with a sodium acetate gradient. However, some material was too strongly bound to the column to be eluted with 2 M NaOAc. These populations were eluted from the column with 0.25 and 0.5 M NaOH. The 1 and 4 M KOH fractions isolated from green olive fruit had similar elution behaviours on DEAE and are, therefore, not shown. The alkali fractions were fractionated with good recoveries. Very small amounts of the 1 and 4 M KOH fractions were not soluble (ca. 1–6%) and removed by centrifugation. Sugar composition analysis revealed that these residues contained mainly xylans in addition to some pectic material. The fractions were pooled as indicated and the yields and sugar compositions of the main pools of 1 and 4 M KOH FIII are given in Tables 4 and 5. Corresponding pools with almost equal sugar compositions could be detected in the 1 and the 4 M KOH fractions, although the yields differed.

The unbound fraction (pool I) represented 12–20% of the sugars present in the 1 M KOH fractions and 51–60% of the 4 M KOH fractions. Pool I consisted mainly of neutral polysaccharides besides small amounts of pectic material. The presence of glucose, xylose, arabinose and galactose gave an indication for arabinogalactoxyloglucans in this pool. Fucose was only present in very small amounts (1 mol%). Fucose has been found as a terminal residue of β-D-Gal*p*-(1,2)-α-D-Xyl*p*-(1,6)-side chains of xyloglucans isolated from rapeseed hulls, apple and onion (Redgwell & Selvendran, 1986; Renard, Lomax & Boon, 1992; York, Van Halbeek, Darvill & Albersheim, 1990). Based on the xylose to glucose ratio of 3/4 we expect in the xyloglucan-rich pool isolated from the 1 M KOH fraction a xyloglucan

Table 5
Yield on sugar basis (%) and sugar composition (mol%) of the DEAE pools of the 4 M KOH FIII fraction from olive fruit (tr = trace amount)

Pool	Yield[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	GlcA	4-*O*-MeglcA	Carbohydrate content[b]
I (Xyloglucan)	61	1	1	8	26	10	11	43	tr	0	0	96
II (Xylan 1)	13	2	tr	8	65	1	5	7	1	0	7	57
III (Pectin)	9	5	tr	29	18	2	7	12	26	tr	tr	11
IV (Xylan 2)	7	2	0	1	78	tr	tr	1	3	2	12	39
V (Xylan 3)	2	4	0	2	65	3	tr	7	6	2	10	12
VI (Xylan 4)	8	3	0	6	68	2	1	5	11	1	3	21

[a] Expressed as % of the sugars recovered in the 6 pools.
[b] Expressed as % (w/w).

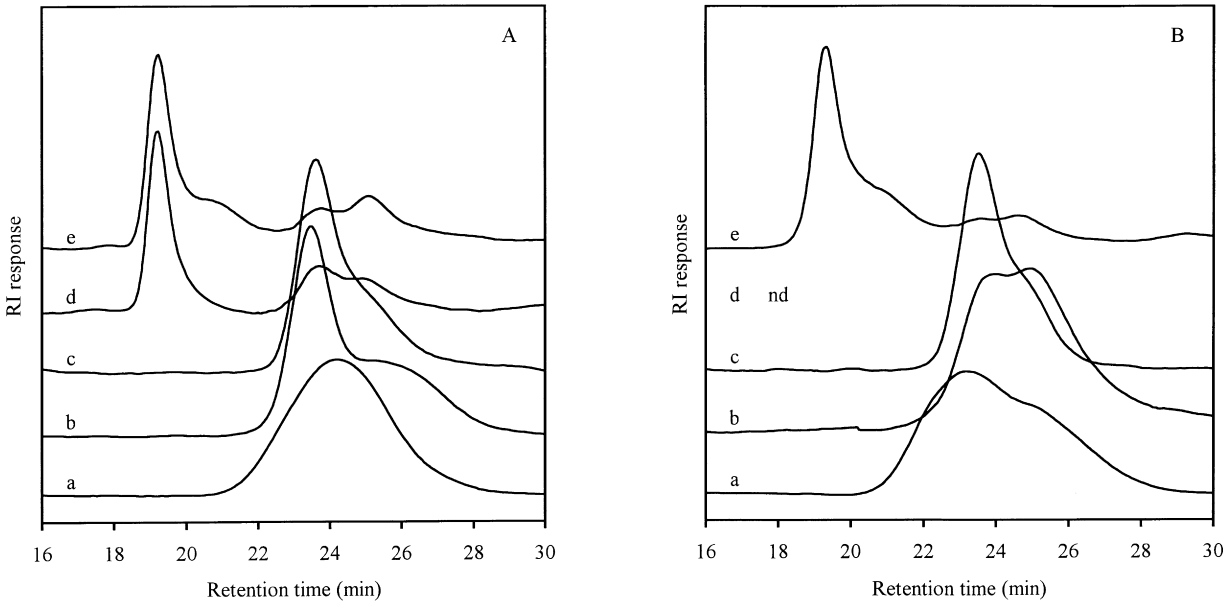

Fig. 5. HPSEC-patterns of the DEAE pools of 1 M KOH FIII (A) and 4 M KOH FIII (B). Pool I (a), pool II (b), pool IV (c), pool V (d) and pool VI (e) nd = not determined.

with a XXXG core which has clusters of three out of four glucose residues branched with xylose residues. Arabinose and galactose were present in equal amounts (11 mol%). The xylose to glucose ratio of the xyloglucan-rich pool isolated from 4 M KOH is 2.4/4. This may indicate that the xyloglucans solubilised by 4 M KOH were less branched than those solubilised by 1 M KOH. Ryden and Selvendran (1990) have also found that less-branched potato xyloglucans require stronger alkali (4 M KOH) for solubilisation and presume that these xyloglucans are more strongly associated with cellulose microfibrils. The difference in the xylose to glucose ratio, which we have found, may also be explained from the fact that not all glucose has to be present in xyloglucans but may also be present in glucomannans. Unfortunately, based on the sugar composition alone it is difficult to draw conclusions on the branching patterns of the xyloglucans in the olive fruit. More information about the substitution patterns of the xyloglucans isolated from the olive fruit can be obtained by incubation with specific endoglucanases. The results of these experiments will be described in a following paper.

The HPSEC elution profiles of the xyloglucan-rich fractions are shown in Fig. 5. The elution patterns of the xyloglucan-rich pools from the 1 M KOH fractions of both ripening stages showed a major population with a molecular mass of about 150 kDa as based on calibration with dextrans. The xyloglucan-rich pools from the 4 M KOH fractions consisted of two populations: a major population of about 150 kDa and in addition a smaller population of about 50 kDa.

Pools II, IV, V and VI consisted mainly of xylans and were designated according to their elution order from the anion-exchange column as xylan 1, 2, 3 and 4, respectively. Xylan 1 was the major xylan-rich fraction of DEAE chromatography and eluted as soon as the acetate gradient was applied. It represented about 40% of the xylans of the 1 and 4 M KOH fractions. The yields of the xylan-rich pools were almost identical for both ripening stages except for the minor fraction xylan 3 which was relatively more abundant in the unripe olive fruit compared to the ripe olive fruit for as well the 1 and 4 M KOH fraction.

Methanolysis with 2 M HCl followed by TFA hydrolysis and enzymatic hydrolysis with (4-*O*-methyl)-α-glucuronidase was used to determine the relative amounts of GalA, GlcA and 4-*O*-MeGlcA of the xylan-rich pools. De Ruiter et al. (1992) have shown that methanolysis with 2 M HCl followed by TFA hydrolysis results in a complete hydrolysis of the very acid resistant uronic acid glycosyl linkages in pectic material. However, methanolysis combined with TFA hydrolysis appeared not to be sufficient for a complete hydrolysis of the glycosyl linkages present in the xylan-rich pools. On HPAEC not only peaks corresponding to monosaccharides but an additional peak resulting from incomplete hydrolysis of the material was also shown. This peak most certainly consisted of the aldobiuronic acid (4-*O*-Me)-GlcA-Xyl because methanolysis combined with TFA hydrolysis followed by incubation with (4-*O*-methyl)-α-glucuronidase resulted in complete hydrolysis of the material.

The quantification of the amount of uronic acids by HPAEC resulted in a significantly lower amount of uronic acids compared to that of sulphuric acid hydrolysis followed by the colorimetric *m*-hydroxy diphenyl assay. Only xylan 4 that contained mainly GalA showed no discrepancy between the two methods. Also, for the pectin-rich fraction (pool III) which contained mainly GalA good results were

obtained. The lack of good standards, the response of GlcA was used to quantify the amount of 4-*O*-MeGlcA could not explain this huge difference (factor 2–3 lower values). The analysis of the neutral sugars on HPAEC agreed well with the amounts of neutral sugars determined as alditol acetates (not shown). In Tables 4 and 5 the uronic acid content determined by the *m*-hydroxy-diphenyl assay is used to express the total amount of uronic acid in the samples. This method was also used to determine the amount of uronic acids of the AISs and the pectin and hemicellulose-rich fractions. The relative amounts of the uronic acids were calculated from the HPAEC analysis.

The ratio of the uronic acids (GalA/GlcA/4-*O*-MeGlcA) differed for each xylan-rich pool. The uronic acids of xylan 1 comprised GlcA and 4-*O*-MeGlcA but also a small amount of GalA from the pectic material that co-eluted. Xylans 2 and 3 contained as xylan 1 mainly 4-*O*-MeGlcA, whereas the uronic acids in xylan 4 appeared to be almost exclusively GalA indicating the presence of pectins. Coimbra et al. (1994) have determined by ^{13}C NMR analysis that most of the GlcA residues of xylans extracted with 1 M KOH from olive fruit carry a *O*-methyl substituent on C-4 and are linked to 1 in 11 of the xylose residues. These results corresponded well with our data except for xylan 1 which was relatively rich in GlcA compared to the other pools (GlcA/4-*O*-MeGlcA ratio is 1/3).

The differences in elution behaviour on DEAE Sepharose of the various xylan-rich pools could not be explained by the (4-*O*-MeGlcA/GlcA) to xylose ratios which were between 5 and 12. A more blockwise distribution of the 4-*O*-MeGlcA or GlcA residues may explain the differences in elution of the various xylans present in the alkali fractions, but other factors may also be involved. Compared to the other xylan-rich pools xylans 1 and 4 contained relatively more arabinose, which may be linked to the xylan backbone. However, these arabinose residues may also originate from pectic material, especially since xylan 4 had a high GalA content.

The HPSEC elution profiles of the xylan-rich fractions are shown in Fig. 5. It appeared that not only the sugar composition of the pools eluting under the same salt conditions resembled well but most of the HPSEC elution patterns were also quite similar. An exception was the elution pattern of xylan 1 from the 4 M KOH FIII fraction compared to the other xylan 1 pools. The elution profiles of xylan 1 showed a major population of about 150 kDa and a small population with a lower molecular mass (about 25 kDa). Only xylan 1 isolated from the 4 M KOH FIII fraction contained both populations in equal amounts. The difference in elution behaviour on HPSEC cannot be explained. Xylan 2 consisted in all the cases of two populations and had an elution pattern quite similar to xylan 1. Xylans 3 and 4 contained a population, which eluted in the void of the column set used and may not represent a distinct population. The molecular mass of these populations was at least 500 kDa. The high molecular mass of the xylans in these pools may be explained by the aggregation of rather linear xylans.

Pool III contained material of mainly pectic origin as was illustrated by the relatively high proportion of rhamnose residues and galacturonic acid.

This study showed that the yield of buffer soluble pectin increased in ripening olive fruit and was accompanied by a diminution of the pectins extracted with stronger solvents (CDTA, 0.05 M NaOH and 6 M NaOH). No major changes were observed in the composition of the hemicellulose-rich fractions. The elution patterns on DEAE Sepharaose Fast Flow were identical for both ripening stages. A xyloglucan-rich fraction and four xylan-rich fractions were obtained which exhibited similar molecular weight distributions and sugar compositions for unripe and ripe olive fruit.

Future research will be directed to a more detailed characterisation of the xyloglucan-rich and xylan-rich fractions by methylation analysis and degradation of the fractions with specific enzymes.

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