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Dimeric calcium complexes of arabinan-rich pectic polysaccharides from *Olea europaea* L. cell walls

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Abstract

The study carried out in this work concerns the pectic polysaccharides of olive cell walls as present in olive pulp and that remained entrapped in the cellulosic residue after sequential extraction of the cell wall material (CWM) with imidazole, carbonate and KOH aqueous solutions. These polymers, obtained after neutralisation and dialysis of an aqueous suspension of the residue (sn-CR fraction), extracted with 4 M KOH, were arabinan-rich pectic polysaccharides. They accounted for 11–19% of the total pectic polysaccharides found in the olive pulp cell walls of fruits collected in two years and in three stages of ripening (green, cherry and black). The analysis by powder X-ray diffraction highlighted the existence, in all sn-CR fractions, of crystalline phases related with the presence of calcium-pectic polysaccharide complexes (CPPC) occurring in an amorphous carbohydrate network. The relative crystallinity of the CPPC varied linearly with the Ca^{2+}/GalA molar ratio until a maximum of 0.57. Size-exclusion chromatography showed that sn-CR fractions possessed a bimodal molecular weight distribution. The lower molecular weight fraction of sn-CR ($M_w = 70$–135 kDa) was independent on the ripening stage of olive fruit, whereas the higher molecular weight fraction showed values of 1.1, 0.6–0.9 and 0.5–0.7 MDa, respectively, for green, cherry and black olives. Treatment of the sn-CR pectic polysaccharides with a 2 M imidazole solution disrupted the CPPC crystalline network showing the loss of low molecular weight galacturonan-rich material during dialysis (12–14 kDa cut off) and the decrease of molecular weight of the polymers to roughly half. These results allowed to infer the presence of oligogalacturonides held within cell walls by calcium ions and that the pectic polysaccharides of sn-CR fraction occurred in olive pulp cell walls as calcium bridged macrodimers.

Keywords: Pectic polysaccharides; Calcium–pectin complexes, Olive; Crystallinity

1. Introduction

Arabinan rich pectic polysaccharides have been reported as one of the major classes of polysaccharides found in olive pulp cell walls (Coimbra, Waldron, & Selvendran, 1994; Huisman, Schols, & Voragen, 1996; Mafra et al., 2001; Mafra et al., 2006; Vierhuis, Schols, Beldman, & Voragen, 2000). Arabinose (Ara) occurs as side chains, linked to the *C-4* of α-(1 → 2)-linked L-rhamnose residues that exist interspersed in the α-(1 → 4)-linked galacturonic acid (GalA) backbone. The GalA residues can bind to calcium ions allowing the pectic polysaccharide chains to assemble by calcium bridges. Ca^{2+}-GalA complexes are commonly found in the middle lamella, playing an important role in cell–cell adhesion and cohesion. Tissue firmness is considerably reduced by Ca^{2+} removal (Parker & Waldron, 1995). As well as calcium, boron is an essential inorganic constituent of plant cell wall pectic polysaccharides (Hu & Brown, 1994). Borate diester-bonding of type-II rhamnogalacturonans (RG-II), forming RG-II dimers,

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promotes cross-linking sites for pectic polysaccharides through apiosyl residues (O'Neill et al., 1996). The interaction of GalA with Ca^{2+} has been reported to have a reinforcing role in the stabilisation of the boron–RG-II complex (Ishii et al., 1999; Kobayashi, Nakagawa, Asaka, & Matoh, 1999), approaching the RG-II monomers for RG-II dimerisation with boron (Perez, Rodríguez-Carvajal, & Doco, 2003).

The sequential extraction of pectic polysaccharides from plant cell walls implies the use of strong chelating agents such as CDTA or imidazole to remove Ca^{2+}-linked pectic polysaccharides. The polysaccharides in these extracts correspond, in the case of olive pulp (Coimbra et al., 1994; Mafra et al., 2001; Mafra et al., 2006), as well as in other fruits, such as apple (Renard, Voragen, Thibault, & Pilnik, 1991), to less than 50% of the total cell wall pectic polysaccharides. A fraction rich in pectic polysaccharides can be also obtained after mild de-esterification of plant cell walls with aqueous dilute carbonate solutions. These carbonate soluble polysaccharides are, however, more branched than those solubilised with the chelating agents (Selvendran, 1985). Additional pectic polysaccharides can be extracted from the residue by sequential extraction with stronger alkali solutions of 0.5, 1 and 4 M KOH (Coimbra, Delgadillo, Waldron, & Selvendran, 1996; Selvendran & Ryden, 1990). However, the final cellulosic residue (CR) still contains variable amounts of highly branched pectic polysaccharides. By collecting the supernatant obtained after neutralisation and dialysis of the alkali extracted residue it is possible to recover some of the CR pectic material (Coimbra et al., 1994). As these pectic polysaccharides enmeshed within the cellulose matrix (sn-CR), to our knowledge, have never been studied, the aim of this paper is to contribute to the elucidation of the structural features of these polymers obtained from olive pulp cell walls. Olive fruits collected in two different harvests (years) and in different stages of ripening were used in this work.

2. Materials and methods

2.1. Samples

Olive fruits (*Olea europaea* L. cv 'Negrinha do Douro') harvested in 1997 and 1998 with an average length of 2.0 cm and a diameter of 1.2 cm were provided by Maçarico Lda, Praia de Mira, Portugal, at different stages of ripening: mature green (2.1 and 2.9 g pulp/fruit), changing colour (cherry – 2.3 and 3.2 g pulp/fruit), and mature black (3.2 and 3.9 g pulp/fruit) for harvests of 1997 and 1998, respectively.

2.2. Preparation of the cell wall material and sequential extraction

The cell wall material (CWM) was prepared according to the method described by Mafra et al. (2001). The CWM was sequentially extracted with imidazole, Na_2CO_3, and KOH solutions of increasing strength (0.5 M KOH + 20 mM $NaBH_4$, for 2 h at 4 °C; 1 M KOH + 20 mM $NaBH_4$, for 2 h at 4 °C; 1 M KOH + 20 mM $NaBH_4$, for 2 h at 20 °C; 4 M KOH + 20 mM $NaBH_4$, for 2 h at 20 °C and 4 M KOH + 3.5% H_3BO_3 + 20 mM $NaBH_4$, for 2 h at 20 °C) to leave a final cellulosic residue (CR). The CR obtained after the alkali extractions was suspended in water, acidified (pH 5–6) with glacial acetic acid and dialysed against distilled water. The supernatant from the dialysis of the CR (sn-CR) was collected separately from the residue by centrifugation and filtration. The sn-CR fraction was then concentrated under reduced pressure, frozen under liquid nitrogen, and freeze-dried.

2.3. Carbohydrate analysis

Neutral sugars were released by Saeman hydrolysis (Selvendran, March, & Ring, 1979) and analysed as their alditol acetates by gas chromatography (Blakeney, Harris, Henry, & Stone, 1983; Harris, Blakeney, Henry, & Stone, 1988) using a Carlo Erba 6000 with a split injector (split ratio 1:60) and a flame ionisation detector as described by Mafra et al. (2001). The column was a DB-225 (J & W, USA) with 30 m × 0.25 mm and film thickness of 0.25 μm and the oven temperature program was: 220 °C for 5 min and then the temperature was raised at 20 °C min^{-1} to 230 °C and maintained at this temperature for another 6 min. The flow rate of the carrier gas (H_2) was set at 1 mL min^{-1} at 220 °C. The injector temperature was 220 °C and the flame ionisation detector temperature was 230 °C. The hydrolysis of all samples was done in triplicate and each one was injected twice. Results with less than 5% variability in the major component cell wall sugars were obtained. Uronic acids were determined colorimetrically by a modification (Coimbra et al., 1996) of the method of Blumenkrantz and Asboe-Hansen (1973). Samples were prepared in triplicate by hydrolysis in 0.2 mL 72% H_2SO_4 for 3 h at 20 °C followed by 1 h in 1 M H_2SO_4 at 100 °C. Calibration was made with D-galacturonic acid. As the FT-IR spectra of the samples were characteristic of pectic polysaccharides (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998), the uronic acids determined were quantitatively accounted as galacturonic acid.

2.4. Size-exclusion chromatography

For determination of the average molecular weight (M_w) by size-exclusion chromatography (SEC), the sn-CR samples were dissolved in an aqueous 0.1 M $NaNO_3$ solution (0.4% w/v). A PL-GPC 110 chromatograph was equipped with a pre-column PLaquagel-OH 15 μm and two SEC columns in series (PLaquagel-OH40 15 μm, 300 × 7.0 mm and PLaquagel-OH60 15 μm, 300 × 7.0 mm). The pre-column, the SEC columns, the injection system, and the refractive index detector were maintained at 36 °C. The eluent (aqueous 0.1 M $NaNO_3$ solution) was pumped at a flow rate of 0.9 mL min^{-1}. The analytical columns were calibrated with

pullulan standards (Polymer Laboratories, UK) in the range of 5.8–1600 kDa. Since the pectic polysaccharides present in the sn-CR fractions are not linear, the molecular weight calculated based on the retention time of pullunan can be overestimated. Anyway, using this methodology, the relative values of the polymers in the samples can be compared.

2.5. Calcium determination

The samples were dispersed in HNO_3 65% (w/w) to a final concentration of pectic polysaccharide sample of 1 mg L^{-1} and heated at 150 °C until the total evaporation of the acid. The residue obtained was then dissolved in 10 mL of a 4% (w/v) HNO_3 solution. Three replicates were prepared for each sample as well as three blanks. The calcium content was then estimated by inductively coupled plasma (ICP)-optical emission spectroscopy (OES) in a Jobin Yvon JY 70 plus with cyclonic spray chamber and Burgener Mira Mist nebuliser. The final concentration was the average of concentrations determined taking in consideration the absorbance of the samples at 396.487 and 422.673 nm.

2.6. Powder X-ray diffraction

The freeze-dried samples of imidazole, carbonate and sn-CR fractions were submitted to a pressure of 800 MPa for 10 min giving rise to homogeneous pellets of 1.2 cm diameter that were screened for crystalline patterns by Powder X-ray diffraction (XRD). XRD data were collected on a X'Pert MPD Philips diffractometer, with a flat-plat sample holder, in a Bragg-Brentano para-focusing optics configuration and using Cu-Kα X-radiation with a constant incidence area of 100 mm^2. Intensity data were collected by the step-counting method (step width, 0.05°/scan) in a range of 2θ 3°–50°. The identification of crystalline phases was made by the Hanawalt method using the *International Centre for Diffraction Data* database PDF-4 (2004).

2.7. De-complexation of pectic polysaccharides

The samples were dispersed in a 2 M imidazole solution to a final concentration of 1 mg mL^{-1}, the pH was adjusted to 7.0 and left under stirring during 24 h at 4 °C. The suspension was then dialysed and frieze-dried. The efficiency of the de-complexation process in disrupting the crystalline structure was checked by X-ray diffraction analysis.

3. Results and discussion

3.1. Sugar composition of sn-CR fractions

Table 1 shows the sugar composition of the pectic polysaccharides fraction enmeshed in olive pulp cellulosic matrix (sn-CR). These are the pectic polysaccharides that remained trapped in the cellulosic network after the sequential removal of the cell wall polymers with imidazole, carbonate and KOH aqueous solutions (Coimbra et al., 1994). Three ripening stages classified, as described by Mafra et al. (2001, 2006), according to the colour of the skin, i.e., green, cherry, and black, and from two harvests (1997 and 1998), were studied. The amount of material recovered in the sn-CR fractions represented 3.4–4.8% of total cell wall material (CWM) and contained 64–79% of polysaccharides, mainly composed of Ara (53-71 mol%) and GalA (17–38 mol%) residues. Galactose (Gal, 4–8%), rhamnose (Rha, 1–4%), xylose (Xyl, 1–2%), and glucose (Glc, 1%) residues were present in small proportions. In both harvests, the ratios Ara/GalA and Gal/GalA (1.4–1.6 and 0.11–0.20, respectively) were similar in green and cherry samples, but both ratios were higher in black samples (2.5–4.2 and 0.32–0.47, respectively). These results suggest that the sn-CR pectic polysaccharides of green and cherry samples contain more neutral sugars than those of black samples. The (Ara + Gal)/Rha ratio was higher in 1998 (36–47) than in 1997 samples (13–27). Assuming that the Rha residues are partly substituted by Ara and Gal

Table 1
Sugar composition and calcium content of the olive pulp sn-CR fractions

sn-CR fraction	Yield[a] (%)	Relative amount[b] (%)	Ca^{2+} (% w/w)	RCI (%)	Cell wall sugars (mol%)						Total sugars[c] (mg g^{-1})
					Rha	Ara	Xyl	Gal	Glc	GalA	
Green olives											
1997	4.7	19 (8)	0.187 (14)	36	3 (17)	56 (1)	1 (18)	4 (2)	1 (12)	34 (6)	785 (7)
1998	4.3	11 (5)	0.164 (7)	47	2 (15)	54 (7)	1 (24)	7 (2)	1 (5)	36 (10)	640 (5)
Cherry olives											
1997	3.4	15 (2)	0.346 (1)	77	4 (19)	53 (5)	1 (19)	4 (14)	1 (9)	38 (3)	758 (2)
1998	4.5	13 (2)	0.521 (2)	100	1 (23)	57 (6)	1 (9)	7 (1)	1 (24)	35 (9)	687 (6)
Black olives											
1997	4.1	11 (6)	0.067 (22)	11	3 (18)	62 (1)	2 (15)	8 (2)	1 (14)	25 (5)	700 (8)
1998	4.8	12 (4)	0.095 (11)	36	2 (12)	71 (1)	1 (14)	8 (13)	1 (11)	17 (9)	760 (6)

Values in brackets are the coefficients of variation obtained from three independent replicates.
[a] Yield is expressed as grams of material per 100 g of CWM.
[b] Percentage of sn-CR pectic polysaccharides in relation to total pectic polysaccharides in the olive pulp cell wall.
[c] Values are expressed as milligrams of anhydrous sugar per gram of dry polymer.

residues, and that the substitution of Rha do not vary with the year of sampling and ripening stage of the fruits, a higher (Ara + Gal)/Rha ratio might indicate that the samples from 1998 contained, in average, longer side chains than those from 1997. For both years, the values of the (Ara + Gal)/Rha ratio were higher in black than in green olives, showing that the neutral side chains could be, in average, longer in the fractions from ripe olives. Further research, such as linkage analysis is still required for a definitive statement about the length of the chains and the degree of branching of these pectic polysaccharides. The ratio GalA/Rha was 9–10 for all samples of the 1997 harvest, however, in samples of 1998, this ratio varied from 21 to 24 in green and cherry to 10 in black olives. This finding seems to indicate that no changes occurred in the relative amount of RG-I and homogalacturonan of these pectic polysaccharides in 1997 samples while in 1998 seems to indicate a decrease of the relative amount of homogalacturonan from cherry to black.

The amount of pectic polysaccharides present in sn-CR fractions corresponded to 11–19% of the total pectic polysaccharides found in the olive pulp cell walls (Table 1). To achieve these numbers, the values previously published by Mafra et al. (2001, 2006) concerning the characterisation of the imidazole, carbonate and KOH extracts from the CWM of the olive pulp samples here studied, were considered. In the olives of 1997, the relative amount of sn-CR pectic polysaccharides decreased with the ripening stage from 19% in green, to 15% in cherry, and to 11% in black olives. However, in the samples of 1998 the relative amount of sn-CR pectic polysaccharides did not vary significantly.

3.2. Calcium quantification and powder X-ray diffraction analysis

The analysis of sn-CR fractions by inductively coupled plasma (ICP) revealed the presence of Ca^{2+} (Table 1). This was only observed in sn-CR fractions, as the dialysed imidazole and carbonate extracts did not show the presence of calcium. Variable percentages of Ca^{2+} were obtained according to the stage of ripening and harvest: 0.19% and 0.16% in green samples, 0.35% and 0.52% in cherry, and 0.067% and 0.095% in black olives of 1997 and 1998 harvests, respectively. The samples with higher percentage of Ca^{2+} were those from cherry olives and those with lower percentage of Ca^{2+} were those from black olives.

To verify the contribution of Ca^{2+} to the crystallinity of the samples, the analysis by powder X-ray diffraction was performed in all of the pectic polysaccharide extracts. The crystallinity was estimated by the sum of the intensities of the peaks from the sn-CR diffractograms that corresponded to crystalline phases. The imidazole and carbonate extracts did not present any crystalline phases. Conversely, all sn-CR fractions exhibited diffractograms (Figs. 1a and b) with a pattern of crystalline phases corresponding to 2θ angles of 29.6°, 36.2°, 39.5°, 43.3°, 47.7° and 48.6°. According to the International Centre for Diffraction Data database (2004), these 2θ angles show crystalline analogy to calcium carbonate. In these samples, the reflections are likely to belong to calcium carboxylates. The broad band spanning at lower 2θ angles (3°–25° 2θ angles, left side of the diffractograms), represent the amorphous halo from sn-CR pectic polysaccharides that did not show crystalline structural organisation.

By plotting the percentage of Ca^{2+} present in the sn-CR extracts (Table 1) against the homology of the obtained diffractograms with the pattern of $CaCO_3$ (Fig. 2), a logarithmic correlation seems to exist between these two parameters ($R^2 = 0.89$). It is, therefore, possible to observe that the homology with the standard pattern is becoming less dependent on the concentration with the increase of the Ca^{2+} percentage, suggesting that the crystallinity does not depend exclusively on this parameter.

Considering as 100% relative crystallinity the sample with the higher value (1998 cherry sn-CR fraction), a relative crystalline index (RCI) was established for all samples

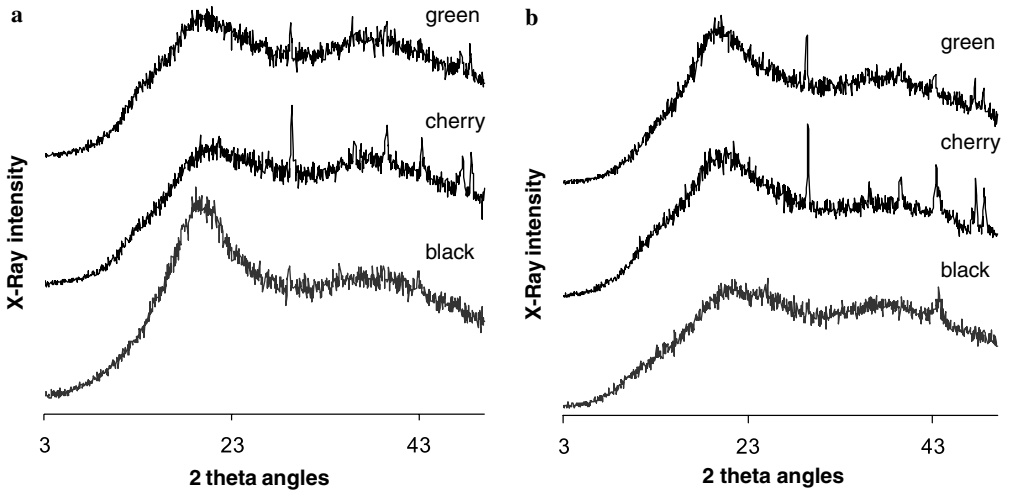

Fig. 1. Powder X-ray diffraction pattern of the sn-CR samples from the three stages of ripening (green, cherry and black). (a) 1997 samples and (b) 1998 samples.

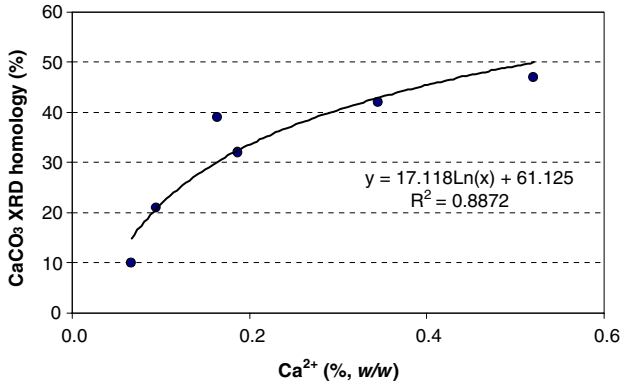

Fig. 2. Percentage of X-ray diffractograms homology of the sn-CR samples with $CaCO_3$ vs. the percentage of Ca^{2+} (w/w) in the samples.

(Table 1). The RCI of the sn-CR pectic polysaccharides showed the same trend with ripening in both harvests: an increase from green to cherry and a decrease from cherry to black. As was observed for the calcium content, the samples with higher crystallinity were those from cherry olives and those with lower crystallinity were those from black olives.

By plotting the RCI of the samples against their percentage of Ca^{2+} (Fig. 3a) it is possible to observe, even though exhibiting a low linear correlation ($R^2 = 0.93$), a direct proportionality between crystallinity and Ca^{2+} content. When considering the Ca^{2+}/GalA molar ratio (Fig. 3b) a similar pattern was observed for the points below the 100% crystallinity, but with a much stronger correlation ($R^2 = 0.97$). The application of the equation given by the linear model ($y = 2033.9x - 14.95$) showed that the maximum relative crystallinity (100%) is reached for a Ca^{2+}/GalA molar ratio of 0.057, suggesting that for higher values, such as the cherry sample from 1998 harvest, the crystallinity remained unchanged. The higher correlation observed for the RCI vs. Ca^{2+}/GalA plot when compared to the RCI vs. Ca^{2+} shows that the crystallinity of the samples was more dependent on the Ca^{2+}/GalA molar ratio than on the Ca^{2+} percentage. This reinforces the hypothesis of the occurrence in the sn-CR fractions of complexes of calcium organised in a crystalline network given by the X-ray analysis. Since the calcium-GalA complexes are known to contribute positively to the structural integrity of the plant cell walls, the identification of such structural arrangements of the pectic polysaccharides recovered in the sn-CR fraction may help to understand their stability and resistance to extraction.

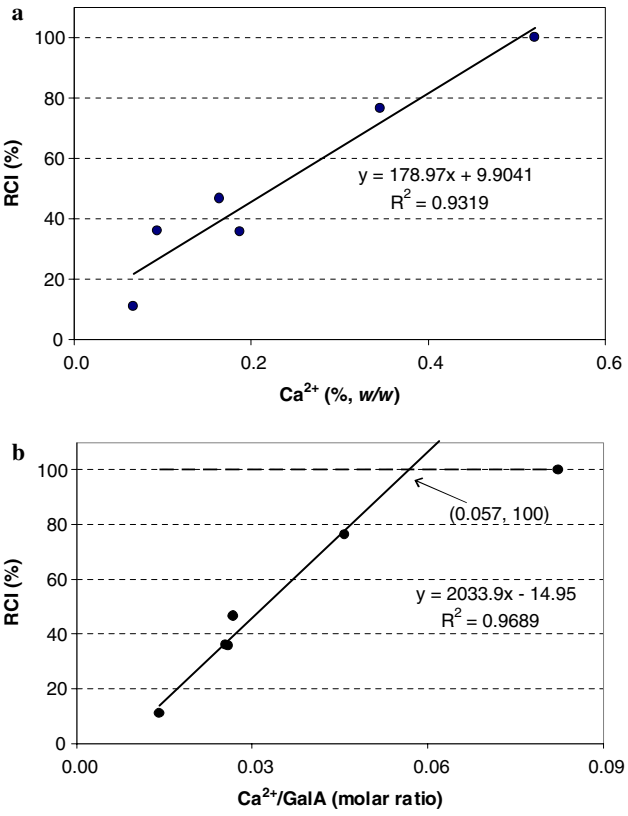

Fig. 3. Relative crystalline index (RCI) vs. the (a) percentage of Ca^{2+} (w/w) in the samples and (b) Ca^{2+}/GalA molar ratio of sn-CR fractions.

The dependency of the crystallinity of the samples with the Ca^{2+}/GalA molar ratio seems to be in accordance with the hyperbolic relation of calcium concentration in the inhibition of cucumber tissue softening observed by McFeeters and Fleming (1989). During the olive ripening, the loss of firmness of the tissues of the pulp is observed (Georget, Smith, & Waldron, 2001), and this seems to be related to the increase of solubilisation of cell wall polysaccharides that have been reported to occur in olive pulp (Huisman et al., 1996; Mafra et al., 2001; Vierhuis et al., 2000). The increase of crystallinity found between the green and cherry stages may be explained by the decrease of the degree of methylesterification of olive pulp cell wall pectic polysaccharides with ripening (Mafra et al., 2001), which would result in an increase of polymeric GalA residues available for complexation. The lower calcium percentage found in the sn-CR fractions of black olives may be related to the lower GalA relative content, when compared to the other samples (Table 1). These samples were also those with higher (Ara + Gal)/Rha ratios. It is possible that the lower relative abundance of GalA associated with higher amount of neutral sugar side chains might contribute to the difference in crystallinity found from cherry to black stages. However, the decrease of the molecular weight of these pectic polysaccharides with ripening (Huisman et al., 1996; Jiménez et al., 2001; Mafra, 2002; Vierhuis et al., 2000), interfering in their organisation in the gel network, might also be important to explain these differences. To study this hypothesis, the molecular weight of the polymers was estimated employing size-exclusion chromatography.

3.3. Molecular weight estimation

The analysis of sn-CR fractions by size-exclusion chromatography shows that they possess a bimodal molecular weight distribution (Fig. 4). M_w of sn-CR polysaccharides

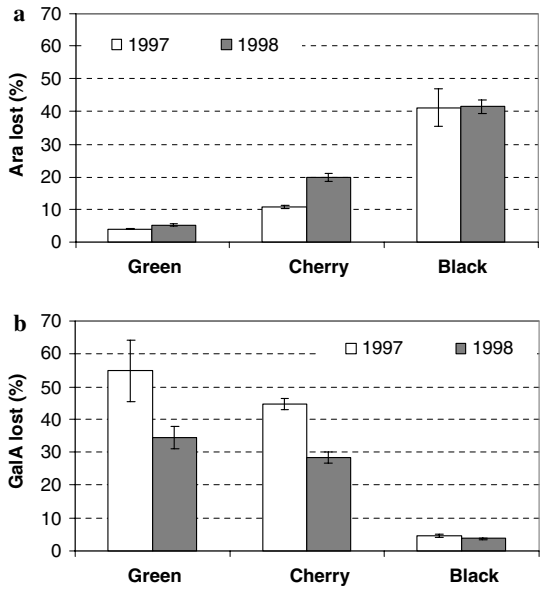

Fig. 4. Percentage of sugars lost by de-complexation and dialysis of the pectic polysaccharides from sn-CR fractions. (a) Arabinose residues; (b) galacturonic acid residues.

were similar in both harvests, but varied according to the stage of ripening (Table 2). In the green stage, the higher molecular weight fraction of sn-CR was 1054–1072 kDa and corresponded to 79–80% of the pectic polysaccharides. That fraction decreased to 47–66% in cherry and black samples of both harvests, accompanied by a decrease in their M_w, which was more pronounced in the samples of 1997 (606 and 543 kDa for cherry and black, respectively) than in the samples of 1998 (882 and 703 kDa for cherry and black, respectively). The less abundant low molecular fraction of sn-CR showed a M_w of about 100 kDa (70–135 kDa), which did not seem to vary with ripening. This fraction corresponded to 20–21% of total sn-CR pectic polysaccharides in the green stage and 35–53% in the cherry and black stages.

Table 2
Molecular weight of olive pulp sn-CR pectic polysaccharides before and after treatment with imidazole

sn-CR fraction	Native		Ca^{2+}-free material	
	M_w (kDa)	Abundance (%)	M_w (kDa)	Abundance (%)
Green olives				
1997	1072	79	737	73
	100	21	62	27
1998	1054	80	659	80
	135	20	53	20
Cherry olives				
1997	606	64	444	60
	78	36	49	40
1998	882	65	475	58
	97	35	59	42
Black olives				
1997	543	47	382	80
	70	53	49	20
1998	703	66	329	72
	103	34	58	28

The M_w decrease of the sn-CR pectic polysaccharides with ripening is consistent with the behaviour already observed for olive polysaccharides found in other pectic fractions (Huisman et al., 1996; Jiménez et al., 2001; Mafra, 2002; Vierhuis et al., 2000), as well as in other fruits, such as the melon (McCollum, Huber, & Cantlife, 1989), the apple (Hobbs, Easterbrook, & Melton, 1991), the papaya (Paull, Gross, & Qiu, 1999) and the grape (Yakushiji, Sakurai, & Morinaga, 2001).

Considering the occurrence of Ca^{2+}-GalA complexes in these pectic polysaccharides, to estimate the M_w of the polymers, the fractions were treated with imidazole, a reagent that is able to disrupt the complexes by the removal of calcium ions (Mort, Moerschbacher, Pierce, & Maness, 1991). The disruption of the crystalline structure of the sn-CR pectic polysaccharides by the applied de-complexation procedure was confirmed by X-ray diffraction. The Ca^{2+}-free material obtained after dialysis of the imidazole treated fractions corresponded to 72–80% of the initial material (Table 3). From the amount of polymeric material and polysaccharides determined before (Table 1) and after de-complexation (Table 3), a loss of 18–35% of polysaccharides can be estimated to occur during this procedure. This loss of polysaccharides is 18–28% for the green and cherry stages, and 34–35% for the black stage. Also, for the green and cherry stages of both harvests, an increase in the molar percentage of Ara and a decrease of GalA in the Ca^{2+}-free material (Table 3) were observed when compared to the native material (Table 1). In the black stage there was a decrease in the percentage of Ara residues accompanied by an increase of GalA. The amounts of Ara and GalA in the samples in the three stages of ripening (Fig. 5) evidence that the diffusion of oligosaccharides during dialysis (12–14 kDa cut off) due to the de-complexation of these pectic polysaccharides resulted in the preferential loss of galacturonan-rich material for the samples in the early stages of ripening, and in the preferential loss of arabinan-rich material for the samples in the late stage of ripening. The release of oligogalacturonides is involved with the ripening process of fruits (Ridley, O'Neill, & Mohnen, 2001). Most biological responses to the oligogalacturonides are attributed to molecules with the degree of polymerisation from 10 to 16 GalA residues. Interestingly, the minimum size required for physiological activity and the minimum size required for the formation of a Ca^{2+}-dependent "egg box" conformation are coincident, and equal to 10 (Ridley et al., 2001). It is possible that the loss of galacturonan-rich material during dialysis is associated to the release of the oligogalacturonides held in cell walls by calcium ions.

The M_w of polymers recovered as Ca^{2+}-free material showed to be roughly half of the values obtained in the

Table 3
Sugar composition of the olive pulp sn-CR Ca^{2+}-free fractions

sn-CR fraction	Yield[a] (%)	Sugar recovery[b] (%)	Cell wall sugars (mol%)						Total sugars[c] (mg/g)
			Rha	Ara	Xyl	Gal	Glc	GalA	
Green olives									
1997	80	75	2 (34)	72 (5)	1 (0)	3 (22)	1 (25)	21 (17)	735 (3)
1998	88	82	2 (16)	63 (4)	–	6 (14)	1 (25)	28 (8)	594 (12)
Cherry olives									
1997	74	72	1 (19)	65 (5)	1 (31)	3 (27)	1 (43)	29 (10)	738 (4)
1998	78	75	2 (30)	60 (4)	1 (27)	6 (20)	–	31 (7)	664 (6)
Black olives									
1997	73	66	3 (28)	55 (14)	1 (37)	5 (17)	1 (12)	36 (17)	624 (11)
1998	73	65	2 (31)	58 (5)	–	6 (6)	– (17)	34 (8)	679 (5)

Values in brackets are the coefficients of variation obtained from three independent replicates.
[a] Yield is expressed as g of dialysed Ca^{2+}-free material per gram of initial sn-CR material.
[b] Sugar recovery is expressed as grams of dialysed Ca^{2+}-free pectic polysaccharides per gram of initial sn-CR pectic polysaccharides.
[c] Values are expressed as milligrams of anhydrous sugar per gram of dry polymer.

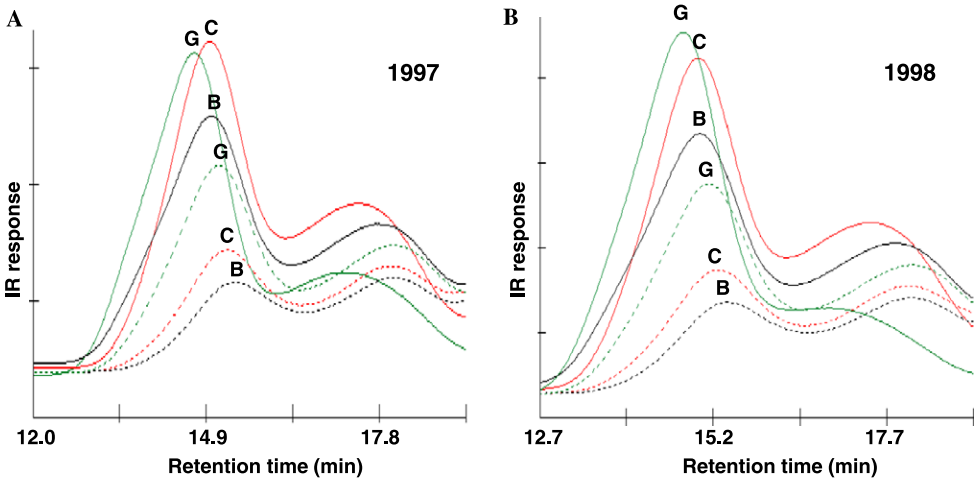

Fig. 5. Size-exclusion chromatograms of the native (thick lines) and Ca^{2+}-free (hatched lines) sn-CR pectic polysaccharides from (a) 1997 and (b) 1998 samples. G, green; C, cherry; B, black.

starting material before the de-complexation (Table 2). This was observed for both molecular fractions of sn-CR for the three stages of ripening (green, cherry and black) and for the two harvests (1997 and 1998). These results indicate that the pectic polysaccharides in all sn-CR fractions are structurally organised as Ca^{2+} complexes. Although the large and variable amount of neutral sugar side chains could confer uncertainty in the estimation of the M_w of the pectic polysaccharides present in the sn-CR fractions, it can be hypothesised that they are composed of two polysaccharide macromers, i.e., in the form of macrodimers. Although pectic polysaccharides are characterised by molar mass heterogeneity, homogeneous molar mass has been reported for homogalacturonans obtained from pectic polysaccharides of lime (Hellín, Ralet, Bonnin, & Thibault, 2005). Galacturonan chains, paired in the 2_1 helical conformation (two residues of GalA per turn of the helix) with calcium ions, fitted between them like eggs in a egg box, and have been shown to occur in dried calcium pectate gels, as well as in pectin-containing plant cell walls (Jarvis & Apperley, 1995). Although the organisation of pectic polysaccharides in the cell wall architecture is still under discussion (Vincken et al., 2003), the occurrence of these calcium complexes could be accounted and explained in all the proposed models, namely by their presence in the homogalacturonan moieties of the middle lamella region (Willats et al., 2001) or associated to the reinforcing role in stabilisation of RG-II dimers (Ishii et al., 1999; Kobayashi et al., 1999). Monovalent cations, such as K^+ used in alkali extractions of sn-CR fractions, have been reported to prevent the conformation transition of calcium pectate gels from the "egg box" twofold helical (2_1) to the 3_1 conformation (Jarvis & Apperley, 1995). This conformational change could allow the diffusion of pectic polysaccharides upon neutralisation of the extraction solution but, because these transitions were reversible, the removal of K^+ in the form of potassium acetate during dialysis of the extracts would lead to the native polymer conformation.

4. Conclusions

This study highlights that the arabinan-rich pectic polysaccharides that remained in the cell wall matrix after extensive extraction with imidazole, carbonate and KOH aqueous solutions are present as calcium complexes, giving rise to Ca^{2+}-pectic polysaccharide crystalline structures. The relative crystallinity of the Ca^{2+}-pectic polysaccharide complexes was directly related to the Ca^{2+}/GalA molar ratio. Based on de-complexation experiments and analysis by size-exclusion chromatography, these pectic polysaccharides were inferred to occur in the form of dimers and revealed a bimodal molecular weight distribution. The lower molecular weight fraction (70–135 kDa) was independent from the fruit ripening stage, whereas the higher molecular weight fraction varied from 1.1 MDa to 0.6–0.9 MDa, and 0.5–0.7 MDa for green, cherry and black olives, respectively. The de-complexation of these pectic polysaccharides resulted in the preferential loss during the dialysis of galacturonan-rich material for the samples in the early stages of ripening, and in the preferential loss of arabinan-rich material for the samples in the late stage of ripening. These oligogalacturonides were held in cell walls by calcium ions.

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