Structural characteristics of polysaccharides from olive fruit cell walls in relation to ripening and processing

Promotor

Prof. dr. ir. A.G.J. Voragen
Hoogleraar in de Levensmiddelenchemie

Co-promotor

Dr. H.A. Schols
Universitair docent bij de leerstoelgroep Levensmiddelenchemie

Promotiecommissie

Prof. dr. ir. M.A.J.S. van Boekel (Wageningen Universiteit)
Prof. dr. R.G.F. Visser (Wageningen Universiteit)
Dr. M.A. Coimbra (University of Aveiro, Portugal)
Dr. J. Maat (Unilever Research Vlaardingen)

Structural characteristics of polysaccharides from olive fruit cell walls in relation to ripening and processing

Esther Vierhuis

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 3 april 2002
des namiddags te half twee in de Aula.

Vierhuis, Esther

Structural characteristics of polysaccharides from olive fruit cell walls in relation to ripening and processing

Ph.D. thesis Wageningen University - 2002

ISBN: 90-5808-594-5

Key words: Olive fruit; olive oil; pectic polysaccharides; xyloglucans; xylans; enzyme preparations; phenolic compounds; processing; ripening

Stellingen

1. De rol van endogene celwandafbrekende enzymen is verwaarloosbaar tijdens de productie van olijfolie.
   Dit proefschrift

2. De aanwezigheid van arabinose-(1→6)-xylose zijketens is niet alleen voorbehouden aan xyloglucanen geïsoleerd uit planten van de nachtschadenfamilie.
   Dit proefschrift

3. Gezuiverde enzymen en substraten zijn niet per definitie zuiver.

4. Statistische toetsing is voor veel auteurs geen kritische controle meer, maar een soort bewijs van goed gedrag, waarmee ze zichzelf tekort doen.
   De Volkskrant, 21 april 2001

5. Goede voeding is altijd functioneel.
   Consumentengids, februari 2001

6. Niet het aantal treinen, maar het aantal reizigers dat op tijd is, is een goede maat voor de punctualiteit van de Nederlandse Spoorwegen. Immers, treinen hebben geen last van vertragingen, maar de passagiers wel.

7. Ongedocumenteerd bewaren is een omslachtige manier van weggooien.

8. Een maatschappelijk draagvlak is een randvoorwaarde om nieuwe wetenschappelijke technologieën in de praktijk te kunnen toepassen.

Stellingen behorende bij het proefschrift

Structural characteristics of polysaccharides from olive fruit cell walls in relation to ripening and processing

Esther Vierhuis
Wageningen, 3 april 2002

Abstract

Technical enzyme preparations can be used as processing aids in the olive oil industry to obtain a higher yield and a better quality of the oil. These technical enzyme preparations degrade the plant cell wall, thus enhancing the permeability for oil. However, still very little is known about the specific role of the various constituent enzymes present in the preparations towards the polysaccharides in the plant cell wall. This study deals with this subject and describes the structural characteristics of the polysaccharides in the cell wall of olive fruit in relation to processing for oil extraction.

The major polysaccharides in the cell wall of olive fruit were found to be the pectic polysaccharides and the hemicellulosic polysaccharides xyloglucan and xylan. The pectic polysaccharides were highly methyl esterified and rich in arabinose. The xyloglucans had a backbone with three out of four glucose residues substituted with xylose residues and had a very specific substitution pattern, because galactose as well as arabinose residues could be linked to the xylose residues. The presence of arabinose residues linked to xylose residues is a common feature of xyloglucans produced by solanaceous plants, but has not been demonstrated for other dicotylodonous plants. Incubation of the cell wall material from olive fruit with pectin degrading enzymes in combination with endo-glucanases revealed that *O*-acetyl groups were not only linked to pectic material in olive fruit but also to xyloglucan. It was established that the arabinose residues of the xyloglucan could carry one or two *O*-acetyl groups. The cell wall material of olive fruit contained besides xyloglucan also considerable amounts of the hemicellulose xylan. These xylans appeared to be very low in substitution. Less than 10% of the xylose residues were mono substituted, mainly with 4-*O*-methyl-glucuronic acid residues.

The use of technical enzyme preparations during processing affected only a relatively small part of the polysaccharides in the cell wall. About 8-10% of the cell wall polysaccharides were extracted with cold and hot buffer and appeared to be modified during enzymatic processing. The changes of the buffer soluble pectic material were reflected by an increase in yield, a change in molecular weight distribution, a decrease in methyl esterification and a degradation of the (1→4)-linked galactan chains. No differences were observed in the composition of the arabinan chains during enzymatic processing. Analysis of the olive oil revealed that the use of enzyme preparations increased the concentration of phenolic compounds in the oil. Especially, the contents of secoiridoid derivatives such as the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol and an isomer of oleuropein aglycon increased significantly. These two phenolic compounds have high antioxidant activities.

The effect of enzyme treatment during the mechanical extraction of olive oil depends on the stage of maturity of the olive fruit. From this perspective the structural characteristics of the cell wall polysaccharides during ripening have also been investigated. During ripening the degree of methyl esterification and acetylation decreased and the solubility of the pectic polymers in buffer markedly increased. No distinct differences could be noticed with regard to the sugar composition and the profile of the molecular weight distribution of the pectic and hemicellulosic polymers during fruit development.

Contents

If walls could talk

Chapter 1

General introduction

Background

Olive fruit has been cultivated for thousands of years in the countries surrounding the Mediterranean Sea and has played an important role in the diet of the people in this area as well as their economy and culture (Boskou, 1996). Currently, more than 95% of the world's olive trees grow in the Mediterranean Basin with Spain, Greece and Italy as leading producers of olive fruit (Macrae *et al.*, 1993; Ensminger *et al.*, 1995; Firestone *et al.*, 1996). The annual olive fruit production is about 9.4 million tonnes and approximately 90% of the production is used for the extraction of olive oil. The remaining 10% is consumed as table olives (Macrea *et al.*, 1993). Most of the world olive oil is consumed in the producer countries themselves. This is due to economic factors as well as dietary habits and preferences acquired through tradition since olive oil is a native product (Boskou, 1996). However, consumption of olive oil is increasing in non-Mediterranean areas such as the United States, Canada, Russia, Australia and Japan because of the growing interest in the Mediterranean diet (Visioli & Galli, 1998). Epidemiological evidence shows that the Mediterranean diet, which is rich in olive oil and fresh fruit and vegetables, is associated with a lower incidence of coronary heart disease and certain tumours (prostate and colon cancer). The high intake of oleic acid (18:1(9)) from olive oil and the concomitant low intake of saturated fat has been proposed as an important factor for the beneficial effect of the diet. Recent findings also suggest that phenolic compounds found in olive oil are endowed with several biologic activities that may contribute to the lower incidence of coronary heart disease in the Mediterranean area (Visioli *et al.*, 1998; Trichopoulou *et al.*, 1999; Parr & Bolwell, 2000). In addition to the biological role, phenolic compounds give olive oil a particular resistance to the development of rancidity and are important for its unique flavour and taste (Boskou, 1996; Galli & Visioli, 1999).

In the olive oil industry technical enzyme preparations can be used as processing aids to increase the extraction output of the oil. This favourable effect of enzymatic treatment on the extraction of olive oil has already been observed in the 1950s. Furthermore, enzymatic treatment can have a beneficial influence on quality in particular as far as the phenolic content and stability by oxidation are concerned (Christensen, 1991; Servili *et al.*, 1992; Di Giovacchino, 1993; Montedoro *et al.*, 1993a; Domínguez *et al.*, 1994; Ranalli & Serraoiocco, 1995; Ranalli & De Mattia, 1997). The technical enzyme preparations developed to be used for the extraction of olive oil degrade the plant cell walls, thus enhancing the permeability for oil. However, although several studies have been carried out on the effect of enzymatic treatment on the yield and the quality characteristics of oil from olive fruit, very little is known about the effect of these enzyme preparations on the plant cell wall structure. This thesis deals with this subject and describes the structural characterisation of polysaccharides from olive fruit cell walls in relation to processing for oil extraction. In addition to this, ripening related changes in the structure of the cell wall polysaccharides have been studied, because knowledge of this aspect is still limited and, because the ripening stage of the olive fruit is very important in relation to olive oil quality. A short overview of the processing of olive fruit and the structure of plant cell walls is given in this chapter.

The olive

Olive oil is obtained by mechanical extraction from the fruit of the *Olea europaea* (family, Oleaceae) tree. The olive fruit is a meaty, intensely bitter, stoned fruit. Its shape is round and generally elongated. The colour of fresh green olives is due to the presence of chlorophylls. As ripening progresses, olives become pink to purple or black. This colour change is due to the formation of anthocyanins. The physical and chemical characteristics of olive fruit depends on many factors, including variety, ripeness at the time of harvest, geographical location, type of cultivation, etc (Garrido Fernández *et al.*, 1995). Table 1.1 gives an overview of the composition of the fruit, which consists of a pericarp and an endocarp. The pericarp includes an epicarp (skin) and a mesocarp (pulp) surrounding the endocarp (woody pit) in which the seed is enclosed (Fernández Díez, 1992; Firestone *et al.*, 1996).

Olive oil

The processing of olive fruit for the extraction of oil is mainly performed in large modern plants but small traditional oil mills are also still in use. The extraction of the oil from the olive fruit requires several processing steps (Fig. 1.1). The olives are first washed to remove dirt and agricultural contaminants. Then the process of releasing oil from the olive fruit begins by crushing the olives into a coarse paste. The objective of this step is to tear flesh cells to let the oil run out of the vacuoles, thus permitting the formation of larger drops that can be

Table 1.1
The composition of the fruit of the olive tree and of the fresh pulp (Fernández Díez, 1992)

	Weight (%)
Fruit	
Pericarp — Epicarp	
Pericarp — Mesocarp — Pulp	70-90
Endocarp — Stone	9-27
Endocarp — Seed	1-3
Pulp	
Moisture	50-75
Lipids (oil)	6-30
Reducing sugars, soluble	2-6
Non-reducing sugars, soluble	0.1-0.3
Crude protein (N x 6.25)	1-3
Fibre	1-4
Phenolic compounds	1-3
Organic acids	0.5-1
Pectic substances	0.3-0.6
Ash	0.6-1
Others	3-7

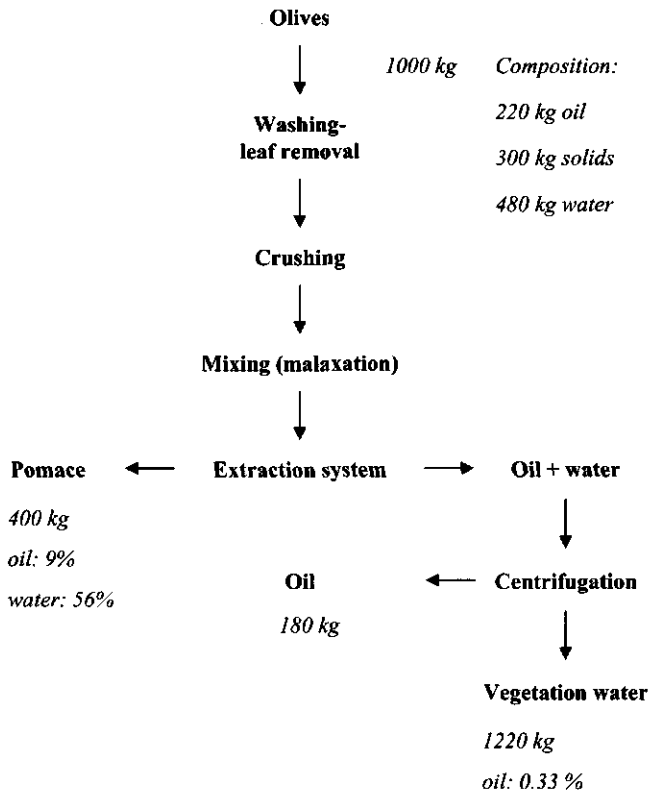

Fig. 1.1. Diagram of the extraction of olive oil including a mass balance. During the process diluting water (paste/water ratio 1:1 (w/v)) is added (Garrido Fernández *et al.*, 1995; Boskou, 1996; Firestone *et al.*, 1996).

separated from other phases. After it is crushed, the olive paste is mixed during 30-60 min (called malaxation), a process that entails stirring the paste slowly and continuously. The objective of this stage is to increase the amount of oil that is released; first, by helping the droplets to merge into large drops that can be separated into a continuous liquid phase and, second, by breaking up the oil/water emulsion. Finally, the oil is separated from the other phases in the paste by pressure, centrifugation or percolation. In addition to oil, a solid residue (pomace) and vegetation water are produced during the extraction. The oil yield per 100 kg of fruit is about 18 kg (Boskou, 1996; Firestone *et al.*, 1996; Williams & Hron, 1996).

The oil obtained from sound fruit by pressing without further treatment is called virgin olive oil. After the first pressing, the residual pulp is still rich in oil and is usually re-crushed or re-pressed. Oils obtained from the second pressing tend to have a more intense colour and a higher acid content, as well as a weaker aroma. This latter oil together with inferior virgin oil is further subjected to refining treatments such as neutralisation, deodorisation and bleaching; resulting in removing acid, odour and colour. The oil so obtained is called refined olive oil and is largely used for mixing with the first extraction to produce edible grades. If olive oil is

used as a salad oil, it is also winterised. Meaning that the oil is cooled to a low temperature and any crystallised material is removed from the liquid oil (Abraham & Hron, 1992; Macrea *et al.*, 1993).

As mentioned in the first section of this chapter, enzymatic treatment can be used to enhance the oil recovery from olive fruit. The enzymes act upon the plant cell walls, thus favouring the permeability for oil. The increase of the oil yield depends on the variety and ripeness of the olive fruit, the enzymes used, the temperature, the pH and the dosage of enzyme applied. Typically there is a 5-10% increase in the oil yield. The effect of the enzymes is most pronounced during processing of unripe or less ripe olives early in the harvest (Christensen, 1991; Di Giovacchino, 1993; Domínguez *et al.*, 1994; Olsen, 1995). The use of technical enzyme preparations during processing can also increase the content of phenolic compounds, which is related to the sensory quality and the oxidative stability of the virgin olive oil. Among the phenolic compounds identified in olive oils are the hydroxycinnamic acids (caffeic acid, vanillic acid, *p*-coumaric acid, syringic acid and ferulic acid) and the phenyl alcohols, *p*-hydroxyphenyl-ethanol (*p*-HPEA) and 3,4-dihydroxyphenyl-ethanol (3,4-DHPEA) and oleuropein aglycon (Gutfinger, 1981; Tsimidou, 1998). However, the prevalent phenolic compounds in the virgin olive oil are secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA) or *p*-HPEA (*p*-HPEA-EDA) and an isomer of the oleuropein aglycon (3,4-DHPEA-EA) (Montedoro *et al.*, 1993b).

The contribution of individual phenols to oil stability and the importance of the presence of phenolic compounds to the sensory quality of the oil have not been fully investigated (Tsimidou, 1998). The most important compound for the high oxidative resistance of the oil seems to be 3,4-DHPEA (Gutfinger, 1981), but also 3,4-DHPEA-EDA and 3,4-DHPEA-EA show important antioxidant activities (Servili *et al.*, 1996). Although phenolic compounds have important antioxidant activities, enrichment of an olive oil with these compounds has also some limitations due to their adverse contribution to the sensory quality of the oil. A very high load of phenols may result in an excessive and unpleasant bitterness and may not be acceptable by the consumers (Tsimidou, 1998; Galli & Visioli, 1999).

Structure of plant cell walls

The enzymes used to enhance oil recovery and quality act upon the plant cell walls. Cell walls are a major component of plant material and are composed of complex polysaccharides and structural proteins (Carpita & Gibeaut, 1993; McCann & Roberts, 1994). Cellulose is the most abundant plant polysaccharide and forms the microfibrillar foundation. Cellulose is an assembly of several dozen unbranched (1→4)-linked β-D-glucan chains that are hydrogen bonded along their length to form paracrystalline cables. In the primary cell wall of dicotyledonous and non-graminaceous monocotyledonous plants xyloglucan is thought to be capable of hydrogen-bonding to cellulosic microfibrils and thus cross-linking them together (Fig. 1.2). Xyloglucans are linear chains of (1→4)-linked β-D-glucose residues, but, unlike cellulose, they have D-xylose residues attached to C-6 of the D-glucose residues. The framework of cellulose microfibrils and xyloglucan polymers is embedded in a matrix of

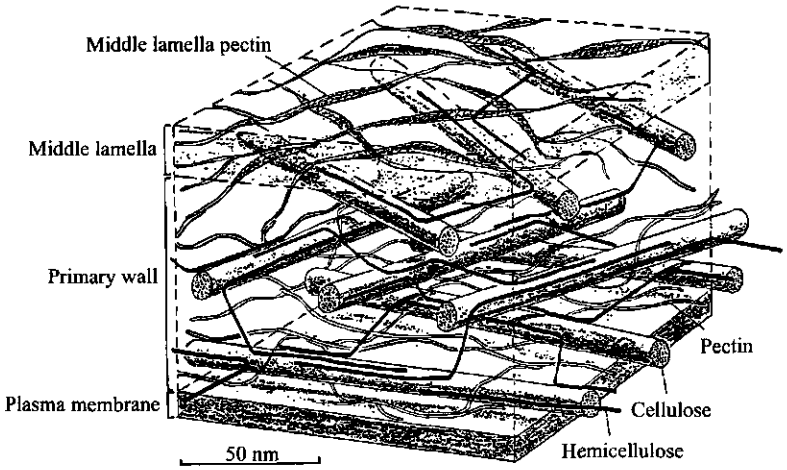

Fig. 1.2. An extremely simplified and schematic representation of the onion parenchyma cell wall. There are two main interpenetrating networks, one of cellulose and hemicellulose and one of pectin. No protein is shown as onion has very little protein in its cell wall (McCann & Roberts, 1991).

pectic polysaccharides (McCann & Roberts, 1991; Carpita & Gibeaut, 1993). Pectic polysaccharides are rich in D-galacturonic acid, L-rhamnose, L-arabinose and D-galactose and form a very complex group of heteropolysaccharides. In essence, they comprise two families of covalently interlinked acidic polymers; these are galacturonans and highly ramified rhamnogalacturonans (Voragen *et al.*, 2001) and will be discussed later. In some cells a third interactive network of structural proteins such as the hydroxyproline-rich glycoprotein extensin might be present (McCann & Roberts, 1991; McCann & Roberts, 1994).

The main experimental approach used to study the cell wall involves the purification of the cell wall followed by the extraction of specific polymers from the cell walls with chemical solvents. The isolated polymers are subsequently analysed for sugar and glycosidic linkage composition and by size-exclusion or ion-exchange chromatography and nuclear magnetic resonance (NMR) spectroscopy (Brett & Waldron, 1990; Selvendran & Ryden, 1990; McCann *et al.*, 1995). Plant-cell-wall-degrading enzymes can also be used as valuable analytic tools in the elucidation of the fine structure of polysaccharides. Pure and well-characterised enzymes can cleave large polysaccharides to yield characteristic oligomers. Subsequent chemical analysis of the released fragments gives a reasonable idea of the general sequence and conformation of very large polymers (Carpita & Gibeaut, 1993; Voragen *et al.*, 1993; Schols & Voragen, 1996). Microscopic techniques can be used to obtain information on the overall appearance and morphology of the cell wall. At the light microscopy level, the distribution of particular components is seen by the use of histochemical stains and probes such as antibodies. Antibodies coupled to colloidal gold can be used at the electron microscope level to show the distribution of components within a single wall. The architecture of the cell walls can also be directly visualised in the electron microscope (Brett & Waldron, 1990; McCann *et al.*, 1995). Fourier transform infrared (FTIR) microspectrometry can be used to detect specific

chemical bonds within the wall, and in combination with polarisers, to detect their orientation (transversely or longitudinally with respect to the long axis of the cell). Fourier transform Raman (FT-Raman) microspectroscopy provides complementary spectral information (McCann *et al.*, 1995).

Composition and structure of pectic polysaccharides

The primary cell walls of dicotyledonous plants contain a high proportion of pectic polysaccharides. The composition of the pectic polysaccharides is very complex and varies with the conditions of extraction, the type of plant tissue and the state of development of the wall (Selvendran, 1985). Despite of the structural diversity, pectin can be described by the structures schematically presented in Fig. 1.3. In this model the pectin molecule consists of unsubstituted ("smooth") regions containing almost exclusively D-galacturonic acid residues and blocks of more highly substituted ("hairy") rhamnogalacturonan regions (Voragen *et al.*, 1995).

The smooth galacturonan regions consist of linear chains of (1→4)-linked α-D-galacturonic acid residues. Other sugars are absent or present only in low amounts (Brett & Waldron, 1990; O'Neill *et al.*, 1990). The galacturonic acid residues in the galacturonan regions can be methyl esterified at the carboxyl group and *O*-acetylated at C-2 and/or C-3 (Voragen *et al.*, 1995; Ishii *et al.*, 1997).

The hairy regions include rhamnogalacturonan I and II. Rhamnogalacturonan I is a major component of the middle lamella and the primary cell wall of dicotyledonous plants. The backbone of rhamnogalacturonan I is composed of repeating (1→4)-α-D-galacturonosyl acid-(1→2)-α-L-rhamnosyl units. The galacturonic acid residues in the rhamnogalacturonan parts of the pectic molecule can be *O*-acetylated at both C-2 and C-3 and methyl esterified (Komalavilas & Mort, 1989; Brett & Waldron, 1990; Ishii *et al.*, 1997). About 20% to 80% of the rhamnose residues are substituted at C-4 with arabinose- and galactose-rich side chains depending on the source of the polysaccharide. These side chains are often quite large and are similar in structure to the arabinan, galactan and arabinogalactan type I and II molecules described later (Brett & Waldron, 1990; Carpita & Gibeaut, 1993; Albersheim *et al.*, 1996).

Rhamnogalacturonan II is a low molecular, structurally well-defined, complex pectic polysaccharide and is composed of a backbone of at least seven (1→4)-linked α-D-galacturonic acid residues. Rhamnogalacturonan II has four structurally well-defined oligosaccharide side chains that are attached to the galacturonic acid residues of the backbone at C-2 and/or C-3. Characteristic for rhamnogalacturonan II is the presence of unusual or unique sugars like apiose, 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid), 3-deoxy-D-*manno*-octulosonic acid (KDO) and 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA) and also the seldom observed methyl-esterified sugars 2-*O*-methyl-L-fucose and 2-*O*-methyl-D-xylose, besides the more common sugar residues L-rhamnose, L-fucose, L-arabinose, D-galactose, D-galacturonic acid and D-glucuronic acid (McNeil *et al.*, 1984; O'Neill *et al.*, 1990; Whitcombe *et al.*, 1995; Pellerin *et al.*, 1996).

Another structural element of pectin is xylogalacturonan. Xylogalacturonan consists of a

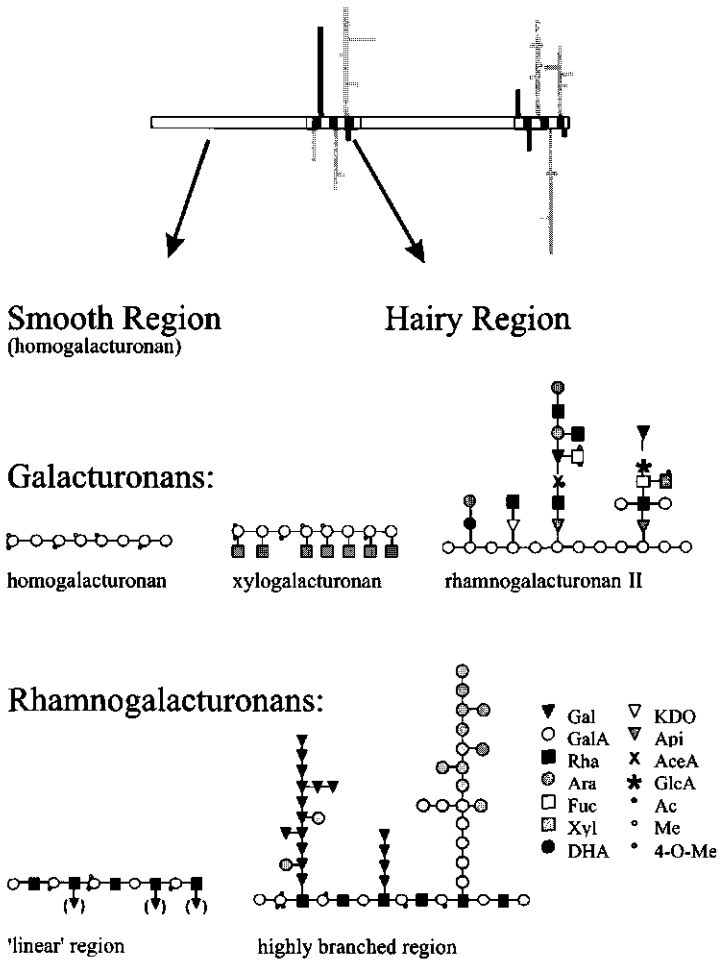

Fig. 1.3. Schematic structure of the pectin molecule. Occurrence, amount and chemical fine structure of the individual segments may vary significant depending on the origin of the pectin (Voragen *et al.*, 2000).

(1→4)-α-D-galacturonan backbone with some L-rhamnose inserted and single unit side chains of D-xylose residues linked to part of the D-galacturonic acid residues at C-3 (Voragen *et al.*, 1993). Part of the galacturonic acid residues is methyl esterified (Schols *et al.*, 1995).

Composition and structure of hemicellulosic polysaccharides

In contrast to the pectins, the hemicelluloses vary greatly in different cell types and in different species. In most cell types one hemicellulose predominates with others present in smaller amounts. For example, xyloglucan is the principal hemicellulose of the primary cell walls of dicotyledonous plants, while the primary walls of monocotyledonous plants include,

as a major hemicellulose, arabinoxylan (McNeil *et al.*, 1984; Brett & Waldron, 1990).

Generally, xyloglucans comprise as much as 20-25% of the primary walls of dicotyledonous plants. It consists of a backbone of β-(1→4)-linked glucose residues in which up to 75% of the glucose residues can be substituted at C-6 with xylose residues (Fig. 1.4). Some xylose residues are further substituted at C-2 with a monosaccharide (galactose or arabinose) or disaccharide (fucose-(1→2)-galactose). Species and tissue specific differences occur in the distribution of the side chains. In addition, *O*-acetyl groups can present on the galactose and arabinose residues of the side chains and on the glucose residues of the backbone (McNeil *et al.*, 1984; Fry, 1989; Hayashi, 1989; Vincken *et al.*, 1997; Pauly *et al.*, 2001). The primary cell walls of monocotyledonous plants contain small amounts of xyloglucan, with in general a lower xylose to glucose ratio compared to dicotylodonous plants. Xyloglucans are also found as storage polysaccharides in some seed endosperm cell walls. In these tissues, the structure generally lacks fucose (Brett & Waldron, 1990).

Xylans have a backbone of β-(1→4)-linked xylose residues. The xylose residues of the backbone can be substituted at C-2 or C-3 with arabinose residues, glucuronic acid residues (or its 4-*O*-methyl derivative) and acetyl esters. Other, longer side chains containing xylose, arabinose and galactose have also been reported. The absolute and relative amounts of these substituents is species and tissue specific. The principal hemicellulosic polysaccharide of the primary cell walls of monocotyledonous plants is an arabinoxylan, while acidic xylan is generally present in the lignified tissues of both monocotyledonous and dicotyledonous plants (McNeil *et al.*, 1984; Brett & Waldron, 1990).

Arabinans are branched polysaccharides with a backbone of α-(1→5)-linked arabinose residues substituted at C-2 and/or C-3 with arabinose. They are present in the cell wall either as independent molecules or as side chains attached to rhamnogalacturonan I (Beldman *et al.*, 1997).

Arabinogalactans occur in two structurally different forms. Arabinogalactan type I is a linear chain of β-(1→4)-linked galactose residues with 20 to 40% arabinose residues (1→5)-linked in side chains connected in general to C-3 of the galactose residues. Arabinogalactan type II is a highly branched polysaccharide with ramified chains of galactose residues joined by β-(1→3)- and β-(1→6)-linkages. The (1→3)-linkages predominate in the interior chains and the (1→6)-linkages occur mainly in the exterior chains. Most of the side chains are terminated with arabinofuranosyl residues and to some extent arabinopyranosyl residues occur (Beldman *et al.*, 1997). Arabinogalactans type I and II can be present in the cell wall as covalently linked side chains of rhamnogalacturonan I or as separate polysaccharides. In addition, type II arabinogalactans are also often found to be associated with hydroxyproline containing proteins (O'Neill *et al.*, 1990; Voragen *et al.*, 1995). Some primary cell walls also contain β-(1→4)-linked galactans with little or no additional sugar material present in the molecule, but they are relatively rare (Brett & Waldron, 1990).

Other types of hemicellulosic polysaccharides are mannans, which are present in a wide range of cell walls and β-(1→3)-, β-(1→4)-linked glucans present in the cell walls of the Gramineae (McNeil *et al.*, 1984; Brett & Waldron, 1990).

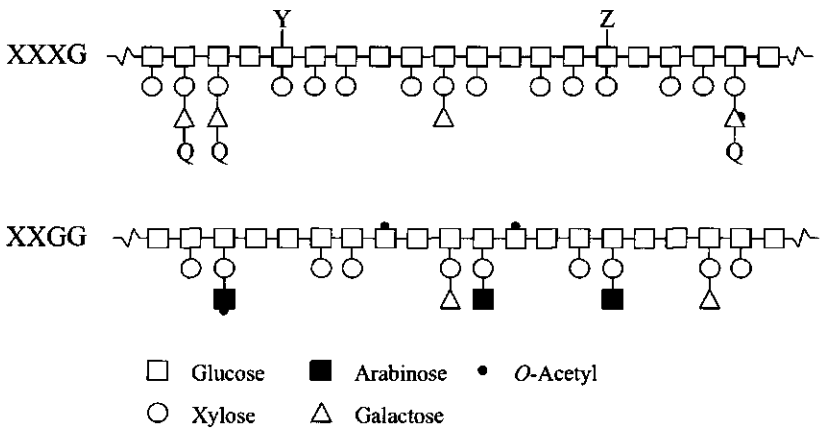

Fig. 1.4. An overview of different branching patterns of xyloglucan. □, β-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; Δ, β-D-Gal*p*-(1→2)-; and ■, α-L-Ara*f*-(1→2)-. Position Q can be substituted with an α-L-Fuc*p*-(1→2)- residue or an α-L-Gal*p*-(1→2)-. Position Y can be substituted with a β-D-Xyl*p*-(1→2)- an α-L-Ara*f*-(1→2)-, or an α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→2)- side chain. Z can be substituted with a β-D-Xyl*p*-(1→2)- residue. *O*-acetyl groups are indicated by closed half circles (Vincken *et al.*, 1997).

Aim and outline

The aim of the study described in this thesis is to obtain more information about the structural characteristics of the polysaccharides present in the cell walls of olive fruit in relation to ripening and enzymatic processing for olive oil extraction. Pectin is a major polysaccharide in the cell walls of olive fruit and the structural characteristics of this polysaccharide are the subject of research in chapters 2 and 6. The pectic polysaccharides were isolated from the cell wall by sequential extraction with specific solvents and sugar (linkage) analysis and degradation studies with specific enzymes were performed to obtain information about their structural characteristics. In addition to the pectic polysaccharides the cell wall contains significant amounts of xylan and xyloglucan. A more detailed study of these hemicellulosic polysaccharides is described in chapter 3. The results indicated a xyloglucan in olive fruit with a specific substitution pattern. So, xyloglucan oligosaccharides were prepared by endo-glucanase digestion of the alkali-extractable xyloglucan from olive fruit and the two most abundant oligosaccharides were isolated and their structure characterised by NMR spectroscopy and mass spectrometry. This research is described in chapter 4 and revealed that olive fruit xyloglucan is mainly built from two novel arabinose-containing oligosaccharides.

Ripening related changes in the composition of the cell wall material from olive fruit are described in chapter 2. Olive fruit was harvested at four specific moments during the season and cell wall material was isolated and sequentially extracted to yield four pectin-rich fractions and two hemicellulose-rich fractions. Subsequently, the ripening related changes in the cell wall material and the pectic and hemicellulosic fractions were examined.

Processing related changes in the composition of the cell wall material from olive fruit

were investigated in order to obtain more insight in the mechanisms by which the use of cell-wall-degrading enzyme preparations lead to a higher yield of the oil (chapters 5 and 6). In addition to the effect of processing on the composition of the cell wall polysaccharides, the effect of processing on the composition of the phenolic compounds has been studied. This, because cell-wall-degrading-enzyme preparations are not only used as processing aids in the olive oil industry to improve the extraction yield but also to improve product quality, which is directly related to the content of phenolic compounds in the olive oil.

Finally, in the concluding remarks (chapter 7) an overview of the thesis work is given and the implications of our work are discussed.

References

Abraham, G. & Hron, R.J. Oilseeds and their oils. In: *Encyclopedia of food science and technology, Vol. 3.* Y.H. Hui (ed), New York: Wiley (1992) 1901-1910.

Albersheim, P., Darvill, A.G., O'Neill, M.A., Schols, H.A. & Voragen, A.G.J. A hypothesis: the same six polysaccharides are components of the primary cell walls of all higher plants. In: *Progress in Biotechnology, Vol. 14: Pectins and pectinases, proceedings of an international symposium.* J. Visser & A.G.J. Voragen (eds), Amsterdam: Elsevier (1996) 47-55.

Beldman, G., Schols, H.A., Pitson, S.M., Searle- van Leeuwen, M.J.F. & Voragen, A.G.J. Arabinans and arabinan degrading enzymes. In: *Advances in macromolecular carbohydrate research, Vol. 1.* R.J. Sturgeon (ed), London: JAI Press (1997) 1-64.

Boskou, D. *Olive oil: chemistry and technology.* Campaign: AOCS Press (1996) 161p.

Brett, C. & Waldron, K. *Physiology and biochemistry of plant cell walls.* Boston: Hyman (1990) 1-45.

Carpita, N.C. & Gibeaut, D.M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth, *Plant J.* 3 (1993) 1-30.

Christensen, F.M. Extraction by aqueous enzymatic processes, *Inform* 2 (1991) 984-987.

Di Giovacchino, L. The use of enzymes in the extraction of olive oil by continues systems of centrifugation. Note 1: Results of tests made over many years, *Riv. Ital. Sostanze Grasse* LXX (1993) 279-287.

Domínguez, H., Núñez, M.J. & Lema, J.M. Enzymatic treatment to enhance oil extraction from fruits and oilseeds: a review, *Food Chem.* 49 (1994) 271-286.

Ensminger, A.H., Ensminger, M.E., Konlande, J.E. & Robson, J.R.K. *The Concise Encyclopedia of Food and Nutrition.* Boca Raton: CRC Press (1995) 801-806.

Fernández Diez, M.J. Olives. In: *Encyclopedia of food science and technology, Vol. 3.* Y.H. Hui (ed), New York: Wiley (1992) 1910-1925.

Firestone, D., Fedeli, E. & Emmons, E.W. Olive oil. In: *Bailey's Industrial Oil and Fat Products: Edible oil and fat products: oils and oilseeds, Vol. 2.* Y.H. Hui (ed), New York: Wiley (1996) 241-270.

Fry, S.C. The structure and functions of xyloglucan (review article), *J. Exp. Bot.* 40 (1989) 1-11.

Galli, C. & Visioli, F. Antioxidant and other activities of phenolics in olive/olive oil, typical components of the Mediterranean diet, *Lipids* 34 (1999) S23-S26.

Garrido Fernández, A., García García, P. & Brenes Balbuena, M. Olive fermentations. In:

Biotechnology: a multi-volume comprehensive treatise, Vol. 9: Enzymes, biomass, food and feed. G. Reed & T.W. Nagodawithana (eds), Weinheim: VCH (1995) 593-628.

Gutfinger, T. Polyphenols in olive oils, *J. Am. Oil Chem. Soc.* 58 (1981) 966-968.

Hayashi, T. Xyloglucans in the primary cell wall, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40 (1989) 139-168.

Ishii, T. *O*-acetylated oligosaccharides from pectins of potato tuber cell walls, *Plant Physiol.* 113 (1997) 1265-1272.

Komalavilas, P. & Mort, A.J. The acetylation at *O*-3 of galacturonic acid in the rhamnose-rich portion of pectins, *Carbohydr. Res.* 189 (1989) 261-272.

Macrae, R., Robinson, R.K. & Sadler, M.J. *Encyclopaedia of Food Science, Food Technology and Nutrition, Vol. 5: Malt-Pesticides and Herbicides.* London: Academic Press (1993) 3353-3358.

McCann, M.C. & Roberts, K. Architecture of the primary cell wall. In: *The cytoskeletal basis of plant growth and form.* C.W. Lloyd (ed), London: Academic Press (1991) 109-129.

McCann, M.C. & Roberts, K. Plant cell walls: murals and mosaics, *Agro-Food-Industry Hi-Tech* 5 (1994) 43-46.

McCann, M., Roberts, K., Wilson, R.H., Gidley, M.J., Gibeaut, D., Kim, J-B. & Carpita, N.C. Old and new ways to probe plant cell-wall architecture, *Can. J. Bot.* 73 (1995) S103-S113.

McNeil, M., Darvill, A.G., Fry, S.C. & Albersheim, P. Structure and function of the primary cell walls of plants, *Annu. Rev. Biochem.* 53 (1984) 625-663.

Montedoro, G.F., Begliomini, A.L., Servili, M., Petruccioli, M., & Federici, F. Pectinase production from olive vegetation waters and its use in the mechanical olive oil extraction process to increase oil yield and improve quality, *Ital. J. Food Sci.* 5 (1993a) 355-362.

Montedoro, G., Servili, M., Baldioli, M., Selvaggini, R., Miniati, E. & Macchioni, A. Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterizations of the secoiridoid derivatives, *J. Agric. Food Chem.* 41 (1993b) 2228-2234.

Olsen, H.S. Use of enzymes in food processing In: *Biotechnology: a multi-volume comprehensive treatise, Vol. 9: Enzymes, biomass, food and feed.* G. Reed & T.W. Nagodawithana (eds), Weinheim: VCH (1995) 663-736.

O'Neill, M., Albersheim, P. & Darvill, A. The pectic polysaccharides of primary cell walls. In: *Methods in plant biochemistry, Vol. 2: Carbohydrates.* P.M. Dey (ed), London: Academic press (1990) 415-441.

Parr, A.J. & Bolwell, G.P. Review: Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile, *J. Sci. Food Agric.* 80 (2000) 895-1012.

Pauly, M., Qin, Q., Greene, H., Albersheim, P., Darvill, A. & York, W.S. Changes in the structure of xyloglucan during cell elongation, *Planta* 212 (2001) 842-850.

Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J-M. & O'Neill, M.A. Structural characterization of red wine rhamnogalacturonan II. *Carbohydr. Res.* 290 (1996) 183-197.

Ranalli, A. & Serraiocco, A. Effect induced by a pectolytic adjuvant in olive oil extraction by the present technological systems; Pluriannual research results, *Riv. Ital. Sostanze Grasse* LXXII (1995) 355-364.

Ranalli, A. & De Mattia, G. Characterization of olive oil produced with a new enzyme processing aid, *J. Am. Oil Chem. Soc.* 74 (1997) 1105-1113.

Schols, H.A., Bakx, E.J., Schipper D. & Voragen A.G.J. A xylogalacturonan subunit present in the modified hairy regions of apple pectin, *Carbohydr. Res.* 279 (1995) 265-279.

Schols, H.A. & Voragen, A.G.J. Complex pectins: Structure elucidation using enzymes. In: *Progress*

in Biotechnology, Vol. 14: Pectins and pectinases, proceedings of an international symposium. J. Visser & A.G.J. Voragen (eds), Amsterdam: Elsevier (1996) 3-19.

Selvendran, R.R. Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers, *J. Cell. Sci. Suppl.* 2 (1985) 51-88.

Selvendran, R.R. & Ryden, P. Isolation and analysis of plant cell walls. In: *Methods of plant biochemistry, Vol. 2.* P.M. Dey & J.B. Harborne (eds), London: Academic Press (1990) 549-579.

Servili, M., Begliomini, A.L., Montedoro, G., Petruccioli, M. & Federici, F. Utilisation of a yeast pectinase in olive oil extraction and red wine making processes, *J. Sci. Food Agric.* 58 (1992) 253-260.

Servili, M., Baldioli, M., Miniati, E. & Montedoro, G.F. Antioxidant activity of new phenolic compounds extracted from virgin olive oil and their interaction with α-tocopherol and β-carotene, *Riv. Ital. Sostanze Grasse* LXXIII (1996) 55-59.

Trichopoulou, A.M.D., Lagiou, P.M.D. & Papas, A.M. Mediterranean diet: are antioxidants central to its benefits? In: *Antioxidant status, diet, nutrition and health.* A.M. Papas (ed), Boca Raton: CRC Press (1999) 107-118.

Tsimidou, M. Review: Polyphenols and quality of virgin olive oil in retrospect, *Ital. J. Food Sci.* 10 (1998) 99-116.

Vincken, J-P., York, W.S., Beldman, G. & Voragen, A.G.J. Two general branching patterns of xyloglucan, XXXG and XXGG: update on cell walls, *Plant Physiol.* 114 (1997) 9-13.

Visioli, F., Bellomo, G. & Galli, C. Free radical-scavenging properties of olive oil polyphenols, *Biochem. Biophys. Res. Commun.* 247 (1998) 60-64.

Visioli, F. & Galli, C. The effect of minor constituents of olive oil on cardiovascular diseases: new findings, *Nutr. Rev.* 56 (1998) 142-147.

Voragen, A.G.J., Schols, H.A. & Gruppen, H. Structural studies of plant cell wall polysaccharides using enzymes. In: *Plant polymeric carbohydrates.* F. Meuser, D.J. Manners & W. Seibel (eds), Berlin: The royal society of chemistry (1993) 3-15.

Voragen, A.G.J., Pilnik, W., Thibault, J-F., Axelos, M.A.V. & Renard, C.M.G.C. Pectins. In: *Food polysaccharides and their applications.* A.M. Stephen (ed), New York: Dekker (1995) 287-339.

Voragen, A.G.J., Beldman, G. & Schols, H.A. Chemistry and enzymology of pectins. In: *Advanced dietary fibre technology.* B.V. McCleary & L. Prosky (eds), Oxford: Blackwell Science (2001) 379-398.

Whitcombe, A.J., O'Neill, M.A., Steffan, W., Albersheim, P. & Darvill, A.G. Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II, *Carbohydr. Res.* 271 (1995) 15-29.

Williams, M.A. & Hron, R.J., Sr. Obtaining oils and fats from source materials. In: *Bailey's Industrial Oil and Fat Products: Edible oil and fat products: oils and oilseeds, Vol. 2.* Y.H. Hui (ed), New York: Wiley (1996) 61-155.

Chapter 2

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

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 compostion changed and the level of methyl esters and acetyl groups of 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.

This chapter has been published in Carbohydr. Polym. 43 (2000) 11-21 by the authors Esther Vierhuis, Henk A. Schols, Gerrit Beldman & Alphons G.J. Voragen.

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 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 *et al.*, 1994; Huisman *et al.*, 1996). Besides pectins the cell wall also contains significant amounts of acidic xylans and xyloglucans (Gil-Serrano *et al.*, 1986; Gil-Serrano & Tejero-Mateo, 1988; Coimbra *et al.*, 1994).

Ripening related changes in the composition of olive fruit cell wall have been studied by Heredia *et al.* (1993). They have studied the neutral sugar composition of the cell wall material during the development and ripening of 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 basis of their colour in 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 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.

Experimental

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).

Isolation of alcohol insoluble solids (AIS)

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 *et al.*, 1956). The residue (AIS) was dried by solvent exchange (96% (v/v) ethanol and acetone) at room temperature and ground (particle size < 1 mm).

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.

Ion-exchange chromatography

Ion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column (50 x 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.

Analytical methods

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.

Total uronic acid content

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

The neutral sugar composition

The neutral sugar composition of the AISs and the fractions was determined by gas chromatography according to Englyst & Cummings (1984) using inositol as an 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.

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 *et al.*, (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 *et al.*, 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 standar for (4-*O*-methyl)-glucuronic acid was available. Standards and samples were analysed by HPAEC.

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 x N.

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 *et al.* (1986) and expressed as mol

methyl esters or acetyl groups per 100 mol uronic acid.

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 x 4 mm) in combination with a CarboPac PA guard column (25 x 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. 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.

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 x 7.5 mm) in series (40XL, 30XL and 20XL; Bio-Rad Labs) in combination with a TSK XL guard column (40 x 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 4-500 kDa).

Results and discussion

Composition of the Alcohol Insoluble Solids (AIS)

The AIS from olive fruit (*O. europaea* cv koroneiki) contained glucose, arabinose and xylose as the major neutral sugars (Table 2.1). 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 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%

Table 2.1
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)	59/63	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.

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 good 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 2.1. 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 Voragen *et al.*, 1995; Redgwell *et al.* 1997).

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.

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 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

Table 2.2
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.

Table 2.3
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.

alkali was performed to solublilise 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 2.2 and 2.3 the yields and the sugar composition of the fractions are shown. The recoveries based on 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 was 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 *et al.*, 1988). In the next two sections the pectin and hemicellulose-rich fractions are described in more detail.

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 Voragen *et al.*, 1995; Seymour & Gross, 1996; Redgwell *et al.*, 1997). 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 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 mol% 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 *et al.*, 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 2.1). 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. 2.1. 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

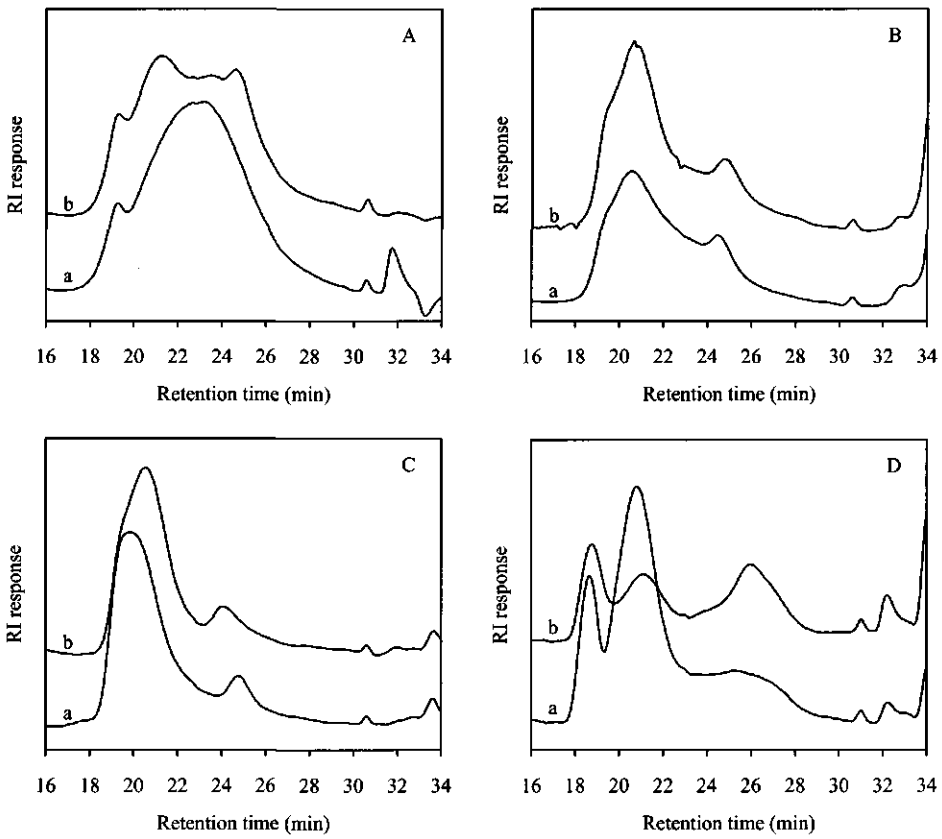

Fig. 2.1. HPSEC-patterns of the pectin-rich fractions from olive fruit. A: HBSS FI (a), HBSS FIII (b), B: ChSS FI (a), ChSS FIII (b), C: 0.05 M NaOH FI (a), 0.05 M NaOH FIII (b), D: 6 M NaOH FI (a), 6 M NaOH FIII (b).

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 which 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 implicate 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.

Hemicellulose-rich fractions

Total hemicellulose as obtained in the 1 and 4 M KOH fractions did not change appreciably during the ripening of the olive fruit (Tables 2.2 and 2.3). 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 part of this fraction (Gil-Serrano *et al.*, 1986; Gil-Serrano & Tejero-Mateo, 1988; Coimbra *et al.*, 1994).

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. 2.2. 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 en 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 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.

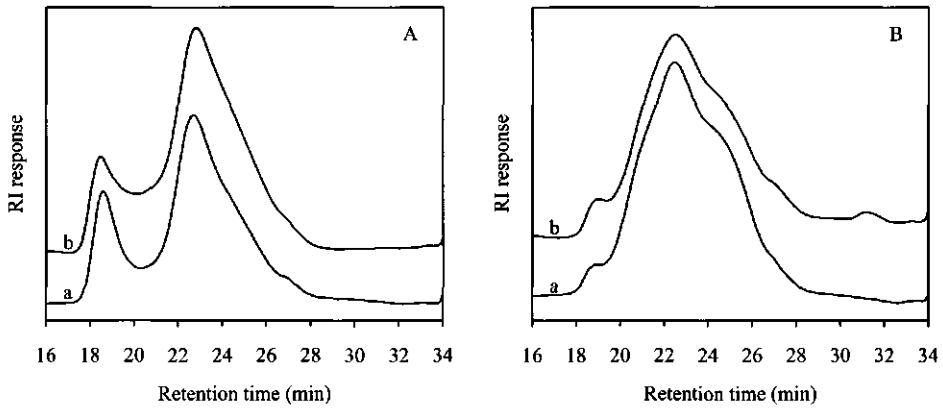

Fig. 2.2. HPSEC-patterns of the hemicellulose-rich fractions from olive fruit. A: 1 M KOH FI (a), 1 M KOH FIII (b), B: 4 M KOH FI (a), 4 M KOH FIII (b).

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 of two ripening stages.

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. 2.3 and 2.4. 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 M 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 (c. 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 2.4 and 2.5. 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

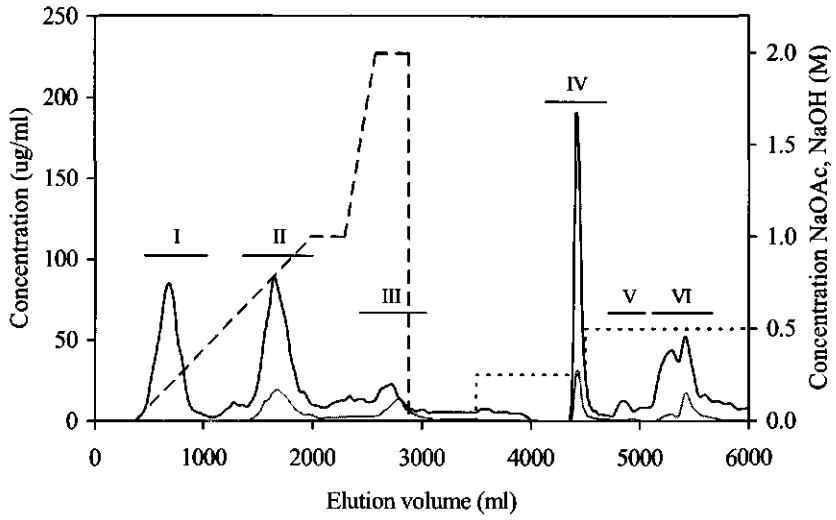

Fig. 2.3. Elution profile of 1 M KOH FIII on DEAE Sepharose Fast Flow. Elution with NaOAc (— —), elution with NaOH (– – –), neutral sugars (——), uronic acids (— —).

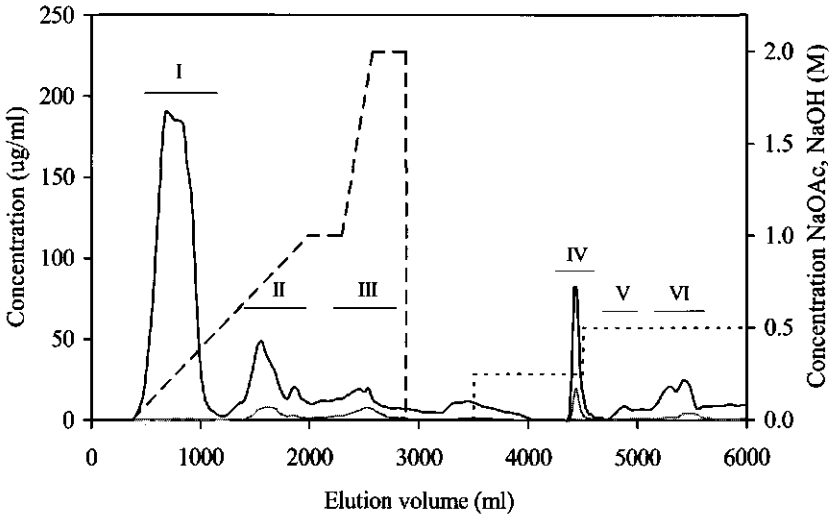

Fig. 2.4. Elution profile of 4 M KOH FIII on DEAE Sepharose Fast Flow. Elution with NaOAc (— —), elution with NaOH (– – –), neutral sugars (——), uronic acids (——).

residue of β-D-Gal*p*-(1→2)-α-D-Xyl*p* side chains of xyloglucans isolated from rapeseed hulls, apple and onion (Redgwell & Selvendran, 1986; York *et al.*, 1990; Renard *et al.*, 1992). 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 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

Table 2.4
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).

Table 2.5
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	3	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).

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 & 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 olive fruit. More information about the substitution patterns of the xyloglucans isolated from 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. 2.5. 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

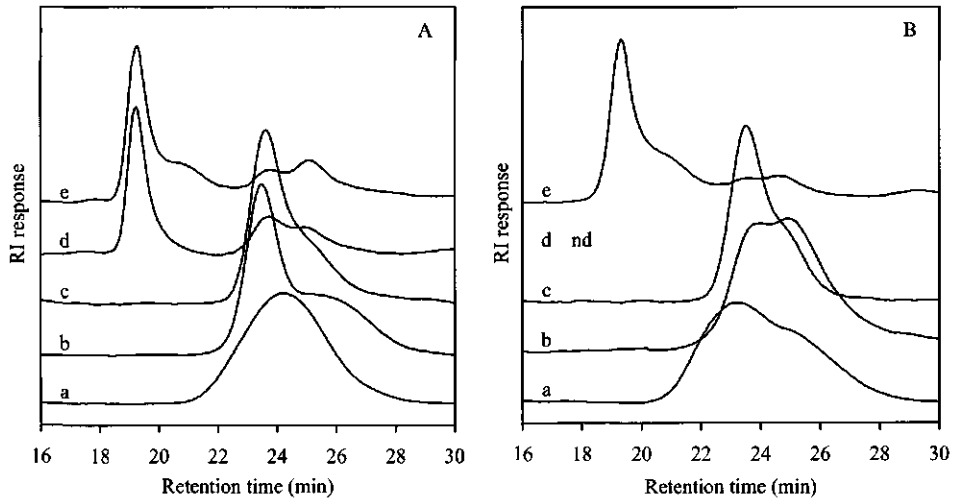

Fig. 2.5. HPSEC-patterns of the DEAE pools of 1 M KOH FIII (A) and 4 M KOH FIII (B). Pool I (a), pool II (b), pool IV (c), pool V (d) and pool VI (e). nd = not determined.

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*-hydroxydiphenyl assay. Only xylan 4 which 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 very well with the amounts of neutral sugars determined as alditol acetates (not shown). In Tables 2.4 and 2.5 the uronic acid content determined by the *m*-hydroxydiphenyl 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 pectic material that co-eluted. Xylan 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 relative 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 xylan 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. 2.5. 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 can not be explained. Xylan 2 consisted in all 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.

Acknowledgements

This project has been supported by the EU (AIR3-CT93-1355).

References

Coimbra, M.A., Waldron, K.W. & Selvendran, R.R. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L.), *Carbohydr. Res.* 252 (1994) 245-262.

De Ruiter, G.A., Schols, H.A., Voragen, A.G.J. & Rombouts, F.M. Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods, *Anal. Biochem.* 207 (1992) 176-185.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350-356.

Englyst, H.N. & Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates, *Analyst* 109 (1984) 937-942.

Fischer, R.L. & Bennett, A.B. Role of cell wall hydrolases in fruit ripening, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42 (1991) 675-703.

Gil-Serrano, A., Mateos-Matos, M.I. & Tejero-Mateo, M.P. Acidic xylan from olive pulp, *Phytochemistry* 25 (1986) 2653-2654.

Gil-Serrano, A. & Tejero-Mateo, P. A xyloglucan from olive pulp, *Carbohydr. Res.* 181 (1988) 278-281.

Heredia, A., Guillén, R., Jiménez, A. & Fernández-Bolaños, J. Activity of glycosidases during development and ripening of olive fruit, *Z. Lebensm. -Unters. -Forsch.* 196 (1993) 147-151.

Huisman, M.M.H., Schols, H.A. & Voragen, A.G.J. Changes in cell wall polysaccharides from ripening olive fruits, *Carbohydr. Polym.* 31 (1996) 123-133.

Kroef, C.H.M., Beldman, G. & Voragen, A.G.J. IX. Hemicellulose hydrolysis. In: *Cellulose hydrolysis and fermentation, proceedings of a CEC workshop* (pp. 94-102) J. Coombs & G. Grassi (eds), Brussels: CPL Press-Newbury (1992).

Massiot, P., Rouau, X. & Thibault, J-F. Characterisation of the extractable pectins and hemicelluloses of the cell wall of carrot, *Carbohydr. Res.* 172 (1988) 229-242.

Mort, A.J., Moerschbacher, B.M., Pierce, M.L. & Maness, N.O. Problems encountered during the extraction, purification, and chromatography of pectic fragments and some solutions to them, *Carbohydr. Res.* 215 (1991) 219-227.

Redgwell, R.J., Fischer, M., Kendal, E. & Macrae, A. Galactose loss and fruit ripening: high-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls, *Planta* 203

(1997) 174-181.

Redgwell, R.J. & Selvendran, R.R. Structural features of cell-wall polysaccharides of onion *Allium cepa*, *Carbohydr. Res.* 157 (1986) 183-199.

Renard, C.M.G.C., Lomax, J.A. & Boon, J.J. Apple-fruit xyloglucans: a comparative study of enzyme digests of whole cell walls and of alkali-extracted xyloglucans, *Carbohydr. Res.* 232 (1992) 303-320.

Roozen, J.P. & Van Boxtel, L. Half-automatische bepaling van stikstof in levensmiddelen, *De Ware(n)-Chemicus* 9 (1979) 192-195.

Ryden, P. & Selvendran, R.R. Structural features of cell wall polysaccharides of potato (*Solanum tuberosum*), *Carbohydr. Res.* 195 (1990) 257-272.

Seymour, G.B. & Gross, K.C. Review article: Cell wall disassembly and fruit softening, *Postharvest News and Information* 7 (1996) 45-52.

Thibault, J-F. Automatisation du dosage des substances pectiques par la methode au meta-hydroxydiphenyl, *Lebensm. -Wiss. -Technol.* 12 (1979) 247-251.

Tollier, M-T. & Robin, J-P. Adaption de la methode a l'orcinol-sulfurique au dosage automatique des glucide neutres totaux: conditions d'application aux extraits d'orgine vegetable, *Ann. Technol. Agric.* 28 (1979) 1-15.

Voragen, A.G.J., Pilnik, W., Thibault, J-F., Axelos, M.A.V. & Renard, C.M.G.C. Pectins. In: *Food polysaccharides and their applications* (pp. 287-339). A.M. Stephen (ed), New York: Dekker (1995).

Voragen, A.G.J., Schols, H.A. & Pilnik, W. Determination of the degree of methylation and acetylation of pectins by h.p.l.c., *Food Hydrocolloids* 1 (1986) 65-70.

York, W.S., Van Halbeek, H., Darvill, A.G. & Albersheim, P. Structural analysis of xyloglucan oligosaccharides by H-nmr spectroscopy and fast-atom-bombardment mass spectrometry, *Carbohydr. Res.* 200 (1990) 9-31.

Chapter 3

Structural characterisation of xyloglucan and xylans present in olive fruit (*Olea europaea* cv koroneiki)

Abstract

Hemicellulose-rich fractions obtained from olive fruit were fractionated by anion-exchange chromatography, which resulted in a xyloglucan-rich pool and four xylan-rich pools. Sugar linkage analyses and degradation studies with specific enzymes were performed to obtain information about the structures. The results indicated a xyloglucan in olive fruit with a specific substitution pattern, which is not commonly found in plant cell walls: XXXG-type building units with both arabinosyl and galactosyl residues linked to it. The xylans present in olive fruit were all very low in substitution with mainly 4-*O*-methyl-glucuronic acid residues. Enzymatic degradation with endo-xylanases resulted in a mixture of neutral and acidic xylo-oligosaccharides. Striking were the identical degradation patterns on HPAEC for all xylan-rich pools while the elution of the pools on DEAE Sepharose differed markedly.

This chapter has been published in Carbohydr. Polym. 44 (2001) 51-62 by the authors Esther Vierhuis, Henk A. Schols, Gerrit Beldman & Alphons G.J. Voragen.

Introduction

The cell wall material of olive fruit contains besides pectic material also considerable amounts of hemicelluloses (Coimbra *et al.*, 1994; Huisman *et al.*, 1996; Vierhuis *et al.*, 2000). Hemicelluloses require relatively strong alkali, typically 1-4 M, for their extraction from the cell wall due to strong hydrogen bonding to cellulose microfibrils. The composition of the hemicelluloses of plant cell walls differs for each species (Brett & Waldron, 1990). The major hemicellulose components in olive fruit are xylans and xyloglucans (Coimbra *et al.*, 1994). Xylans have a backbone of (1→4)-β-linked Xyl residues. Depending on their origin the backbone is substituted with GlcA or its 4-*O*-methylated derivative, Ara and acetyl groups (Wilkie, 1979). Xyloglucan consists of a backbone of (1→4)-β-linked Glc residues branched on C-6 with Xyl residues. Two general types of xyloglucan poly-XXXG and poly-XXGG can be distinguished which differ in the degree of backbone substitution with Xyl residues. Some of the Xyl residues are substituted with Gal or Ara residues or a disaccharide of Fuc 1,2-linked to Gal. In addition, xyloglucans are often *O*-acetylated as reviewed by Vincken *et al.* (1997b).

From cell wall material of olive fruit an acidic xylan and a xyloglucan were isolated and partially characterised by Gil-Serrano *et al.* (1986) and Gil-Serrano & Tejero-Mateo (1988). Coimbra and co-workers (1994) have continued the research on hemicellulose in olive fruit and reported the composition and structural features of hemicellulose-rich fractions. They have described the presence of xylan-xyloglucan complexes in the cell wall of olive pulp in which the xylan and xyloglucan moieties are strongly attached (Coimbra *et al.*, 1995). In chapter 2 we have described the isolation and analysis of hemicellulose-rich fractions from olive fruit. These 1 and 4 M KOH soluble polymers were fractionated by anion-exchange chromatography, which resulted in a xyloglucan-rich pool and four xylan-rich pools. The present study investigates in more detail the polymers present in the 1 and 4 M KOH fractions. Besides analysis of the glycosidic linkage composition also well characterised enzymes will be used to obtain information about the structure of the xyloglucans and xylans present in olive fruit cell walls.

Experimental

Materials

Substates

The xyloglucan-rich pools and xylan-rich pools were obtained by anion-exchange chromatography from 1 and 4 M KOH fractions of purple olive fruit as described by Vierhuis *et al.* (2000). The xylan-rich pools were named according to their order of elution from the anion-exchange column as xylan 1, 2, 3 and 4.

Enzymes

Endo-(1→4)-β-D-xylanase I and III (XylI, XylIII) were purified from a culture filtrate from

Aspergillus Awamori as described by Kormelink *et al.* (1993b). Endo-(1→4)-β-D-glucanase IV and V (EndoIV, EndoV) were purified from a commercial enzyme preparation from *Trichoderma viride* as described by Beldman *et al.* (1985).

Enzyme incubations

The xyloglucan-rich pools and xylan-rich pools were dissolved in a 150 mM NaOAc buffer of pH 5.0 containing 0.01% (w/v) NaN3 and incubated with purified enzyme. The incubations were performed at 40°C for 24 h with a substrate concentration of 1 mg/mL. The amount of enzyme used was 0.2 µg protein/mL and 1.0 µg protein/mL for EndoIV and EndoV, respectively and 0.5 µg protein/mL for XylI and XylIII. The resulting digests were heated for 15 min at 100°C to inactivate the enzymes. The change in molecular weight distribution and the release of oligomeric end-products were studied by HPSEC and HPAEC, respectively. For each enzyme it was checked that the limit of digestion was reached after 24 h for the concentration of the enzyme used.

Isolation and characterisation of XylI-treated polymeric material

Xylan 3 and 4 were incubated with endo-xylanase I (XylI) as described above. After incubation the residual polymeric fraction was separated from the oligosaccharides present in the digest by ultrafiltration (Nominal Molecular Weight Cut-off 30 kDa; Pall Filtron). The polymeric fraction was freeze-dried and analysed for sugar composition using methanolysis combined with a trifluoroacetic acid hydrolysis as described by De Ruiter *et al.* (1992) followed by an enzymatic hydrolysis of the incomplete hydrolysed aldobiuronic acids (Vierhuis *et al.*, 2000). The neutral sugar and uronic acid composition was determined by HPAEC using the gradient for uronic acids described before (Vierhuis *et al.*, 2000) which was also used for the analysis of neutral sugars.

Analytical methods

Sugar linkage composition

Samples were methylated according to a modification of the Hakomori method (Sandford & Conrad, 1966) without a carboxyl reduction and subsequently dialysed against water and dried by evaporation (air stream, room temperature). The methylation step was repeated in order to improve the completeness of the reaction. The methylated polysaccharides were hydrolysed with 2 M trifluoroacetic acid (1 h, 121°C), which was removed by evaporation (air stream, at < 10°C). The released (partially methylated) sugars were converted into their alditol acetates (Englyst & Cummings, 1984), which were quantified by GC-FID and identified by GC-MS. Sodium borodeuteride was used for reduction. Quantification was performed by GC-FID by on-column injections on a fused silica capillary column (30 m x 0.32 mm; wall coated with DB 1701; 0.25 µm film thickness; J & W Scientific) in a Carlo-Erba HRGC 5160 gas chromatograph equipped with a flame ionisation detector set at 280°C. The temperature program was 80→180°C at 20°C/min, 180→230°C at 2°C/min, and 230°C for 3 min.

Derivatives were quantified according to their effective carbon response (Sweet *et al.*, 1975). Identification of the compounds was confirmed by GC-MS using a CP Sil 19 CB capillary column (25 m x 0.25 mm, 0.2 μm; Chrompack) in an HP 6890 gas chromatograph coupled to a HP 5973 mass-selective detector and using a HP Chem Station (Hewlett Packard). The temperature program was 160→185°C at 0.5°C/min, 185→230°C at 10°C/min and 230°C for 5.5 min.

High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed as described previously (Vierhuis *et al.*, 2000). Xyloglucan oligosaccharides were analysed with the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→50 mM NaOAc; 5→45 min, linear gradient of 50→80 mM NaOAc; 45→60 min, linear gradient of 80→260 mM NaOAc; Xylan oligosaccharides were analysed as described by Verbruggen *et al.* (1998b). After each run the column was washed for 5 min with 100 mM NaOH containing 1 M NaOAc, and subsequently equilibrated for 15 min with the starting eluent.

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed as described before (Vierhuis *et al.*, 2000)

Mass spectrometry

Matrix-assisted laser-induced desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) in the reflector mode was performed using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems) equipped with a nitrogen laser operating at 337 nm (3 ns pulse duration), a single stage reflector and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from 100-256 laser shots. Sample preparation: The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxy isoquinoline in 700 μL distilled water and 300 μL acetonitril. A 1 μL volume of this solution was placed on the sample plate and mixed with 1 μL of the enzyme digest and allowed to dry at room temperature. Mass spectra were calibrated with an external standard containing cellodextrins (DP 3-9).

Results and discussion

Chemical characterisation

In chapter 2 we have described the fractionation by anion-exchange chromatography of hemicellulosic polysaccharides extracted from olive fruit with 1 and 4 M KOH. The extracts had similar elution behaviours on DEAE Sepharose and all contained a xyloglucan-rich pool and four xylan-rich pools. The xyloglucan-rich pool represented 20% and 61% of the sugars present in the 1 and 4 M KOH extract, respectively. Glc, Xyl, Ara and Gal were identified as

the major neutral sugar residues of the xyloglucan-rich pools. The four xylan-rich pools all bound to the column and were eluted by a sodium acetate gradient or by alkali. The major fraction bound to the DEAE column was xylan 1, which eluted as soon as the acetate gradient was applied. It comprised 40% of the xylans in the extract. The other xylan-rich pools were designated according to their order of elution from the anion-exchange column as xylan 2, 3 and 4. The xylan-rich pools contained mainly Xyl residues (65-83 mol%) and small amounts of uronic acids (11-18 mol%). The uronic acids of xylan 1, 2 and 3 comprised mainly 4-*O*-MeGlcA, whereas the uronic acids in xylan 4 appeared to be almost exclusively GalA indicating the presence of pectins. The presence of acetyl groups in olive fruit xyloglucan and xylan could not be determined due to the use of alkali to extract the hemicelluloses which saponified the ester-linkages.

The glycosidic linkage compositions of the pools obtained from the 1 M KOH extract are shown in Table 3.1. Unfortunately, no sugar linkage analysis could be performed of xylan 3 because the amount of material was too low. Of this pool only the neutral sugar composition determined by alditol acetates (between brackets) is given. The sugar linkage analysis confirmed the presence of a xyloglucan and xylans. The sugar compositions derived from the sugar linkage analyses were in good agreement with the results from the analyses of alditol acetates. Only the Rha and Gal contents determined by methylation analysis were rather low for all pools. Despite of the good correlation between the sugar composition found after per-*O*-methylation and the composition determined by alditol acetates, the ratio of terminal over branched residues deviated from 1 for a number of pools. This indicates undermethylation, which is generally observed for uronide containing polysaccharides.

The xyloglucan-rich pool contained 1,4- and 1,4,6-linked Glc residues typical for the cellulosic backbone of xyloglucans. About 70% of the Glc residues of the backbone were branched compared to the value of 55% reported previously for a xyloglucan isolated from olive fruit (Gil-Serrano & Tejero-Mateo, 1988). Terminal Xyl and 1,2-linked Xyl residues were present in almost similar amounts. No 1,4-linked Xyl residues typical for the presence of xylans were detected in this pool. Almost all Ara and Gal residues were present as terminal residues. Very small amounts of 1,5-linked Ara and 1,4-linked Gal indicated that the xyloglucan-rich pool was slightly contaminated with arabinan and galactan. The data in Table 3.1 further indicated contamination with a small amount of 1,4-linked Man residues. Terminal Fuc residues suggested that also small amounts of α-L-Fuc*p*- containing side chains (< 1%) could be present in olive fruit. However, the specific Gal 1,2-linkage present in this side chain could not be detected in the sugar linkage analyses. No Glc residues with a substitution at C-2 could be detected. This indicated that substitution of the backbone with α-L-Ara*f*, β-D-Xyl*p* or α-L-Ara*f*-(1→3)-β-D-Xyl*p* at C-2 of the Glc residue (Kiefer *et al.*,1990; Hisamatsu *et al.*,1992) did not occur in olive fruit xyloglucan.

Xylan 1, 2 and 4 were mainly composed of 1,4-linked Xyl residues. Less than 10% of the Xyl residues were mono substituted with branch points at the C-2 or C-3 position. No double branched Xyl residues could be detected in xylan 1 and 2. Xylan 4 consisted for about 14% of unmethylated Xyl residues. These residues could be present as disubstituted Xyl, but it is more likely that some undermethylation has occurred. Especially, since also part of the Ara, Gal and Glc residues appeared to be present as unmethylated alditol acetates. Previous results

Table 3.1
Neutral sugar linkage composition (mol%) of the xyloglucan- and xylan-rich pools of the 1 M KOH extract from olive fruit; within brackets the neutral sugar composition determined by alditol acetates and the uronic acid content determined by *m*-hydroxydiphenyl assay is given (mol per 100 mol neutral sugars)

Sugar linkage	Xyloglucan		Xylan 1		Xylan 2		Xylan 3		Xylan 4	
Rhamnose										
T-Rha*p*	-		-		-				0.3	
Total	-	(1)	-	(2)	-	(2)	-	(2)	0.3	(5)
Fucose										
T-Fuc*p*	0.7		-		-				-	
Total	0.7	(1)	-	(-)	-	(-)	-	(-)	-	(-)
Arabinose										
T-Ara*f*	10.9		5.0		1.1				1.9	
1,5-Ara*f*	0.6		2.2		-				2.9	
1,3,5-Ara*f*	-		-		-				1.0	
1,2,3,5-Ara*f*	-		-		-				3.0	
Total	11.5	(11)	7.2	(9)	1.1	(1)	-	(1)	8.8	(10)
Xylose										
T-Xyl*p*	15.7		3.7		1.8				1.1	
1,4-Xyl*p*	-		77.1		90.6				65.4	
1,2-Xyl*p*	19.3		-		-				-	
1,2,4-Xyl*p*	-		4.8		3.6				1.6	
1,3,4-Xyl*p*	-		1.8		1.3				0.7	
1,2,3,4-Xyl*p*	-		-		-				13.7	
Total	35.0	(31)	87.4	(79)	97.3	(94)	-	(94)	82.5	(74)
Mannose										
1,4-Man*p*	1.4		-		-				-	
Total	1.4	(2)	-	(1)	-	(1)	-	(1)	-	(1)
Galactose										
T-Gal*p*	8.6		0.7		-				0.3	
1,4-Gal*p*	0.6		-		-				-	
1,3,6-Gal*p*	-		2.2		-				-	
1,2,3,4,6-Gal*p*	-		-		-				0.6	
Total	9.2	(11)	2.9	(7)	-	(1)	-	(1)	0.9	(3)
Glucose										
1,4-Glc*p*	13.7		1.3		0.9				4.9	
1,4,6-Glc*p*	28.6		1.1		-				-	
1,2,3,4,6-Glc*p*	-		-		0.7				2.7	
Total	42.3	(43)	2.4	(2)	1.6	(1)	-	(1)	7.6	(7)
Uronic acid										
Total	nd[a]	(1)	nd	(16)	nd	(15)	nd	(12)	nd	(14)
Ratio terminal/ branching	1.25		0.96		0.43		-		0.08	

[a] Not determined.

showed that the xylan-rich pools contain 4-*O*-MeGlcA and GlcA which are expected to be attached to the backbone (Vierhuis *et al.*, 2000). Xylan 1 contains GlcA as well as 4-*O*-MeGlcA residues that are most certainly attached to C-2 of the Xyl residues (Gil-Serrano *et al.*, 1986). Xylan 2 and 3 contain mainly 4-*O*-MeGlcA. The uronic acids of xylan 4 appear to be almost exclusively GalA indicating that pectins are also present in this pool. Only 16% of the uronic acids present in xylan 4 were GlcA and 4-*O*-MeGlcA. The substituents on the xylan backbone are besides 4-*O*-MeGlcA and GlcA residues probably also single unit Ara residues for xylan 1, 2 and 4. The exact amount of branch points was difficult to estimate from the sugar linkage analyses of the xylan-rich pools because the uronic acid residues were not reduced and so not included in the methylation analysis. Probably, only a small part of the very acid resistant 4-*O*-MeGlcA-Xyl and GlcA-Xyl linkages are hydrolysed in the procedure followed since TFA hydrolysis after per-*O*-methylation is not able to completely hydrolyse the acid resistant uronic acid glycosyl linkages (Vierhuis *et al.*, 2000). In general, carboxyl reduction is used to determine the glucuronic and galacturonic acid as deuterated Glc and Gal in the sugar linkage analysis. The reduction makes the glycosyl linkages more susceptible to acid hydrolysis but it is also known that carboxyl reduction is often not complete and gives a poor recovery of the uronic acids (Coimbra *et al.*, 1995; Verbruggen *et al.*, 1995). Despite of the fact that the precise percentages of branch points could not be determined by the method followed, it can be concluded that the xylans extracted from olive fruit were low in substitution using data from a previous study (Vierhuis *et al.*, 2000) for an estimation of the amounts of uronic acids present.

Coimbra *et al.* (1995) have reported the occurrence of a complex containing glucuronoxylan and xyloglucan in the 1 M KOH fraction from olive fruit. The extraction procedure we performed was not exactly identical to their method but we expected that the material they extracted with 1 M KOH at 1°C would appear in our fraction of 1 M KOH at 20°C. However, we were not able to confirm the occurrence of the complex containing glucuronoxylan and xyloglucan. Anion-exchange chromatography of our material resulted in pools that contained either xyloglucan or xylans, but not a mixture of both of them. The xylan-rich pools did contain some (1,4)- and (1,4,6)-linked Glc, but only in very small amounts compared to the amounts described by Coimbra *et al.* (1995).

Enzymatic characterisation

More information about the structure of the xyloglucan present in the 1 and 4 M KOH extract was obtained by incubating them with two endo-glucanases from *Trichoderma viride* having a high xyloglucanase activity, EndoIV and EndoV. Both enzymes cleave the glucan backbone next to an unbranched Glc residue but have different subsites (Vincken *et al.*, 1997a). The HPSEC elution profiles of the incubations are shown in Fig. 3.1. The elution pattern of the xyloglucan-rich pool isolated from the 1 M KOH extract showed a major population with a molecular mass of about 150 kDa as based on calibration with dextrans. Incubation with EndoIV or EndoV degraded the xyloglucan entirely into oligosaccharides. The elution pattern of the xyloglucan-rich pool isolated from the 4 M KOH extract showed a major population with a molecular mass of about 150 kDa and in addition a smaller

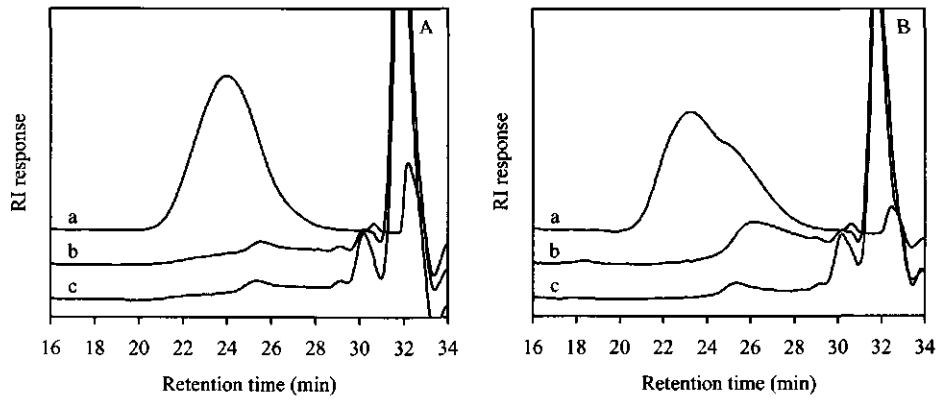

Fig. 3.1. HPSEC-patterns of the xyloglucan-rich pools of the 1 M KOH (A) and 4 M KOH (B) extract from olive fruit (a) before incubation and after incubation with (b) endo-glucanase IV (EndoIV) and (c) endo-glucanase V (EndoV).

population of about 50 kDa. HPSEC analysis of this xyloglucan-rich pool treated with EndoIV showed a residual population which probably consisted of a Man-containing polysaccharide. The same phenomenon has been described for a xyloglucan-rich fraction isolated from potato with a mannan contamination (Vincken *et al.*, 1996a). Incubation with EndoV degraded both populations present in this pool entirely into oligosaccharides. Apparently, EndoV was able to degrade the Man-containing polysaccharide of the xyloglucan-rich pool. It is not sure whether EndoV is able to cleave this polysaccharide or it contains a residual endo-mannanase activity (Vincken *et al.*, 1996a).

The elution patterns on HPAEC of the digests of the xyloglucan-rich pools showed a rather

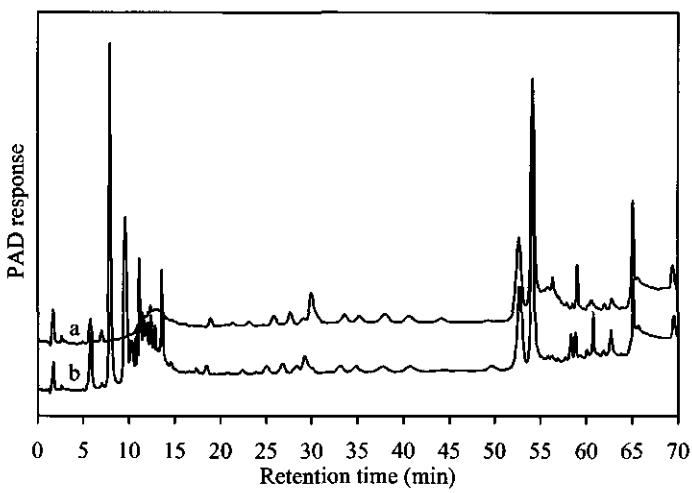

Fig. 3.2. HPAEC-patterns of the xyloglucan-rich pool of the 4 M KOH extract from olive fruit digested with (a) endo-glucanase IV (EndoIV) and (b) endo-glucanase V (EndoV).

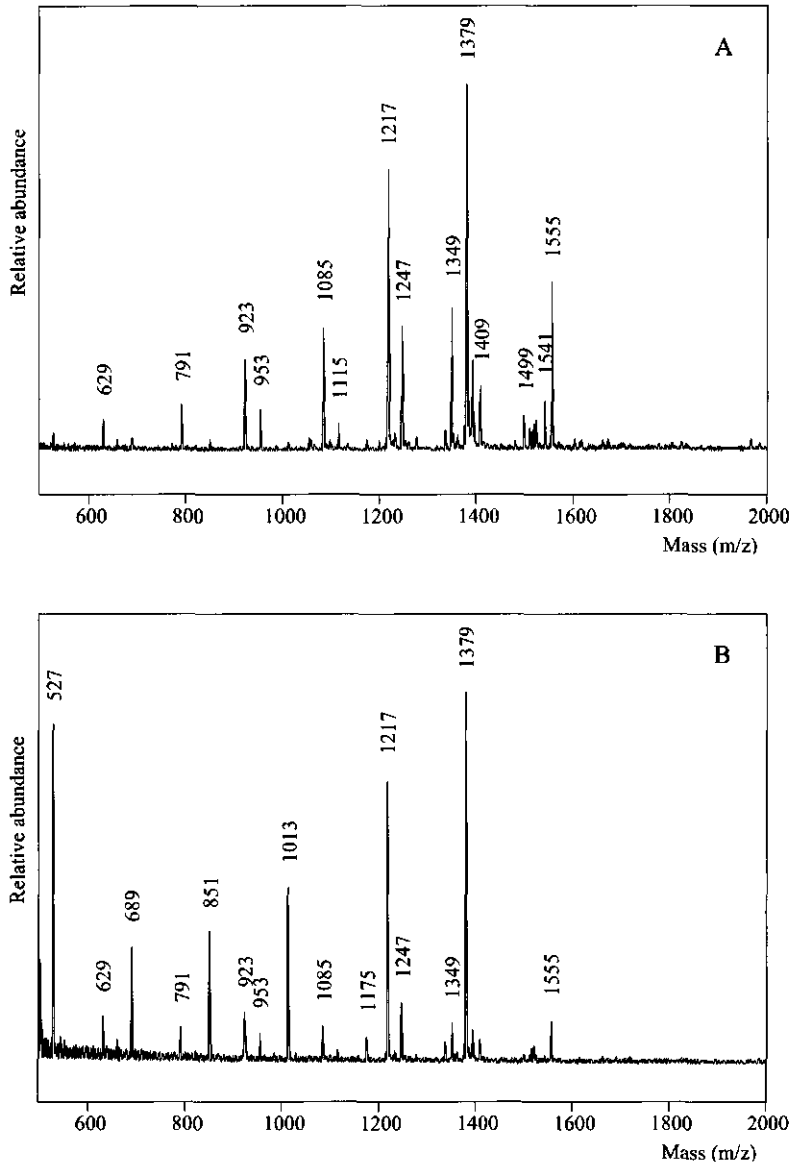

Fig. 3.3. MALDI-TOF mass spectra of the xyloglucan-rich pool of the 4 M KOH extract from olive fruit digested with (A) endo-glucanase IV (EndoIV) and (B) endo-glucanase V (EndoV).

complicated mixture of oligosaccharides with two major peaks at about 52 and 54 min (Fig. 3.2). Products eluting between 7 and 15 min probably originated from the mannan contamination because they mainly appeared in the xyloglucan-rich pool from the 4 M KOH extract incubated with EndoV. Analysis on HPAEC of a galactomannan digest confirmed that Man-containing oligosaccharides eluted in this region. The degradation patterns of the

Table 3.2
Data on MALDI-TOF mass spectra analysis of the xyloglucaan-rich pools of the 1 and 4 M KOH extracts from olive fruit incubated with endo-glucanase IV (EndoIV) and endo-glucanase V (EndoV)

Mass ($M+Na^+$)		1 M KOH		4 M KOH		Tentative structures[a]
		EndoIV	EndoV	EndoIV	EndoV	
527	$(Hex)_3$	nd[b]	+	nd	+++	Mannan oligosaccharide
851	$(Hex)_5$	nd	w	w	++	Mannan oligosaccharide
1013	$(Hex)_6$	nd	w	w	++	Mannan oligosaccharide
1085	$(Hex)_4(Pent)_3$	+	w	+	w	XXXG/G[SX]G/[SX]GG
1217	$(Hex)_4(Pent)_4$	+++	+++	+++	+++	X[SX]G/GSSG/SSGG
1247	$(Hex)_5(Pent)_3$	+	+	++	+	X[LX]G/G[SL]G/[SL]GG
1349	$(Hex)_4(Pent)_5$	+	w	++	w	XSSG
1379	$(Hex)_5(Pent)_4$	+++	+++	+++	+++	X[SL]G/GSSGG
1555	$(Hex)_6(Pent)_3(Deoxyhex)_1$	+	w	++	w	X[LF]G

[a] Nomenclature according to Fry *et al.* (1993) with specific code letters for each segment (G: β-D-Glc*p*-; X: α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; L: β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; S: α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; F: α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-).

[b] +++, ++, +, w and nd denote high, medium, minor, weak and not detectable peaks in the spectra.

xyloglucans isolated from the 1 and 4 M KOH extract were identical between 15 and 55 min. Only a small amount of free Glc was produced on enzymic hydrolysis (c. 1-2% of Glc present). Degradation of the xyloglucan-rich pools by EndoIV and EndoV gave identical products although small differences in the relative amounts of products were noticed. Comparison of the degradation patterns of olive fruit xyloglucan with degradation patterns of xyloglucans with known structural features (apple: XXXG core with Gal residues; potato: XXGG core with Gal and Ara residues in a ratio of 1/1) incubated with the same endo-glucanases (Vincken *et al.*, 1996a; Vincken *et al.*, 1996b) indicated that xyloglucan in olive fruit had a different substitution pattern.

The MALDI-TOF mass spectra of the digests of the xyloglucan-rich pool from the 4 M KOH extract are given in Fig. 3.3. Both spectra showed a diversity of masses. Tentative structures were proposed for the different oligosaccharides considering the glycosidic linkage composition of the xyloglucan-rich pool, the mode of action of the endo-glucanases and the molecular masses of the oligosaccharides in the digests (Table 3.2). The tentative structures of the masses with an accumulated intensity of at least one third of the main peak (*m/z* 1379) in the mass spectra are shown to emphasise the main characteristics of the digests. It should be kept in mind that these intensities do not necessarily have to correlate completely with the amount of material present in the digests although it is thought that peaks with the highest intensities are the most abundant. Also it is not possible to compare the intensities of different spectra because the intensity may be influenced by many factors like concentration on a specific spot, laser intensities, average scans etc.

The mass spectra of the digests showed two major components with a mass that corresponded to a sodium adduct of oligosaccharides from xyloglucans. Oligosaccharide

structures originating from xyloglucans composed of either XXXG-type or XXGG-type building units could be proposed. In principle, there were three possible structures for $(Hexose)_4(Pentose)_4$: X[SX]G, GSSG and SSGG. For $(Hexose)_5(Pentose)_4$ two structures could be proposed: X[SL]G and GSSGG. The structural elements containing Ara (S) and Gal (L) were indicated between brackets to indicate that the exact position of these residues was not known.

Based on the sugar linkage composition the gross formula of $(Hexose)_5(Pentose)_4$ could represent two possible structures X[SL]G and GSSGG as mentioned before. However, from literature it is known that EndoV is able to release Glc from the reducing end of oligosaccharides with two unbranched Glc residues (Vincken *et al.*, 1996a). Consequently, the major peak with *m/z* 1379 was not likely to correspond to GSSGG in the EndoV digest. Re-incubation of the EndoV digest showed that the profile of the mass spectrum did not change which further substantiated that X[SL]G and not GSSGG was present as a major compound in the digest. The fact that very low amounts of free Glc were detected in the fractions treated with endo-glucanases was also in agreement with the above results.

The other major component in the mass spectrum (*m/z* 1217) could represent structures of oligosaccharides from xyloglucan of the XXGG-type as well as the XXXG-type. In case of a structure with XXGG-type building units, SSGG and GSSG could be present as a major component in the digests of EndoIV and EndoV, respectively. In case of XXXG-type building units both enzymes would have released X[SX]G. So, evidence about the branching pattern of the xyloglucans present in olive fruit could not be obtained from this *m/z* value.

For several components in the mass spectra it was not possible to compose a structure of XXXG-type as well as XXGG-type building units. For example, the peak with *m/z* 1349 with a gross formula of $(Hexose)_4(Pentose)_5$ suggested almost certainly an XXXG-type building unit with two Xyl residues substituted with Ara residues. An XXGG-type building unit with a short side chain of Ara residues might be a possibility but has not been published yet (Vincken *et al.*, 1997b). Also for the oligosaccharide with *m/z* 1555 the structure X[LF]G with a XXXG-type building unit is more likely than a structure with an XXGG-type building unit. Oligosaccharides with Fuc residues like XLFG have been reported before in literature. Besides XLFG also XXFG, and XFFG have been described (York *et al.*, 1990; Hisamatsu *et al.*, 1991). The spectrum of the EndoV digest of the xyloglucan-rich fraction from the 4 M KOH extract contained besides the masses characteristic for xyloglucan oligosaccharides also peaks with a high intensity consisting of only hexose residues. These peaks most probably originated from glucomannan or galactomannan oligosaccharides formed by EndoV. The absence of these peaks in the EndoV digest of the 1 M KOH extract and the EndoIV digests confirmed this observation.

The data of the MALDI-TOF mass spectra combined with the sugar linkage composition and the knowledge of the mode of action of both endo-glucanases indicated the presence of a xyloglucan in olive fruit with a different structure compared to xyloglucan from other plant sources. Based on the results it can be concluded that olive fruit contained a xyloglucan with an XXXG core with Gal as well as Ara residues linked to the Xyl residues. The presence of terminal Ara as well as terminal Gal residues are usually described for xyloglucans with an XXGG core which are present in various solanaceous plants (Vincken *et al.*, 1997b).

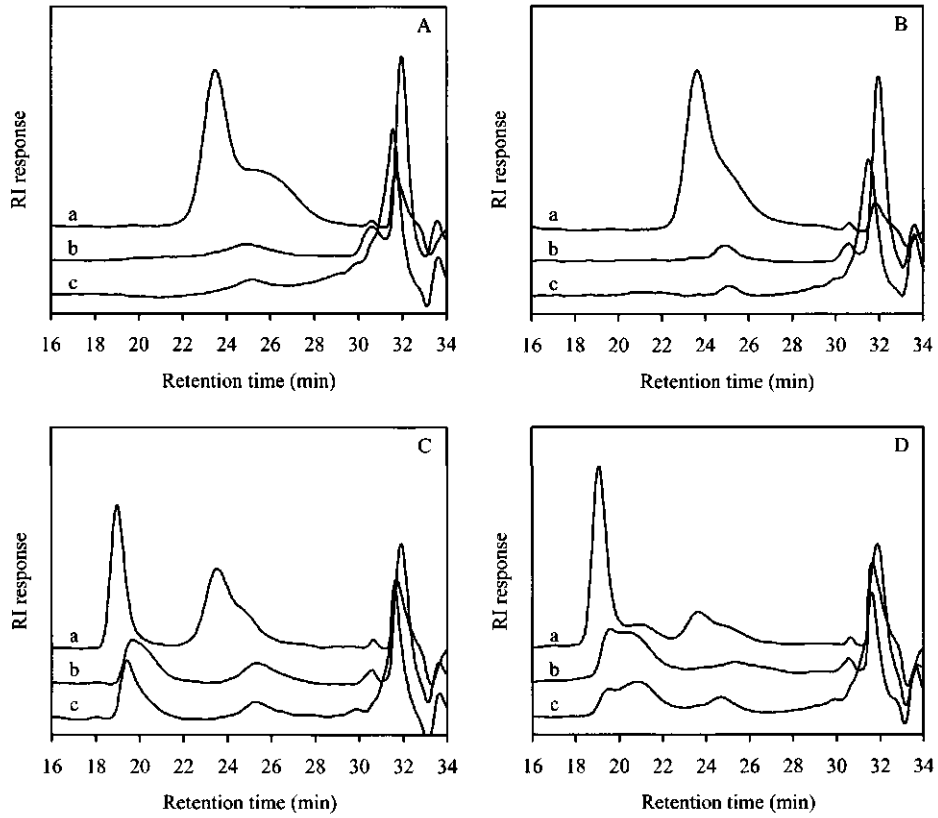

Fig. 3.4. HPSEC-patterns of the xylan-rich pools of the 1 M KOH extract from olive fruit (a) before incubation and after incubation with (b) endo-xylanase I (XylI) and (c) endo-xylanase III (XylIII): (A) xylan 1; (B) xylan 2; (C) xylan 3; (D) xylan 4.

Xyloglucans isolated from other dicotyledonous plants in general have XXXG-type building units and terminal Ara residues attached to Xyl residues have not been described very often. Side chains of α-L-Ara*f*-(1→2)-α-D-Xyl*p*- have been suggested to be present in xyloglucan from non solanaceous plants as runner bean (O'Neill & Selvendran, 1983) and tamarind (Niemann *et al.*, 1997) but no clear evidence has been reported (Vincken *et al.*, 1997b). However, the presence of especially the peak with *m/z* 1379 as a major component in the digest and the other peaks with masses that could only correspond to an oligosaccharide with a structure of the XXXG-type supported the finding that olive fruit xyloglucan consisted of XXXG-type building units with terminal Ara as well as terminal Gal residues linked to it.

More information about the xylan-rich pools isolated from the 1 and 4 M KOH extract was obtained by incubation with two different endo-xylanases from *Aspergillus awamori*, XylI and XylIII. Kormelink *et al.* (1993a) have shown clear differences in the mode of action of these two endo-xylanase. The enzymes are in a different way restricted in the hydrolysis of xylosidic linkages in the vicinity of branch points, which is reflected in many types of

heterogenous oligosaccharides released. In general it can be concluded that XylIII is more hindered by substitution of the xylan backbone than XylI. The digests of xylan-rich pools were subjected to analysis by HPSEC, HPAEC and MALDI-TOF MS. It appeared that the results of the analyses were identical for the xylans isolated from the 1 and 4 M KOH extract, therefore only the results of the xylan-rich pools isolated from the 1 M KOH extract will be discussed in the next paragraphs.

The HPSEC elution pattern of xylan 1 showed a major population with a molecular mass of about 150 kDa which corresponded to a DP of c. 1100 as based on calibration with dextrans (Fig. 3.4). The shoulder of the main peak in the elution pattern indicated the presence of another population in this pool with a molecular mass of about 25 kDa. Incubation with XylI as well as XylIII resulted in a shift to lower molecular mass ranges of the material in this pool. Only 5-10% of the polymers remained as material with a high molecular mass indicating that the substitution pattern of the xylans hardly hindered the action of the endo-xylanases. Xylan 2 had an elution pattern quite similar to xylan 1 and XylI and XylIII were also able to degrade this xylan-rich pool almost entirely. Xylan 3 and 4 both contained a population which eluted in the void volume of the column set used so no exact molecular mass could be determined. The molecular mass of these populations was at least 500 kDa. Unlike the first two xylan-rich pools, xylan 3 and 4 were less degradable to fractions with a low molecular mass. Although XylI is less hindered by substitution than XylIII both enzymes were not able to degrade these pools completely. This might indicate that these pools contained besides low substituted xylans also highly substituted xylans. The xylans with highly branched regions could be interlinked with degradable, more linear regions but the existence of different populations of xylans in the partly degradable pools was also possible.

Xylan 3 and 4 were incubated with XylI on a larger scale to investigate the composition of the undegradable polymers. The residual polymeric material was isolated to yield a XylI-treated xylan 3 and a XylI-treated xylan 4 fraction. Characterisation of the XylI-treated xylan 3 showed that the sugar composition was identical to that of the original xylan 3 (no further data shown). The sugar composition of the XylI-treated xylan 4 contained relatively more pectic material than the original xylan 4 (factor 1.5), but low substituted xylans were still the main part of the polysaccharides in this pool (no further data shown). These data indicated that the structure of the xylans could not explain the restriction of both xylanases towards these substrates but that another factor was involved. Measurement of the A_{280} signal during the elution on the DEAE Sepharose column showed that all xylan-rich pools exhibited UV absorption. Especially xylan 3 and 4 contained a significant amount of UV absorbing material. Therefore, the presence of lignin-like material or proteins (A_{280}) in these pools might explain the incomplete degradability of the residual xylans.

XylI degradation resulted in very similar elution patterns on HPAEC for all xylan-rich pools. The degradation products as well as the relative amounts were identical, showing that the same oligomers were formed in the same amounts. The degree of degradation of the pools by XylI differed: XylI gave a two times higher degree of degradation of xylan 1 and 2 compared to xylan 3 and 4. Analysis of the xylan-rich pools incubated with XylIII on HPAEC showed that also in this case the degradation products as well as the relative amounts were identical for all pools. In Fig. 3.5 it can be seen that XylIII released mainly xylobiose and

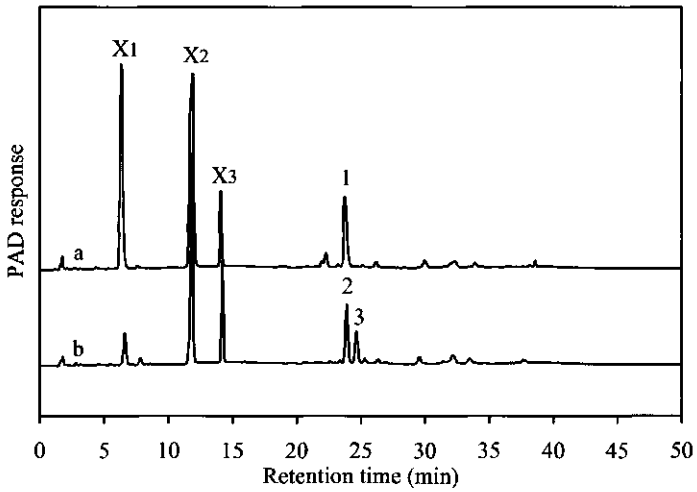

Fig. 3.5. HPAEC-patterns of xylan 1 of the 1 M KOH extract from olive fruit digested with (a) endo-xylanase I (XylI) and (b) endo-xylanase III (XylIII). Xylan oligosaccharides (X1, X2 and X3) are denoted above their corresponding peaks; the peak numbers 1, 2, and 3 correspond to tentative structures discussed in the text.

xylotriose and smaller amounts of Xyl monomer, while XylI degraded the xylan mainly into Xyl monomer and xylobiose. This was in agreement with the results of Kormelink *et al.* (1993a) and confirmed the difference in the mode of action and substrate specificity of XylI and XylIII. Besides the main end-products Xyl monomer, xylobiose and xylotriose, XylI released another major component which eluted around 24 min (peak 1) whilst XylIII released oligomers eluting at 24-25 min (peak 2 and 3).

MALDI-TOF MS was employed to determine the molecular masses of the unknown oligosaccharides. The spectra of xylan 1 incubated with XylI and XylIII are depicted in Fig. 3.6. The spectrum of xylan 1 incubated with XylI showed a main peak at *m/z* 627 that corresponded to a 4-*O*-MeGlcA linked to three pentose residues. Although mass analysis could not differentiate between the pentoses Ara or Xyl, the mode of action of XylI pointed to a xylotriose substituted with 4-*O*-MeGlcA at the non reducing terminus. XylI is not able to remove at least two unsubstituted Xyl residues towards the reducing end adjacent to the Xyl residue substituted with GlcA (Kormelink *et al.*, 1993a; Verbruggen *et al.*, 1998b). Although the mode of action of the XylI towards a 4-*O*-MeGlcA linked to the xylan backbone has not been revealed yet, we expected that this enzyme would act similary on a backbone substituted with a 4-*O*-MeglcA residue instead of a GlcA residue. The main peak at *m/z* 627 in the mass spectrum corresponded almost certainly to peak 1 on HPAEC which was the only major compound detected besides Xyl monomer, xylobiose and xylotriose. Besides the main peaks, the HPAEC patterns as well as the MALDI-TOF MS spectra also contained smaller peaks. Probably of xylo-oligomers containing besides 4-*O*-MeGlcA side groups also Ara side groups. The glycosidic linkage composition of the xylan-rich pools especially xylan 1 gave indications for the presence of Ara residues linked to the xylan backbone. However, no major peaks

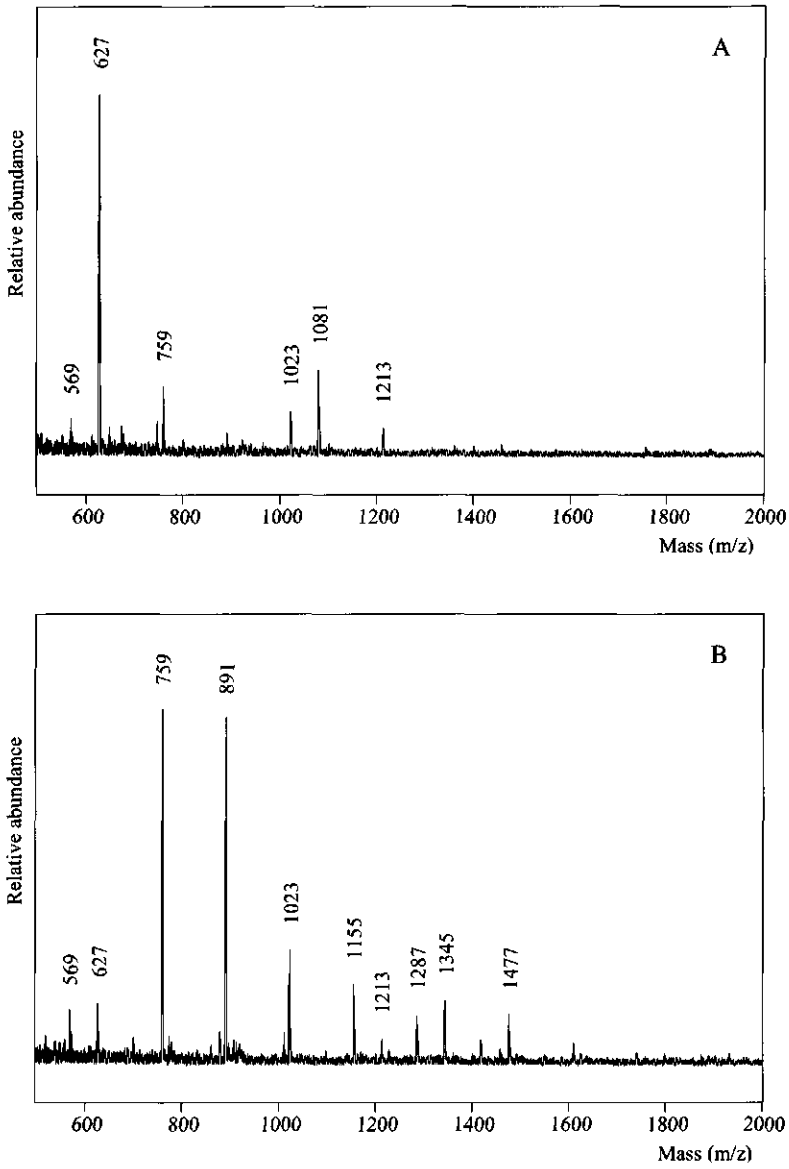

Fig. 3.6. MALDI-TOF mass spectra of xylan 1 of the 1 M KOH extract from olive fruit digested with (A) endo-xylanase I (XylI) and (B) endo-xylanase III (XylIII).

corresponding to Ara-rich oligomers were detected on HPAEC or in the MALDI-TOF MS spectra. Probably several xylo-oligomers with Ara substituents were formed but all in very small amounts. Also part of the undegradable material of xylan 1 might have consisted of an Ara-rich polymer.

The MALDI-TOF MS spectrum of olive fruit xylan incubated with XylIII showed two

main peaks which probably corresponded to peak 2 and 3 on HPAEC. The masses were *m/z* 759 and *m/z* 891 equal to an oligomer with a 4-*O*-MeGlcA linked to four and five pentoses, respectively. XylIII is not able to remove at least two unsubstituted Xyl residues adjacent to a substituted Xyl residue towards the reducing end (Kormelink *et al.*, 1993a). Thus, it could be concluded that these oligomers consisted most certainly of 4-*O*-MeGlcA residues linked to the third Xyl residue counting from the reducing end of a xylotetraose and xylopentaose.

Although clear differences in the mode of action of XylI and XylIII were observed towards wheat and barley arabinoxylans (Kormelink *et al.*, 1993a) and sorghum glucurono-arabinoxylan (Verbruggen *et al.*, 1998a), both enzymes were able to degrade olive fruit glucuronoxylan to the same extend. The substitution of the backbone with 4-*O*-MeGlcA residues and perhaps Ara residues did not hinder both endo-xylanases which suggested that the substituents were distributed very evenly over the xylan backbone. The results also confirmed earlier observations that the xylans extracted from olive fruit are low in substitution.

Conclusions

The glycosidic linkage composition of the xyloglucan-rich pools and the enzymatic degradation with endo-glucanases indicated the presence of an arabinogalactoxyloglucan with an XXXG core in olive fruit. The substitution of Xyl residues with Ara as well as Gal residues is commonly described for xyloglucans belonging to the poly-XXGG group but has only been suggested before in literature for xyloglucans belonging to the poly-XXXG group. No differences in the branching patterns of the xyloglucans extracted with a 1 or 4 M KOH solution could be noticed.

Based on the sugar linkage composition and the incubation with two endo-xylanases it can be concluded that the xylans in olive fruit were low in substitution. Although anion-exchange chromatography of the 1 and 4 M KOH fractions resulted in four xylan-rich pools, the structures of these xylans were almost identical as concluded from the results of the degradation studies with endo-xylanases. The 4-*O*-MeGlcA substituents of the xylans in olive fruit were probably rather regular distributed which resulted in identical patterns on HPAEC for all endo-xylanase digests of the xylan-rich pools. The differences in elution on DEAE Sepharose were most likely explained by the presence of proteins or lignin-like material (A_{280}), which did not only affected the elution on DEAE Sepharose but also the degradability of two of the xylan-rich pools.

Acknowledgements

The authors would like to thank Dr Jean-Paul Vincken for valuable discussions. This project has been supported by the EU (AIR3-CT93-1355).

References

Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M. & Voragen, A.G.J. The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all dectable endoglucanases, exoglucanases and β-glucosidases, *Eur. J. Biochem.* 146 (1985) 301-308.

Brett, C. & Waldron, K. *Physiology and biochemistry of plant cell walls,* Boston: Hyman (1990) 255.

Coimbra, M.A., Rigby, N.M., Selvendran, R.R. & Waldron, K.W. Investigation of the occurrence of xylan-xyloglucan complexes in the cell walls of olive pulp (*Olea europaea*), *Carbohydr. Polym.* 27 (1995) 277-284.

Coimbra, M.A., Waldron, K.W. & Selvendran, R.R. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L.), *Carbohydr. Res.* 252 (1994) 245-262.

De Ruiter, G.A., Schols, H.A., Voragen, A.G.J. & Rombouts, F.M. Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods, *Anal. Biochem.* 207 (1992) 176-185.

Englyst, H.N. & Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates, *Analyst* 109 (1984) 937-942.

Fry, S.C. Fry, York W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J. & White, A.R. An unambiguous nomenclature for xyloglucan derived oligosaccharides, *Physiol. Plant.* 89 (1993) 1-3.

Gil-Serrano, A., Mateos-Matos, M.I. & Tejero-Mateo, M.P. Acidic xylan from olive pulp, *Phytochemistry* 25 (1986) 2653-2654.

Gil-Serrano, A. & Tejero-Mateo, P. A xyloglucan from olive pulp, *Carbohydr. Res.* 181 (1988) 278-281.

Hisamatsu, M., Impallomeni, G., York, W.S., Albersheim, P. & Darvill, A.G. A new undecasaccharide subunit of xyloglucans with two α-L-fucosyl residues, *Carbohydr. Res.* 211 (1991) 117-129.

Hisamatsu, M., York, W.S., Darvill, A.G. & Albersheim, P. Characterization of seven xyloglucan oligosaccharides containing from seventeen to twenty glycosyl residues, *Carbohydr. Res.* 227 (1992) 45-71.

Huisman, M.M.H., Schols, H.A. & Voragen, A.G.J. Changes in cell wall polysaccharides from ripening olive fruits, *Carbohydr. Polym.* 31 (1996) 123-133

Kiefer, L.L., York, W.S., Albersheim, P. & Darvill, A.G. Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan, *Carbohydr. Res.* 197 (1990) 139-158.

Kormelink, F.J.M., Gruppen, J., Vietor, R.J. & Voragen, A.G.J. Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans, *Carbohydr. Res.* 249 (1993a) 355-367.

Kormelink, F.J.M., Searle-Van Leeuwen, M.J.F., Wood, T.M. & Voragen, A.G.J. Purification and characterization of three endo-(1,4)-β-xylanases and one β-xylosidase from *Aspergillus awamori*, *J. Biotechnol.* 27 (1993b) 249-265.

Niemann, C., Carpita, N.C. & Whistler, R.L. Arabinose containing oligosaccharides from tamarind xyloglucan, *Starch* 4 (1997) 154-159.

O'Neill, M. & Selvendran, R.R. Isolation and partial characterisation of a xyloglucan from the cell walls of *Phaseolus coccineus*, *Carbohydr. Res.* 111 (1983) 239-255.

Sandford, P.A. & Conrad, H.E. The structure of the *Aerobacter aerogenes* A3 (SI) polysaccharide. I. A reexamination using improved procedures for methylation analysis, *Biochemistry* 5 (1966) 1508-1517.

Sweet, D.P., Shapiro, R.H. & Albersheim, P. Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates, *Carbohydr. Res.* 40 (1975) 217-225.

Verbruggen, M.A., Beldman, G. & Voragen, A.G.J. The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions, *J. Cereal Sci.* 21 (1995) 271-282.

Verbruggen, M.A., Beldman, G. & Voragen, A.G.J. Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures, *Carbohydr. Res.* 306 (1998a) 275-282.

Verbruggen, M.A., Spronk, B.A., Schols, H.A., Beldman, G., Voragen, A.G.J., Thomas, J., Kamerling, J.P. & Vliegenthart, J.F.G. Structures of enzymically derived oligosaccharides from sorghum glucuronoarabinoxylan, *Carbohydr. Res.* 306 (1998b) 265-274.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J. Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv koroneiki) at different ripening stages, *Carbohydr. Polym.* 43 (2000) 11-21.

Vincken, J-P., Wijsman, A.J.M., Beldman, G., Niessen, W.M.A. & Voragen, A.G.J. Potato xyloglucan is built from XXGG-type subunits, *Carbohydr. Res.* 288 (1996a) 219-232.

Vincken, J-P., Beldman, G., Niessen, W.M.A. & Voragen, A.G.J. Degradation of apple fruit xyloglucan by endoglucanase, *Carbohydr. Polym.* 29 (1996b) 75-85.

Vincken, J-P., Beldman, G. & Voragen, A.G.J. Substrate specificity of endoglucanases: what determines xyloglucanase activity?, *Carbohydr. Res.* 298 (1997a) 299-310.

Vincken, J-P., York, W.S., Beldman, G. & Voragen, A.G.J. Two general branching patterns of xyloglucan, XXXG and XXGG: update on cell walls, *Plant Physiol.* 114 (1997b) 9-13.

Wilkie, K.C.B. The hemicelluloses of grasses and cereals, *Adv. Carbohydr. Chem. Biochem.* 36 (1979) 215-264.

York, W.S., Halbeek, H. van, Darvill, A.G. & Albersheim, P. Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and fast-atom-bombardment mass spectrometry, *Carbohydr. Res.* 200 (1990) 9-31.

Chapter 4

Structural analyses of two arabinose containing oligosaccharides derived from olive fruit xyloglucan: XXSG and XLSG

Abstract

Xyloglucan oligosaccharides were prepared by endo-(1→4)-β-D-glucanase digestion of alkali-extractable xyloglucan from olive fruit and purified by a combination of gel-permeation (Bio-Gel P-2) chromatography and high-performance anion-exchange chromatography. The two most abundant oligosaccharides were converted to the corresponding oligoglycosyl alditols by borohydride reduction and structurally characterised by NMR spectroscopy and post-source decay (PSD) fragment analysis of matrix-assisted laser-induced desorption/ionisation time-of-flight (MALDI-TOF) mass spectra. The results revealed that olive fruit xyloglucan is mainly built from two novel oligosaccharides: XXSG and XLSG. The structure of the oligosaccharides confirmed the presence of a specific xyloglucan in olive fruit with α-L-Ara*f*-(1→2)-α-D-Xyl*p* side chains as was suggested previously. The presence of such side chains is a common feature of xyloglucans with an XXGG core produced by solanaceous plants but has not been demonstrated for other dicotyledonous plants, which have in general an XXXG core. Direct treatment of cell wall material from olive fruit with pectin degrading enzymes in combination with endo-(1→4)-β-D-glucanase revealed that some of the arabinose residues of the oligosaccharides XXSG and XLSG are substituted with either 1 or 2 *O*-acetyl groups.

This chapter has been published in Carbohydr. Res. 332 (2001) 285-297 by the authors Esther Vierhuis, William S. York, V.S. Kumar Kolli, Jean-Paul Vincken, Henk A. Schols, Gert-Jan W.M. Van Alebeek & Alphons G.J. Voragen.

Introduction

Xyloglucans are hemicellulosic polysaccharides found in the primary cell wall of plants and are closely associated with cellulose microfibrils by hydrogen bonds. These noncovalent cross-links between cellulose microfibrils and xyloglucan molecules play a major role in defining the structural properties of plant cell walls and the regulation of growth and development of dicotyledonous plants (Hayashi, 1989; Carpita & Gibeaut, 1993; Levy *et al.*, 1997). In addition to this structural role, xyloglucans can be present as a food reserve in seeds and they can be broken down to oligosaccharides that may act as signal molecules (York *et al.*, 1984; Fry, 1989; McDougall & Fry, 1991).

Xyloglucans have a cellulose-like main chain composed of β-(1→4)-linked D-Glc*p* residues in which up to 75% of the β-D-Glc*p* residues can be substituted at C-6 with α-D-Xyl*p* residues. Some of the α-D-Xyl*p* residues are extended at C-2 with a monosaccharide (β-D-Gal*p* or α-L-Ara*f*) or disaccharide (α-L-Fuc*p*-(1→2)-β-D-Gal*p*). The distribution of the side chains is species and tissue specific (Kooiman, 1961; Fry, 1989; York *et al.*, 1990; York *et al.*, 1996; Pauly *et al.*, 2001). Comparison of known xyloglucan structures revealed that most xyloglucans are composed of either XXXG-type or XXGG-type building units (Vincken *et al.*, 1997). The letters 'G' and 'X' refer to an unbranched Glc residue and a Xyl-Glc segment, respectively (Fry *et al.*, 1993).

Sugar linkage analyses and degradation studies with endo-(1→4)-β-D-glucanases indicated a xyloglucan in olive fruit with a specific substitution pattern. It probably consists of XXXG-type building units with both Ara and Gal residues linked to it (Vierhuis *et al.*, 2001). So far, Ara-containing side chains have been described as a common feature for xyloglucans isolated from solanaceous plants which consist of XXGG-type building units (Vincken *et al.*, 1997). In our previous study, we were not able to establish the exact position of the specific Ara-containing side chain on the backbone of the xyloglucan oligosaccharides. We now describe the purification and rigorous characterisation of the two most abundant oligosaccharides present in the endo-glucanase digest of xyloglucan from olive fruit.

Experimental

Materials

Olive (*Olea europaea* cv koroneiki) cell wall material was obtained from purple olive fruit by ethanol extraction as described previously (Vierhuis *et al.*, 2000). The xyloglucan-rich fraction was obtained by anion-exchange chromatography from a 4 M KOH extract of purple olive fruit (Vierhuis *et al.*, 2000). Endo-(1→4)-β-D-glucanase V (EndoV) was purified from a commercial enzyme preparation from *Trichoderma viride* (Beldman *et al.*, 1985). Endo-polygalacturonase (PG) originated from *Kluveromyces fragiles* and pectin methyl esterase (PME) from *Aspergillus niger* (Schols *et al.*, 1990). Pectin lyase (PL) was purified from a commercial enzyme preparation of *A. niger* (Van Houdenhoven, 1975). The pectin lyase preparation contained residual endo-glucanase side activities. These side activities have an

identical mode of action towards the xyloglucan-rich fraction obtained from the 4 M KOH extract as EndoV isolated from *T. viride*.

Preparation of olive xyloglucan oligosaccharides from a 4 M KOH extract

The xyloglucan-rich fraction (50 mg) was dissolved in a 50 mM NaOAc buffer of pH 5.0 containing 0.01% (w/v) NaN_3 and incubated with EndoV. The incubation was performed at 40°C for 48 h with a substrate concentration of 5 mg/mL. The resulting digest was heated for 15 min at 100°C to inactivate the enzymes. The xyloglucan digest was applied on a Bio-Gel P-2 column (87 x 2.6 cm, 200-400 mesh, Bio-Rad) and eluted with distilled water at a flow rate of 0.5 mL/min at 60°C. Fractions of 2.6 mL were collected and analysed for neutral sugar content using colorimetric methods (Vierhuis *et al.*, 2000). Appropriate fractions were combined and freeze dried. Fractions 7 and 8 were subjected to preparative high-performance anion-exchange chromatography (HPAEC) using a Thermo Quest P4000 quaternaire gradient pump equipped with a CarboPac PA-1 column (250 x 22 mm; Dionex). Elution took place at 20°C at a flow rate of 25.0 mL/min. The fractions were subjected to the following NaOAc gradient in 100 mM NaOH: 0→5 min, linear gradient of 0→50 mM NaOAc; 5→35 min, linear gradient of 50→70 mM NaOAc; 35→60 min, linear gradient of 70→74 mM NaOAc, 60→80 min, linear gradient of 74→90 mM NaOAc. After each run the column was washed for 5 min with 100 mM NaOH containing 1 M NaOAc and subsequently equilibrated with the starting eluent for 15 min. 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. The effluent was neutralised by on-line addition of 5 M HOAc and appropriate fractions (c. 6.25 mL) were combined. To desalt the HPAEC fractions, the oligosaccharides were adsorbed on a Sep-Pac C18 reversed phase cartridge (Waters). The cartridge was washed extensively with distilled water and subsequently eluted with methanol (80% v/v). The purity of the oligosaccharides was checked by analytical HPAEC (Vierhuis *et al.*, 2001).

Preparation of xyloglucan oligoglycosyl alditols

Xyloglucan oligosaccharides were converted to the corresponding oligoglycosyl alditol derivatives by reduction with $NaBH_4$ (10 mg/mL in 1 M NH_4OH), and the products were isolated by reverse-phase chromatography on an octadecyl silica cartridge (Supelclean LC-18, Supelco), as previously described (York *et al.*, 1996).

Preparation of olive xyloglucan oligosaccharides from cell wall material

Cell wall material of olive fruit (150 mg) was suspended in 6.5 mL of a 50 mM NaOAc buffer of pH 5.0 containing 0.01% (w/v) NaN_3 and treated with a combination of PG, PME and PL to digest the pectic polymers. Oligosaccharides from xyloglucan were released by the

residual endo-glucanase side activity in the PL preparation The incubation was performed at 40°C for 24 h. The resulting digest was heated for 15 min at 100°C to inactivate the enzymes. To remove residual polymeric material from the digest an ethanol precipitation (60% v/v) was performed. Subsequently, the supernatant was dried, dissolved in 2 mL distilled water and applied on a Sep-Pac C18 reversed phase cartridge (Waters). The cartridge was washed extensively with distilled water to remove salts and eluted with methanol (20% v/v).

Analytical methods

The neutral sugar composition

The neutral sugar composition of the fractions was determined by gas chromatography according to Englyst & Cummings (1984) using inositol as an internal standard. The samples were treated with 1 M sulphuric acid for 3 h at 100°C. The released constituent sugars were analysed as their alditol acetates.

Mass spectrometry

Matrix-assisted laser-induced desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) in the reflector mode was performed using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems) equipped with a nitrogen laser operating at 337 nm (3 ns pulse duration), a single stage reflector and delayed extraction. The accelerating voltage used was 12 kV and the delay time setting was 200 ns. Each spectrum was produced by accumulating data from over 100 laser shots.

MALDI-TOF mass spectra used to determine the glycosyl sequence of oligoglycosyl alditols XXSGol and XLSGol were recorded with post-source decay (PSD) using a Kratos SEQ spectrometer equipped with a nitrogen laser operating at 337 nm (3 ns pulse duration), a curved field reflectron and delayed extraction. Each spectrum represents the accumulated data from over 100 laser shots, smoothed using the 'average' function. Alternatively, MALDI-TOF mass spectra used to determine the positions of the *O*-acetyl groups on the native XXSG and XLSG were recorded with PSD using the Voyager-DE RP Biospectrometry Workstation mentioned above.

Sample preparation: The matrix solution was prepared by dissolving 9 mg 2,5-dihydroxybenzoic acid (DHB) and 3 mg 1-hydroxy isoquinoline in 700 µL distilled water and 300 µL acetonitrile. A 1 µL volume of this solution was placed on the sample plate and mixed with 1 µL of the enzyme digest and allowed to dry at room temperature. Mass spectra were calibrated with an external standard containing cellodextrins (DP 3-9).

Nuclear magnetic resonance spectroscopy (NMR Spectroscopy)

The oligoglycosyl alditol samples were dissolved in 99.6% isotopically enriched ^2H_2O (Cambridge Isotope Laboratories) and lyophilised to replace exchangeable protons with deuterons. The residue was dissolved in 99.96% enriched ^2H_2O (600 µL) and transferred to a 5 mm NMR tube. COSY (Bax & Freeman, 1981), TOCSY (Bax & Davis, 1985), NOESY (States *et al.*, 1982), and HSQC (Bodenhausen & Ruben, 1980) spectra were recorded at 298K with a Varian Inova 600 NMR spectrometer. The TOCSY mixing time was 94 ms. NOESY

spectra were recorded with mixing times of 500 ms. COSY and HSQC were recorded using pulsed field gradients for coherence selection (Keeler *et al.*, 1994). In a typical 2-dimensional (^{1}H-^{1}H) spectrum, 512 transients of 1024 data points were recorded with a spectral width of 1800 Hz in both dimensions, and the data were processed with zero filling to obtain a 2048 x 2048 matrix. Chemical shifts were measured relative to internal acetone at δ 2.225.

Results and discussion

Preparation of olive xyloglucan oligosaccharides from a 4 M KOH extract

The xyloglucan-rich pool obtained by alkali extraction of cell wall material from olive fruit was digested with endo-(1→4)-β-D-glucanase (EndoV). In order to isolate the Ara-containing xyloglucan oligosaccharides of interest (i.e. with three out of four Glc residues bearing a side chain), the xyloglucan digest was fractionated on Bio-Gel P-2, as shown in Fig. 4.1. The eluate was pooled into 11 fractions (1: monomers; 11: void volume). The sugar composition of the four main fractions 7, 8, 10 and 11 are given in Table 4.1. Fractions 7 and 8 contained the oligosaccharides of interest. The MALDI-TOF mass spectrum of fraction 7 showed two main $[M + Na]^+$ ions at m/z 1217 and m/z 1379, corresponding to the glycosyl composition $Hexose_4 \cdot Pentose_4$ and $Hexose_5 \cdot Pentose_4$, respectively. The tentative structures X[XS]G and X[LS]G seem most likely for these two ions based on characteristics of the xyloglucan-rich pool, the mode of action of two endo-glucanases towards the substrate, and the molecular masses of the oligosaccharides (Vierhuis *et al.*, 2001). The structural elements S and L, containing Ara and Gal, respectively, are indicated in brackets because the exact position of these residues was not known. In principle, the structural elements S and L could also be

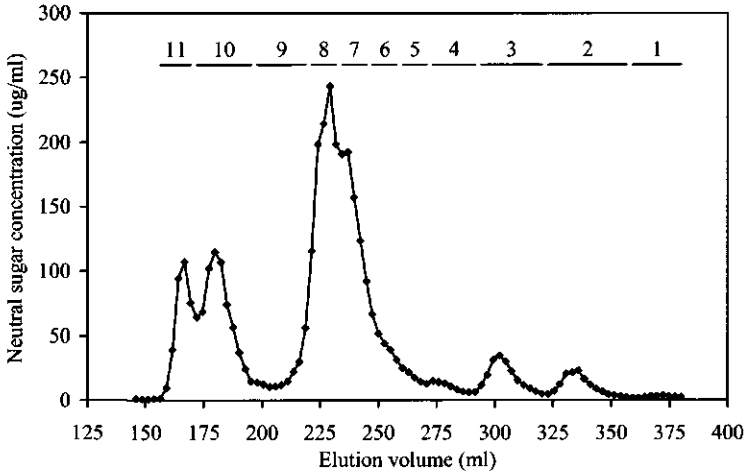

Fig. 4.1. Elution profile of the EndoV digest of an alkali-extracted xyloglucan-rich fraction from olive fruit on Bio-Gel P-2.

Table 4.1
Neutral sugar composition (mol%) of Bio-Gel P-2 fractions 7, 8, 10 and 11 and the purified xyloglucan oligosaccharides obtained from an alkali-extracted xyloglucan-rich fraction from olive fruit after treatment with EndoV (tr = trace amount)

Sample	Fuc	Ara	Xyl	Man	Gal	Glc
Bio-Gel P-2 fractions						
Fraction 7	tr	11	27	8	8	46
Fraction 8	tr	11	35	3	10	41
Fraction 10	1	11	34	5	10	39
Fraction 11	1	12	40	1	10	36
Purified oligosaccharides						
m/z 1217	0	13	36	0	0	51
m/z 1379	0	11	34	0	10	45

located at the non-reducing terminus of the xyloglucan oligosaccharides, but the existence of these elements at that position has never been reported before. In addition to the two main $[M + Na]^+$ ions at *m/z* 1217 and *m/z* 1379, an ion of intermediate intensity corresponding to an oligosaccharide consisting of six hexosyl residues was present. This oligosaccharide, which lacks pentosyl residues, most probably originated from glucomannan or galactomannan oligosaccharides released by EndoV (Vierhuis *et al.*, 2001). The MALDI-TOF mass spectrum of fraction 8 revealed the presence of one main component with *m/z* 1379. This ion is likely to correspond to X[LS]G.

The elution patterns on HPAEC of Bio-Gel P-2 fractions 7 and 8 are shown in Fig. 4.2. Besides the major peaks of the oligosaccharides of interest (at 45 and 49 min) the fractions also contained a large number of other oligosaccharides. Further fractionation by preparative

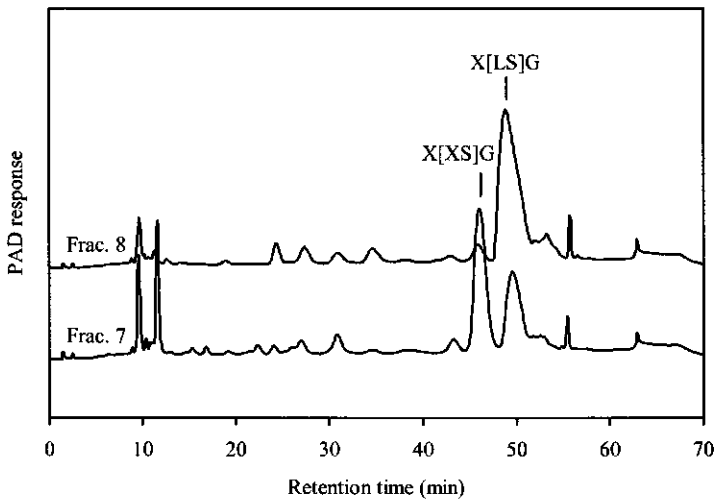

Fig. 4.2. HPAEC-pattern of Bio-Gel P-2 fractions 7 and 8 obtained from an alkali-extracted xyloglucan-rich fraction from olive fruit after treatment with EndoV.

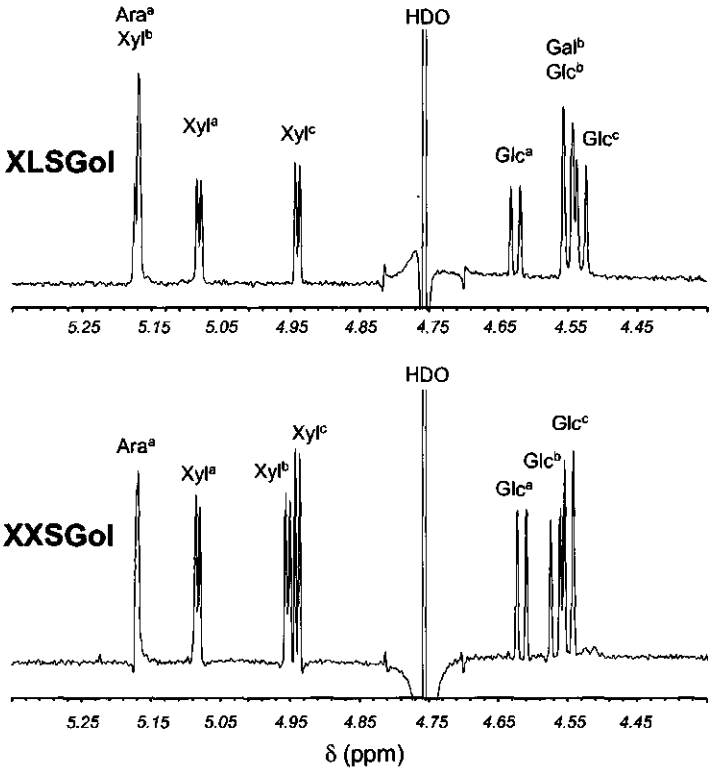

Fig. 4.3. Anomeric regions of the 1D NMR spectra of the xyloglucan oligoglycosyl alditols XLSGol and XXSGol. The superscript letters (a, b, c) indicate the positions of the glycosyl residues within the oligomer (See footnotes of Table 4.2).

HPAEC yielded an oligosaccharide with a Glc:Xyl:Gal:Ara ratio of 4:3:1:1 and an oligosaccharide with a Glc:Xyl:Ara ratio of 4:3:1 (Table 4.1). The molecular masses of the oligosaccharides, as determined by MALDI-TOF MS, were consistent with these compositions. The oligosaccharides were further characterised using NMR spectroscopy and PSD MALDI-TOF MS in order to determine the exact position of the Ara and Gal residues.

Fraction 10 contained mainly 'dimers' of xyloglucan oligosaccharides, each four Glc residues in its backbone, as determined by HPAEC and MALDI-TOF MS (not shown). Fraction 10 was further digested with EndoV, and MALDI-TOF MS indicated that the major product had a mass (*m/z* 1379 for the $[M + Na]^+$ ion) that is consistent with the structure X[LS]G. Apparently, EndoV releases X[LS]G from xyloglucan more slowly than X[XS]G, which indicates that the distribution of side chains substantially affects the rate at which this enzyme attacks specific sites on the xyloglucan backbone.

The xyloglucan-rich pool was not completely degraded by EndoV, as some material eluted in the void volume of the column (Fig. 4.1; fraction 11). Sugar analysis of fraction 11 (Glc:Xyl:Gal:Ara ratio of 36:40:10:12) indicated the presence of larger oligomeric xyloglucan fragments and a small amount of xylan. Further digestion of this fraction with EndoV also produced a fraction with one main component (*m/z* 1379), which confirmed the tendency of

Table 4.2
NMR resonance assignments* for the xyloglucan oligoglycosyl alditols XLSGol and XXSGol

	H-1'	H-1	H-2	H-3	H-4	H-5	H-5e	H-6 pro-R	H-6 pro-S
XLSGol									
Glcol	3.687	3.743	3.92	3.88	3.955	3.956	---	3.740	3.852
		(na)[d]	(na)	(na)	(na)	(na)		(na)	
		63.3	73	70.2	81.0	72.1		62.8	
Glc[a]		4.626	3.421	3.675	3.685	3.796	---	3.922	3.972
		(8.0)	(9)	(sc)[e]	(10)	(na)		(na)	
		103.2	73.7	75.9	80.4	74.6		67.5	
Glc[b]		4.548	3.404	3.664	3.655	3.885	---	3.92	3.969
		(8)	(9)	(sc)	(na)	(sc)			
		103.3	73.5	74.9	80.6	74.4		67.5	
Glc[c]		4.531	3.336	3.51	3.52	3.694	---	3.937	3.778
		(8.0)	(9)	(sc)	(na)	(5, <3)			
		103.8	73.7	76.3	70.3	75.1		66.7	
Xyl[a]		5.081	3.572	3.782	3.76	3.57	3.730	---	---
		(4.0)	(10)	(9)	(10, 5)	(11)			
		99.5	79.6	72.9	70.1	62.0			
Xyl[b]		5.172	3.667	3.914	3.670	3.570	3.725	---	---
		(4)	(10)	(9)	(10, na)	(11)			
		99.5	81	72.7	70.1	62.0			
Xyl[c]		4.940	3.538	3.736	3.617	3.544	3.712	---	---
		(4.0)	(10)	(9)	(na, 5)	(11)			
		99.1	72.3	73.8	70.2	62.0			
Gal[b]		4.550	3.615	3.66	3.922	3.67	---	3.77	3.77
		(8)	(10)	(<4)	(<2)	(na)		(na)	
		105.3	71.9	73.4	69.4	73.4		61.7	
Ara[a]		5.169	4.194	3.936	4.074	3.849	3.711	---	---
		(<2)	(4)	(7)	(3, 6)	(12)			
		110.1	81.8	77.2	84.5	62.0			

* See footnotes of Table 4.2, continued.

EndoV to release X[LS]G more slowly than X[XS]G.

NMR spectroscopy of the olive xyloglucan oligoglycosyl alditols

The presence of two different anomeric forms at the reducing end of the olive xyloglucan oligosaccharides complicated their analysis by NMR spectroscopy, and a complete NMR assignment of these reducing oligosaccharides was not achieved. Therefore, the

Table 4.2
NMR resonance assignments* for the xyloglucan oligoglycosyl alditols XLSGol and XXSGol, continued

	H-1'	H-1	H-2	H-3	H-4	H-5	H-5e	H-6 pro-R	H-6 pro-S
XXSGol									
Glcol	na (na)	na (na)	na (na)	na (na)	3.949 (na)	na (na)		na (na)	na (na)
Glc^a		4.617 (8.0)	3.412 (9.2)	3.657 (9)	3.678 (10)	3.784 (na)	---	3.924 (na)	3.978
Glc^b		4.568 (8.0)	3.388 (9.4)	3.672 (10)	3.733 (na)	3.815 (na)	---	4.012 (na)	3.895
Glc^c		4.549 (7.8)	3.338 (9)	3.51 (sc)	3.52 (sc)	3.699 (6, <3)	---	3.937 (12)	3.781
Xyl^a		5.083 (3.6)	3.576 (10)	3.783 (9)	3.681 (na)	3.57 (na)	3.728	---	---
Xyl^b		4.953 (3.6)	3.546 (10)	3.729 (9)	3.629 (na)	3.568 (na)	3.72	---	---
Xyl^c		4.940 (3.6)	3.543 (10)	3.733 (9)	3.621 (na)	3.55 (na)	3.711	---	---
Ara^a		5.170 (1.5)	4.190 (4.0)	3.938 (6.3)	4.077 (6.3, 3.2)	3.851 (12)	3.712	---	---

* Data includes: ^1H chemical shifts of protons, ^3J for scalar interaction with next higher numbered proton (in parenthesis), and ^{13}C chemical shifts for the directly attached carbon (XLSGol only).

[a,b,c] The position of the glycosyl residue with respect to the alditol moiety (i.e., $Glc^c \rightarrow Glc^b \rightarrow Glc^a \rightarrow$Glcol; Xyl^a is linked to Glc^a; Ara^a is linked to Xyl^a).

[d] Chemical shifts and scalar coupling constants that were not assigned are indicated as "na".

[e] Strongly coupled proton pairs are indicated as "sc".

oligosaccharides were converted to the corresponding oligoglycosyl alditols (OAs) by reduction with $NaBH_4$. The 1D (Fig. 4.3) and 2D homonuclear NMR spectra (COSY, NOESY, TOCSY) of both OAs, and the ^1H-^{13}C HSQC spectrum of the Gal-containing OA were recorded. Analysis of these spectra, in light of a large number of previously reported assignments (York *et al.*, *1990*; York *et al.*, 1993; York *et al.*, 1996) for structurally related OAs, allowed all of the ^1H and ^{13}C resonances of the Gal-containing OA and most of the ^1H resonances of the other OA to be assigned (Tables 4.2 and 4.3). The general approach was to assign the resonances of the isolated spin systems (each corresponding to a glycosyl residue of the oligomer) by measuring homonuclear scalar coupling constants and tracing scalar connectivity in the COSY and TOCSY spectra. The ^{13}C-resonances of the Gal-containing OA were then assigned by virtue of one-bond heteronuclear (^1H-^{13}C) scalar coupling observed in the HSQC spectrum. Glycosidic linkages between residues of the OAs were established by

Table 4.3
Crosspeaks in the NOESY spectra of the xyloglucan oligoglycosyl alditols XLSGol and XXSGol

From	To			
XLSGol				
5.172	3.969	3.92	3.669	
Xyl^b H-1	Glc^b H-6S	Glc^b H-6R	Xyl^b H-2	
5.169	3.574			
Ara^a H-1	Xyl^a H-2			
5.081	3.974	3.923	3.572	
Xyl^a H-1	Glc^a H-6S	Glc^a H-6R	Xyl^a H-2	
4.940	3.935	3.778	3.539	
Xyl^c H-1	Glc^c H-6R	Glc^c H-6S	Xyl^c H-2	
4.626	3.954	3.797	3.677	
Glc^a H-1	Glcol H-4	Glc^a H-5	Glc^a H-3	
4.550	3.67	3.884	3.972	
4.548	Gal^b H-3	Glc^b H-5	Glc^a H-6S	
Gal^b H-1	Gal^b H-5			
Glc^b H-1	Glc^b H-3			
	Glc^a H-4			
	Xyl^b H-2			
4.531	3.969	3.693	3.655	3.514
Glc^c H-1	Glc^b H-6S	Glc^c H-5	Glc^b H-4	Glc^c H-3
3.937	3.777			
Glc^c H-6R	Glc^c H-6S			
3.912	3.569			
Xyl^b H-3	Xyl^b H-5			

analysis of their NOESY spectra (as described next).

Dipolar ^1H-^1H interactions in the OAs were detected as crosspeaks in the NOESY spectra of the OAs. The observation of a crosspeak in the NOESY spectrum indicates that the two interacting protons are in close proximity to each other, which usually occurs because they reside within a single glycosyl residue or within two residues that are glycosidically linked to each other. Although an inter-residue crosspeak in the NOESY spectrum is evidence for a glycosidic linkage, it cannot be taken as proof of the linkage, as NOEs are occasionally observed for pairs of protons that are in close proximity even though they reside within residues that are not glycosidically linked to each other. Nevertheless, all of the NOE crosspeaks listed in Table 4.3 are consistent with the proposed OA structures XXSGol and XLSGol.

It should be noted that, although the β-Glc*p* residues in the backbone of xyloglucan OAs are connected via (1→4)-linkages, a relatively weak NOE is often observed between H-1 of one β-Glc*p* residue and one or both of the protons on C-6 of the β-Glc*p* residue to which it is

Table 4.3
Crosspeaks in the NOESY spectra of the xyloglucan oligoglycosyl alditols XLSGol and XXSGol, continued

From	To				
XXSGol					
5.170	3.576				
Ara[a] H-1	Xyl[a] H-2				
5.083	3.576	3.922	3.978		
Xyl[a] H-1	Xyl[a] H-2	Glc[a] H-6R	Glc[a] H-6S		
4.953	3.544	3.893	4.013		
Xyl[b] H-1	Xyl[b] H-2	Glc[b] H-6S	Glc[b] H-6R		
4.940	3.544	3.782(w)			
Xyl[c] H-1	Xyl[c] H-2	Glc[c] H-6S			
4.617	3.949	3.785	3.655	virtual coupling	
Glc[a] H-1	Glcol H-4	Glc[a] H-5	Glc[a] H-3	to Glc[a] H-4	
4.568	3.814	3.670	3.681	Glc[b] H-3 and Glc[a] H-4 overlap	
Glc[b] H-1	Glc[b] H-5	Glc[b] H-3	Glc[a] H-4		
4.549	4.013	3.893	3.735	3.699	3.51
Glc[c] H-1	Glc[b] H-6R	Glc[b] H-6S	Glc[b] H-4	Glc[c] H-5	Glc[c] H-3

[a,b,c] The positions of the glycosyl residue within the oligomer (see footnotes of Table 4.2).

linked (W.S. York, unpublished results). These NOEs are consistent with the hypothesis that the xyloglucan backbone is (at least transiently) 'twisted' in solution, as has been proposed on the basis of conformational energy calculations (Levy *et al.*, 1991). If the backbone were flat (like crystalline cellulose) H-1 would *not* be close enough to H-6' to generate the observed NOE (W.S. York, unpublished results). The occurrence of NOEs from H-1 to H-6' thus provides information about the sequence of β-Glc*p* residues in the OA backbone that would otherwise be difficult to establish because the H-1 to H-4' NOEs (normally used to establish the (1→4) linkages in the backbone) are often in a crowded region of the ^{1}H-NMR spectrum. (See, e.g. the crosspeaks between δ 4.55 and δ 3.67 in the NOESY spectrum of XLSGol, Table 4.3). Therefore, H-1 to H-6' NOEs observed in the NOESY spectra of the xyloglucan OAs described herein (Table 4.3) were interpreted as evidence to support the proposed glycosyl sequences for these oligomers.

The chemical shift assignments given in Table 4.2 are fully consistent with previously published (York *et al.*, 1990; York *et al.*, 1993; York *et al.*, 1996) data for related OAs. Furthermore, these assignments make it possible to deduce additional correlations between the chemical shifts of diagnostic ^{1}H-resonances and specific structural features in xyloglucan OAs. Specifically, these new assignments will facilitate the rapid structural assignment of Ara-containing xyloglucan OAs in which three contiguous β-Glc*p* residues in the backbone bear an α-Xyl*p* residue at C-6.

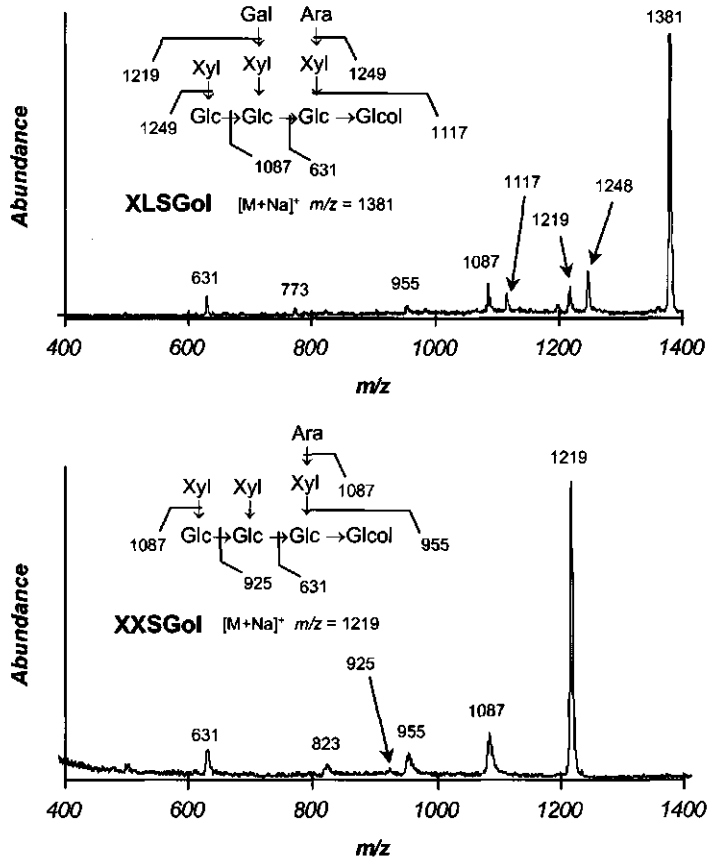

Fig. 4.4. Post-Source Decay MALDI-TOF spectra of xyloglucan oligoglycosyl alditols XLSGol and XXSGol. The oligoglycosyl alditols have a mass that is 2 Da greater than the oligosaccharides from which they were derived. The diagnostic ion at *m/z* 631 indicates that the Ara-containing side chain is adjacent to the alditol in both of these oligomers.

MALDI-TOF MS of the olive xyloglucan oligoglycosyl alditols

The glycosyl sequences of the two OAs were confirmed by MALDI-TOF mass spectrometry, with detection of fragment ions formed by post-source decay (PSD) (Fig. 4.4). The PSD technique results in the formation of fragment ions derived from both ends of the oligomer and from multiple fragmentation processes (Yamagaki *et al.*, 1998). However, the conditions used to record the MALDI-TOF PSD mass spectra reported here resulted in a fragmentation pattern dominated by sequence-specific ions derived from the alditol end of the oligomers. This fragmentation pattern is analogous to that previously reported (York *et al.*, 1990) for the negative-ion FAB mass spectra xyloglucan OAs recorded using 1-amino-2,3-dihydroxy propane as the liquid matrix. The main difference between the two techniques is

that deprotonated 'Y-type' fragment ions (Domon & Costello, 1988) dominate the negative-ion FAB spectra while sodiated 'Y-type' ions dominate the MALDI-TOF PSD spectra. In any case, the dominance of 'Y-type' ions in these spectra facilitate assignment of the glycosyl sequence of the OAs, which would be more difficult if the spectra also contained abundant ions derived from the non-reducing end and/or from multiple fragmentation processes. Although double fragment ions (e.g. at *m/z* 823 in the spectrum of XXSGol) and ions derived from the non-reducing end (e.g. at *m/z* 773 in the spectrum of XLSGol) are visible in the MALDI-TOF PSD spectra of the olive OAs (Fig. 4.4), interpretation of the fragmentation patterns is straightforward and supports the glycosyl sequences proposed on the basis of NMR spectrometry. For example, the diagnostic $[Y + Na]^+$ ion at *m/z* 631 ($Pentose_2$·Hexose·Hexitol) in these spectra indicates that the Ara-containing side chain is adjacent to the alditol moiety in both of the olive OAs.

Structural analyses of olive xyloglucan oligosaccharides substituted with O-acetyl groups from cell wall material

The strong alkaline conditions used to solubilise xyloglucan from cell wall material hydrolyses all ester linkages and consequently de-*O*-acetylates the polymer. To determine if xyloglucan present in olive fruit is *O*-acetylated, cell wall material was treated directly with enzymes to release xyloglucan oligosaccharides. Pectinases were included to increase the accessibility of the cellulose-xyloglucan network for the glucanases (Vincken *et al.*, 1997). Analysis of the partially purified xyloglucan oligosaccharides on MALDI-TOF MS showed ions with *m/z* values corresponding to the two xyloglucan oligosaccharides studied by NMR (*m/z* 1217 and 1379) and also mono-*O*-acetylated and di-*O*-acetylated forms of these xyloglucan oligosaccharides (Fig. 4.5).

MALDI-TOF PSD analysis of the *O*-acetylated xyloglucan oligosaccharides XLSG (Fig. 4.6) and XXSG (not shown) present in the partially purified digest revealed that the *O*-acetyl groups were located on the Ara residue. All fragments containing the Ara residue increased in *m/z* value with 42 or 84 corresponding to either 1 or 2 *O*-acetyl groups, respectively, whereas fragments without the Ara residue did not change in *m/z*. The fragment ions without the Ara residue at *m/z* 773, 1115 and 1247 were very diagnostic, as they showed where the acetate(s) were not located. In particular the ion at *m/z* 1115, because it clearly indicated that the acetate(s) were present on the side chain containing two pentosyl residues. The ions at *m/z* 713 (two acetates), 671 (one acetate) and 629 (no acetates) confirmed the interpretation of the spectra of the oligoglycosyl alditols (Fig. 4.4; i.e. that the dipentosyl side chain is attached to the glucosyl residue next to the alditol in XLSGol) and showed that the dipentosyl side chain was attached to the glycosyl residue next to the reducing glucose in XLSG. The difference of 2 Da between the ion at *m/z* 629 for the reducing oligosaccharide (XLSG, no acetates) and *m/z* 631 for the oligoglucosyl alditol (XLSGol) confirmed that these ions were derived from the reducing and alditol end, respectively, of these oligomers. PSD analysis could not reveal the exact position of the *O*-acetyl groups on the Ara residue.

NMR analysis of the partially purified digest indicated that *O*-acetyl substituents were present on the xyloglucan oligosaccharides, but also failed to establish their positions, as the

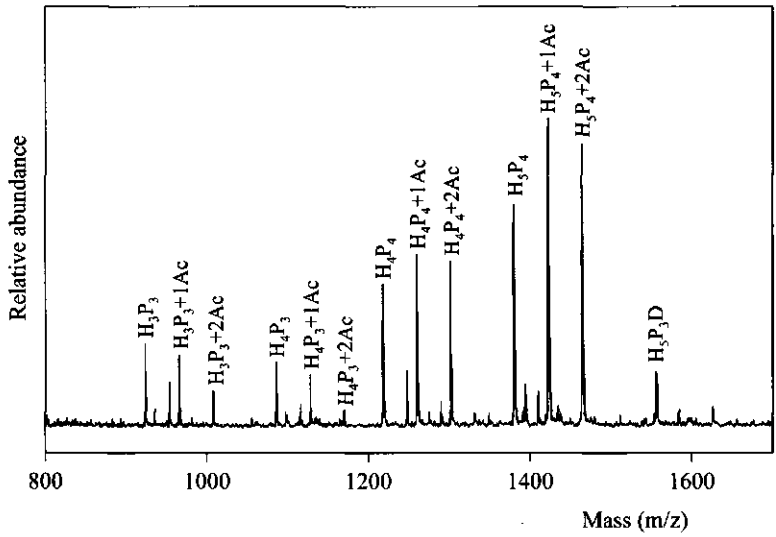

Fig. 4.5. MALDI-TOF mass spectrum of (*O*-acetylated) xyloglucan oligomers released from olive fruit cell wall material by digestion with pectin degrading enzymes in combination with endo-glucanase; H = hexose, P = pentose, D = deoxyhexose.

complexity of the spectra was too great. This complexity was probably due to the presence of a large number of differently *O*-acetylated xyloglucan oligomers (York *et al.*, 1988).

Correlations between xyloglucan structure and taxonomy

The primary cell walls of most plants analysed thus far contain fucosylated xyloglucans composed of oligosaccharide subunits (e.g. XXFG) with one unbranched glucosyl residue followed by three branched glucosyl residues (Vincken *et al.*, 1997) (see Fig. 4.7). This type of branching structure is observed in xyloglucans from a wide range of species, including gymnosperms and both monocotyledonous and dicotyledonous angiosperms. However, the cell walls of some plant species contain xyloglucans with atypical structures. For example, xyloglucans produced by most of the *Poaceae* (grasses) appear to lack Fuc. Another example are dicotyledonous species of the subclass *Asteridae*, some of which produce xyloglucans with α-Ara*f*-(1→2)-α-Xyl*p* side chains. These include the members of the order Solanales, such as tobacco, potato and tomato, in which the xyloglucan backbone has an unusual branching structure, with two unbranched glucosyl residues followed by two branched glucosyl residues (e.g. XXGG and XSGG) (York *et al.*, 1996). As described here, olive fruit (order Lamiales) contains a xyloglucan whose side chains contain Ara and lack Fuc, but whose branching structure is more typical, with three out of four glucosyl residues bearing a side chain. Not all of the *Asteridae* produce unusual xyloglucans, as at least one species (*Arctium lappa*, group euasterids II, order Asterales) produces a typical, fucosylated xyloglucan, in which three of every four glucosyl residues bear side chains (Kato & Watanabe, 1993). The structural features of xyloglucan are known to be tissue-specific, so

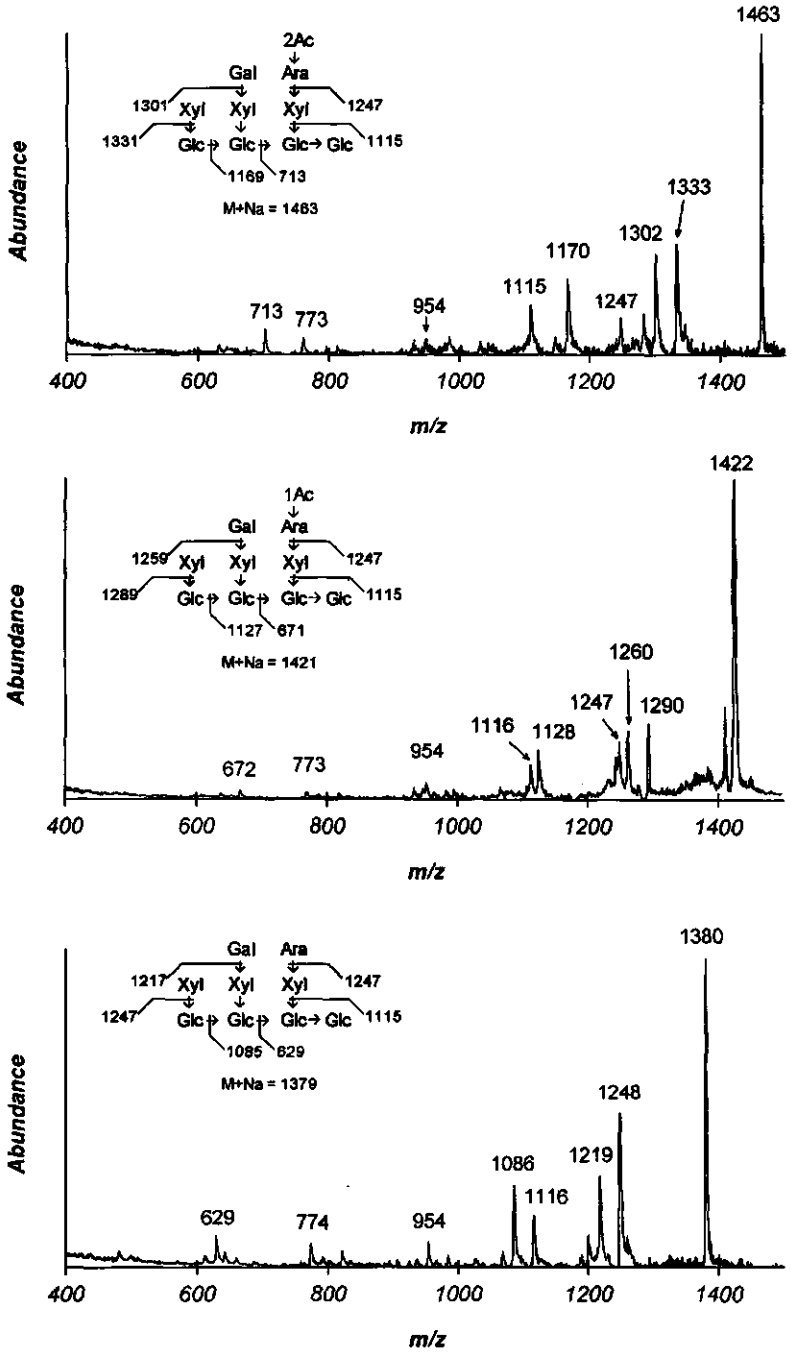

Fig. 4.6. Post-Source Decay MALDI-TOF spectra of the xyloglucan oligomer XLSG without *O*-acetyl groups (lower panel), with one (middle panel), and two *O*-acetyl groups (upper panel). DHB (50 mg/mL) was used as a matrix and additional NaCl (50 nmol) was added for optimal cationisation.

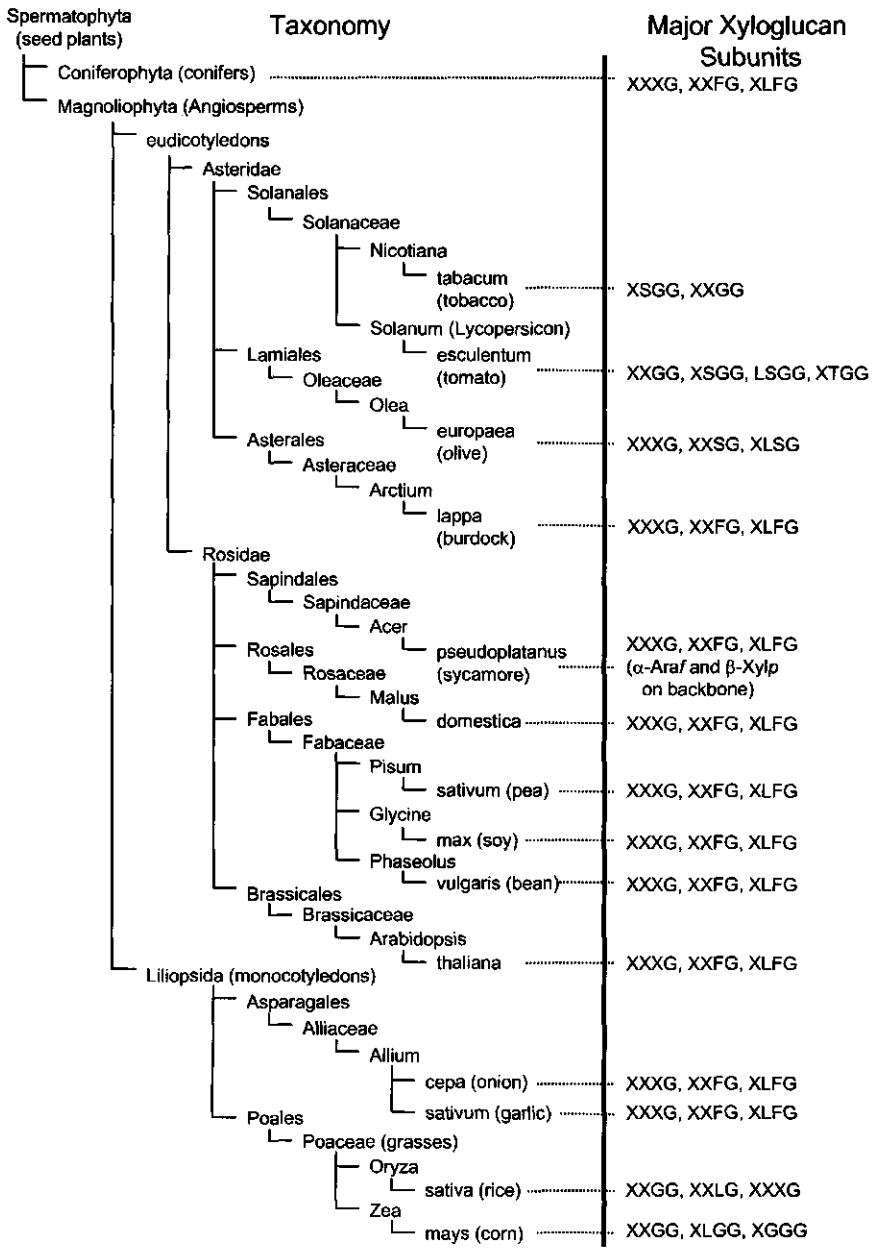

Fig. 4.7. Phylogenetic relationships of xyloglucan oligosaccharide subunit structures. Each oligosaccharide structure is represented as described in Fry *et al.* (1989) with specific code letters for each segment (G: β-D-Glc*p*-; X: α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; L: β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; S: α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; F: α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-; T: β-Ara*f*-(1→3)-α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*-). Phylogenetic relationships are based on the NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/).

care should be exercised when using these features to determine taxonomic relationships. For example, seed xylogucans often lack fucosyl residues, while leaf tissue from the same plant contains fucosylated xyloglucans (Pauly *et al.*, 2001). Nevertheless, structural features that are common to the xyloglucans from olive fruit, tomato cells and tobacco cells, for example, are consistent with the inclusion of the orders Lamiales and Solanales in the same taxonomic group, euasterids I. That is, these species all produce xyloglucans with α-Ara*f*-(1→2)-α-Xyl*p* side chains, which are relatively uncommon. Of these, species in the order Solanales have been shown to produce xyloglucans with an unusual branching pattern that has not been observed in the order Lamiales.

Acknowledgements

This research was supported by the EU (AIR3-CT93-1355) and in part by funds from the National Science Foundation grant MCB-9974673 and by the DOE-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG02-93ER20097). The authors would like to thank the National Institutes of Health, grant no. S10 RR12013 to Dr. Ron Orlando (CCRC), for providing the funds to acquire the Kratos SEC mass spectrometer.

References

Bax, A. & Freeman, R. Investigation of complex networks of spin-spin coupling by two-dimensional NMR. *J. Magn. Reson.* 44 (1981) 542-561.

Bax, A. & Davis, D.G., MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 65 (1985) 355-360.

Beldman, G., Searle-Van Leeuwen, M.F., Rombouts, F.M. & Voragen, A.G.J. The cellulase of *Trichoderma viride*. Purification, characterization and comparison of all dectable endoglucanases, exoglucanases and β-glucosidases. *Eur. J. Biochem.* 146 (1985) 301-308.

Bodenhausen, G. & Ruben, D.J. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem. Phys. Lett.* 69 (1980) 185-189.

Carpita, N.C. & Gibeaut, D.M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3 (1993) 1-30.

Domon, B. & Costello, C.E. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconj. J.* 5 (1988) 397-409.

Englyst, H.N. & Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* 109 (1984) 937-942.

Fry, S.C. The structure and functions of xyloglucan (review article). *J. Exp. Bot.*, 40 (1989) 1-11.

Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J. & White, A.R. An unambiguous nomenclature for xyloglucan derived oligosaccharides. *Physiol. Plant.* 89 (1993) 1-3.

Hayashi, T. Xyloglucans in the primary cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40 (1989) 139-168.

Kato, Y. & Watanabe, T. Isolation and characterization of a xyloglucan from Gobo (*Arctium lappa* L.). *Biosci. Biotechnol. Biochem.* 57 (1993) 1591-1592.

Keeler, J., Clowes, R.T., Davis, A.L. & Laue, E.D. Pulsed field gradients - theory and practice. *Meth. Enzymol.* 239 (1994) 145-207.

Kooiman, P. The constitution of Tamarindus-amyloid. *Recl. Trav. Chim. Pays-Bas* 80 (1961) 849-865.

Levy, S., York, W.S., Stuike-Prill, R., Meyer, B. & Staehelin, L.A. Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *The Plant J. Cell Mol. Biol.* 1 (1991) 195-215.

Levy, S., Maclachlan, G. & Staehelin, L.A. Xyloglucan sidechains modulate binding to cellulose during in vitro binding assays as predicted by conformational dynamics simulations. *Plant J.* 11 (1997) 373-386.

McDougall, G.J. & Fry, S.C. Xyloglucan nonasaccharide, a naturally-occurring oligosaccharin, arises in vivo by polysaccharide breakdown. *J. Plant Physiol.* 137 (1991) 332-336.

Pauly, M., Qin, Q., Greene, H., Albersheim, P., Darvill, A. & York, W.S. Changes in the structure of xyloglucan during cell elongation. *Planta* 212 (2001) 842-850.

Schols, H.A., Posthumus, M.A. & Voragen, A.G.J. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydr. Res.* 206 (1990) 117-129.

States, D.J., Haberkorn, R.A. & Ruben, D.J. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.* 48 (1982) 286-292.

Van Houdenhoven, F.E.A. *Studies on pectin lyase, Ph.D. thesis,* Wageningen: Wageningen Agricultural University (1975) 100 p.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J. Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv koroneiki) at different ripening stages. *Carbohydr. Polym.* 43 (2000) 11-21.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J. Structural characterisation of xyloglucan and xylans present in olive fruit (*Olea europaea* cv koroneiki). *Carbohydr. Polym.* 44 (2001) 51-62.

Vincken, J-P. Van den Broek, L.A.M., Van der Lei, D.D., Beldman, G. & Voragen, A.G.J. Fungal and plant xyloglucanases may act in concert during liquefaction of apples. *J. Sci.Food Agric.* 73 (1997) 407-416.

Vincken, J-P., York, W.S., Beldman, G. & Voragen, A.G.J. Two general branching patterns of xyloglucan, XXXG and XXGG: update on cell walls. *Plant Physiol.* 114 (1997) 9-13.

Yamagaki, T., Mitsuishi, Y. & Nakanishi, H. Determination of structural isomers of xyloglucan octasaccharides using post-source decay fragment analysis in MALDI-TOF mass spectrometry. *Tetrahedr. Lett.* 39 (1998) 4051-5054.

York, W.S., Darvill, A.G. & Albersheim, P. Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiol.* 75 (1984) 295-297.

York, W.S., Oates, J.E., Van Halbeek, H., Darvill, A.G. & Albersheim, P. Location of the *O*-acetyl substituents on a nonasaccharide repeating unit of sycamore extracellular xyloglucan. *Carbohydr. Res.* 173 (1988) 113-132.

York, W.S., Van Halbeek, H., Darvill, A.G. & Albersheim, P. Structure of plant cell walls XXIX.

Structural analysis of xyloglucan oligosaccharides by ^{1}H-NMR spectroscopy and fast-atom-bombardment mass spectrometry. *Carbohydr. Res.* 200 (1990) 9-31.

York, W.S., Harvey, L.K., Guillén, R., Albersheim, P. & Darvill, A.G. Structural analysis of tamarind seed xyloglucan oligosaccharides using β-galactosidase digestion and spectroscopic methods. *Carbohydr. Res.* 248 (1993) 285-301.

York, W.S., Kolli, V.S.K., Orlando, R., Albersheim, P. & Darvill, A.G. The structures of arabinoxyloglucans produced by solanaceous plants. *Carbohydr. Res.* 285 (1996) 99-128.

Chapter 5

Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides

Abstract

The effect of the use of cell-wall-degrading-enzyme preparations during the mechanical extraction process of virgin olive oil on the phenolic compounds and polysaccharides was investigated. The use of the enzyme preparations increased the concentration of phenolic compounds in the paste, oil and by-products. Especially, the contents of secoiridoid derivatives such as the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol (3,4-DHPEA-EDA) and an isomer of oleuropein aglycon (3,4-DHPEA-EA), which have high antioxidant activities increased significantly in the olive oil. Furthermore, the use of an N_2 flush during processing strongly increased the phenolic concentration. Analyses of the pectic polymers present in the paste showed that the use of pectinolytic enzyme preparations increased the yield of the buffer soluble pectins and the proportion of molecules with a lower molecular mass. Also, the content of uronic acids in the buffer soluble extract increased considerably due to the use of the enzyme preparations. Analysis of the polymeric carbohydrates in the vegetation waters showed the presence of mainly pectic polymers. The addition of commercial enzyme preparations increased the uronic acid content of the polysaccharides in the vegetation water substantially compared to the blank. This study showed that the addition of cell-wall-degrading enzymes did improve the olive oil quality; however, mechanisms remained unclear.

This chapter has been published in J. Agric. Food Chem. 49 (2001) 1218-1223 by the authors Esther Vierhuis, Maurizio Servili, Maura Baldioli, Henk A. Schols, Alphons G.J. Voragen & GianFrancesco Montedoro.

Introduction

Secoiridoids such as oleuropein and demethyloleuropein are the predominant phenolic compounds of olive fruit (Panizzi *et al.*, 1960; Ragazzi *et al.*, 1973; Servili *et al.*, 1999b). In addition, olive fruit also contains verbascoside and low amounts of other classes of phenolic compounds including phenyl alcohols such as 3,4-dihydroxyphenylethanol (3,4-DHPEA) and *p*-hydroxyphenylethanol (*p*-HPEA), phenyl acids and flavonoids (Macheix *et al.*, 1990; Brenes-Balbuena *et al.*, 1992; Mazza & Miniati, 1993; Servili *et al.*, 1999a). The phenol alcohols 3,4-DHPEA and *p*-HPEA are also present in virgin olive oil. However, the prevalent phenolic compounds in the oil are the secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to 3,4-DHPEA or *p*-HPEA (3,4-DHPEA-EDA or *p*-HPEA-EDA, respectively) and an isomer of oleuropein aglycon (3,4-DHPEA-EA) (Montedoro *et al.*, 1993a).

The concentration of phenolic compounds present in virgin olive oil is strongly affected by the extraction conditions used during processing (Servili *et al.*, 1994; Servili *et al.*, 1998; Morales *et al.*, 1999). In this ambit, the loss of secoiridoids and phenyl-alcohols in the oil during malaxation is well-known (Servili *et al.*, 1996; Servili *et al.*, 1999a). So far, however, the mechanism that explains the quantitative modification of secoiridoids in the oil during malaxation is unknown. Enzymatic oxidation by endogenous oxidoreductases such as polyphenoloxidase (PPO) and peroxidase (POD) may promote oxidation of phenolic compounds (Sciancalepore, 1985; Servili *et al.*, 1996) but nonenzymatic oxidation processes are also involved (Servili *et al.*, 1998).

Interactions between polysaccharides and phenolic compounds present in the olive pastes may also be involved in the loss of phenols during processing. Previous work has shown that polyphenols are able to complex with certain types of polysaccharides, that most probably results from the ability of the polysaccharide to form a structure which encapsulates the polyphenol (McManus *et al.*, 1985; Ozawa *et al.*, 1987; Haslam & Lilley, 1988; Spencer *et al.*, 1988). The interaction of the phenolic compounds with polysaccharides may reduce their release in the oil during crushing and malaxation. In fact, it is shown that the use of technical preparations containing cell-wall-degrading-enzyme activities during processing can improve the phenolic concentration in the oil (Siniscalco & Montedoro, 1988; Siniscalco & Montedoro, 1989; Servili *et al.*, 1992; Montedoro *et al.*, 1993b; Ranalli & Serraiocco, 1996; Ranalli & De Mattia, 1997).

Although the changes in the composition of the phenolic compounds due to the use of enzyme preparations have been studied before, very little is known about the effect of these enzyme preparations on the cell wall structure. This paper reports both elements and also the effect of malaxation under N_2 flush to reduce the oxidative degradation of phenolic compounds in pastes during malaxation.

Experimental

Materials

Olives from the cultivar moraiolo were used. Olivex is an enzyme preparation rich in pectolytic enzymes and in addition hemicellulolytic and cellulolytic side activities produced from *Aspergillus aculeatus* and especially suitable for application in the extraction of vegetable oils. Novoferm 12 is a pectolytic enzyme preparation of *A. niger* origin, which in addition contains β-glucosidase activity. Both commercial enzyme preparations were kindly provided by Novo Nordisk Ferment Ltd. (Dittingen, Switzerland).

Sample preparation

Olives (90 kg) were crushed with a hammer mill (α-LAVAL, Lund, Sweden) and slowly mixed (malaxed) for 60 min at 30°C. Extraction of the oil was performed with an α-LAVAL Decanter UVNX-414 with a working capacity of 1.00 ton/h (paste/water ratio 1 : 0.15 (w/v)). Samples of the pastes and vegetation waters were taken and immediately frozen in liquid nitrogen to inhibit enzymatic activity, freeze-dried and stored at -30°C before analysis. The enzyme preparations were added at the beginning of malaxation (500 mg/kg of pastes). To study the effect of O_2 during processing, malaxation was performed with and without an N_2 flush. The oxygen value in the pastes was measured, during malaxation, using a Mettler Toledo oxygen sensor model 4100 (Greifensee, Switzerland).

Extraction, purification and separation of phenolic compounds

Pastes. Freeze-dried paste (10 g) was mixed with 50 mL of 80% methanol containing 20 mg/L sodium diethyldithiocarbamate (DIECA) at -25°C to inhibit PPO. The mixture was homogenized in an Omni-mixer (Sorvall) for 30 s at 16000 rpm and filtered using a Buchner funnel apparatus. The procedure was repeated six times, and the extracts were collected. The methanol was evaporated in a vacuum at 35°C under a flow of nitrogen. The water extract (2 mL) was loaded on a 5 g/20 mL Extract-Clean high-load C18 cartridge (Alltech Associates Inc., Deerfield, IL) and eluted with methanol (600 mL). The eluate was collected and the organic solvent evaporated in a vacuum at 35°C under a flow of nitrogen. The residue was dissolved in methanol (1 mL) and analysed by HPLC (Servili *et al.*, 1999b).

Vegetation waters. Freeze-dried vegetation water was rehydrated with water containing DIECA (20 mg/L) and loaded (2 mL) on a 5 g/20 mL Extract-Clean high-load C18 cartridge (Alltech Associates Inc.) and eluted with ethyl ether (600 mL). The concentration and analysis on HPLC were carried out as reported previously (Servili *et al.*, 1999b).

Virgin olive oil. Phenols were extracted with 80% methanol from the virgin olive oil, the crude oil from the mechanical extraction, and evaluated by HPLC according to the procedure performed by Montedoro *et al.* (1992).

Reference compounds

3,4-DHPEA was synthesised in the laboratory according to the procedure of Baraldi *et al.* (1983). 3,4-DHPEA-EDA, 3,4-DHPEA-EA and verbascoside were extracted from virgin olive oil and olive fruit, respectively, and the chemical structures were verified by NMR according to the method of Montedoro *et al.* (1993b). Oleuropein glucoside was obtained from Extrasynthèse Co. (Genay, France). *p*-HPEA was obtained from Janssen Chemical Co. (Beerse, Belgium), luteolin-7-glycoside was obtained from Roth Co. (Karlsruhe, Germany) and rutin was obtained from BDH Co. (Poole, U.K.).

Statistical analysis

Experiments to determine the content of the phenolic compounds were performed in triplicate and the means ± standard deviations are reported in Tables 5.1-5.3. One way analysis of variance (ANOVA) using the Tukey test was performed to evaluate the significance of differences between mean values among three or more different experimental groups. Statgraphics Version 6.1 (Statistical Graphics Corp., 1992, Manugistics, Rockville, MD) was used to perform the statistical analyses.

Isolation of alcohol insoluble solids (AIS)

The freeze-dried pastes were defatted by (Soxhlet) extraction with petroleum ether (40-60). A sieve of 425 μm was used to separate the stones from the pulp. 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 *et al.*, 1956). The residue (AIS) was dried by solvent exchange (96% (v/v) ethanol and acetone) at room temperature. The freeze-dried vegetation waters were defatted by extraction with petroleum ether (40-60) and extracted with 70% (v/v) ethanol as described above to isolate the AIS.

Extraction of AIS

The AISs prepared from olive pulp (75 mg) were extracted with 2.25 mL of 0.05 M NaOAc buffer, pH 5.2 at 70°C for 1 h (hot buffer soluble solids). The solubilised polymers were separated from the insoluble residue by centrifugation. The extracts were analysed by high-performance size-exclusion chromatography (HPSEC) and the sugar composition of the polysaccharides present in the extracts was determined.

Enzyme incubation of AIS with Olivex

The AISs prepared from vegetation water were dissolved (2.5 mg/mL) in 0.05 M NaOAc buffer, pH 5.0 and incubated with the commercial enzyme preparation Olivex (0.1% (w/v)) at 40°C for 24 h. After incubation the samples were heated for 15 min at 100°C to inactivate the

enzymes. The change in molecular weight distribution of the samples was studied by HPSEC.

Analytical methods

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. Analysis of the uronic acid content was performed in triplicate. The mean values are reported in Tables 5.4 and 5.5 and have a standard deviation ≤ 1.

Neutral sugar composition

The neutral sugar composition of the AISs and the polysaccharides present in the hot buffer extracts was determined by gas chromatography according to the method of Englyst & 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 converted into their alditol acetates and analysed. Cellulosic glucose in the AISs was calculated as the difference between the glucose contents determined with and without pretreatment with 72% (w/w) sulphuric acid. Analysis of the neutral sugar composition was performed in triplicate. The mean values are reported in Tables 5.4 and 5.5 and have a standard deviation ≤ 1.

High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed as described previously (Vierhuis *et al.*, 2000). Calibration was performed using dextrans (M_w 4-500 kDa).

Results and Discussion

The crushed paste showed a high concentration of the secoiridoid derivative 3,4-DHPEA-EDA, whereas oleuropein, demethyloleuropein and verbascoside were present in small amounts (Table 5.1). The high concentration of 3,4-DHPEA-EDA is due to the hydrolysis of oleuropein and demethyloleuropein catalyzed by endogenous glycosidases during the crushing of olive fruit (Servili *et al.*, 1996). The virgin olive oil obtained from the crushed paste contained a high concentration of the aglycon derivatives 3,4-DHPEA-EDA and 3,4-DHPEA-EA (Table 5.2). Oleuropein and demethyloleuropein where not present in the olive oil. The phenolic composition of vegetation waters was very different from the composition of the oil as the vegetation waters contained secoiridoid glucosides and other polar compounds such as luteolin 7-glucoside and rutin that were not detected in virgin olive oil. The secoiridoid derivative 3,4-DHPEA-EDA was the predominant phenolic compound of vegetation waters (Table 5.3).

Malaxation reduced the concentration of phenolic compounds in the pastes, oil and vegetation waters (Tables 5.1-5.3) and is the most critical point in the mechanical extraction

Table 5.1
Phenolic composition of olive pastes with and without enzymatic treatment during processing (malaxation was performed in the absence and presence of N_2)

	Crushed paste blank	Malaxed paste blank	Malaxed paste + NF12/Olivex	Malaxed paste under N_2 flush	Malaxed paste under N_2 flush + NF12/Olivex
O_2[a] (ppm)	-	6.9 ± 0.7^a	6.6 ± 0.5^a	0.16 ± 0.07^b	0.18 ± 0.08^b
Phenolic compounds[b] *(mg/g of dry weight)*					
3,4-DHPEA	0.54 ± 0.01^a	0.04 ± 0.01^b	0.03 ± 0.01^b	0.04 ± 0.01^b	0.03 ± 0.01^b
p-HPEA	0.32 ± 0.02^a	0.04 ± 0.01^b	0.05 ± 0.01^b	0.03 ± 0.01^b	0.09 ± 0.01^c
Demethyloleuropein	1.54 ± 0.02^a	0.30 ± 0.01^b	0.51 ± 0.04^c	0.71 ± 0.04^d	0.90 ± 0.02^e
Verbascoside	3.3 ± 0.2^a	1.3 ± 0.1^b	1.2 ± 0.2^b	2.4 ± 0.2^c	2.8 ± 0.4^{ac}
3,4-DHPEA-EDA	20.8 ± 0.9^a	5.7 ± 0.3^b	7.5 ± 0.5^c	10.6 ± 0.5^d	13.9 ± 0.1^e
Oleuropein	2.3 ± 0.1^a	0.75 ± 0.05^b	0.75 ± 0.07^b	1.19 ± 0.03^c	1.89 ± 0.02^d
Luteolin-7-glucoside	0.22 ± 0.02^a	0.13 ± 0.01^b	0.12 ± 0.01^b	0.25 ± 0.01^a	0.18 ± 0.01^c
Rutin	0.16 ± 0.01^a	0.11 ± 0.01^{bc}	0.09 ± 0.01^b	0.17 ± 0.01^a	0.12 ± 0.01^c

[a] The O_2 concentration is the mean value of six independent determinations evaluated during malaxation ± standard deviation.
[b] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another.

process of oil. In fact, the nutritional value of oil is directly related to the concentration of its natural antioxidants such as 3,4-DHPEA, 3,4-DHPEA-EA and 3,4-DHPEA-EDA (Petroni *et al.*, 1995; Visioli *et al.*, 1995). The use of commercial enzyme preparations with activity towards polysaccharides during processing increased significantly the concentration of 3,4-DHPEA-EDA in the malaxed paste. Also, the release of secoiridoid derivatives, such as 3,4-DHPEA-EDA and 3,4-DHPEA-EA, into the oil improved greatly. The vegetation waters showed a significant increase of 3,4-DHPEA-EDA, oleuropein and demethyloleuropein. The use of an N_2 flush during malaxation reduced the O_2 level in the pastes and greatly increased the phenolic concentration in the oil, paste and vegetation waters (Tables 5.1-5.3).

To study the effect of the commercial enzyme preparations on the polysaccharides present in the cell wall, cell wall material was isolated from the olive pastes before and after malaxation and analysed for sugar composition (Table 5.4). Glucose, xylose and arabinose were the major neutral sugars in the olive pastes. About 94% of the glucose in the paste represented cellulose. The uronic acid content was 14-15 mol%. The results revealed that the sugar composition of the paste hardly changed during processing without commercial enzyme preparations added. However, the addition of commercial enzyme preparations during processing also hardly changed the sugar composition of the paste. This may indicate that the degradation of the polysaccharides present in the cell wall by endogenous enzymes or the addition of exogenous enzymes was very limited, despite the positive effect of the latter on the level of phenolic compounds.

The enzyme preparations used during processing were rich in pectolytic enzymes.

Table 5.2
Phenolic composition of virgin olive oil (mg/kg) with and without enzymatic treatment during processing (malaxation was performed in the absence and presence of N_2)

	Crushed paste blank	Malaxed paste blank	Malaxed paste + NF12/Olivex	Malaxed paste under N_2 flush	Malaxed paste under N_2 flush + NF12/Olivex
3,4-DHPEA[a]	2.7 ± 0.3^{a}	0.7 ± 0.1^{b}	1.9 ± 0.1^{c}	2.0 ± 0.2^{ac}	1.7 ± 0.3^{c}
p-HPEA	2.3 ± 0.4^{a}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	2.6 ± 0.3^{a}	0.81 ± 0.03^{b}
3,4-DHPEA-EDA	515 ± 23^{a}	317 ± 16^{c}	439 ± 16^{d}	504 ± 6^{a}	556 ± 13^{b}
p-HPEA-EDA	24.8 ± 1.9^{a}	25.8 ± 1.4^{ab}	29.4 ± 0.8^{c}	28.4 ± 1.4^{bc}	31.4 ± 1.0^{c}
p-HPEA derivative	32.5 ± 1.4^{a}	24.2 ± 0.8^{b}	28.5 ± 0.9^{c}	21.6 ± 1.3^{b}	22.4 ± 0.9^{b}
3,4-DHPEA-EA	357 ± 13^{a}	177 ± 8^{b}	218 ± 8^{c}	242 ± 5^{c}	299 ± 10^{d}

[a] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another.

Table 5.3
Phenolic composition of vegetation waters (mg/g of dry weight) with and without enzymatic treatment during processing (malaxation was performed in the absence and presence of N_2)

	Crushed paste blank	Malaxed paste blank	Malaxed paste + NF12/Olivex	Malaxed paste under N_2 flush	Malaxed paste under N_2 flush + NF12/Olivex
3,4-DHPEA[a]	1.52 ± 0.06^{a}	0.37 ± 0.03^{b}	0.44 ± 0.01^{b}	0.37 ± 0.02^{b}	0.55 ± 0.04^{c}
p-HPEA	0.14 ± 0.01^{a}	nd[b]	0.04 ± 0.01^{c}	0.04 ± 0.01^{c}	0.07 ± 0.01^{d}
Demethyloleuropein	1.39 ± 0.06^{a}	0.47 ± 0.06^{b}	0.82 ± 0.01^{c}	0.95 ± 0.10^{c}	1.8 ± 0.1^{d}
Verbascoside	nd[b]	nd	nd	nd	nd
3,4-DHPEA-EDA	98.6 ± 8.6^{a}	17.5 ± 1.1^{b}	31.9 ± 2.6^{c}	35.4 ± 0.7^{cd}	44.2 ± 1.2^{d}
Oleuropein	2.8 ± 0.3^{a}	0.60 ± 0.04^{b}	1.1 ± 0.1^{c}	1.1 ± 0.1^{c}	1.2 ± 0.2^{c}
Luteolin-7-glucoside	0.20 ± 0.01^{a}	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.14 ± 0.01^{c}	0.17 ± 0.01^{d}
Rutin	0.19 ± 0.01^{a}	0.06 ± 0.01^{b}	0.10 ± 0.01^{c}	0.08 ± 0.01^{bc}	0.18 ± 0.01^{a}

[a] The phenolic content is the mean value of three independent experiments ± standard deviation. Values in each row bearing the same superscripts are not significantly ($P < 0.05$) different from one another.
[b] Not detected.

Therefore, an extraction with hot buffer was performed to study whether processing or the addition of enzyme preparations had an effect on the buffer soluble pectic material in olive paste. The extracts were analysed for their sugar composition (Table 5.5) and the molecular weight distribution was determined by HPSEC (Fig. 5.1A). It appeared that processing without the addition of enzyme preparations hardly changed the sugar composition or the solubility of the pectic material extracted with hot buffer. Also, the molecular weight distributions as determined by HPSEC were identical for the buffer soluble pectins from the crushed paste and from the malaxed paste. Although olive fruit contains endogenous enzymes

Table 5.4
Sugar composition expressed as mol% of cell wall material (AIS) from pastes and vegetation waters

Sample	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	% (w/w)
Crushed paste blank	1	13	31	1	4	36	14	45
Malaxed paste blank	2	13	30	1	4	36	14	46
Malaxed paste + NF12/Olivex	2	12	30	2	3	37	15	42
Vegetation water blank	3	24	3	1	21	8	39	32
Vegetation water + NF12/Olivex	3	20	1	1	11	2	63	47

such as glycosidases and polygalacturonase and enzymes with cellulolytic activity (Heredia *et al.*, 1991; Heredia *et al.*, 1993; Fernàndez-Bolaños *et al.*, 1995), the enzymes had hardly any effect on the polysaccharides during malaxation. It may be suggested that the high concentration of phenolic compounds present in olive pulp inhibited these enzymes (Heredia *et al.*, 1990; Knee *et al.*, 1991; Servili *et al.*, 1999a; Servili *et al.*, 1999b).

The addition of commercial enzyme preparations increased the extractability of pectic material with hot buffer and also the sugar composition of the extractable polysaccharides changed compared to the blank. The relative amount of uronic acids extracted with buffer was substantially higher and consequently the ratio neutral sugars to uronic acids decreased. Also, the elution profile of the extracted material on HPSEC changed due to the addition of commercial enzyme preparations. The paste obtained by malaxation with commercial enzyme preparations added during processing had a relatively larger proportion of molecules with a lower molecular mass (c. 100 kDa).

The polysaccharides present in the vegetation waters were also studied. The polysaccharides were isolated by ethanol precipitation and the sugar composition was determined (Table 5.4). The presence of the neutral sugars arabinose and galactose and the high content of uronic acid in both vegetation waters indicated that mainly pectins were present. Pectic polymers consist of regions of linear galacturonan and regions of more highly substituted rhamnogalacturonan with complex chains of arabinose and galactose linked to the rhamnose residues (Voragen *et al.*, 1995). Therefore, the ratio of the sugars arabinose and galactose to uronic acid gives an indication of the degree of branching of the pectin molecules present in the vegetation water. The ratio was 1.2 for the polysaccharides present in the vegetation water of the blank and 0.5 for the polysaccharides present in the vegetation water with Novoferm 12 and Olivex added during processing. These ratios indicate that the addition

Table 5.5
Yield and sugar composition (mol%) of polysaccharides solubilised with hot buffer from crushed and malaxed pastes

Sample	Yield[a]	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids
Crushed paste blank	7.0	3	23	2	1	14	4	53
Malaxed paste blank	7.0	3	22	3	1	14	4	53
Malaxed paste + NF12/Olivex	9.5	4	24	1	1	8	2	60

[a] Percent of total polysaccharides solubilised.

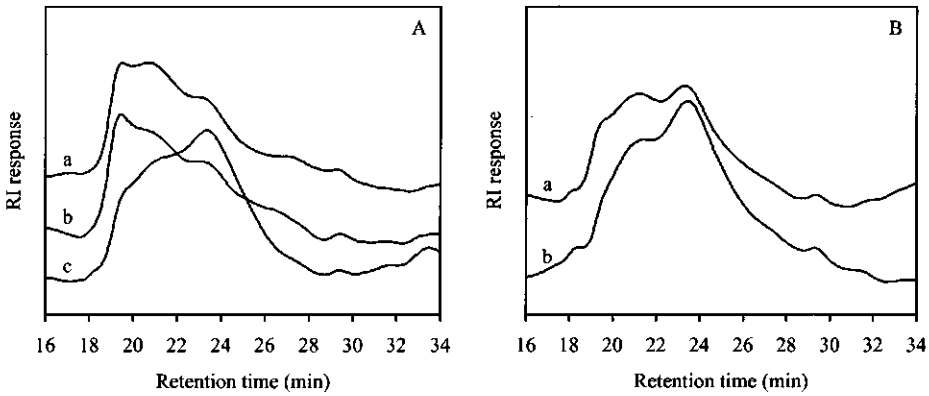

Fig. 5.1. HPSEC-patterns of polysaccharides extracted with hot buffer from crushed and malaxed pastes (A) and polysaccharides present in vegetation waters (B). A: crushed paste blank (a), malaxed paste blank (b), malaxed paste plus NF12/Olivex (c); B: vegetation water blank (a), vegetation water plus NF12/Olivex (b).

of commercial enzyme preparations partly debranched the pectic material or solubilised additional pectic substances with a high galacturonic acid content.

The HPSEC elution patterns showed almost identical molecular weight distributions for the polysaccharides isolated from both vegetation waters despite the differences in the sugar composition (Fig. 5.1B). Only a slight shift toward lower molecular weight ranges could be observed. Polymeric material was still present in the vegetation waters and was not completely degraded to oligosaccharides or their constituent monosaccharides even though enzyme preparations were added during processing. To check whether the structure of the pectins restricted the action of the enzymes, the pectic polymers isolated from the vegetation waters were incubated with the enzyme preparation Olivex, which was also used during processing. The HPSEC elution patterns revealed that the polysaccharides could be degraded to a substantial extent (not shown). About 90% of the polysaccharides were degraded to oligomers (molecular mass < 4 kDa) and monosaccharides. Therefore, the structures of the polysaccharides or the enzyme preparations used were not limiting for the degradation. Probably, the enzymes present in the commercial enzyme preparations were partially inactivated during processing due to phenolic compounds present in the pastes, as was suggested before.

Concluding remarks

It is known that the addition of cell-wall-degrading-enzyme preparations during the mechanical extraction of olive oil can improve the release of phenolic compounds in the oil. However, it is the first time that the effect of the addition enzyme preparations has been studied analysing both the phenolic compounds and polysaccharides present in the pastes and vegetation waters. In fact, previous papers reported only results about quantitative and

qualitative modifications on the phenolic composition of oil when enzymatic preparations were used during processing (Siniscalco & Montedoro, 1989; Servili *et al.*, 1992; Montedoro *et al.*, 1993b; Ranalli & Serraiocco, 1996; Ranalli & De Mattia, 1997). However, although the results of this study revealed that the use of commercial enzyme preparations changed part of the cell wall structure, it is difficult to conclude how these changes affected the phenol content of the oil. The addition of commercial enzyme preparations might have reduced the complexation of the phenolic compounds with the polysaccharides, thus improving the concentration of free phenols in the pastes and their release in the oil and vegetation water during processing. Also, the addition of pectolytic enzymes could have resulted in weakening and disruption of the cell wall, thus facilitating the release of phenolic compounds from the fruit. However, the content of phenolic compounds in the oil is also influenced by enzymatic and nonenzymatic oxidation. In fact, the inhibition of the oxidation process by reducing the O_2 level in the paste during malaxation strongly improved the concentration 3,4-DHPEA-EDA and 3,4-DHPEA-EA, which have high antioxidant activities (Baldioli *et al.*, 1996) and are mainly affected by oxidative degradation. In conclusion, the addition of commercial enzyme preparations improved the release of phenolic compounds in the oil but the oxidative reactions during malaxation have also an important impact on the final concentration in the oil and vegetation waters. Work is in progress to define the interaction between the oxidative degradation of phenolic compounds and the activity of endogenous oxidoreductases such as PPO and POD during the mechanical extraction of olive oil.

Acknowledgements

We thank Dr. Roberto Selvaggini and Roberto Santibacci for technical assistance. This research work was supported by the European Economic Community, under project AIR3-CT93-1355.

References

Baldioli, M., Servili, M., Perretti, G. & Montedoro, G.F. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil, *J. Am. Oil Chem. Soc.* 73 (1996) 1589-1593.

Baraldi, P.G., Simoni, D., Manfredini, F. & Menziani, E. Preparation of 3,4-dihydroxy-1-benzeneethanol: a reinvestigation, *Liebigs Ann. Chem.* (1983) 684-686.

Brenes-Balbuena, M.& Garcia-Garcia, P. & Garrido-Fernández, A. Phenolic compounds related to the black colour formed during the processing of ripe olive, *J. Agric. Food Chem.* 40 (1992) 1192-1196.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350-356.

Englyst, H.N. & Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates, *Analyst* 109 (1984) 937-942.

Fernández-Bolaños, J., Rodríguez, R., Guillén, R., Jimenéz, A. & Heredia, A. Activity of cell wall-

associated enzymes in ripening olive fruit, *Physiol. Plant.* 93 (1995) 651-658.

Haslam, E. & Lilley T.H. Natural astringency in foodstuffs - a molecular interpretation, *Crit. Rev. Food Sci. Nutr.* 27 (1988) 1-40.

Heredia, A., Fernández-Bolaños, J. & Guillén, R. Cellulase inhibition by polyphenols in olive fruits, *Food Chem.* 38 (1990) 69-73.

Heredia, A., Fernández-Bolaños, J. & Guillén, R. Identification of endoglucanases in olives (*Olea europaea* arolensis), *Z. Lebensm. -Unters. -Forsch.* 193 (1991) 554-557.

Heredia, A., Guillén, R., Jiménez, A. & Fernández-Bolaños, J. Activity of glycosidases during development and ripening of olive fruit, *Z. Lebensm. -Unters. -Forsch.* 196 (1993) 147-151.

Knee, M., Paull, R.E., Ben Arie, R. & Hawker, J.S. Enzymes in fruit. In: *Food enzymology*. P.F. Fox (ed), London: Elsevier (1991) 545-598.

Macheix, J.J., Fleuriet, A. & Billot, J. *Fruit phenolics.* Boca Raton: CRC Press (1990) 1-126.

Mazza, G. & Miniati, E. *Anthocyanins in fruits, vegetables and grains.* Boca Raton: CRC Press (1993) 64-67.

McManus, J.P., Davis, K.G., Beart, D.J.E., Gaffney, S.H., Lilley, T.H. & Haslam, E. Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc., Perkin Trans.* II (1985) 1429-1438.

Montedoro, G.F., Servili, M., Baldioli, M. & Miniati, E. Simple and hydrolyzable compounds in virgin olive oil. 1. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC, *J. Agric. Food Chem.* 40 (1992) 1571-1576.

Montedoro, G.F., Servili, M., Baldioli, M., Selvaggini, R., Miniati, E. & Macchioni, A. Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterization of the secoiridoids derivatives, *J. Agric. Food Chem.* 41 (1993a) 2228-2234.

Montedoro, G.F., Begliomini, A.L., Servili, M., Petruccioli, M. & Federici, F. Pectinase production from olive vegetation waters and its use in the mechanical olive oil extraction process to increase oil yield and improve quality, *Ital. J. Food Sc.* 5 (1993b) 355-362.

Morales, M.T., Angerosa, F. & Aparicio, R. Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications, *Grasas Aceites* 50 (1999) 114-121.

Ozawa, T., Lilley, T.H. & Haslam, E. Polyphenol interactions: astringency and the loss of astringency in ripening fruit, *Phytochemistry* 26 (1987) 2937-2942.

Panizzi, L., Scarpati, M.L. & Oriente, G. Chemical structure of oleuropein, bitter glucoside of olive with hypotensive activity, *Gazz. Chim. Ital.* 90 (1960) 1449-1485.

Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedoro, G.F. & Galli, C. Inhibition of platelet aggregation and eicosanoid production by phenolic component of olive oil, *Thromb. Res.* 78 (1995) 151-160.

Ragazzi, E., Veronese, G. & Guitto, A. The demethyloleuropein, a new glucoside extracted from ripe olives, *Ann. Chim.* 63 (1973) 13-20.

Ranalli, A. & De Mattia, G. Characterization of olive oil production with a new enzyme processing aid, *J. Am. Oil Chem. Soc.* 74 (1997) 1105-1113.

Ranalli, A. & Serraiocco, A. Quantitative and qualitative effects of a pectolytic enzyme in olive oil production, *Grasas Aceites* 47 (1996) 227-236.

Sciancalepore, V. Enzymatic browning in five olive varieties, *J. Food Sci.* 50 (1985) 1194-1195.

Servili, M., Begliomini, A.L., Montedoro, G.F., Petruccioli, M. & Federici, F. Utilisation of a yeast pectinase in olive oil extraction and red wine making processes, *J. Sci. Food Agric.* 58 (1992) 253-260.

Servili, M., Baldioli, M. & Montedoro, G.F. Phenolic composition of virgin olive oil in relationship

to some chemical and physical aspects of malaxation, *Acta Hortic.* 1 (1994) 331-336.

Servili, M., Baldioli, M., Mariotti, F. & Montedoro G.F. Secoiridoids of virgin olive oil: modification during the oil mechanical extraction process. In: *Proceedings of the world conference and exhibition on oil seed and edible oils processing, Istanbul, Oct 6-10.* Istanbul (1996).

Servili, M., Baldioli, M., Selvaggini, R., Mariotti, F., Federici, E. & Montedoro, G.F. Effect of malaxation under N_2 flush on phenolic and volatile compounds of virgin olive oil, In: *Advances in plant lipid research, the proceedings of the 13th international symposium on plant lipids.* Sevilla (1998) 307-310.

Servili, M., Baldioli, M., Mariotti, F. & Montedoro, G.F. Phenolic composition of olive fruit and virgin olive oil: distribution in the constitutive parts of fruit and evolution during oil mechanical extraction process, *Acta Hortic.* 474 (1999a) 609-619.

Servili, M., Baldioli, M., Selvaggini, R., Miniati, E. & Montedoro G.F. HPLC evaluation of phenols in olive fruit, virgin olive oil, vegetation water and pomace and 1D- and 2D-NMR characterization, *J. Am. Oil Chem. Soc.* 76 (1999b) 873-882.

Siniscalco, V. & Montedoro, G.F. Mechanical extraction of olive oil using technological adjuvants. Note 1: Drains and enzymes, *Riv. Sostanze Grasse* 65 (1988) 675-678.

Siniscalco, V. & Montedoro, G.F. Mechanical extraction of olive oil using technological adjuvants. Note 2: Extraction of integral and stoned pastes by the percolation-centrifugation system, *Riv. Sostanze Grasse* 66 (1989) 85-90.

Spencer, C.M., Cai, Y., Russel, M., Gaffney, S.H., Goulding, P., Magnolato, D., Lilley, T.H. & Haslam, E. Polyphenol complexation some thoughts and observations, *Phytochemistry* 27 (1988) 2397-2409.

Thibault, J-F. Automatisation du dosage des substances pectiques par la methode au meta-hydroxydiphenyl. *Lebensm. -Wiss. -Technol.* 12 (1979) 247-251.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J. Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv koroneiki) at different ripening stages, *Carbohydr. Polym.* 43 (2000) 11-21.

Visioli, F., Bellomo, G., Montedoro, G.F. & Galli, C. Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents, *Atherosclerosis* 117 (1995) 25-32.

Voragen, A.G.J., Pilnik, W., Thibault, J-F., Axelos, M.A.V. & Renard, C.M.G.C. Pectins. In: *Food polysaccharides and their applications.* A.M. Stephen (ed), New York: Dekker (1995) 287-339.

Chapter 6

Structural characteristics of pectic polysaccharides from olive fruit (*Olea europaea* cv moraiolo) in relation to processing for oil extraction

Abstract

In the olive oil industry technical enzyme preparations are used as processing aids to improve the extraction yield and product quality. In order to obtain more insight in the mechanisms by which these enzyme preparations lead to a higher yield and a better quality, the effect of these preparations on the structure of the pectic polysaccharides present in the cell wall of olive fruit was investigated. Four pectin-rich fractions were isolated from the cell wall material of non-enzyme treated and enzyme treated olive fruit by successive extractions with cold buffer, hot buffer, chelating agents and diluted alkali and analysed. The results revealed that the use of technical enzyme preparations during processing mainly affected the pectic material present in the cold and hot buffer fractions. The structures of the arabinose-rich pectic polysaccharides solubilised by extraction with chelating agents and diluted alkali were barely affected by the use of enzyme preparations. The changes of the buffer soluble pectic material were reflected by a decrease in methyl esterification, a change in molecular weight distribution and a degradation of the (1→4)-linked galactan chains. No differences were observed in the composition of the arabinan chains. Also, the structural features of the polysaccharides present in the vegetation waters, the liquid by-product of olive processing, were studied. The sugar composition indicated the presence of mainly material of pectic origin in the vegetation waters. As a result of enzymatic processing the degree of methyl esterification decreased, the profile of the molecular weight distribution changed and the (1→4)-linked galactan chains were degraded as was also shown for the buffer soluble pectic polysaccharides.

This chapter has been submitted to Carbohydr. Polym. by the authors Esther Vierhuis, Mandy Korver, Henk A. Schols & Alphons G.J. Voragen and has been accepted for publication.

Table 6.1
Neutral sugar linkage composition (mol%) of the polysaccharides isolated from the vegetation waters of non-enzyme treated paste (VW^-) and enzyme treated paste (VW^+); within brackets the neutral sugar composition determined by alditol acetates and the uronic acid content determined by the *m*-hydroxydiphenyl assay is given (mol per 100 mol neutral sugars)

Sugar linkage	Vegetation water$^-$ (VW^-)		Vegetation water$^+$ (VW^+)	
Rhamnose				
T-Rha*p*	1.2		1.0	
1,2-Rha*p*	0.1		0.1	
1,2,4-Rha*p*	0.1		0.2	
1,2,3,4-Rha*p*	0.4		0.2	
Total	1.8	(6)	1.5	(7)
Fucose				
T-Fuc*p*	0.2		0.2	
Total	0.2	(-)	0.2	(-)
Arabinose				
T-Ara*f*	20.1		22.8	
1,2-Ara*f*	0.4		0.2	
1,3-Ara*f*	1.4		2.3	
1,5-Ara*f*	20.8		26.7	
1,2,5-Ara*f*	1.1		1.1	
1,3,5-Ara*f*	5.4		9.3	
1,2,3,5-Ara*f*	1.6		1.0	
Total	50.8	(39)	63.4	(53)
Xylose				
T-Xyl*p*	2.9		1.7	
1,2-Xyl*p*	3.5		2.1	
1,2,3,4-Xyl*p*	0.3		0.6	
Total	6.7	(5)	4.4	(3)
Mannose				
1,4-Man*p*	0.3		0.3	
1,2,3,4,6-Man*p*	0.4		0.5	
Total	0.7	(2)	0.8	(3)
Galactose				
T-Gal*p*	2.0		2.4	
1,3-Gal*p*	4.3		4.3	
1,4-Gal*p*	5.4		0.1	
1,6-Gal*p*	2.1		4.6	
1,3,4-Gal*p*	0.2		0.2	
1,3,6-Gal*p*	12.3		13.1	
1,2,3,4,6-Gal*p*	0.1		0.2	
Total	26.4	(35)	24.9	(28)
Glucose				
1,4-Glc*p*	7.6		4.2	
1,4,6-Glc*p*	3.2		0.2	
1,2,3,4,6-Glc*p*	2.6		0.4	
Total	13.4	(13)	4.8	(6)
Uronic acid				
Total	-[a]	(64)	-	(167)
Ratio terminal/branching	0.73		0.90	

[a] Not included in the sugar linkage analysis.

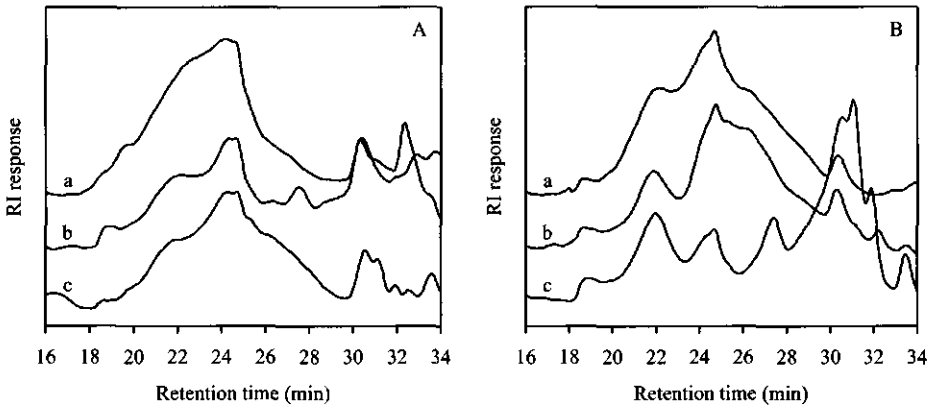

Fig. 6.1. HPSEC-patterns of the polysaccharides isolated from the vegetation waters of (A) non-enzyme treated paste, VW^- and (B) enzyme treated paste, VW^+ (a) before incubation and after incubation with (b) pectin lyase and (c) endo-polygalacturonase.

low degree of branching of the arabinans it is very likely that a relative large proportion of the terminal arabinose residues originated from the side chains of (1→3)/(1→6)-linked galactans. (1→3)/(1→6)-Linked galactans are often found to be associated with hydroxyproline-containing proteins (O'Neill *et al.*, 1990). However, they can also be present as covalently linked side chains of rhamnogalacturonan I or as separate polysaccharides (Voragen *et al.*, 1995). About 20% of the galactose residues was present as (1→4)-linked galactan in VW^-.

The glycosidic linkage composition showed that the use of enzymes during processing resulted in an almost complete degradation of the (1→4)-linked galactan chains. No major changes of the composition of the other neutral sugar linkages were noticed due to the use of enzyme preparations. The various arabinose linkages were present in almost similar ratios in both vegetation waters. Furthermore, the sugar composition showed that the galacturonic acid content of the polysaccharides present in VW^+ was relatively high compared to polysaccharides present in VW^-. The use of commercial enzyme preparations might have solubilised additional pectic material with a high galacturonic acid content or part of the pectic material may have been partly debranched. The level of methyl esters of the pectic material present in the vegetation waters decreased due to the use of commercial enzyme preparations. The degree of methyl esterification was 75 and 39 for VW^- and VW^+, respectively. The degree of acetylation was about 20 for both samples.

The HPSEC elution patterns of the pectic material present in the vegetation waters are shown in Fig. 6.1. Both samples contained material with a broad molecular weight distribution. The use of enzyme preparations changed the profile and shifted the molecular weight distribution of the material towards the lower molecular weight ranges, but most pectic material in VW^+ remained present as material with a high molecular mass.

Treatment of the polysaccharides from the vegetation waters with pectin lyase, endo-polygalacturonase and pectin methyl esterase

To obtain more information on the structure of the galacturonic acid backbone, the pectic polymers present in the vegetation waters were studied by incubation with pectin lyase and endo-polygalacturonase. Pectin lyases split glycosidic linkages between methyl esterified galacturonide residues by a trans-elimination reaction and therefore have a preference for high-methyl esterified pectins. Endo-polygalacturonases preferentially hydrolyse low-methyl esterified pectins or pectic acid because these enzymes can only split glycosidic linkages adjacent to galacturonic acid residues with free carboxyl groups (Voragen *et al.*, 1995).

Analysis of the pectin lyase digests by HPSEC showed that pectin lyase was able to degrade the polymers in VW^- to a larger extend than those present in VW^+ (Fig. 6.1). This agreed with the higher degree of methyl esterification of the former substrate. Analysis of the digests by HPAEC showed the presence of mainly unsaturated galacturonic acid oligomers with a degree of polymerisation (DP) of 2-10. Also small quantities of saturated oligomers were detected. The saturated oligogalacturonic acids were most certainly released by an endo-polygalacturonase impurity in the pectin lyase preparation. Pectin lyase was able to release ten times higher amounts of oligosaccharides from VW^- compared to VW^+ as calculated from the release of oligomers analysed by HPAEC expressed as percentage of the total amount of galacturonic acid present. However, despite of the large decrease in molecular mass on HPSEC, the amount of oligomeric degradation products in the digest of VW^- was rather low. Apparently, relatively large degradation products remained after incubation with pectin lyase, which could not be further degraded to smaller oligosaccharides to be analysed on HPAEC.

Analysis of the endo-polygalacturonase digests by HPSEC showed that the pectic material present in VW^- was rather resistant to the pectic enzyme, which agreed with the degree of methyl esterification of 75 (Fig. 6.1). The incubation of the low methyl esterified VW^+ with endo-polygalacturonase resulted in a drastic shift in retention times on HPSEC. Analysis of the endo-polygalacturonase digests on HPAEC showed that the degradation products were monogalacturonic acid and galacturonic acid oligomers in the range of DP 2-10. The analysis on HPAEC confirmed that endo-polygalacturonase was more active towards VW^+ than VW^- based on the amount of oligomers released expressed as percentage of the total amount galacturonic acid present in the sample. About three times higher amounts of oligosaccharides were released from VW^+ compared to VW^-. An exact quantification of the oligomers was not possible, because the PAD-response factors were not known for all galacturono-oligosaccharides. The pectic material was also incubated with endo-polygalacturonase in combination with pectin methyl esterase. Pectin methyl esterase is able to remove ester-linked methoxyl groups from the pectins, which form structural barriers for endo-polygalacturonase (Voragen, 1990). When endo-polygalacturonase was combined with pectin methyl esterase, the amount of oligomers present in the digests increased. The amount of galacturono-oligosaccharides released was about equal for VW^- and VW^+ and estimated to be 10-15% of the total amount of galacturonic acid present in the sample based on the peak areas. After removal of the methyl esters and *O*-acetyl groups by chemical saponification the substrates were degraded even further by endo-polygalacturonase to resistant polymeric fractions and

mono-, di- and trigalacturonic acid oligomers. Also in this case the amounts of galacturono-oligosaccharides released were about equal for VW^- and VW^+ and estimated to be 15-20% of the total amount of galacturonic acid present in the sample.

Treatment of the polysaccharides from the vegetation waters with endo-arabinanase, arabinofuranosidase B, endo-galactanase and exo-galactanase

The sugar composition revealed that a considerable part of the pectic material present in VW^- and VW^+ consisted of arabinans and galactans. More information about these structures was obtained by incubation with specific and well-characterised enzymes. The digests were analysed by HPSEC and HPAEC.

HPAEC analysis of the VW^- digest showed that endo-arabinanase was hardly able to degrade the pectic arabinan side chains present. About 0.5% of the total amount of arabinose residues present was released as monomeric arabinose and very few oligomeric degradation products were detected in the digest. The endo-arabinanase used in this experiment is known to be especially active towards linear (1→5)-α-L-arabinan and shows little activity towards highly branched arabinans like beet arabinan. The results of the sugar linkage analyses revealed that the arabinans present in olive fruit were less branched than beet arabinan, but apparently the degree of branching was still too high and restricted the activity of endo-arabinanase. Arabinofuranosidase B is able to linearise branched arabinans to enhance the degradation of the arabinan by endo-arabinanase (Beldman *et al.*, 1997). Incubation with arabinofuranosidase B showed that this enzyme was able to release about 30% of the arabinose present as monomeric arabinose. Endo-arabinanase in combination with arabinofuranosidase B enhanced the degradation of the polymer and about 40-50% of the arabinose present was analysed as monomeric arabinose on HPAEC. The removal of the arabinofuranose side chains by arabinofuranosidase B improved the degradability of the substrate by endo-arabinanase. Although arabinofuranosidase B is able to split besides (1→5)- also (1→2)- and (1→3)-α-L-linked arabinose (Rombouts *et al.*, 1988) the enzyme was not able to completely degrade the arabinan. A time course study of the degradation of the arabinan present in the vegetation water by arabinofuranosidase B as analysed by HPAEC revealed that after 4 h already half of the maximum amount of arabinan that could be degraded had been released as monomeric arabinose. Incubation with a 10 fold higher amount of enzyme hardly affected the degradation of the polymer. So, apparently the amount of enzyme was not limiting and probably the structure of the substrate restricted the activity of arabinofuranosidase B. No significant differences were noticed in the degradability of the arabinans present in VW^+ compared to VW^- for the arabinan degrading enzymes used.

The pectic material present in the vegetation waters was also incubated with endo-galactanase and exo-galactanase. Both enzymes are only active towards (arabino)galactans with a backbone consisting of (1→4)-linked galactopyranose residues (Van de Vis *et al.*, 1991). Unfortunately, no 'purified' enzymes having activity towards (1→3)/(1→6) galactans were available to study arabinogalactan type II, which was also present in the vegetation waters. HPAEC analysis of the reaction products present in the VW^- digest after incubation with endo-galactanase showed that galactose and galactobiose were released. Based on the

peak areas it was estimated that about 50% of the (1→4)-linked galactan present had been degraded. Exo-galactanase showed very little activity towards the pectic material present in VW^- and released only monomeric galactose. About 5% of the (1→4)-linked galactan present was degraded. The combination of endo- and exo-galactanase did not enhance the degradation of the (1→4)-linked galactan present in VW^-. Analysis of the digest of VW^+ incubated with endo-galactanase on HPAEC revealed that hardly any degradation products were present. Only 0.4% of the galactose residues present in VW^+ was released as monomeric galactose. Also, exo-galactanase showed very little activity towards the pectic material present in VW^+. These experiments confirmed the data of the sugar linkage analysis, which showed that less than 1% of the galactose residues present in VW^+ originated from a (1→4)-linked galactan.

The interaction between galactanases and arabinanases was also studied. The degradation of the (1→4)-linked (arabino)galactan was not enhanced when arabinofuranosidase B was combined with endo-galactanase and confirmed the results of the sugar linkage analysis, which showed that only a very small part of the (1→4)-linked galactose residues was substituted at C-3.

The digests were also analysed on HPSEC for changes in the molecular weight distribution. Treatment of the material with the enzymes resulted only in very slight shifts towards lower molecular weights compared to the blank (not shown). Apparently, the arabinan and galactan chains present in the vegetation water hardly affected the hydrodynamic volume of the pectic polymers.

Composition of the polysaccharides present in the malaxed pastes

In addition to the effect of the use of commercial enzyme preparations during processing on the water soluble polysaccharides also the effect on the structure of the other cell wall polysaccharides present in the olive paste was investigated. Therefore, the cell wall material present in the non-enzyme treated (MP^-) and the enzyme treated malaxed paste (MP^+) was sequential extracted with different solvents to characterise the polysaccharides. Pectic material was solubilised by extraction with cold buffer, hot buffer, chelating agent and diluted alkali. Subsequently, a fractionation with 4 M alkali was performed to solubilise the hemicelluloses. When olive oil is extracted from the malaxed paste with a decanter, the water soluble polysaccharides in olive fruit turn up in the vegetation waters. Consequently, the pectic material solubilised by the extraction with cold buffer from the malaxed paste is expected to show the same characteristics as the pectic material in the vegetation waters. So, especially the pectin-rich fractions extracted with hot buffer, chelating agent and diluted alkali are of interest in the next part of this chapter, because these fractions will give additional information on the effect of the enzyme preparations towards pectins present in the cell wall material, not soluble in the vegetation waters. The sugar compositions of the AISs and the obtained fractions are presented in Tables 6.2 and 6.3 for MP^- and MP^+, respectively.

Arabinose and uronic acid accounted for more than 75% of the sugars present in the pectin-rich fractions from MP^-. Especially, the 0.05 M NaOH fraction contained a large amount of arabinose, which indicated the presence of pectins with a considerable amount of arabinan or arabinogalactan side chains in this fraction. The galactose content was relatively low for all

Table 6.2
Yield on sugar basis (%) and sugar composition (mol%) of the fractions and the residue of AIS isolated from the non-enzyme treated malaxed paste, MP^- of olive fruit (tr = trace amount)

Sample	Yield	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	OMe/OAc[a]	Carbohydrate content[b]
AIS	100	2	13	30	1	4	36	14	79/114	46
$CBSS^-$	6.6	2	15	3	1	14	3	62	56/17	53
$HBSS^-$	1.3	4	27	3	1	11	4	50	65/32	34
$ChSS^-$	2.2	4	30	1	tr	6	1	58	62/27	34
0.05 M $NaOH^-$	2.5	6	57	3	tr	9	3	22	0/0	33
4 M KOH^-	9.6	2	10	48	5	7	16	12	0/0	41
RES^-	72.3	2	8	34	1	2	46	8	0/0	50

[a] Expressed as mol methyl esters or *O*-acetyl groups per 100 mol uronic acid.
[b] Expressed as % (w/w).

Table 6.3
Yield on sugar basis (%) and sugar composition (mol%) of the fractions and the residue of AIS isolated from the enzyme treated malaxed paste, MP^+ of olive fruit (tr = trace amount)

Sample	Yield	Rha	Ara	Xyl	Man	Gal	Glc	Uronic acids	OMe/OAc[a]	Carbohydrate content[b]
AIS	100	2	12	30	2	3	37	15	76/123	42
$CBSS^+$	9.0	3	19	2	1	8	1	66	35/20	58
$HBSS^+$	1.1	5	36	3	2	11	4	39	49/20	44
$ChSS^+$	1.3	5	34	1	1	6	1	52	49/22	40
0.05 M $NaOH^+$	2.2	6	55	4	tr	9	3	23	0/0	38
4 M KOH^+	7.4	2	10	50	5	6	16	11	0/0	53
RES^+	78.5	2	5	36	1	1	47	8	0/0	49

[a] Expressed as mol methyl esters or *O*-acetyl groups per 100 mol uronic acid.
[b] Expressed as % (w/w).

pectin-rich fractions (6-14%). The 4 M KOH fraction consisted mainly of xylose, which accounted for 48 mol% of the sugars present. The residue consisted for about 46-47 mol% of glucose of which the main part (95%) could be designated as cellulose. Besides glucose the residue still contained a considerable proportion of xylose, and small amounts of arabinose and uronic acids. Apparently, the solvents used to extract the AIS were not able to solubilise all pectic and hemicellulosic substances as was observed before (Coimbra *et al.*, 1994, Vierhuis *et al.*, 2000). The sugar composition of the pectin-rich fractions CBSS, HBSS and ChSS from MP^- and MP^+ were quite similar. Some differences were found for the amounts of uronic acid, arabinose and galactose present in the samples but no clear trends were noticed. The composition of the 0.05 M NaOH and the 4 M KOH fraction from MP^- and MP^+ were practically identical. Also the sugar composition of the residues from both pastes appeared to be very similar.

More information about the structure of the pectic polysaccharides can be obtained by

sugar linkage analysis. However, the results of the sugar linkage analysis of the pectin-rich fractions revealed high proportions of unmethylated sugar residues for almost all of the fractions (not shown). Calculation of the ratio between terminal and branched residues suggested that under-methylation had occurred, even though the methylation procedure was repeated to improve the completeness of the methylation of the free hydroxyl groups. Nevertheless, further study of the results of the sugar linkage analysis indicated that the relative proportions of the individual partially methylated sugars in an under-methylated sample were quite similar to those in a comparable sample with negligible under-methylation as was also shown by Düsterhöft *et al.* (1992). So, although no exact data could be obtained from the methylation analysis, it was possible to give some trends. In all pectin-rich fractions from MP^- arabinose was essentially present as 1,5-linked and terminal residues. The relative amount of 1,3,5-linked arabinose increased when stronger solvents were used to extract the pectic polymers. Since several samples were under-methylated the amount of disubstituted arabinose was difficult to determine but is expected to be negligible as was also shown for the pectins present in the vegetation waters and by Coimbra *et al.* (1994) for a CDTA and a Na_2CO_3 fraction from olive fruit cell wall material. The major part of the galactose residues in the CBSS and HBSS fractions was present as (1→3)/(1→6)-linked galactans. About 20-30% of the galactose arose from (1→4)-linked galactan. The ChSS and the 0.05 M NaOH fractions contained both relatively small amounts of galactose residues, which were also for the major part present as (1→3)/(1→6)-linked galactans. The use of commercial enzyme preparations during processing considerably decreased the relative amount of linkages associated with (1→4)-linked galactan for CBSS and HBSS, which agreed with the results of the analysis of the pectic polysaccharides present in the vegetation waters. The addition of commercial enzyme preparations did not affect the composition of the galactan polysaccharides present in the ChSS and 0.05 M NaOH fractions. Also, no significant degradative effect was noticed for the arabinans present in the various pectin-rich fractions from MP^+.

The CBSS, HBSS and ChSS fractions from MP^- were all highly methyl esterified (DM 56-65) and the degree of acetylation varied from 17 to 32. The polymers solubilised by (diluted) alkali were de-esterified by the conditions of their extraction. However, based on the high content of methyl esters and *O*-acetyl groups in the cell wall material (AIS) from MP^- it is likely that the polymers present in the 0.05 M NaOH and 4 M KOH fractions and the residue were highly esterified as well. In the CBSS, HBSS and ChSS fractions only about 30% of the methyl esters and about 10% of the *O*-acetyl groups were recovered. The remainder of the methyl esters were most likely linked to galacturonic acid residues of the pectic material present in the 0.05 M NaOH and 4 M KOH fractions or the pectic material which remained in the residue. The remainder of the *O*-acetyl groups could have been located on the pectic material as well as on the xyloglucans present in olive fruit (Kiefer *et al.*, 1989; Sims *et al.*, 1996; York *et al.*, 1996; Vierhuis *et al.*, 2001b). Xylans can also carry *O*-acetyl groups but the presence of these groups has mainly been described for hardwood xylans (Ishii, 1991; Puls *et al.*, 1991; Ross *et al.*, 1992; Van Hazendonk *et al.*, 1996; Bardet *et al.*, 1997). The level of methyl esters appeared to be lower for the pectin-rich fractions isolated from MP^+ than the fractions from MP^-. The degree of methyl esterification of the CBSS, HBSS and ChSS fractions from MP^+ varied from 35 to 49. The lower contents of methyl esters present in the

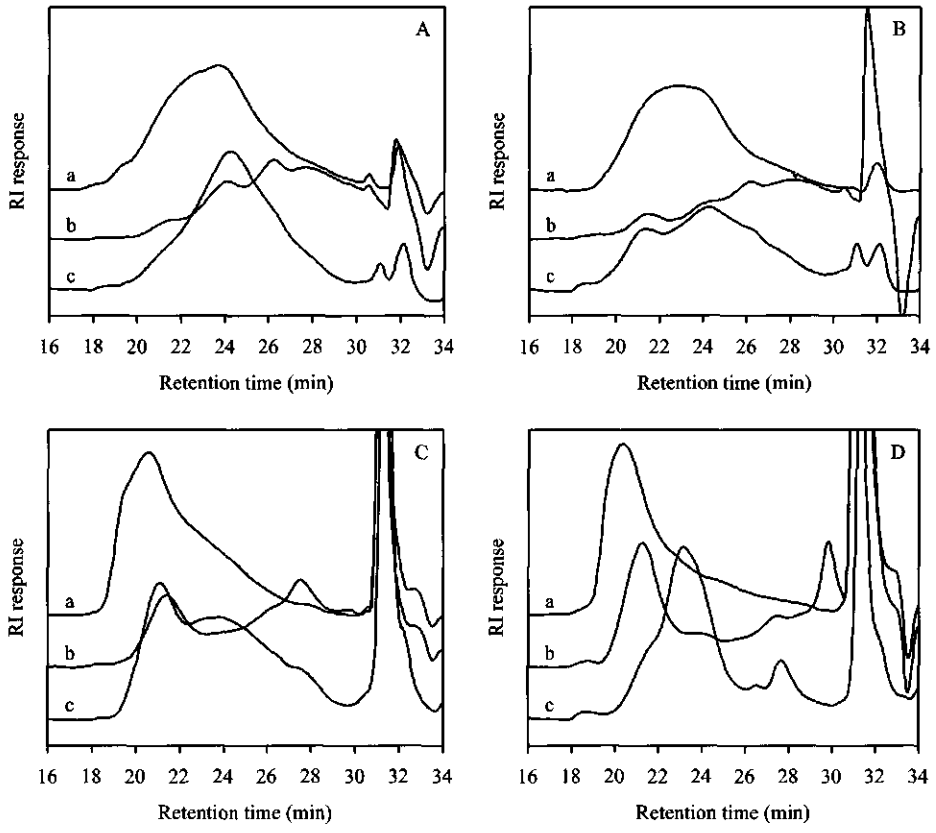

Fig. 6.2. HPSEC-patterns of the pectin-rich pools obtained from non-enzyme treated malaxed paste, MP^- (a) before incubation and after incubation with (b) pectin lyase and (c) endo-polygalacturonase: (A) CBSS; (B) HBSS; (C) ChSS; and (D) 0.05 M NaOH.

pectin-rich fractions from MP^+ were consistent with the lower content of methyl esters present in the pectic material isolated from VW^+ compared to VW^-. The degree of acetylation of the pectin-rich fractions from MP^+ was about 20 and in general lower than the degree of acetylation of the MP^- fractions, but no clear trend could be ascertained.

The HPSEC elution patterns of the pectin fractions are shown in Figs. 6.2 and 6.3. The pectin-rich fractions consisted of high molecular weight material with a broad molecular weight range. The use of enzyme preparations during the olive oil extraction procedure changed the profile of the pectins present in the CBSS and HBSS fractions. The CBSS and HBSS fractions from MP^- seemed to be one population, while in the elution pattern of the CBSS and HBSS fractions from MP^+ two populations could be distinguished. A shift of the molecular weight distribution to lower molecular weight ranges as was seen for the pectins present in the vegetation waters was not noticed. The pectins extracted with chelating agents from MP^- and MP^+ showed a similar HPSEC elution behaviour, which was also the case for the 0.05 M NaOH fractions.

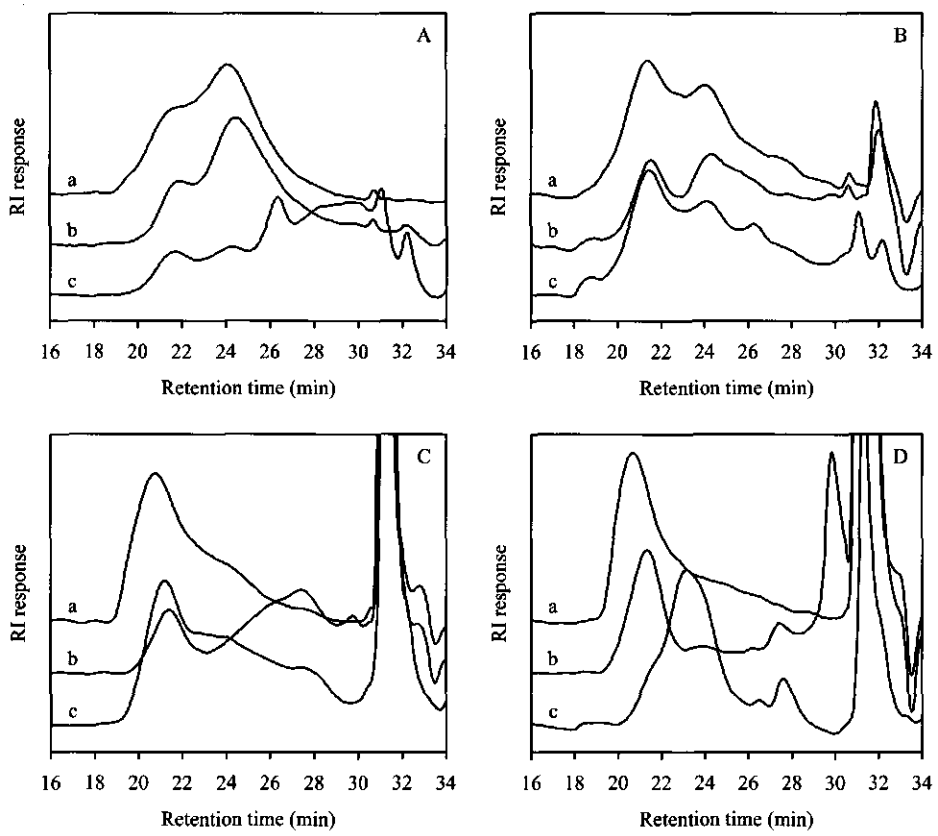

Fig. 6.3. HPSEC-patterns of the pectin-rich pools obtained from enzyme treated malaxed paste, MP^+ (a) before incubation and after incubation with (b) pectin lyase and (c) endo-polygalacturonase: (A) CBSS; (B) HBSS; (C) ChSS; and (D) 0.05 M NaOH.

Treatment of the various pectin fractions from the malaxed pastes with pectin lyase, endo-polygalacturonase and pectin methyl esterase

A more detailed study was performed for the pectin-rich fractions from MP^- and MP^+. The galacturonic acid backbone of the pectin fractions from MP^- and MP^+ was studied by incubation with pectic enzymes specific for high- and low-methyl esterified pectins and the digests were analysed by HPSEC and HPAEC. The HPSEC elution patterns before and after enzymatic treatment with pectin lyase are shown in Figs. 6.2 and 6.3. Incubation of the CBSS fraction from MP^- with pectin lyase showed that this enzyme degraded the polymers to a large extend and the molecular weight distribution shifted towards lower molecular weight ranges. By contrast, the pectic material present in the CBSS fraction from MP^+ was rather resistant to pectin lyase and only a slight decrease of the molecular mass was observed. The limited action of this pectic enzyme towards the CBSS fraction from MP^+ agreed with the degree of methyl esterification of the fraction of only 35, which is in general too low for pectin lyase activity

(Voragen *et al.*, 1995). The degree of methyl esterification of the HBSS fraction from MP^- and MP^+ were 65 and 49, respectively and high enough for pectin lyase to act upon the galacturonic backbone. Also the pectic polymers present in both ChSS fractions were degraded to a large extend by pectin lyase. The saponified pectins present in the 0.05 M NaOH fractions were expected to be very resistant to degradation with pectin lyase. Still, a small shift in the molecular weight distribution was noticed which could not be explained.

Analysis of the endo-polygalacturonase digests by HPSEC revealed that the incubation of $CBSS^+$ fraction (DM of 35) with endo-polygalacturonase resulted in a larger shift in the molecular weight distribution than the $CBSS^-$ fraction (DM of 56) as shown in Figs. 6.2 and 6.3. Incubation of the $CBSS^-$ fraction with in addition to endo-polygalacturonase pectin methyl esterase, which is able to remove the methoxyl groups, made the substrate more susceptible for endo-polygalacturonase. The addition of endo-polygalacturonase to the HBSS fraction from MP^- resulted only in slight shifts in retention times and also no obvious shifts in the molecular weight distribution of the HBSS fraction from MP^+ were noticed. Apparently, the degree of methyl esterification of these substrates was too high. Also the pectic polymers present in both ChSS fractions showed no obvious shift in the molecular weight distribution. The polymeric material present in the 0.05 M NaOH fractions also appeared to be very resistant to endo-polygalacturonase even though the degrees of methyl esterification and acetylation were zero due to the extraction procedure. The average molecular mass of the population decreased, but most of the polymeric fraction remained present as a polymeric material. This has also previously been observed for a 0.05 M NaOH fraction isolated from apple cell wall material (Schols *et al.*, 1995) and can be explained by the fact that most of the galacturonic acid residues in the 0.05 M NaOH fractions are present in rhamnogalacturonan-type segments instead of homogalacturonan segments.

Analysis by HPAEC of the endo-polygalacturonase digests showed that the release of oligomers (DP 2-10) was relatively small. The amount of galacturonan oligomers released was estimated to be 5% of the total amount of galacturonic acids present in the sample based on the peak areas. Even the enormous decrease in molecular weight of the CBSS fraction from MP^+ incubated with endo-polygalacturonase did not result in large amount of oligomeric end products. The combination of endo-polygalacturonase and pectin methyl esterase increased the amounts of galacturonic acid oligomers in the digests of the pectin-rich fractions CBSS, HBSS and ChSS and chemical saponification of the methyl esterified pectin-rich fractions made the substrates even more susceptible. The amount of galacturono-oligosaccharides released after chemical saponification was estimated to be 15-20% of the total amount of galacturonic acid present in the CBSS, HBSS and ChSS fractions. From the 0.05 M NaOH fractions only 3-4% of the total amount of galacturonic acid residues was released as mono-, di- and trigalacturonic acid. No marked differences were noticed between the degradation products and relative amounts of the oligomers for the pectins isolated from the non-enzyme treated paste, MP^- and the enzyme treated paste, MP^+ by HPAEC.

In addition to the analyses on HPAEC at high pH, the endo-polygalacturonase digests of the pectin-rich fractions were also analysed on HPAEC with a sodium gradient at pH 5.0 to be able to distinguish between the non-esterified oligomers and the methyl esterified oligomers. The endo-polygalacturonase digests of VW^- and VW^+, which were described in the first part

of this chapter were not analysed at HPAEC at pH 5.0, but are expected to show similar characteristics as the HPAEC-patterns of $CBSS^-$ and $CBSS^+$. The HPAEC-pattern of the analysis at pH 5.0 revealed that the endo-polygalacturonase digest of the CBSS fraction from MP^- contained mainly non-methyl esterified mono- and digalacturonic acid and a small amount of trigalacturonic acid (Fig. 6.4). Incubation of endo-polygalacturonase combined with pectin methyl esterase made the CBSS fraction more susceptible for degradation. Predominantly non-methyl esterified galacturonic acids were released, but also partially methyl esterified galacturonic acid oligomers were detected in the digest. Endo-polygalacturonase was shown to release the largest amounts of mono-, di-, and trigalacturonic acid when the pectic material was chemically saponified. Although a complete saponification was expected, the chromatogram showed also small amounts of partially methyl esterified oligomers. The CBSS fraction from MP^+ incubated with endo-polygalacturonase contained besides non-methyl esterified galacturonic acid oligomers also partially methyl esterified oligomers, this in contrast to the CBSS fraction from MP^-, which contained only non-methyl esterified oligomers. Daas *et al.* (2000) have concluded from their study of the methyl ester distribution of pectins that endo-polygalacturonase can only release methyl esterified oligomers from pectin when it contains non-esterified galacturonic acid sequences (so-called blocks) close enough to each other. When the enzyme degradable blocks are located too far from each other relatively large methyl esterified degradation products remain and these can not be detected by the HPAEC method at pH 5.0. So apparently, the use of enzyme preparations during processing demethylated part of the CBSS present in the malaxed paste in such a way that it contained more clustered endo-polygalacturonase degradable blocks.

The proportion in which the non-esterified mono-, di- and trigalacturonic acid were released were practically identical for both CBSS fractions and appeared not to be affected by the use of commercial enzyme preparations during processing. The endo-polygalacturonase digests of the CBSS fractions from MP^- and MP^+ contained both predominantly mono- and digalacturonic acids, which indicated that the pecins present in the fractions contained mainly relatively small non-esterified blocks (Daas *et al.*, 2000). The endo-polygalacturonase used for our study is 'most likely' able to cleave in non-esterified galacturonic acid sequences of four and more residues (Daas *et al.*, 1999). As a result, endo-polygalacturonase has to degrade blocks close to the limit of its mode of action and will only release small (mono- and di-) galacturonic acid molecules. The proportion of trigalacturonic acid in the digests increased, as expected, when endo-polygalacturonase was used in combination with pectin methyl esterase and increased even further when the pectins were saponified before incubation with endo-polygalacturonase.

The profile of the endo-polygalacturonase digest on HPAEC of the HBSS fraction from MP^- contained non-methyl esterified and partially methyl esterified galacturonic acid oligomers and the profile was comparable to the endo-polygalacturonase digest of the CBSS fraction from MP^+. The HBSS fraction from MP^+ showed a similar HPAEC pattern as the fraction from MP^-. The pectins present in the ChSS fraction are believed to participate in calcium cross-linking in the cell walls. To be able to participate in calcium cross-linking these pectins have to contain relatively large blocks of non-methyl esterified homogalacturonan compared to the pectins present in the CBSS and HBSS fractions. So, the relative proportion

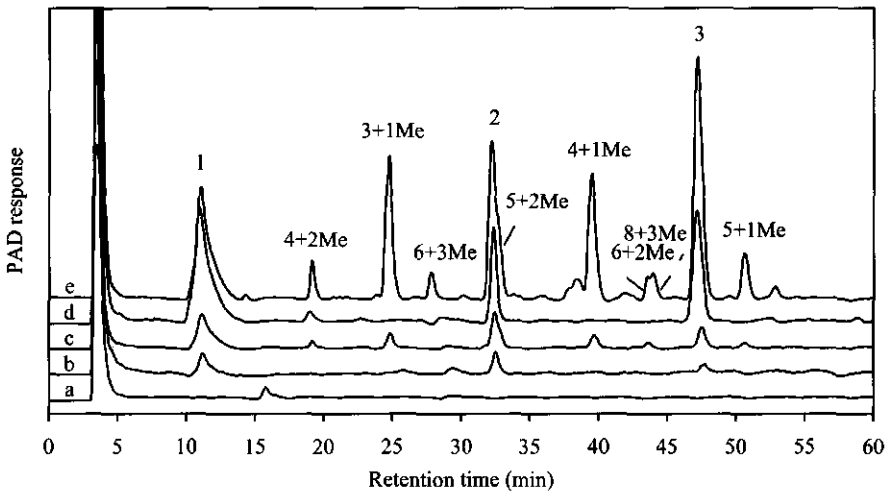

Fig. 6.4. HPAEC-patterns (pH 5.0) of the CBSS fraction obtained from non-enzyme treated malaxed paste (CBSS$^-$; a) before incubation and after incubation with (b) endo-polygalacturonase and (c) endo-polygalacturonase combined with pectin methyl esterase; (d) chemically saponified CBSS$^-$ after incubation with endo-polygalacturonase; (e) standard of a pectin polygalacturonase digest. The composition of the galacturonic acid oligomers (for example 3+1Me: a trimer of galacturonic acid having one methyl ester group) are indicated above the corresponding peaks as determined by Daas *et al.* (1998).

of trigalacturonic acid to mono- and digalacturonic acid should be higher in the ChSS digests compared to the CBSS and HBSS fractions because endo-polygalacturonase is less hindered by methyl esters. However, the relative proportion of trigalacturonic acid hardly increased. Apparently, in addition to methyl esters also other factors were involved in the degradation of the homogalacturonan by endo-polygalacturonase. Renard & Jarvis (1999), for example, have shown that endo-polygalacturonase is inhibited by the presence of *O*-acetyl groups on chemically acetylated homogalacturonan. In general, it is stated that the homogalacturonan regions of pectins are only slightly *O*-acetylated or not at all, and that *O*-acetylation is mostly confined to the rhamnogalacturonan regions (Komalavilas & Mort, 1989; Ishii, 1995; Renard & Jarvis, 1999). However, high amounts of *O*-acetyl groups in the homogalacturonan regions have been described for sugar beet pectin with a degree of acetylation of 35 (Voragen *et al.*, 2001). The *O*-acetyl content of the pectins extracted from olive fruit was also high (17 to 32), and may indicate that the pectins present in olive fruit have also *O*-acetyl groups linked to the galacturonic acid residues in the homogalacturonan regions. MALDI-TOF mass spectrometry was performed to investigate the presence of *O*-acetyl groups on oligomers present in a pectin fraction digested with endo-polygalacturonase in combination with pectin methyl esterase (Fig. 6.5). The MALDI-TOF mass spectrum showed that part of the partially methyl esterified oligomers released from the homogalacturonan regions were indeed (mono)-*O*-acetylated and indicated that the presence of *O*-acetyl groups in the homogalacturonan regions could have hindered endo-polygalacturonase. The relative proportion of trigalacturonic acid to mono- and

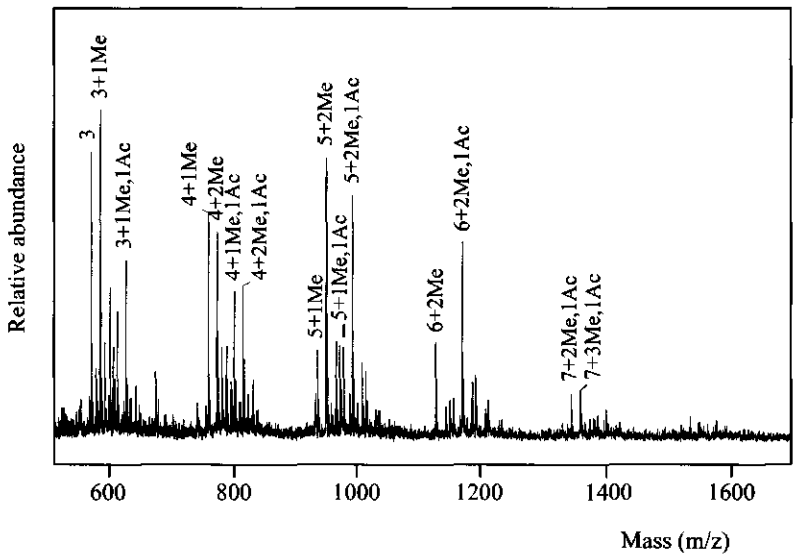

Fig. 6.5. MALDI-TOF mass spectrum of the CBSS fraction obtained from non-enzyme treated malaxed paste ($CBSS^-$) digested with endo-polygalacturonase combined with pectin methyl esterase. The composition of the galacturonic acid oligomers (for example 3+1Me,1Ac: a trimer of galacturonic acid having one methyl ester group and one *O*-acetyl group) are indicated above the corresponding peaks.

digalacturonic acid increased when the pectins of the ChSS fraction were saponified but endo-polygalacturonase could still not produce mono-, di- and trigalacturonic acid in the proportions, determined when a commercial polygalacturonic acid was used as a substrate. When a commercial polygalacturonic acid is used as a substrate endo-polygalacturonase is not hindered by any methyl esters or *O*-acetyl groups and produces mono-, di- and trigalacturonic acid in proportions strictly determined by the mode of action of the enzyme. So, apparently the enzyme was still hindered in the saponified ChSS fraction even though the methyl esters substituents and *O*-acetyl groups were not present anymore. It might be that, for example, the presence of blocks of the more highly substituted rhamnogalacturonan regions, which are hardly present in commercial polygalacturonic acid hindered the enzyme. No differences were distinguished between the ChSS fractions from MP^+ and MP^-. The HPAEC patterns of the ChSS fractions from MP^+ and MP^- showed the same characteristics. The NaOH fractions from MP^- and MP^+ showed also similar elution patterns and contained, as expected, only non-methyl esterified mono-, di- and trigalacturonic acid.

Treatment of the various pectin fractions from the malaxed pastes with endo-arabinanase, arabinofuranosidase B, endo-galactanase and exo-galactanase

The arabinan and galactan chains present in the pectin fractions from MP^- and MP^+ were further studied by incubation with specific arabinan and galactan degrading enzymes. The digests were analysed by HPSEC and HPAEC. Incubation of the pectin-rich fractions from

MP^- with endo-arabinanase resulted in small amounts of mono- and oligomeric reaction products. Incubation of the CBSS fraction from MP^- with arabinofuranosidase B showed that this enzyme was able to degrade the arabinan chains present in CBSS to a large extend (70%). For the other pectin-rich fractions from MP^- (HBSS, ChSS and 0.05 M NaOH) the degradation of the arabinan side chains by arabinofuranosidase B was limited till 30-40%. Incubation of the CBSS fraction with a combination of arabinofuranosidase B and endo-arabinanase enhanced the degradation and resulted in an almost complete degradation of the arabinan side chains. Arabinose monomer was the major degradation product, but also small amounts of oligomeric products were released. The combination of both arabinan degrading enzymes increased also the degradation of the arabinan side chains present in HBSS and ChSS (till 60-70%) and 0.05 M NaOH (till 40%). Supplementation of the arabinan degrading enzymes with the galactan degrading enzymes endo-galactanase and exo-galactanase showed no additional effect on the release of arabinose. No differences were distinguished between pectin-rich fractions from MP^+ compared to MP^- for the enzymes we used for our research.

The degree of degradation of the galactan side chains differed markedly for the fractions isolated from MP^- compared to MP^+. Exo-galactanase was able to hydrolyse 19% of the total amount of galactose present in the CBSS fraction from MP^-, while incubation of the CBSS fraction from MP^+ resulted in relatively very small amounts of galactose hydrolysed. Only 3% of the galactan present in this fraction was degraded to galactose monomer. The same trends were observed for the HBSS and ChSS fractions (Table 6.4). The enzymatic degradability of the 0.05 M NaOH fractions with exo-galactanase was practically identical for both processing procedures. Also endo-galactanase degraded the pectin-rich fractions CBSS, HBSS and ChSS from the non-enzyme treated paste, MP^- to a large extend, while very small amounts of degradation products were present in the endo-galactanase digests of CBSS, HBSS and ChSS from MP^+. The results of the incubations with exo- and endo-galactanase confirmed that the use of enzyme preparations during processing fragmented the (1→4)-linked galactan chains as shown by the data of the sugar linkage analysis.

The incubation of the pectin-rich fractions with the enzymes described resulted only in rather slight shifts in the molecular weight distribution on HPSEC (not shown). Even when considerable amounts of arabinose were released from the pectic polysaccharides, the effect of this enzyme on the molecular weight distribution of the polysaccharides was negligible. Apparently, the arabinan chains had a rather small effect on the hydrodynamic volume of the polysaccharide.

Table 6.4

Release of galactose from the pectin-rich extracts after 24 h incubation with exo-galactanase and endo-galactanase expressed as percentage of total galactose present.

	Non-enzyme treated paste (MP^-)		Enzyme treated paste (MP^+)	
	Exo-galactanase	Endo-galactanase	Exo-galactanase	Endo-galactanase
CBSS	19	35	3	5
HBSS	14	37	5	5
ChSS	10	24	6	11
0.05 M NaOH	10	18	11	14

Concluding remarks

Based on the results of this study it can be concluded that the composition of the water soluble pectins present in the vegetation waters and especially the buffer soluble pectic material isolated from the malaxed paste changed due to the use of enzyme preparations during processing. This means that the enzymes affected only a relatively small part of the cell wall material. Previous research has shown that the action of the enzyme preparations is probably restricted during processing by the high concentration of phenolic compounds present in olive pulp (Vierhuis *et al.*, 2001c). It appeared that re-incubation of the isolated polymeric material present in VW^+ with a fresh batch of Olivex resulted in an almost complete degradation of the polymers to oligosaccharides and monosaccharides. So, apparently the structure of the polysaccharides present in VW^+ or the enzyme preparations used are not limiting for the degradation but other factors like, for example, phenolic compounds affect the activity of the enzymes in the preparations. However, not all enzymes in the enzyme preparations seemed to be restricted in their activity during processing. For example, the use of enzyme preparations during processing resulted in an almost complete degradation of the (1→4)-linked galactan chains in VW^+. This suggests, that the enzymes involved in the degradation of the (1→4)-linked galactan chains were contrary to other enzymes present in the preparations less affected by the phenolic compounds present in the olive pulp.

The polysaccharides present in the cold buffer fraction from the malaxed paste and the vegetation waters for the non-enzyme treated and enzyme treated samples showed almost similar characteristics. This was also expected, because the vegetation water is obtained from the malaxed pastes as a liquid by-product of the process to extract the olive oil. Only the uronic acid content differed for $CBSS^+$ and VW^+, which might be caused by the preparation of the samples.

Acknowledgements

The authors would like to thank Piet Daas for valuable discussions and suggestions. This research was supported by the EU (AIR3-CT93-1355).

References

Bardet, M., Emsley, L. & Vincedon, M. Two-dimensional spin-exchange solid-state NMR studies of C-13-enriched wood, *Solid-State-Nuclear-Magnetic-Resonance* 8 (1997) 25-32.

Beldman, G., Schols, H.A., Pitson, S.M., Searle- van Leeuwen, M.J.F. & Voragen, A.G.J. Arabinans and arabinan degrading enzymes. In: *Advances in macromolecular carbohydrate research, Vol. 1.* R.J. Sturgeon (ed), London: JAI Press (1997) 1-64.

Christensen, F.M. Extraction by aqueous enzymatic processes, *Inform* 2 (1991) 984-987.

Coimbra, M.A., Waldron, K.W. & Selvendran, R.R. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L.), *Carbohydr. Res.* 252 (1994) 245-262.

Daas, P.J.H., Arisz, P.W., Schols, H.A., Ruiter, G.A. & Voragen, A.G.J. Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Anal. Biochem.* 257 (1998) 195-202.

Daas, P.J.H., Meyer-Hansen, K., Schols, H.A. De Ruiter, G.A. & Voragen, A.G.J. Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase, *Carbohydr. Res.* 318 (1999) 135-145.

Daas, P.J.H., Schols, H.A. & Voragen, A.G.J. Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase, *Carbohydr. Res.* 326 (2000) 120-129.

Domínguez, H., Núñez, M.J. & Lema, J.M. Enzymatic treatment to enhance oil extraction from fruits and oilseeds: a review, *Food Chem.* 49 (1994) 271-286.

Düsterhöft, E-M., Posthumus, M.A. & Voragen, A.G.J. Non-starch polysaccharides from sunflower (*Helianthus Annuus*) meal and palm-kernel (*Elaeis Guineensis*) meal; II. Investigation of the structure of major polysaccharides, *J. Sci. Food Agric.* 59 (1992) 151-160.

Englyst, H.N. & Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates, *Analyst* 109 (1984) 937-942.

Ishii, T. Acetylation at *O*-2 of arabinofuranose residues in feruloylated arabinoxylan from bamboo shoot cell-walls, *Phytochemistry* 30 (1991) 2317-2320.

Ishii, T. Isolation and characterization of acetylated rhamnogalacturonan oligomers liberated from bamboo shoot cell-walls by Driselase, *Mokuzai Gakkaishi* 41 (1995) 561-572.

Kiefer, L.L,. York, W.S., Darvill, A.G. & Albersheim, P. Xyloglucan isolated from suspension-cultured sycamore cell walls is *O*-acetylated, *Phytochemistry* 28 (1989) 2105-2107.

Komalavilas, P. & Mort, A.J. The acetylation at *O*-3 of galacturonic acid in the rhamnose-rich portion of pectins, *Carbohydr. Res.* 189 (1989) 261-272.

Montedoro, G.F., Begliomini, A.L., Servili, M., Petruccioli, M. & Federici, F. Pectinase production from olive vegetation waters and its use in the mechanical olive oil extraction process to increase oil yield and improve quality, *It. J. Food Sci.* 5 (1993) 355-362.

O'Neill, M., Albersheim, P. & Darvill, A. The pectic polysaccharides of primary cell walls. In: *Methods in plant biochemistry, Vol. 2: Carbohydrates,* P.M. Dey (ed), London: Academic press (1990) 415-441.

Puls, J., Tenkanen, M., Korte, H.E. & Poutanen, K. Products of hydrolysis of beechwood acetyl-4-*O*-methylglucuronoxylan by a xylanase and an acetyl xylanesterase, *Enzyme Microb. Technol.* 13 (1991) 483-486.

Ranalli, A. & De Mattia, G. Characterization of olive oil produced with a new enzyme processing aid, *J. Am. Oil Chem. Soc.* 74 (1997) 1105-1113.

Ranalli, A. & Serraiocco, A. Effect induced by a pectolytic adjuvant in olive oil extraction by the present technological systems; Pluriannual research results, *Riv. Ital. Sostanze Grasse* LXXII (1995) 355-364.

Renard, C.M.G.C. & Jarvis, M.C. Acetylation and methylation of homogalacturonans 1: optimisation of the reaction and characterisation of the products, *Carbohydr. Polym.* 39 (1999) 201-207.

Rombouts F.M., Voragen A.G.J., Searle-van Leeuwen, M.F., Geraeds C.C.J.M., Schols H.A. & Pilnik W. The arabinanases of *Aspergillus niger* - Purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinanase, *Carbohydr. Polym.* 9 (1988) 25-47.

Ross, N.W., Johnson, K.G., Braun, C., MacKenzie, C.R. & Schneider, H. Enzymic hydrolysis of water-soluble lignin-carbohydrate complexes from *Populus deltoides*: Effects of combinations of

ß-mannanases, xylanase, and acetyl xylan esterase, *Enzyme Microbiol. Technol.* 14 (1992) 90-95.

Schols, H.A., Posthumus, M.A. & Voragen, A.G.J. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process, *Carbohydr. Res.* 206 (1990) 117-129.

Schols, H.A., Vierhuis, E., Bakx, E.J. & Voragen, A.G.J. Different populations of pectic hairy regions occur in apple cell walls, *Carbohydr. Res.* 275 (1995) 343-360.

Servili, M., Begliomini, A.L., Montedoro, G., Petruccioli, M. & Federici, F. Utilisation of a yeast pectinase in olive oil extraction and red wine making processes, *J. Sci. Food Agric.* 58 (1992) 253-260.

Sims, I.M., Munro, S.L.A., Currie, G., Craik, D. & Bacic, A. Structural characterisation of xyloglucan secreted by suspension-cultured cells of *Nicotiana plumbaginifolia*, *Carbohydr. Res.* 293 (1996) 147-172.

Thibault, J-F. Automatisation du dosage des substances pectiques par la methode au meta-hydroxy-diphenyl, *Lebensm. -Wiss. Technol.* 12 (1979) 247-251.

Van Hazendonk, J.M., Reinerink, E.J.M., De Waard, P. & Van Dam, J.E.G. Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linum usitatissimum* L), *Carbohydr. Res.* 291 (1996) 141-154.

Van Houdenhoven, F.E.A. *Studies on pectin lyase, Ph.D. thesis,* Wageningen: Wageningen Agricultural University (1975) 100 p.

Van de Vis, J.W., Searle- van Leeuwen, M.J.F., Siliha, H.A., Kormelink, F.J.M. & Voragen, A.G.J. Purification and characterization of endo-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: Use in combination with arabinanases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan, *Carbohydr. Polym.* 16 (1991) 167-187.

Van de Vis, J.W. *Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans, Ph.D. thesis,* Wageningen: Wageningen Agricultural University (1994) 161 p.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J. Isolation and characterisation of cell wall material from olive fruit (*Olea europaea* cv koroneiki) at different ripening stages, *Carbohydr. Polym.* 43 (2000) 11-21.

Vierhuis, E., Schols, H.A., Beldman, G. & Voragen, A.G.J., Structural characterisation of xyloglucan and xylans present in olive fruit (*Olea europaea* cv koroneiki) *Carbohydr. Polym.* 44 (2001a) 51-62.

Vierhuis, E., York, W.S., Kolli, V.S.K., Vincken, J-P., Schols, H.A., Van Alebeek, G-J., & Voragen, A.G.J., Structural analyses of two arabinose containing oligosaccharides derived from olive fruit xyloglucan: XXSG and XLSG, *Carbohydr. Res.* 332 (2001b) 285-297.

Vierhuis, E., Servili, M., Baldioli, M., Schols, H.A., Voragen, A.G.J. & Montedoro, G.F. Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides, *J. Agric. Food Chem.*, 49 (2001c) 1218-1223.

Voragen, A.G.J., Schols, H.A. & Pilnik, W. Determination of the degree of methylation and acetylation of pectins by h.p.l.c., *Food Hydrocolloids* 1 (1986) 65-70.

Voragen, A.G.J. Enzymatic modification of plant cell walls. In: *Agricultural Biotechnology in focus in the Netherlands.* J.J. Dekkers, H.C. Van der Plas & D.H. Vuijk (eds), Wageningen: Pudoc (1990) 175-182.

Voragen, A.G.J., Pilnik, W., Thibault, J-F., Axelos, M.A.V. & Renard, C.M.G.C. Pectins. In: *Food polysaccharides and their applications.* A.M. Stephen (ed), New York: Dekker (1995) 287-339.

Voragen, A.G.J., Beldman, G. & Schols, H.A. Chemistry and enzymology of pectins. In: *Advanced dietary fibre technology.* B.V. McCleary & L. Prosky (eds), Oxford: Blackwell Science (2001)

379-398.

York, W.S., Kolli, V.S.K., Orlando, R., Albersheim, P. & Darvill, A.G. The structures of arabinoxyloglucans produced by solanaceous plants. *Carbohydr. Res.* 285 (1996) 99-128.

Chapter 7

Concluding remarks

Introduction

The favourable effect of the use of technical enzyme preparations in the olive oil industry has already been observed in the 1950s. Initially, enzyme preparations were mainly used as processing aids to enhance the extraction yield, but at present research is also conducted to improve the product quality (Servili *et al.*, 1992; Montedoro *et al.*, 1993; Di Giovacchino, 1993; Domínguez *et al.*, 1994; Ranalli & Serraoiocco, 1995; Ranalli & De Mattia, 1997). The technical enzyme preparations developed to be used for the extraction of olive oil act upon the plant cell walls, thus favouring the permeability for the oil. However, although several studies have been carried out on the effect of enzymatic treatment on the yield and quality characteristics of olive oil, very little is known about the specific role of the various constituent enzymes present in the preparations towards the polysaccharides in the plant cell wall. This thesis deals with this subject and the first part of this chapter describes the structural characteristics of the polysaccharides in the cell wall of olive fruit based on the results from the preceding chapters. In the second part the ripening related changes in the structure of the cell wall polysaccharides are discussed, because knowledge of this aspect is still limited and, because the ripening stage of the olive fruit is very important in relation to olive oil quality. Finally, we will go more deeply into the processing related changes of the cell wall polysaccharides in order to obtain more insight in the mechanisms by which the use of enzyme preparations leads to a higher yield and better quality.

Structural characteristics of polysaccharides present in olive fruit

Pectin in olive fruit

Pectic polysaccharides are important components of the plant cell wall of dicotyledonous plants as olive fruit (Table 7.1). Although the composition of pectins varies with conditions of extraction, the type of plant tissue and many other environmental factors (Selvendran, 1985), the dominant structural feature is a linear chain of (1→4)-linked α-D-galacturonic acid residues. In addition, blocks of more highly substituted rhamnogalacturonan regions are present (Voragen *et al.*, 2001). These rhamnogalacturonan regions can be substituted with neutral side chains like arabinans, galactans and arabinogalactans of various configurations and sizes (Carpita & Gibeaut, 1993). To characterise the pectins present in olive fruit, cell wall material was isolated from defatted olive pulp and sequentially extracted with various solvents. These were a cold and hot NaOAc buffer to extract the buffer soluble pectins, a solution of chelating agents to extract the calcium bound pectins and finally diluted alkali to extract pectins bound in the cell wall by ester linkages and hydrogen bonding. To elucidate the fine structure of the polysaccharides present in the pectin-rich extracts the sugar and glycosidic linkage composition were determined and immunodot-binding assays with defined monoclonal antibodies were performed. In addition, pure and well-characterised enzymes were used as analytical tools (chapters 2 and 6).

The sugar composition of the pectins extracted with buffer and chelating agents revealed

Table 7.1
Cell wall polysaccharide composition of olive fruit (*Olea europaea* cv koroneiki); the composition of the polysaccharides in the cell wall has been calculated on weight basis as described by Mafra *et al.* (2001) and is based on the sugar composition of the cell wall material of green and purple olive fruit and the extracts described in chapters 2 and 3 (tr = trace amount)

	Olive fruit composition (%)
Pectic polysaccharides	39
Galacturonan	*(20)*
Arabinan	*(13)*
Rhamnogalacturonan backbone	*(3)*
Galactan	*(3)*
Xyloglucan	15
Glucuronoxylan	14
Mannan	2
Ara-rich glycoprotein	tr
Cellulose	30

that these pectins consisted for an important part of linear homogalacturonan regions. The pectins extracted with diluted alkali had a relatively low content of homogalacturonan. These pectins consisted mainly of the more highly substituted rhamnogalacturonan regions and were particularly rich in arabinose. The pectins extracted with buffer and chelating agents were all highly methyl esterified (DM 56-65) and the degree of acetylation varied from 17 to 32. The pectins solubilised by diluted alkali were de-esterified by the conditions of their extraction. However, based on the high content of methyl esters and *O*-acetyl groups of the cell wall material (AIS) it is likely that these pectins were highly esterified as well.

The homogalacturonan regions of the pectins extracted with cold and hot buffer and chelating agents were further studied by incubation with pectin lyase, a homogalacturonan-degrading enzyme specific for high-methyl esterified pectins (chapter 6). Analysis of the pectin lyase digests by size exclusion chromatography (HPSEC) showed that pectin lyase was able to degrade these pectins to a large extend, as expected, because of the high degree of methyl esterification of these three extracts. However, despite of the large decrease of the molecular mass on HPSEC relatively few oligomeric degradation products (DP 2-10) were present in the digests of the pectins degraded by pectin lyase. Apparently, relatively large degradation products remained in the pectin lyase digest, which could not be further degraded to smaller oligosaccharides. Based on the findings in chapter 6 it is very likely that the homogalacturonan regions could not be further degraded by pectin lyase because the enzyme was hindered by the presence of *O*-acetyl groups. In general, it is stated that the homogalacturonan regions of pectins are only slightly *O*-acetylated or not at all and that *O*-acetylation is mostly confined to the rhamnogalacturonan regions (Komalavilas & Mort, 1989; Schols & Voragen, 1994; Ishii, 1995; Renard & Jarvis 1999). However, high amounts of *O*-acetyl groups in the homogalacturonan regions have been described for sugar beet pectin with a degree of acetylation of 35 (Voragen *et al.*, 2001). The *O*-acetyl content of the pectins extracted from olive fruit was also relatively high (17 to 32), which may indicate that also the

homogalacturonan regions of the pectins present in olive fruit were *O*-acetyled. This was confirmed by MALDI-TOF mass spectrometry of a pectin fraction digested with endo-polygalacturonase in combination with pectin methyl esterase (Fig. 6.5). The MALDI-TOF mass spectrum showed that part of the partially methyl esterified oligomers released from the homogalacturonan regions were indeed (mono)-*O*-acetylated, which also indicated that the presence of *O*-acetyl groups in the homogalacturonan regions could have hindered pectin lyase.

Defined monoclonal antibodies against homogalacturonan regions were used to complement the study of the pectic material extracted with buffer and chelating agents. The three antibodies used were JIM5, JIM7 and PAM1. JIM5 is a monoclonal antibody that binds weakly to a completely non-methyl esterified homogalacturonan, but binding is greatly increased by the presence of methyl esterified galacturonic acid residues up to a level of about 40%. At a degree of methyl esterification greater than this JIM5 binding is reduced (Willats *et al.*, 2000a). JIM7 recognises methyl esterified homogalacturonan with degrees of esterification ranging from 15% to 80% (Willats *et al.*, 2000a) and PAM1 binds to long (>30 residues) contiguous stretches of non-methyl esterified galacturonic acid residues (Willats *et al.*, 1999). For JIM5 and JIM7 the patterns (blockwise or random) of methyl esterification do not significantly influence the binding to pectin, this in contrast to PAM1 (Willats *et al.*, 2000a). The results of the immunodot-binding assay showed JIM5 as well as JIM7 binding to the buffer soluble pectic material and the pectic material extracted with chelating agents. PAM1 binding only occurred for the chelating agents soluble pectins. Based on the binding of PAM1 to the pectic material extracted with chelating agents it could be concluded that only these pectins contained long stretches of non-methyl esterified homogalacturonan. Binding of PAM1 to the pectic material extracted with chelating agents was also expected, because pectins extracted with chelating agents are believed to participate in calcium cross-linking in the cell walls and therefore, have to contain relatively large blocks of non-methyl esterified homogalacturonan. These 'blocks' of non-methyl esterified homogalacturonan regions are besides of their role in calcium pectate gel formation also of particular interest in cell to cell adhesion (Willats *et al.*, 2000b). The results of the experiments described in chapter 6 showed that despite of the presence of relatively large blocks of non-methyl esterified homogalacturonan, endo-polygalacturonase could hardly degrade these regions, because the enzyme was inhibited by the presence of *O*-acetyl groups. It is known that also PAM1 binding can be completely inhibited by the presence of *O*-acetyl groups, but the exact pattern or degree of *O*-acetylation which influences the binding is not known (Willats *et al.*, 2000b). So, apparently, the pectins extracted with chelating agents from olive fruit cell wall material contained blocks of non-methyl esterified homogalacturonan with a pattern or degree of *O*-acetylation, which did not hinder the binding of PAM1.

The pectic polysaccharides in olive fruit, especially the pectins extracted with diluted alkali, were rich in arabinose. Sugar linkage analysis was performed to determine the degree of branching of the arabinans. The results revealed that the arabinans in the cold buffer soluble extract had a relatively low degree of branching and were mainly composed of (1→5)-linked arabinose residues. About 30% of the (1→5)-linked arabinose residues of the backbone were substituted, mainly at C-3. The relative amount of (1→5)-linked arabinose residues

branched at C-3 increased till 70% when stronger solvents were used to extract the pectic polymers. The amount of disubstituted arabinose residues was negligible as was also shown by Coimbra *et al.* (1994).

Two types of galactans were found in the pectin-rich extracts of olive fruit. Most of the galactose residues in the buffer soluble extracts appeared to be present as (1→3)/(1→6) galactans, which are often found to be associated with hydroxyproline-containing proteins (O'Neill *et al.*, 1990). However, they can also be present as covalently linked side chains of rhamnogalacturonan I or as separate homopolysaccharides (Voragen *et al.*, 1995). About 20-30% of the galactose residues were present as (1→4)-linked galactan.

The solvents, which were used to extract the pectic material from the cell wall material were not able to solubilise all pectins. Even after a final fractionation with 6 M alkali to solubilise residual pectins, still 30% of the total amount of pectins present in the cell wall material remained in the residue (chapter 2). The (highly branched) pectic material, which remained 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 have caused some of the material to become insoluble (Massiot *et al.*, 1988).

Xyloglucan in olive fruit

Xyloglucans are linear chains of (1→4)-linked glucan, in which the glucose residues can be substituted at the C-6 position with xylose residues. Comparison of known xyloglucan structures revealed that, in principle, two general branching patterns can be distinguished, XXXG and XXGG (Vincken *et al.*, 1997). The letters 'G' and 'X' refer to an unbranched glucose residue and a xylose-(1→6)-glucose segment, respectively (Fry *et al.*, 1993). Xyloglucans with an XXXG branching pattern are present in most angiosperms and gymnosperms. The xylose residues of these xyloglucans can be substituted at C-2 with a galactose residue or a disaccharide of fucose-(1→2)-galactose. Xyloglucans with an XXGG branching pattern have, so far, only been reported for solanaceous plants, such as potato, tomato and tobacco. The xylose residues of xyloglucans with an XXGG branching pattern can be substituted with predominantly arabinose or arabinose and galactose residues (Vincken *et al.*, 1997). The presence of arabinose residues linked to xylose residues of xyloglucans with an XXXG branching pattern had not (yet) been demonstrated. However, the results of degradation studies with endo-(1→4)-β-D-glucanases revealed that this uncommon substitution pattern of xyloglucan is present in olive fruit. Xyloglucan from olive fruit appeared to consist of an XXXG branching pattern with arabinose residues linked to xylose residues (chapters 3 and 4). To establish the exact position of the specific arabinose-containing side chain on the backbone, the two most abundant oligosaccharides present in the endo-(1→4)-β-D-glucanase digest of xyloglucan were structurally characterised by NMR spectroscopy and PSD fragment analysis of MALDI-TOF mass spectra. The results revealed that olive fruit xyloglucan was mainly built from two novel oligosaccharides: XXSG and XLSG. Both xyloglucan oligosaccharides have three out of four glucose residues substituted with xylose residues and the letters 'S' and 'L' refer to arabinose-(1→2)-xylose-(1→6)-glucose and galactose-(1→2)-xylose-(1→6)-glucose segments, respectively. The tentative

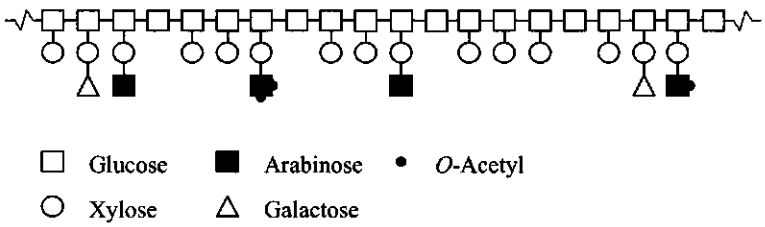

Fig. 7.1. Tentative structure of xyloglucan from olive fruit. □, β-D-Glc*p*-(1→4)-; ○, α-D-Xyl*p*-(1→6)-; Δ, β-D-Gal*p*-(1→2)-; and ■, α-L-Ara*f*-(1→2)-.

structure of xyloglucan present in olive fruit is shown in Fig. 7.1. This model is based on the results of the degradation studies with endo-(1→4)-β-D-glucanases described in chapters 3 and 4.

Xyloglucans can carry *O*-acetyl groups, but the *O*-acetyl groups are usually not detected in the molecules isolated from cell walls because of their instability in the strong alkaline conditions used for solubilisation (Fry *et al.*, 1989; Sims *et al.*, 1996). For that reason, most data on *O*-acetylation of xyloglucans are obtained from studies of enzyme digests of whole cell walls and from studies of xyloglucans present in suspension cultures. To investigate the presence of *O*-acetyl groups on xyloglucan from olive fruit, cell wall material was treated with pectin degrading enzymes in combination with endo-glucanases to release (*O*-acetylated) xyloglucan oligosaccharides. Analysis of the oligosaccharides revealed that both mono-*O*-acetylated and di-*O*-acetylated xyloglucan oligomers were present in the digest and that the *O*-acetyl groups were located on the arabinose residues (chapter 4). *O*-acetyl groups attached to arabinose residues have also been found on xyloglucan isolated from tobacco (Sims *et al.*, 1996; W.S. York, unpublished results). Tobacco xyloglucan contains besides *O*-acetyl groups attached to arabinose residues also *O*-acetyl groups attached to the glucose residues of the backbone (Sims *et al.*, 1996; York *et al.*, 1996). However, *O*-acetylation of the unbranched glucose residues of the backbone of olive fruit xyloglucan was not found. This would have decreased the susceptibility of the xyloglucan to attack by endo-glucanases (York *et al.*, 1996), causing larger xyloglucan fragments to accumulate in the digest, which were not detected.

The role of *O*-acetylation on xyloglucans is not understood. The presence of *O*-acetyl substituents does not seem to affect the amount of xyloglucan that binds to cellulose *in vitro* (Pauly, 1999). However, *O*-acetylation may reduce the ability of xyloglucan chains to act as a substrate for xyloglucan endotransglycosylase (XET), and thus play a role in controlling cell wall expansion. In addition, *O*-acetylation of the side chains has been shown to block the action of exo-glycosylhydrolases (Sims *et al.*, 1996) and *O*-acetylation of the backbone affects the accessibility to endo-glucanase. On the other hand, not all enzyme activity is hindered by the presence of *O*-acetyl groups. For example, *O*-acetyl groups on the galactose or arabinose residues of the side chains do not provide resistance against the attack of endo-glucanases (York *et al.*, 1996; Vincken *et al.*, 1997).

Xylan in olive fruit

The cell wall material of olive fruit contained besides xyloglucans also considerable amounts of the hemicellulose xylan (chapter 2). In general, the presence of xylans in edible fruit is very restricted because these polysaccharides are related to secondary growth in the cell wall (Jiménez *et al.*, 2001). However, it is very likely that a large proportion of the glucuronoxylans in the 1 and 4 M KOH fractions from olive fruit is derived from the lignified sclereids of the olive pulp, as is the case in stone cells of pear. In addition, a small proportion may have originated from the parenchyma tissues (Coimbra *et al.*, 1994).

Xylans have a backbone of (1→4)-linked xylose resides, which can be substituted with glucuronic acid residues or its 4-*O*-methylated derivative, arabinose residues and *O*-acetyl groups. The absolute and relative amounts of the substituents depend on the origin of the xylan. The xylans present in olive fruit appeared to be very low in substitution. Less than 10% of the xylose residues were mono substituted with branch points at C-2 or C-3. The xylans were mainly substituted with 4-*O*-methyl-glucuronic acid residues, but besides 4-*O*-methyl-glucuronic acids, also glucuronic acid residues were linked to the backbone (chapters 2 and 3). The presence of *O*-acetyl groups could not be determined because the strong alkaline conditions used to solubilise the xylans from the cell wall material saponified the ester-linkages. Also, direct treatment of cell wall material with pectin degrading enzymes in combination with endo-xylanases could not give information about the occurrence of *O*-acetyl groups on xylans in olive fruit, because hardly any xylan oligosaccharides were released. From literature it is known that xylans can carry *O*-acetyl groups, but the presence of these groups has mainly been described for hardwood xylans (Timel, 1964; Ishii, 1991; Puls *et al.*, 1991; Ross *et al.*, 1992; Bardet *et al.*, 1997).

Ripening of olive fruit

Modifications of the polysaccharides

An important factor that influences the quality of olive oil is the time of harvest of the olive fruit (Montedoro *et al.*, 1986; Boskou, 1996). When harvesting the olives for the production of olive oil the majority of the fruit should be at the right stage of maturity to obtain an acceptable quality of oil that also guarantees a satisfactory yield. The effect of the technical enzyme preparations which are used in the olive oil industry also depends on the time of harvest and is most pronounced during the processing of unripe or less ripe olives. The technical enzyme preparations act upon the polysaccharides present in the cell walls and from this perspective the structural characteristics of the polysaccharides in the cell walls during fruit development have been investigated in chapter 2.

The ripening of fruit is associated with several modifications of the polysaccharides present in the cell wall. Changes to the pectic polymers are the most common and can involve an increased solubility, depolymerisation, de-esterification and a loss of neutral sugar associated side chains. In some cases a reduction in the molecular mass of polymers present in the

hemicellulose fractions has also been reported (References in Voragen *et al.*, 1995; Seymour & Gross, 1996; Redgwell *et al.*, 1997). In chapter 2 the ripening related changes in the composition of the polysaccharides from the cell wall of olive fruit have been described. The olive fruit was harvested at specific moments during the season and the cell wall material of four different ripening stages was studied (F0 immature green, FI green, FII turning and FIII purple olives). During ripening the yield of the cell wall material expressed as % of fresh weight of destoned olive pulp decreased from 8.9 to 4.7% and the amount of polysaccharides isolated from the cell wall material expressed as % of fresh fruit decreased from 3.3 to 1.5%. However, the amount of polysaccharides per olive fruit remained almost constant during ripening and was about 10 mg per fruit. The oil content expressed as % of fresh weight of destoned olive fruit increased from 23 to 38% during ripening, which is equal to an increase of the amount of oil from 70 to 270 mg per fruit.

No distinct differences in the sugar composition of the cell wall material could be noticed during fruit development. Further analysis of the cell wall material showed that the level of methyl esters and acetyl groups in the cell wall decreased at the end of the harvesting period. The degree of methyl esterification was about 68 during the first three ripening stages and decreased to 59 when the olives became purple. This de-esterification of the polygalacturonic backbone may facilitate the depolymerisation of pectins by polygalacturonase during ripening. The degree of acetylation was also constant in the first three ripening stages and then decreased from 72 to 63 for purple olives.

For further study the cell wall material from green and purple olive fruit was fractionated by successive extractions with specific solvents. The obtained pectic and hemicellulosic fractions were compared on the basis of yield, sugar composition and molecular weight distribution. It appeared that the solubility of pectin in hot NaOAc buffer increased markedly from 40% of the total extractable pectin for green olive fruit to 60% of the total extractable pectin for purple olive fruit. The total amount of pectins as solubilised from the cell wall material with hot buffer, chelating agent in buffer, diluted alkali and 6 M NaOH hardly changed during ripening of olive fruit. So, 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 implicate 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 considerable increase in the amount of pectic material extracted with NaOAc buffer may result from cleavage of cross-linkages between pectins and other cell wall components in the ripening process or from the hydrolysis of large pectin polymers during ripening. However, it should be noted that modification of cell wall material during ripening is a dynamic process in which not only degradation of the existing polymers is involved but also the role of synthetic processes should be considered (Seymour & Gross, 1996). Although the amount of pectic material extracted with hot buffer increased, the sugar composition and the molecular weight distribution of the pectic polymers hardly changed during ripening.

Analysis of the hemicelluloses present in the 1 and 4 M KOH fractions showed that their yield and sugar composition hardly changed during ripening. Also, no distinct differences could be noticed between the two ripening stages for the molecular weight distributions.

Table 7.2
Relative amounts of the *O*-acetylated xyloglucan oligosaccharides XXSG and XLSG in the endo-glucanase digest of cell wall material isolated form olive fruit at different ripening stages. The relative amounts are based on the accumulated intensities of the oligosaccharides in the MALDI-TOF mass spectra

	Immature green olives (F0)	Green olives (FI)	Turning olives (FII)	Purple olives (FIII)
XXSG				
Non-acetylated	1	1	1	1
Acetylated	3.2	3.2	2.5	1.4
XLSG				
Non-acetylated	1	1	1	1
Acetylated	5.5	4.6	3.8	2.8

However, study of xyloglucan oligosaccharides released by direct treatment of the cell wall material with endo-glucanases in combination with pectin degrading enzymes revealed that the *O*-acetyl content of the released oligosaccharides changed during ripening of the fruit. Analysis of the MALDI-TOF mass spectra of the digests showed that the accumulated intensity of the peaks of the *O*-acetylated xyloglucan oligomers decreased compared to the accumlated intensity of the peaks of xyloglucan oligomers without acetyl groups. The increase of the relative amount of xyloglucan oligomers without *O*-acetyl groups during fruit development may result from the synthesis of new polymers or modification of the existing polymers by endogenous enzymes. The accumulated intensities of the two most abundant xyloglucan oligosaccharides in the digest, XXSG and XLSG, are shown in Table 7.2. The ratio of the accumulated intensities of the non-acetylated and acetylated peaks in the MALDI-TOF mass spectra do not necessarily have to correlate with the ratio of the amounts of the (*O*-acetylated) xyloglucan oligomers, but it is thought that peaks with the highest intensities are the most abundant. The MALDI-TOF mass spectra showed besides xyloglucan oligomers also unsaturated galacturonic acid oligomers with a degree of polymerisation of 3-6. Oligomers with a single *O*-acetyl group attached to it as well as non-acetylated oligomers were detected but the ratio of the accumulated intensities of the non-acetylated and acetylated peaks in the MALDI-TOF mass spectra did not change during fruit development.

The effect of ripening on the polysaccharides present in the cell wall material of olive fruit has also been studied by other groups (Heredia *et al.*, 1993; Huisman *et al.* 1996, Jímenez *et al.*, 2001a; Jímenez *et al.*, 2001b; Mafra *et al.* 2001). In general, the sugar compositions of the cell wall material (mol%) obtained from olive fruit at the green, turning and purple ripening stages were quite similar for all groups. However, the results of the analysis of the polysaccharides present in the extracts obtained during the preparation of the cell wall material and by sequential extraction of the cell wall material differed for the ripening stages studied. Comparison of the changes to the pectic polymers during ripening revealed that the changes described by Jímenez *et al.* (2001a) and Mafra *et al.* (2001) were quite different from the effects of ripening to the pectic polymers described in this thesis and the ripening related changes described by Huisman *et al.* (1996). For example, the amount of pectic polymers

solubilised during ripening is much higher in the studies described by Jímenez *et al.* (2001a) and Mafra *et al.* (2001) and, in addition, the pectic polymers in their extracts became significantly richer in arabinose during ripening, while the galacturonic acid content decreased. The differences of the results may be due to the use of, for example, different varieties, but also the season (year) in which the fruit is harvested seems to effect the results (Heredia *et al.*, 1993; Mafra *et al.*, 2000). In addition, it should be remarked that although the study of compositional changes of carbohydrates by cell wall fractionation has revealed very significant changes in many fruits during development, more subtle changes in cell wall structure may be overlooked by this method (Gross, 1990). Besides the chemical analyses of fractions from the cell wall, light or electron microscopy can be used to further investigate ripening related changes and may give important additional information (Seymour & Gross, 1996; Steele *et al.*, 1997).

Endogenous enzymes in olive fruit

Several studies have been directed towards establishing a relationship between changes in the composition of cell wall polysaccharides during ripening and the activity of endogenous enzymes (Hobson, 1981; Brady, 1987; Fisher & Bennett, 1991; Heredia *et al.*, 1993; Seymour & Gross, 1996). In general, the most apparent changes in wall structure occur in the pectic fraction during fruit development and therefore, much attention has been focussed on the correlation between the degradation of cell wall pectins during ripening and the presence of polygalacturonase in the fruit. However, several fruit tissues also appear to soften in the absence of endo-polygalacturonase activity. So, other mechanisms must also be involved in regulating cell wall turnover and tissue softening (Gross, 1990). Furthermore, the existence of wall enzymes does not prove that they act in vivo. They could be at a site that precludes access to the postulated substrate, or inhibitors of the enzyme could be present (Fry, 1995).

The presence and activities of several endogenous enzymes have been reported in olive fruit. Several glycosidases are detected like α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase, α-arabinosidase and α-xylosidase as well as Cx-cellulase (endo-β-(1→4)-glucanase) and endo-polygalacturonase (Heredia *et al.*, 1992; Heredia *et al.*, 1993; Fernández-Bolaños *et al.*,1997). The results of our study also showed the presence of several glycosidases in olive fruit with activity towards *p*-nitrophenyl substrates. The highest glycosidase activities found were α-galactopyranosidase and α-mannopyranosidase. In addition, a β-galactopyranosidase was detected. The role of α-galactopyronosidase and α-mannopyranosidase is still unclear, since a possible site of action towards cell wall polysaccharides is not known. β-Galactopyranosidase might be able to modify specific pectic regions (RG-I) or xyloglucans present in olive fruit, although it is not clear what significant effect the trimming of a few galactose residues from polysaccharides could have. Perhaps the relevant product is free galactose, an agent which affects fruit ripening (Fry, 1995). In addition to the activity of the endogenous enzymes towards *p*-nitrophenyl substrates, the activity towards several purified and characterised polysaccharides and oligosaccharides was also studied. The results of this study showed neither exo- nor endo-activity towards xyloglucan, arabinan, galactan, arabinogalactan, galactomannan and oligomers made thereof. Also no

activity towards polygalacturonan or carboxymethylcellulose could be detected despite of an extensive screening.

Although the enzymes present in the extracts were not active towards the cell wall polysaccharides expected to be present in olive fruit, they were active towards other polysaccharides like starch, an α-(1→4)-linked glucan, laminarin, a β-(1→3)-linked glucan, a β-(1→3)/(1→6) glucan from yeast and a β-(1→3)/(1→4) glucan from barley. These results indicated the presence of a β-amylase and a β-(1→3)-endoglucanase or laminarinase in olive fruit. Laminarin was a better substrate for the β-(1→3)-endoglucanases than the β-(1→3) glucans from barley and yeast. Probably, because laminarin has relative less other bonds (1→4 and 1→6) in its structure, which can hinder the action of the enzyme. β-(1→3)-Endoglucanase might play a role in olive fruit in the induction of plant defence reactions in response to fungal infection (Keen & Yoshikawa, 1982). β-Amylase is able to split dimers from α-(1→4)-linked glucans and can play a role in the degradation of starch granules. However, the presence of starch in olive fruit has not been described yet.

As mentioned, no activity could be determined in our study towards the cell wall polysaccharides, which are expected to be present in olive fruit. It might be that the enzymes, which are active towards these polysaccharides were inactivated during the enzyme extraction procedure or inhibited by the polyphenolic compounds present in olive fruit (Heredia *et al.*, 1990; Knee *et al.*, 1991), but other (unknown) factors might also be involved.

Processing of olive fruit

Several processing steps are required for the extraction of olive oil (Fig. 1.1). The olive fruit is ground with a hammer crusher and the oily paste is slowly mixed at room temperature during 30-60 min (called malaxation) before the oil can be separated from the other phases in the paste with a decanter. The objective of malaxation is to increase the amount of oil that is released by helping the droplets to merge into larger drops that can be separated into a continuous liquid phase and by breaking up the oil/water emulsion. In addition, during the mechanical extraction process several enzymes are activated like endogenous oxidoreductases such as polyphenoloxidase, peroxidase and lipoxygenase, which affect the virgin olive oil quality (Servili *et al.*, 1998). Lipoxygenases, for example, catalyse the formation of saturated and unsaturated C_6 and C_9 alcohols and aldehydes to which the "green flavour" of green virgin olive oil is correlated (Olias *et al.*, 1993). Polyphenoloxidase and peroxidase, on the contrary, may promote the oxidation of phenolic compounds and the activities of these enzymes may be related to the loss of phenolic compounds during malaxation (chapter 5). In addition to the oxidoreductases also endogenous glycosidases are present in olive fruit, which catalyse the formation of secoiridoid derivatives during processing (Servili *et al.*, 1999). However, an effect of endogenous glycosidases or other endogenous enzymes on the structural characteristics of the polysaccharides in the cell wall, in specific the pectic polysaccharides, was not noticed during processing (chapter 5). It is very likely that the various endogenous enzymes present in olive fruit, such as glycosidases, polygalacturonases and enzymes with cellulolytic activities (Heredia *et al.*, 1991; Heredia *et al.*, 1993; Fernàndez-

Bolaños *et al.*, 1995; research described in this chapter) were inhibited during malaxation by the high concentrations of polyphenolic compounds in olive fruit (Heredia *et al.*, 1990). But, in addition, the time period of malaxation is probably also too short for the endogenous enzymes to act on the cell wall polysaccharides, especially, because their activities are rather low in the fruit.

As mentioned before, technical enzyme preparations can be added to the malaxer when the olives are ground to obtain a higher yield and a better quality of the oil. Olivex is one of the commercial pectolytic enzyme preparations developed to be used in the olive oil industry and this preparation was also used for our research to investigate the effect of enzyme preparations on the phenolic compounds and polysaccharides present in the olive fruit (chapter 5 and 6). Analysis of the olive oil revealed that the use of Olivex as a processing aid increased the concentration of the phenolic compounds in the oil. Especially, the contents of secoiridoid derivatives such as the dialdehydic form of elenolic acid linked to 3,4-dihydroxy-phenylethanol (3,4-DHPEA-EDA) and an isomer of oleuropein aglycon (3,4-DHPEA-EA), which have high antioxidant activities increased significantly in the olive oil (chapter 5). Also the structure of the polysaccharides present in the cell wall were modified due to the use of the enzyme preparation Olivex. To be able to study the effect of the enzyme preparations on the pectic polysaccharides in more detail four pectin-rich fractions were isolated from the cell wall material present in the malaxed paste by successive extractions with cold buffer, hot buffer, chelating agents and diluted alkali (chapter 6). The structural characteristics of the pectins present in the enzyme treated malaxed olive paste were compared with the characteristics of the pectins present in the malaxed paste from a process without enzyme preparations added. The pectin-rich fractions were compared on the basis of yield, sugar (linkage) composition, degree of methyl esterification and acetylation and the molecular weight distribution. Also well-characterised enzyme were used to elucidate the structure of the pectins. Analysis of the fractions revealed that mainly the buffer soluble pectic material was affected by the use of enzyme preparations. The structure of the pectic polysaccharides solubilised by extraction with chelating agents and diluted alkali was barely affected by the use of enzyme preparations during processing. The changes of the buffer soluble pectic material were reflected by an increase in the yield, a change in the molecular weight distribution, a decrease in methyl esterification and a degradation of the (1→4)-linked galactan chains. No differences were observed in the composition of the arabinan chains.

Based on the results of this study it can be concluded that the enzymes used during processing affected only a relatively small part of the polysaccharides present in the cell wall material. About 8-10% of the cell wall polysaccharides was extracted with cold and hot buffer and appeared to be modified during processing. Apparently, the action of the enzymes is restricted during processing. This aspect will be further discussed in the next section on the structural characteristics of the polysaccharides present in the vegetation waters, a by-product of olive oil processing.

About 5-10% of the polysaccharides present in olive fruit were soluble in water and turned up in the vegetation waters when the oil was extracted from the malaxed paste by decantation. The sugar composition of the polysaccharides present in the vegetation waters indicated the presence of mainly material of pectic origin. The use of commercial enzyme preparations

during processing resulted in a relatively higher galacturonic acid content of the polysaccharides present in the vegetation waters. This led to a decrease of the ratio of the neutral sugars arabinose and galactose to uronic acids indicative for the length of the side chains. Apparently, the addition of enzyme preparations partly debranched the pectic material and/or solubilised additional pectic substances with a high galacturonic acid content. Furthermore, as a result of processing the degree of methyl esterification decreased and the (1→4)-linked galactan chains were degraded as was also shown for the buffer soluble material isolated from the pastes.

The HPSEC elution pattern revealed that polymeric material was still present in the vegetation waters and that the pectins were not completely degraded to oligosaccharides and their constituent monosaccharides even though enzyme preparations were added during processing. To check whether the structure of the pectins restricted the action of the enzymes, the pectic polymers isolated from the vegetation waters were incubated with a fresh batch of Olivex. The results showed that the polysaccharides could be degraded to a substantial extent. About 90% of the polysaccharides were degraded to oligomers (molecular mass < 4 kDa). Apparently, the structures of the polysaccharides or the enzyme preparations used were not limiting for the degradation. As mentioned before, polyphenolic compounds inhibit enzymes in their activity (Heredia *et al.*, 1990; Knee *et al.*, 1991). So, it is very likely that the high concentrations of phenolic compounds in olive fruit restricted also the activity of the enzymes in the preparations during processing, but other (unknown) factors might also be involved. However, not all enzymes in the enzyme preparations seemed to be restricted in their activity to the same extent during processing. For example, the use of enzyme preparations during processing resulted in an almost complete degradation of the (1→4)-linked galactan chains present in the vegetation waters. This suggests, that the enzymes involved in the degradation of the (1→4)-linked galactan chains were, contrary to other enzymes present in the preparations, less affected by the phenolic compounds present in the olive pulp.

Although the results of this work gave more insight in the action of the commercial enzyme preparation Olivex towards the pectic polysaccharides present in the cell wall, it is still difficult to conclude how these changes affected the phenol content of the oil. Further research will be necessary on this aspect. Especially, because it is known that the use of commercial enzyme preparations in the olive oil industry not always assures a significant increase of the quality aspects or the yield of the oil (Ranalli & Serraiocco, 1995). An adequate strategy to efficiently carry out enzymatic treatment remains difficult to give, because the effect of enzymatic treatment depends on several factors in which not only technological aspects, but also agronomic aspects, like variety, geographical location, etc are expected to play an important role.

Acknowledgements

We thank J.P. Knox and W.G.T. Willats for providing the defined monoclonal antibodies and their assistance with the immunodot-binding assays and Marit van Beest for her help with the experiments on endogenous enzymes in olive fruit during her graduate research.

References

Bardet, M., Emsley, L. & Vincendon, M. Two-dimensional spin-exchange solid-state NMR studies of C-13-enriched wood, *Solid State Nucl. Magn. Reson.* 8 (1997) 25-32.

Boskou, D. *Olive oil: chemistry and technology.* Campaign: AOCS Press (1996) 161p.

Brady, C.J. Fruit ripening, *Annu. Rev. Plant Physiol.* 38 (1987) 155-178.

Carpita, N.C. & Gibeaut, D.M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth, *Plant J.* 3 (1993) 1-30.

Coimbra, M.A., Waldron, K.W. & Selvendran, R.R. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L.), *Carbohydr. Res.* 252 (1994) 245-262.

Di Giovacchino, L. The use of enzymes in the extraction of olive oil by continues systems of centrifugation. Note 1: Results of tests made over many years, *Riv. Ital. Sostanze Grasse* LXX (1993) 279-287.

Domínguez, H., Núñez, M.J. & Lema, J.M. Enzymatic treatment to enhance oil extraction from fruits and oilseeds: a review, *Food Chem.* 49 (1994) 271-286.

Fernández-Bolaños, J., Rodríguez, R., Guillén, R., Jimenéz, A. & Heredia, A. Activity of cell wall-associated enzymes in ripening olive fruit, *Physiol. Plant.* 93 (1995) 651-658.

Fernández-Bolaños, J., Heredia, A., Vioque, B., Castellano, J.M. & Guillén, R. Changes in cell-wall-degrading enzyme activities in stored olives in relation to respiration and ethylene production. Influence of exogenous ethylene, *Z. Lebensm. -Unters. -Forsch.* 204 (1997) 293-299.

Fischer, R.L. & Bennett, A.B. Role of cell wall hydrolases in fruit ripening, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42 (1991) 675-703.

Fry, S.C. The structure and functions of xyloglucan (review article). *J. of Exp. Bot.* 40 (1989) 1-11.

Fry, S.C., York, W.S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J-P., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J. & White, A.R. An unambiguous nomenclature for xyloglucan derived oligosaccharides, *Physiol. Plant.* 89 (1993) 1-3.

Fry, S.C. Polysaccharides-modifying enzymes in the plant cell wall, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46 (1995) 497-520.

Gross, K.C. Recent developments on tomato fruit softening, *Postharvest News and Information* 1 (1990) 109-112.

Heredia, A., Fernández-Bolaños, J. & Guillén, R. Cellulase inhibition by polyphenols in olive fruits, *Food Chem.* 38 (1990) 69-73.

Heredia, A., Fernández-Bolaños, J. & Guillén, R. Identification of endoglucanases in olives (*Olea europaea* arolensis), *Z. Lebensm. -Unters. -Forsch.* 193 (1991) 554-557.

Heredia, A., Guillén, R., Jiménez, A. & Fernández-Bolaños, J. Olive fruit glycosidases: factors affecting their extraction, *Z. Lebensm. -Unters. -Forsch.* 194 (1992) 561-565.

Heredia, A., Guillén, R., Jiménez, A. & Fernández-Bolaños, J. Activity of glycosidases during development and ripening of olive fruit, *Z. Lebensm. -Unters. -Forsch.* 196 (1993) 147-151.

Hobson, G.E. Enzymes and texture changes during ripening. In: *Recent advances in the biochemistry of fruit and vegetables.* J. Friend & M.J.C. Rhodes (eds.), London: Academic Press (1981) 123-132.

Huisman, M.M.H., Schols, H.A. & Voragen, A.G.J. Changes in cell wall polysaccharides from ripening olive fruits, *Carbohydr. Polym.* 31 (1996) 123-133.

Ishii, T. Acetylation at *O*-2 of arabinofuranose residues in feruloylated arabinoxylan from bamboo shoot cell-walls, *Phytochemistry* 30 (1991) 2317-2320.

Ishii, T. Isolation and characterization of acetylated rhamnogalacturonan oligomers liberated from

bamboo shoot cell-walls by Driselase, *Mokuzai Gakkaishi* 41 (1995) 561-572.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños J. & Heredia, A. Olive fruit cell wall: Degradation of pectic polysaccharides during ripening, *J. Agric. Food Chem.* 49 (2001a) 409-415.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños J. & Heredia, A. Olive fruit cell wall: Degradation of cellulosic and hemicellulosic polysaccharides during ripening, *J. Agric. Food Chem.* 49 (2001b) 2008-2013.

Keen, N.T. & Yoshikawa, M. Beta-1,3-endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls *Glycine max*, defense reactions to pathogens, *Phytophthora* spp. *Plant physiol.* 71 (1982) 460-465.

Knee, M., Paull, R.E., Ben Arie, R. & Hawker, J.S. Enzymes in fruit. In: *Food enzymology.* P.F. Fox (ed), London: Elsevier (1991) 545-598.

Komalavilas, P. & Mort, A.J. The acetylation at *O*-3 of galacturonic acid in the rhamnose-rich portion of pectins, *Carbohydr. Res.* 189 (1989) 261-272.

Mafra, I., Reis, A., Barros, A., Nunes, C., Guedes, S., Vitorino, R., Saraiva, J. & Coimbra, M.A. Ripening-related changes of olive fruit cell wall polysaccharides and associated enzymes in two consecutive harvests. In: *Plant Polysaccharides 2000; 10th International Symposium.* Wageningen: Wageningen University (2000).

Mafra, I., Lanza, B., Reis, A., Marsilio, V., Campestre, C., De Angelis, M. & Coimbra, M.A. Effect of ripening on texture, microstructure and cell wall polysaccharide composition of olive fruit (*Olea europaea*), *Physiol. Plant.* 111 (2001) 439-447.

Massiot, P., Rouau, X. & Thibault, J-F. Characterisation of the extractable pectins and hemicelluloses of the cell wall of carrot, *Carbohydr. Res.* 172 (1988) 229-242.

Montedoro, G.F., Garofolo, L. & Bertuccioli, M. Factors shaping the quality characteristics of an olive oil, *Industrie Alimentari* 25 (1986) 549.

Montedoro, G.F., Begliomini, A.L., Servili, M., Petruccioli, M. & Federici, F. Pectinase production from olive vegetation waters and its use in the mechanical olive oil extraction process to increase oil yield and improve quality, *It. J. Food Sci.* 5 (1993) 355-362.

Olias, J.M., Perez, A.G., Rios, J.J. & Sanz, L.C. Aroma of virgin olive oil: Biogenesis of the "Green" odour notes. *J. Agric. Food Chem.* 41 (1993) 2368-2373.

O'Neill, M., Albersheim, P. & Darvill, A. The pectic polysaccharides of primary cell walls. In: *Methods in plant biochemistry, Vol. 2: Carbohydrates.* P.M. Dey (ed), London: Academic press (1990) 415-441.

Pauly, M. *Development of analytical tools to study plant cell wall xyloglucan. Ph.D. thesis*, Aachen: Shaker-Verlag (1999).

Puls, J., Tenkanen, M., Korte, H.E. & Poutanen, K. Products of hydrolysis of beechwood acetyl-4-*O*-methylglucuronoxylan by a xylanase and an acetyl xylan esterase, *Enzyme Microb. Technol.* 13 (1991) 483-486.

Ranalli, A. & Serraiocco, A. Effect induced by a pectolytic adjuvant in olive oil extraction by the present technological systems; Pluriannual research results, *Riv. Ital. Sostanze Grasse* LXXII (1995) 355-364.

Ranalli, A. & De Mattia, G. Characterization of olive oil produced with a new enzyme processing aid, *J. Am. Oil Chem. Soc.* 74 (1997) 1105-1113.

Redgwell, R.J., Fischer, M., Kendal, E. & Macrae, A. Galactose loss and fruit ripening: high-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls, *Planta* 203 (1997) 174-181.

Renard, C.M.G.C. & Jarvis, M.C. Acetylation and methylation of homogalacturonans 1: optimimisation of the reaction and characterisation of the products, *Carbohydr. Polym.* 39 (1999)

Summary

Technical enzyme preparations can be used as processing aids in the olive oil industry to increase the extraction output of the oil and to improve the quality of the product. The technical enzyme preparations degrade the plant cell walls, thus enhancing the permeability for oil. However, although several studies have been carried out on the effect of enzyme treatment on the yield and the quality characteristics of the olive oil, very little is known about the effect of these enzyme preparations on the polysaccharides present in the plant cell wall. This thesis deals with this subject and describes the structural characterisation of polysaccharides from olive fruit cell walls in relation to enzymatic processing for oil extraction. In addition to this, the structural characteristics of the cell wall polysaccharides during ripening have been studied, because knowledge of this aspect is still limited and because the ripening stage of the olive fruit is very important in relation to olive oil quality. In chapter 1 a short overview of the processing of olive fruit for the extraction of the oil is given and the structure of plant cell walls is described.

In chapter 2 the structural characteristics of polysaccharides from olive fruit cell walls in relation to ripening are described. The olive fruit was harvested at four specific moments during the season and the composition of the cell wall material was studied. The results revealed that the level of methyl esters and acetyl groups in the cell wall decreased at the end of the harvesting period, but no distinct differences in the sugar composition could be noticed during fruit development. For further study the cell wall material of green (unripe) and purple (ripe) olive fruit was fractionated by successive extractions with specific solvents. The obtained pectic and hemicellulosic fractions were compared on the basis of yield, sugar composition and molecular weight distribution. It appeared that during ripening the solubility of pectin in hot buffer considerably increased from 40% of the total extractable pectin for green olive fruit to 60% of the total extractable pectin for purple olive fruit. The sugar composition and the molecular weight distribution of the pectic polymers showed little change throughout development. Analysis of the hemicellulosic fractions showed that their yield and sugar composition hardly changed during ripening. Also no distinct differences could be noticed between the two ripening stages for the molecular weight distribution.

A more detailed study of the hemicellulosic polysaccharides is described in chapter 3. The hemicellulosic fractions obtained from olive fruit were fractionated by anion-exchange chromatography, which resulted in a xyloglucan-rich pool and four xylan-rich pools. Sugar linkage analyses and degradation studies with specific enzymes were performed to obtain information about the structures. The results indicated a xyloglucan in olive fruit with a specific substitution pattern, which is not commonly found in plant cell walls. The xyloglucans had a backbone with three out of four glucose residues substituted with xylose residues (XXXG-type building units) and arabinose as well as galactose residues linked to the xylose residues. The presence of arabinose residues linked to the xylose residues is a common feature of xyloglucans with XXGG-type building units produced by solanaceous plants, but has not been demonstrated for other dicotyledonous plants, which have in general XXXG-type building units. The xylans present in olive fruit appeared to be very low in substitution. Less than 10% of the xylose residues were mono substituted, mainly with 4-*O*-methyl-glucuronic

acid residues.

In chapter 4 the purification and rigorous characterisation of two arabinose containing oligosaccharides from olive fruit xyloglucan is described. Xyloglucan oligosaccharides were prepared by endo-glucanase digestion of alkali-extractable xyloglucan from olive fruit and purified by a combination of gel-permeation chromatography and high-performance anion-exchange chromatography. The two most abundant oligosaccharides were structurally characterised by NMR spectroscopy and mass spectrometry. The results revealed that olive fruit xyloglucan is mainly built from two novel oligosaccharides: XXSG and XLSG. Both xyloglucan oligosaccharides have three out of four glucose residues substituted with xylose residues and the letters 'S' and 'L' refer to α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* and β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* segments, respectively. The structure of the oligosaccharides confirmed the presence of a specific xyloglucan in olive fruit with α-L-Ara*f*-(1→2)-α-D-Xyl*p* side chains as was suggested in chapter 3. Direct treatment of cell wall material from olive fruit with pectin degrading enzymes in combination with endo-glucanase was performed to investigate the presence of *O*-acetyl groups on xyloglucan from olive fruit. Analysis of the oligosaccharides revealed that the arabinose residues of the oligosaccharides XXSG and XLSG can be substituted with either none, one or two *O*-acetyl groups.

As mentioned, technical enzyme preparations can be used as processing aids during the extraction of olive oil to obtain a higher yield and a better quality of the oil. In order to obtain more insight in the mechanisms by which these enzyme preparations lead to a higher yield and a better quality, the effect of these preparations on the concentration of phenolic compounds and the structure of the polysaccharides was investigated in chapter 5. Analysis of the olive oil revealed that the use of enzyme preparations increased the concentration of phenolic compounds in the oil. Especially, the contents of secoiridoid derivatives such as the dialdehydic form of elenolic acid linked to 3,4-dihydroxyphenylethanol (3,4-DHPEA-EDA) and an isomer of oleuropein aglycon (3,4-DHPEA-EA) significantly increased in the olive oil. These two compounds have high antioxidant activities. Analysis of the pectic polymers present in the olive paste revealed that due to the use of enzyme preparations the solubility of the pectic polysaccharides in buffer increased and the profile of the molecular weight distribution changed. Also, the content of uronic acids in the buffer soluble extract considerably increased due to the use of enzyme preparations. Analysis of the polymeric carbohydrates in the vegetation waters, a by-product of olive oil processing, indicated that the use of commercial enzyme preparations during processing also resulted in a relatively higher uronic acid content of the pectic polysaccharides present in the vegetation waters.

The effect of enzyme preparations on the structure of the pectic polysaccharides in olive paste was further investigated in chapter 6. Four pectin-rich fractions were isolated from the cell wall material by successive extractions with cold buffer, hot buffer, chelating agents and diluted alkali and analysed. The results revealed that the use of technical enzyme preparations during processing affected only a relatively small part of the polysaccharides present in the cell wall. About 8-10% of the cell wall polysaccharides were extracted with cold and hot buffer and appeared to be modified during enzymatic processing. The structures of the arabinose-rich pectic polysaccharides solubilised by extraction with chelating agents and diluted alkali from the cell wall material were barely affected by the use of enzyme

Samenvatting: Structuurkenmerken van polysachariden uit de celwand van de olijf, gerelateerd aan het rijpen van de vrucht en het winnen van olijfolie.

In de olijfolie-industrie kunnen commerciële enzympreparaten worden gebruikt om een hogere opbrengst en een betere kwaliteit van de olijfolie te verkrijgen. Deze commerciële preparaten bevatten enzymen die de celwanden van planten kunnen afbreken, waardoor de olie gemakkelijker geëxtraheerd kan worden. Diverse studies hebben het effect van het toevoegen van enzympreparaten op de opbrengst en de kwaliteitskarakteristieken van de olie bestudeerd. Echter, er is nog weinig bekend over het effect van de enzymen in deze preparaten op de structuur van de polysachariden in de celwanden van de plant. In dit proefschrift wordt dit onderwerp bestudeerd en worden de structuurkenmerken van de polysachariden en de veranderingen in de structuur van de polysachariden door het gebruik van enzympreparaten beschreven. Daarnaast worden ook de veranderingen in de structuur van polysachariden bestudeerd tijdens het rijpen van de olijf. Dit, omdat er nog weinig kennis is op dit gebied en omdat het rijpingsstadium van de olijf erg belangrijk is voor de uiteindelijke kwaliteit van de olijfolie. In hoofdstuk 1 wordt in het kort beschreven hoe olijfolie uit olijven wordt gewonnen en wordt vervolgens ingegaan op de structuur van de celwanden van planten.

In hoofdstuk 2 worden de veranderingen in de structuur van de polysachariden in de celwand beschreven tijdens het rijpen van de olijf. Om de veranderingen in de structuur te kunnen bestuderen werden de olijven geoogst op vier momenten tijdens het seizoen, waarna het celwandmateriaal werd geïsoleerd en vervolgens gekarakteriseerd. Uit de resultaten van het onderzoek bleek dat het aantal methyl esters en *O*-acetylgroepen in de celwand afnam aan het eind van het oogstseizoen. In de suikersamenstelling van het celwandmateriaal werden geen duidelijke verschillen waargenomen. Voor verdere studie werden fracties geïsoleerd uit het celwandmateriaal van groene (onrijpe) en paarse (rijpe) olijven door gebruik te maken van specifieke extractiemiddelen. De pectinerijke en hemicelluloserijke fracties die op deze manier werden verkregen, zijn vergeleken op basis van opbrengst, suikersamenstelling en molecuulgewichtsverdeling. Uit de analyses bleek dat de oplosbaarheid van pectine in buffer toenam van 40% van het totaal geëxtraheerde pectine voor de groene olijven tot 60% van het totaal geëxtraheerde pectine voor de paarse olijven. De suikersamenstelling en de molecuulgewichtsverdeling van de pectinerijke fracties veranderden nauwelijks tijdens het rijpen van de vrucht. Ook kwamen er geen duidelijke verschillen naar voren tussen de twee rijpingsstadia met betrekking tot de opbrengst, de suikersamenstelling en de molecuulgewichtsverdeling van de hemicelluloserijke fracties.

Een meer gedetailleerde studie van de hemicelluloserijke fracties is beschreven in hoofdstuk 3. De hemicelluloserijke fracties werden gefractioneerd met behulp van anionenuitwisselingchromatografie. Dit resulteerde in een xyloglucaanfractie en vier xylaanfracties. Deze fracties werden gekarakteriseerd op basis van hun suiker- en bindingstypesamenstelling en door gebruik te maken van specifieke enzymen. Uit de resultaten bleek dat het xyloglucaan in olijven een structuur heeft met een specifiek substitutiepatroon, dat niet algemeen voorkomt in de celwanden van planten. Xyloglucaan uit olijven heeft een hoofdketen van glucose eenheden, waarvan drie van de vier eenheden vertakt zijn met een xylose eenheid (XXXG-blokken). Aan deze xylose eenheden kan zowel een arabinose als een galactose

eenheid gebonden zijn. Een xylose eenheid met daaraan gebonden een arabinose eenheid is een belangrijk kenmerk van xyloglucanen, die een structuur hebben bestaande uit XXGG-blokken en geïsoleerd zijn uit planten van de nachtschadenfamilie. Echter, een arabinose eenheid gebonden aan een xylose eenheid is nog niet eerder aangetoond voor andere dicotyle planten, die over het algemeen een structuur hebben bestaande uit XXXG-blokken. De xylanen in de olijf bleken laag vertakt te zijn. Minder dan 10% van de xylose eenheden was enkelvoudig gesubstitueerd met hoofdzakelijk 4-*O*-methylglucuronzuur eenheden.

In hoofdstuk 4 wordt de zuivering en karakterisering van twee arabinose bevattende xyloglucaanoligosachariden beschreven. Xyloglucaan werd geïncubeerd met het enzym endo-glucanase, waarna de xyloglucaanoligomeren werden gezuiverd door gebruik te maken van een combinatie van gel-permeatiechromatografie en anionenuitwisselingchromatografie. De twee oligosachariden die het meest aanwezig waren in het incubatiemengsel werden vervolgens gekarakteriseerd met behulp van NMR spectroscopie en massa spectrometrie. Uit de resultaten bleek dat het xyloglucaan in olijven hoofdzakelijk is opgebouwd uit twee nieuwe oligosachariden: XXSG en XLSG. Beide xyloglucaanoligomeren hebben drie van de vier glucose eenheden vertakt met een xylose eenheid. De letters 'S' en 'L' verwijzen naar het α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*, respectievelijk het β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment. De structuur van deze twee oligosachariden bevestigde dat het xyloglucaan in olijven een specifieke structuur heeft met α-L-Ara*f*-(1→2)-α-D-Xyl*p* zijketens zoals werd gesuggereerd in hoofdstuk 3. Door het celwandmateriaal van olijven te incuberen met pectineafbrekende enzymen in combinatie met endo-glucanases werd meer informatie verkregen over de aanwezigheid van *O*-acetylgroepen op xyloglucaan. Uit de analyse van de vrijgekomen oligosachariden bleek dat de arabinose eenheden van de oligosachariden XXSG en XLSG gesubstitueerd kunnen zijn met één of twee *O*-acetylgroepen.

Zoals beschreven in de eerste alinea, kan in de olijfolie-industrie gebruik worden gemaakt van commerciële enzympreparaten om een hogere opbrengst en een betere kwaliteit van de olijfolie te verkrijgen. Om meer inzicht te krijgen in de mechanismen waardoor het gebruik van enzympreparaten leidt tot een hogere opbrengst en een betere kwaliteit van de olie, werd het effect van deze preparaten op de concentratie van fenolen en de structuur van de polysachariden bestudeerd in hoofdstuk 5. Uit de analyse van de olijfolie bleek dat door het gebruik van enzympreparaten de hoeveelheid fenolen in de olie toenam. Voornamelijk het gehalte van de secoiridoid derivaten, zoals de dialdehyde vorm van elenoolzuur gekoppeld aan 3,4 dihydroxyfenylethanol (3,4-DHPEA-EDA) en een isomeer van het oleuropein aglycon (3,4-DHPEA-EA) nam sterk toe. Deze twee componenten hebben beide goede anti-oxidatieve eigenschappen. Verder bleek uit de analyse van de polysachariden in de olijvenpasta dat door het gebruik van enzympreparaten de oplosbaarheid van pectine in buffer toenam en de molecuulgewichtsverdeling van de pectines veranderde. Ook het gehalte aan uronzuren nam sterk toe in de met buffer geëxtraheerde pectine fractie. Uit de analyse van de polysachariden in het vegetatiewater, een bijproduct van de olijfoliewinning, bleek dat door het gebruik van enzympreparaten ook een relatief hoger gehalte aan uronzuren in het vegetatiewater aanwezig was.

Het effect van het gebruik van enzympreparaten op de structuur van pectine in de olijf is verder bestudeerd in hoofdstuk 6. Vier pectinerijke fracties werden geïsoleerd door het

celwandmateriaal te extraheren met achtereenvolgens een koude en warme buffer, een buffer met daarin chelatoren en een verdunde loog oplossing. Uit de analyse van de fracties bleek, dat door het gebruik van enzympreparaten slechts een klein deel van de polysachariden in de celwand gemodificeerd werd. Ongeveer 8-10% van de polysachariden in de celwand werd geëxtraheerd met de koude en warme buffer en was door het gebruik van enzympreparaten tijdens de winning van olijfolie van structuur veranderd. De structuur van de arabinoserijke pectines, die geëxtraheerd waren met buffer met daarin chelatoren en de verdunde loog oplossing was nauwelijks veranderd. De structuurveranderingen van de pectines geëxtraheerd met koude en warme buffer waren als volgt: een afname van het aantal methyl esters, een verandering van de molecuulgewichtsverdeling en een afbraak van de (1→4)-gebonden galactaanketens. De structuur van de arabinaanketens van de pectines geëxtraheerd met koude en warme buffer was niet veranderd door het gebruik van enzympreparaten. Daarnaast is in hoofdstuk 6 ook de structuur van de pectines bestudeerd, die aanwezig waren in het vegetatiewater. Door het gebruik van enzymen tijdens de winning van de olie veranderde de molecuulgewichtsverdeling van de polysachariden in het vegetatiewater en nam het aantal methyl esters af. Daarnaast bleken de (1→4)-gebonden galactaanketens afgebroken te zijn. Deze veranderingen in de structuur kwamen overeen met de structuurveranderingen van de pectines geëxtraheerd met buffer uit het celwandmateriaal.

Ten slotte wordt in hoofdstuk 7 een overzicht gegeven van de belangrijkste resultaten van het onderzoek, dat beschreven is in dit proefschrift. Op basis van de resultaten uit de voorgaande hoofdstukken worden de structuurkenmerken van de polysachariden in de celwand van de olijf beschreven. Daarnaast worden in dit hoofdstuk de veranderingen in de structuur van de polysachariden beschreven gerelateerd aan het rijpen van de vrucht en het gebruik van enzympreparaten tijdens de winning van de olijfolie.

Nawoord

Het proefschrift is geschreven en de afronding van mijn promotieonderzoek is nu echt in zicht! Echter, zonder de hulp van een aantal mensen was dit zeker niet gelukt.

In de eerste plaats wil ik mijn promotor Fons Voragen en co-promotor Henk Schols bedanken voor hun begeleiding. Fons, bedankt voor je interesse in het project, het nuttige commentaar op mijn manuscripten en het vertrouwen dat ik het wel zou redden. Henk, bedankt voor je enthousiaste begeleiding en de enorme betrokkenheid bij mijn onderzoek. Voor vragen en hulp kon ik altijd bij je terecht en dat heb ik zeer gewaardeerd. Gerrit Beldman, met name in het begin van mijn onderzoek ben je betrokken geweest bij mijn project. De opbouwende kritiek en de goede suggesties die je plaatste bij de eerste verslagen die ik schreef over mijn onderzoek en je belangstelling voor mijn project nadien heb ik als zeer waardevol ervaren.

I would like to thank Professor GianFrancesco Montedoro and Dr. Maurizio Servili for the opportunity they gave me to work at their laboratory at the University of Perugia, Italy. I have enjoyed the visit to your lab and have learned a lot from you about the processing of olive fruit. Thanks again for all your kindness and efforts to make my stay in Italy a pleasant one.

I also acknowledge Dr. Panayotis Rodis, Niki Proxenia, Dr. Maura Baldioli, Dr. Evangelia Stefanoydaki, Dr. Dimitris Iconomou, Dr. Kurt Dörreich, Hans Obergföll, Dr. José Alba Mendoza and Dr. Rafael Guillén for co-operation within the project of the EC. I have really enjoyed the annual meetings and the trips we made and I wish you all good luck in the future.

Jean-Paul Vincken, bedankt voor alle hulp bij het interpreteren van de resultaten van mijn experimenten. Waar een korte vraag over een HPAEC-diagram van xyloglucaanoligomeren niet toe kan leiden! Jouw enthousiasme en verhelderende blik op de resultaten waren zeer waardevol. Bedankt daarvoor! Will York, I have really appreciated your help with the elucidation of the structures of the xyloglucan oligomers by NMR spectroscopy and mass spectrometry and the constructive criticism you gave on the joined manuscript. I have enjoyed working with you! Gert-Jan van Alebeek, ook jij natuurlijk bedankt voor alle hulp bij de analyses van de xyloglucaanoligomeren.

Toos Kroef, bedankt voor je hulp bij de adsorptie-experimenten voor het EG-project. Ik vond het erg leuk om met je samen te werken.

Ook de doctoraalstudenten Marit van Beest, Liesbeth Fung A Foek en Mandy Korver wil ik bedanken voor hun enthousiaste bijdrage aan het onderzoek.

Edwin Bakx, Margaret Bosveld en Jan Cozijnsen, jullie zorgden ervoor dat de HPLC en GLC apparatuur altijd in prima staat verkeerden voor de vele analyses. Bedankt daarvoor!

En dan natuurlijk mijn kamergenoten, Miranda Huisman en Willemiek van Oostveen. Vanaf het begin van mijn promotieonderzoek hebben we naast een kamer, "van alles en nog wat" gedeeld, zowel op het wetenschappelijk als het niet-wetenschappelijk vlak. Bedankt voor de leuke en ontzettend gezellige tijd!

Ook alle andere collega's die ik hier nog niet heb genoemd wil ik bedanken. Ik kijk met heel veel plezier terug op de bijna vijf jaar onderzoek bij de vakgroep. Bedankt voor alle gezelligheid tijdens het werk!

Verder is dit ook de plaats om alle mensen in mijn naaste omgeving te bedanken voor hun interesse en belangstelling in mijn onderzoek. Het was jullie misschien niet altijd duidelijk wat mij zo fascineerde aan olijven, maar ziehier het resultaat.

Pap en mam, bedankt voor alle vrijheid en het vertrouwen dat jullie mij altijd gegeven hebben om mijn eigen weg te kiezen. Jullie interesse en betrokkenheid bij mijn promotie-onderzoek waren erg belangrijk voor me.

Ten slotte, lieve Marco, jij hebt me altijd gestimuleerd en gemotiveerd om dit onderzoek af te ronden en jij verdiend daarom zeker een speciale vermelding! Bedankt voor alles!

Esther

Curriculum vitae

Esther Vierhuis werd geboren op 27 december 1971 te Zwolle. Na het behalen van haar VWO diploma in 1990 aan het Lambert Franckens College te Elburg, begon zij in september van dat jaar met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. In het kader van deze studie deed ze tijdens de doctoraalfase afstudeeronderzoeken bij de leerstoelgroepen Levensmiddelenchemie (dr. H.A. Schols; prof. dr. ir. A.G.J. Voragen) en Levensmiddelenmicrobiologie (dr. A. Verheul; dr. T. Abee). Daarnaast liep ze stage bij het Institut d'Oenologie in Bordeaux, Frankrijk (dr. A. Lonvaud-Funel). In januari 1996 behaalde zij het doctoraal diploma.

Van januari 1996 tot januari 2000 deed zij een promotieonderzoek bij de leerstoelgroep Levensmiddelenchemie aan de Wageningen Universiteit, onder begeleiding van prof. dr. ir. A.G.J. Voragen, dr. H.A. Schols en dr. G. Beldman. Het onderzoek dat in deze periode werd uitgevoerd staat beschreven in dit proefschrift.

Sinds november 2000 is zij werkzaam als statistisch-wetenschappelijk onderzoeker bij het Centraal Bureau voor de Statistiek te Voorburg.

Addendum

The research described in this thesis was performed at the Laboratory of Food Chemistry at the Department of Agrotechnology and Food Sciences of the Wageningen University, The Netherlands, and was financially supported by the European Community (AIR3-CT93-1355). The research was part of the research programme of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Cover: the picture on the cover was taken from "The Concise Encyclopedia of Foods and Nutrition" from Ensminger, A.H., Ensminger, M.E., Konlande, J.E. & Robson, J.R.K. Boca Raton: CRC Press (1995) p. 801.