Temporal sequence of cell wall disassembly in boysenberry, raspberry and blueberry fruit

by

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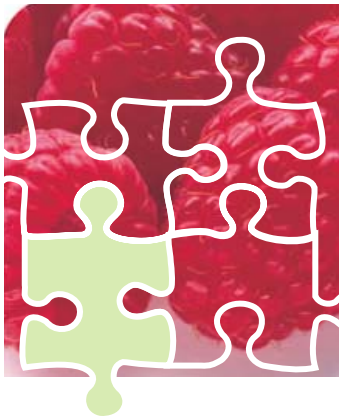
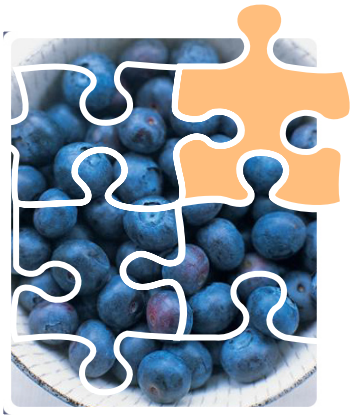
TEMPORAL SEQUENCE OF CELL WALL DISASSEMBLY IN BOYSENBERRY, RASPBERRY AND BLUEBERRY FRUIT


…los pensamientos […] reciben vida y calor de los ideales, sin cuya influencia yacerían inertes y los siglos serían mudos. Los hechos son puntos de partida: los ideales son faros luminosos que de trecho en trecho alumbran la ruta.

José Ingenieros. El Hombre Mediocre.

DEDICATION

This thesis is dedicated the love of my life Victoria without whom none of this would have been even possible. It is also dedicated to my family for their love and encouragement throughout the extended period physically far from them. In memory of my cousin Javi.

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CONTENTS

I. LIST OF FIGURES

Figure 7: Proposed model for cell wall changes accompanying boysenberry fruit development and softening.

Figure 8: Changes in raspberry fruit weight (A), firmness (B), cell wall yield (AIR) (C) during development. The standard deviation is shown.

Figure 9: Changes in raspberry total uronic acids (A) neutral sugars (B) and monosaccharide composition (C), during development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color. The standard deviation is shown.

Figure 10: Changes in uronic acids (left panels) and neutral sugars (right panels) in the water (top panel), CDTA (middle panels) and Na_2CO_3 soluble fraction (lower panels) throughout raspberry fruit development. 25 %R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown.

Figure 11: Neutral sugar composition (mol %) of raspberry water (A) and Na_2CO_3 (B) soluble fraction throughout fruit development. 25 %R (25 % surface red color); 75 %R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown.

Figure 12: Size exclusion chromatography profiles of pectic polymers throughout raspberry fruit development. The samples were fractionated on HW65. Column fractions (2 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Vo, void volume; Vt, total volume. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction.

Figure 13: Changes in 4 % (A) and 24 % KOH (B) soluble polymers throughout raspberry fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color).The least significant difference (LSD) at P= 0.05 is shown. The standard deviation is shown.

Figure 14: Neutral sugar composition (mol %) of raspberry 4 % KOH (A) 24 % KOH (B) soluble fraction throughout fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown

Figure 15: Gel-filtration profiles of 4 % (A) and 24 % KOH (B) soluble polysaccharides derived from five developmental stages of raspberry fruit and fractionated on Sepharose CL-4B. Column fractions (1.5 mL) were assayed for neutral sugars using the the anthrone method (Yemm and Willis, 1954). Vo, void volume; Vt, total volume.

Figure 16: Proposed model for cell wall changes accompanying raspberry fruit development and softening.

Figure 17: Changes in blueberry fruit firmness (A), and alcohol insoluble residue (B) during development. The standard deviation is shown.

Figure 18: Changes in blueberry fruit uronic acids (A), neutral sugars (B), and cell wall monosaccharide composition (C) during development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

Figure 19: Changes in uronic acids (left panels) and neutral sugars (right panels) in the water (top panel), CDTA (middle panels) and Na_2CO_3 soluble fraction (lower panels) throughout blueberry fruit development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

Figure 20: Neutral sugar composition (mol %) of blueberry water (A) CDTA (B) and Na_2CO_3 (C) soluble fraction throughout fruit development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

Figure 21: Size exclusion chromatography profiles from pectins throughout blueberry fruit development, fractionated on HW65. Column fractions (2 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Vo, void volume; Vt, total volume. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction.

Figure 22: Changes cross-linking glycans throughout blueberry fruit development. A: 4 % KOH soluble fraction. B: 24 % KOH soluble fraction. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The least significant difference (LSD) at P= 0.05 is shown.

Figure 23: Neutral sugar composition (mol %) of raspberry 4 % KOH (top panel) 24 % KOH soluble fraction throughout fruit development. G: large green; 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color); BR: Blue ripe. The standard deviation is shown.

Figure 24: Gel-filtration profiles of 4 % (A) and 24 % (B) KOH-soluble polysaccharides derived from five developmental stages of blueberry fruit and fractionated on Sepharose CL-4B. Column fractions (1.5 mL) were assayed for neutral sugars using the phenol-sulfuric acid method (Dubois et al., 1956). Vo, void volume; Vt, total volume.

Figure 25: Proposed model for cell wall changes accompanying blueberry fruit development and softening. 25 % B (25 % surface blue color); 75 % R (75 % surface blue color); 100 % R (100 % surface blue color).

II. LIST OF TABLES

III. OBJECTIVES

- **To determine the modifications in cell wall structure and composition in boysenberries, raspberries and blueberries during development.**

- **To review the recent advances related to cell wall structure and metabolism and the potential implications of these findings in fruit softening control.**

- **To analyze the main strategies available to control postharvest spoilage in soft fruits.**

IV. SCHEMATIC ORGANIZATION OF THE WORK

PROBLEMS IN POSTHARVEST OF SOFT FRUITS

EXCESSIVE SOFTENING

DECAY

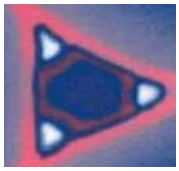
STUDY OF CELL WALL CHANGES ACOMPANYING FRUIT DEVELOPMENT

Cell wall changes accompanying <u>boysenberry</u> development

Cell wall changes accompanying <u>raspberry</u> development

Cell wall changes accompanying <u>blueberry</u> development

<u>APPENDIX I:</u> Recent findings in cell wall structure and metabolism



<u>APPENDIX II:</u> Control of postharvest spoilage in soft fruit

Abstract

Fruit growth and ripening involve a series of changes in appearance like size, shape, color and sensory attributes such as flavor, aroma texture. Excessive fruit softening is one of the main factors limiting postharvest storage of many horticultural commodities. This is particularly true in the case of 'soft fruits' such as raspberries, boysenberries blueberries and strawberries. Reduction of firm texture affects fruit quality and postharvest storage, reducing consumer acceptability, limiting transportation and, perhaps also increasing decay due to reduced strength of the cell wall, a main barrier against tissue colonization by plant pathogens. Fruit textural changes are thought to be, at least in part, a consequence of changes in the composition and architecture of the cell wall. There have been many studies analyzing changes in cell wall on different fruits showing that pectins, hemicelluloses, and possibly the amorphous regions of cellulose undergo structural modifications during fruit development and ripening (Brummell and Harpster, 2001). However, so far only a few works have been done to evaluate cell wall disassembly in other 'soft fruits' besides strawberry. The objective of this work was to analyze temporal sequence cell wall disassembly in blueberry, raspberry and boysenberry. Fruit was harvested at five different developmental stages from green to commercial maturity and the modification in the cell wall structure and composition were analyzed. Cell walls were isolated, fractionated and changes in pectin and hemicelluloses solubilization, depolymerization and monosaccharide composition were determined. The results showed that there are differences in cell wall disassembly among the fruits studied and also with other fruits that have already been studied. In the three fruits analyzed an increased solubilization of arabinose was found during development. This is coincident with previous reports in strawberries but it is distinctly different to what has been described in other fruits such as watermelon, plum, pepper, melon and tomato. In the case of boysenberry cell wall disassembly accounts for at least 3 stages: an early stage associated with cellulose loss and solubilization of cross-linking glycans, an intermediate period characterized by a large increase in pectin solubilization without depolymerization in which arabinose is lost and a final stage characterized mainly by a reduction of galactose and a dramatic increase in pectin depolymerization. Raspberry showed a similar pattern in general terms being also much higher the changes observed in the pectic matrix than in the cross-linking glycans. The main difference was that in this case the highest pectin solubilization and depolymerization occurred in late ripening. The changes

occurring during blueberry cell wall disassembly are quite different from the findings in boysenberries and raspberries and also from other fruit models studied to date. At early stages of development the main changes include increased pectin and hemicelluloses solubilization but without depolymerization and a late stage characterized mainly by an increased arabinose solubilization and a downshift in hemicelluloses but not pectin molecular size. From a biotechnological perspective enzymes involved in pectin matrix disassembly seem to be the better candidates to affect boysenberry and raspberry fruit late softening by genetic intervention. In the case of blueberries genes associated with hemicellulose metabolism such as endoglucanases (EGases) and/or expansins (Exps) seem to be better targets. Because all three fruit, raspberry, blueberry, boysenberry pectins are enriched in arabinose relative to galactose, in contrast to tomato and melon pectins, strategies considering α-ara as a potential target to reduce late softening by directly affecting pectin solubilization and indirectly pectin depolymerization and or cross linking glycans disassembly should also consider the potential negative side effects of altering arabinan metabolism in a fruit in which enlargement occurs simultaneously with ripening.

1.INTRODUCTION

1.1. 'Soft fruits'

The term 'soft fruit' groups several different commodities such as strawberries, raspberries, blueberries blackberries and their hybrids (Manning, 1993). This grouping does not have a botanical basis and consequently includes species from different families and with very diverse fruit structures. However, these fruits have several characteristics that make the grouping useful at least from a postharvest technological perspective. They are characterized by a high metabolic rate and are very short shelf life (Kader, 1992). Fresh 'soft fruits' are highly accepted by consumers, but their high perishability determines that a significant proportion of the production is destined for processing including jams, yoghurts, sauces juices and other products. Most 'soft fruits' typically have blue or red color due to the accumulation of anthocyanins. Although anthocyanins are more or less ubiquitous in horticultural crops, soft fruits contain high levels of antocyanins (Dugo et al., 2001). Besides their role determining the esthetic properties of the fruits anthocyanins are antioxidants (Wang et al., 1996) and several works have suggested that soft fruit consumption could have beneficial effects in the prevention of several chronic and degenerative diseases associated with oxidative damage (Heinonen et al., 1998; Meydani, 2001). In addition soft fruits are in general rich in ascorbic acid (Lee and Kader, 2000) and phenolic acids (Zadernowski et al., 2005) which have also been associated with disease prevention (Cozzi et al., 1997). Blueberry and raspberry fruit rank high in antioxidant activity among fresh fruits (Wu et al., 2004). Tau et al. (2005) showed that blueberry supplementation was beneficial in both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior. It has been reported that low dietary intake of fruits and vegetables doubles the risk of most types of cancer and also markedly increases the risk of heart disease and cataracts as compared to high intake (Ames et al., 1993). This has led in the last year to a much higher concern of consumers about food nutritional value and has probably contributed to the increased popularity of 'soft fruits' in the last years. In terms of production strawberry is the most important crop within the group. Its world production is estimated in 3.5 million M Tons (Table 1; FAOSTAT, 2006) although it is thought this value could be underestimated due to the uncertainty of China production volumes. The volume of production of other berries such as blueberries, raspberries and other berries is low relative to total soft fruits production (Table 1) but it has rapidly increased in the last ten years (50 % and 80 % respectively).

Table 1: Strawberry, blueberry and raspberry world production (Thousand M Tons) in the last 10 years (Adapted from FAOSTAT, 2006).

Product/Year	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Strawberry	2,752	2,760	2,867	3,186	3,299	3,204	3,215	3,335	3,546	3,530
Blueberry	137	150	137	211	237	237	222	245	241	241
Raspberry	321	319	355	400	408	432	471	442	485	483
Other berries	510	510	573	463	618	630	626	642	647	652
TOTAL	**3,620**	**3,739**	**3,932**	**4,260**	**4,562**	**4,503**	**4,534**	**4,664**	**4,919**	**4,906**

1.3. Boysenberry, raspberry and blueberry

Blackberries and Raspberries, often termed "Brambles", are a diverse group of species and hybrids in the genus *Rubus* (Rieger, 2005). They are members of the *Rosaceae* family, closely related to strawberry in the subfamily *Rosoideae*. *Rubus* is a highly diverse genus of flowering plants in the world, with 12 subgenera, some of which group hundreds of species (Klemola, 2001). Red raspberries belong to the species *Rubus idaeus* while boysenberries (*Rubus hybrid*) are hybrids between raspberries and blackberries (Bushman et al., 2004). They were discovered by Ralf Boysen in California in 1920. In all brambles, the fruit is an aggregate structure composed of drupelets with a fleshy mesocarp and a lignified endocarp containing inside the true seed held together by a receptacle. Each drupelet is supplied with assimilate and water by a separate vascular supply and contains a single seed (Ianetta et al., 1999). A morphological difference between raspberry and boysenberry is that the drupelets of boysenberry lack the fine hairs on the fruit surface that raspberries have, and appear shiny and smooth. Another difference is that boysenberries retain the receptacle at harvest while it abscised in the case of raspberry. Fruit development occurs rapidly, taking only 30-50 days for most raspberries, and 40-60 days for boysenberries. Most ‘soft fruits’ show low ethylene production and sensitivity and are considered non-climacteric fruit, although there has been some debate about their correct categorization (Lipe, 1978; Walsh et al., 1983; Burdon and Sexton, 1993). In terms of growth pattern there are also significant differences between soft fruits and other commodities. In many fruits ripening is triggered once

growth has finished but boysenberries and raspberries continue to enlarge simultaneously with the ripening process. The fruits are mature when they have completely developed their characteristic color, and are easily detached from the plant. These 2 characteristics are commonly used maturity indices. All brambles require frequent pickings over a period of a few weeks. If the berries are picked too soon, berry size will be reduced. Furthermore since sugars accumulate until late ripening, early harvests will significantly reduce flavor (Mitcham et al., 2002). To harvest at peak quality, berries should be picked every 2–3 days. Harvesting should be done when the fruits are not wet to reduce decay which is one of the main problems limiting postharvest life. The picking should be done gently and the berries should be placed into shallow containers to minimize physical damage. Lack of storage potential is one of the main barriers to fresh fruit production on a large commercial scale. Raspberries and blackberries have an extremely high softening rate and in order to maintain firmness and reduce moisture loss, fungal growth and fruit breakdown they should be cooled within one hour after picking. After cooling storage at 0 ºC and 95 % relative humidity is recommended (Mitcham et al., 2002). However, even under these conditions the fruit is highly perishable; lasting only 2-5 days and most fruit should be destined for processing.

Blueberries are members of the *Ericaceae* family, genus *Vaccinium* (Kron et al., 2002). Demand for blueberries has increased in recent years, and fresh-market prices have been relatively stable. To meet growing consumer demand, commercial blueberry acreage in the US increased more than 60 percent in the past 15 years (Demchak et al., 2001). Production has more than doubled since the late 1970s. Major increases have occurred in Michigan (where more than 40 percent of the commercial acreage is located) and in the southeastern United States (Demchak et al., 2001). Interest in blueberry as a crop has also increased in other countries and the western states of the United States. The fruit is an epigynous or "false" berry (Rieger, 2005). This means that the fruit is berry-like, but derived from an inferior ovary, unlike true berries that derive from superior ovaries. The "button" on the far end of the fruit is actually the calyx scar. Fruit development occurs for about 2 to 3 months after bloom, depending on cultivar, weather, and plant vigor. They have a low ethylene production (0.1-1.0 µl $kg^{-1}hr^{-1}$ at 5 °C) (Mitcham et al., 2002). Fruit size continues to increase after fruit turns blue, due mainly to water uptake. Blue color is currently the most often used indicator of maturity (Mitcham et al., 2002). Berries turn from green to pink, and then gradually to a complete blue color, at which time they are ripe. Ripe berries will remain attached

for several days or weeks, and sugars continue to accumulate. All berries should be harvested near ripe as eating quality does not improve after harvest. Careful handling to reduce physical damage is extremely important. Furthermore, diseased or wounded berries should be removed in order to avoid cross contamination. Diseases are the greatest cause of postharvest losses in berries. In order to reduce them it is crucial to rapidly cool the fruit after harvest. *Botrytis* rot is also the main cause of decay. The longest storage life is attained by storing the fruit 0 ºC and 90-95 % relative humidity (Mitcham et al., 2002). Under these conditions the shelf life is still fairly short (1-2 weeks).

1.6. Firmness loss in 'soft fruits'

Controlled softening is desirable in order to attain consumption maturity, but excessive loss of firmness is a one of the main factors limiting marketing, transportation and retail of fresh 'soft fruits'. Fruit firmness is affected by several factors. For example, it may change due to altered hydrostatic pressure (turgor) within fruit cells (Shackel et al., 1991; King et al., 2000; Salentijn et al., 2003). Membrane damage and dehydration and mesocarp cell enlargement could be involved in textural changes in some fruits (Sexton et al., 1997; Waldron et al., 2003). However, fruit textural changes are thought to be, at least in part, a consequence of changes in the composition and architecture of the cell wall (Brummell and Harpster, 2001). The second main factor that makes soft fruit postharvest management difficult is the high susceptibility to decay. Postharvest diseases can be caused by different fungi such as *Botrytis cinerea*, *Rhizopus stolonifer*, *Mucor mucedo* and *Colletotrichum acutatum*. The last 2 organisms are less common while *Rhizopus* rot could be easily controlled by storing the fruit at 0 ºC since the fungus can not develop at this temperature. However, this is not the case for *Botrytis cinerea* which can continue to grow and cause disease even under low temperature storage. Several studies have been done in order to characterize the infection caused by *Botrytis* in plant tissues (Elad, 2004). Interestingly it has been shown that the fungus produces several cell wall degrading enzymes at early stages of colonization and that some *Botrytis* polygalacturonases (PGs) are virulence factors. For instance mutant *Botrytis* strains lacking *Bcpg1* were shown to be less virulent than wild type *Botrytis* (ten Have et al., 1998). Furthermore, reduced susceptibility to *Botrytis* was observed in plants over-expressing a polygalacturonase inhibiting protein (PGIP) (Powell et al., 2000; Agüero et al., 2005). Consequently both excessive softening and

postharvest decay, the two main problems limiting ‘soft fruit’ postharvest life seem to be associated with cell wall modifications. While extensive degradation of cell wall polymers would lead to an extensive softening of the fruit tissues these changes will perhaps also contribute to increase decay susceptibility by reducing the strength of the cell wall, a main barrier against tissue colonization by plant pathogens (Vorwerk et al., 2004). Plant cell walls are highly complex, dynamic and organized structures composed of polysaccharides, proteins and phenolic compounds, as well as some ions (Carpita and Gibeaut, 1993). The pattern and biochemical basis of this "organized disorganization" varies depending on the species or even the variety considered. Many studies analyzing changes in cell wall of different fruits have reported that pectins, hemicelluloses, and possibly the amorphous regions of cellulose undergo structural modifications during fruit development and ripening (Brummell and Harpster, 2001). The tomato has been used as a model for cell wall metabolism during ripening in part because of its importance as a food crop species, its diploid inheritance, and its ease of seed and clonal propagation (Moore et al., 2002). Furthermore, the efficient sexual hybridization, a relatively short generation period, and the availability of several ripening mutants exhibiting dramatically reduced ripening-associated modifications of the cell wall and arrested softening have contributed to its use as a model system and the subsequent generation of an extensive literature describing tomato softening (Brummell and Harpster, 2001; Steele et al., 1997; Moore et al., 2002). However it is unclear to which extent our understanding of cell wall composition and disassembly in these model fruits could be extrapolated to totally different commodities such as ‘soft fruits’. A main characteristic of these fruit is that differently to tomato fruit, their enlargement period continues throughout development. Consequently a fine and concerted balance between cell wall synthesis and degradation generating a structure that can support the growth of individual cells (Cosgrove, 2002), and then ultimately permit the dramatic disassembly of the structure accompanying the ripening process (Rose et al., 2004). Several studies have been done to understand cell wall metabolism in some ‘soft fruits’ such as strawberries (Neal, 1965; Barnes and Patchett, 1976; Knee et al., 1977; Huber, 1984 Koh and Melton 2002, Rosli et al., 2004). Although strawberry is usually grouped operationally with raspberry, boysenberry and blueberry, and this classification could be pragmatically useful, the fruits are totally different in terms of their botanical origin, morphology and probably many biochemical and physiological changes occurring during their development. For instance, the only study

looking at changes in the cell wall structure in ripe and unripe blackberry shows that there are differences in wall thickness, viscosity and swelling compared to strawberry (Redgwell et al., 1997). Even if we assume that most biochemical changes observed in strawberry fruit cell wall during ripening could be at least in general terms extrapolated to other soft fruits the results reported to date in the literature are quite contradictory. For instance, Huber (1984) reported that hemicelluloses of strawberry fruit are depolymerized during ripening while only small changes are observed in pectin size. Other authors, analyzing ripening-related cell wall changes in three strawberry varieties found that pectin size was reduced, but only slight depolymerization was observed in hemicelluloses (Rosli et al., 2004). Furthermore, Jiménez Bermúdez et al. (2002) reported reduced softening and pectin depolymerization in strawberries with antisense expression of the pectate lyase gene, encoding a protein involved with pectin breakdown.

Various studies have reported the compositional changes of boysenberry, raspberry and blueberry during development and ripening (Given et al., 1986; Monro and Lee, 1987; Perkins-Veazie et al., 2000; Porter, 1988; Plowman, 1991), but very few have looked at the cell wall metabolism (Redgwell et al., 1997). Thus, studies that clarify the differences reported for berry fruit cell wall degradation would be of fundamental interest, in terms of increasing our understanding of the process of cell wall disassembly in these fruits. From a technological perspective unraveling the mechanisms underlying cell wall metabolism in these fruits would also be of great value since softening can be extensive and extremely detrimental to quality maintenance. Furthermore this information could be useful as a guidance to select candidate genes for further studies aimed at trying to reduce softening and decay susceptibility by genetic intervention. In the present work we analyze the changes in cell wall structure and composition and cell wall degrading enzymes in boysenberry, raspberry and blueberry and a model for the temporal sequence of cell wall disassembly in these fruits is discussed.

2. MATERIALS AND METHODS

2.1. Plant material

Boysenberries raspberries and blueberries were harvested at five different developmental stages:

For each fruit the ripening stages defined were the following:

- **Boysenberries:**


Green (G)


25 % red (25 % R)


75 % red (75 % R)


100 % red (100 % R)


Purple

- **Raspberries:**


Green (G)


25 % red (25 % R)


75 % red (75 % R)


100 % red (100 % R)


Red ripe

- **Blueberries:**


Green (G)


25 % blue (25 % B)


75 % blue (75 % R)


100 % blue (100 % R)


Blue ripe

The fruit was taken to the laboratory and immediately processed or frozen and stored at –20 °C until use.

2.2. Fruit weight and water content

One hundred individual fruits were weighed at each developmental stage. For water content measurements, approximately 20 g of fruit were crushed and transferred to a weighed beaker. Samples were then held in a 70 °C oven until no changes in weight were recorded. Fruit water content was expressed as g of water per 100 g of fresh fruit. Measurements were done in triplicate.

2.3. Chlorophylls and anthocyanin

Fruit tissue for every developmental stage was crushed in an Ultraturrax with 5 mL of acetone/water (80:20), stirred and then centrifuged at 9,000 x g for 10 min at 4 °C. The supernatant was used to determine the content of chlorophyll according to Costa et al. (2002). For anthocyanin measurements frozen fruit was ground in a mill and different amounts of the resultant powder depending on the developmental stage were poured into 20 mL of HCl–methanol (1 % v/v) and held at 4 °C for 10 min. The slurry was centrifuged at 9,000 x g for 10 min at 4 °C and the absorbance of the supernatant was measured at 520 nm. For both chlorophylls and anthocyanin measurements, 3 replicates were done at each developmental stage.

2.4. Firmness measurement

Firmness was measured using a texture analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with a 2 mm flat probe. Each drupelet was compressed 2 mm at a rate of 0.5 mm s^{-1} and the maximum force developed during the test was recorded. Eighty measurements were done for each stage analyzed.

2.5. Isolation of cell walls

In the case of boysenberry and raspberry the endocarp and seeds were removed from the individual drupelets and approximately 30 g of the remaining tissue (exocarp plus mesocarp) for each developmental stage was homogenized in an Ultraturrax with 75 mL of 95 % ethanol and boiled for 45 min to ensure the inactivation of enzymes and the extraction of low moecular weight solutes and to prevent autolytic activity. The insoluble material was filtered through Miracloth (Calbiochem) and sequentially washed with 150 mL of boiling ethanol, 150 mL of chloroform:methanol (1:1 v/v), and 150 mL of acetone and dried at 25 °C, yielding the crude cell wall extract (alcohol insoluble residue, AIR). After this, the residue was dried overnight at 37 °C and weighed. Results were expressed as mg AIR per g of fresh fruit. The same procedure was followed in the case of blueberry but in this case the whole fruit was used for the cell wall preparations.

2.6. Cell wall solubilization, uronic acids (UA) and neutral sugar (NS) measurements

Three mg of AIR were solubilized by adding 0.5 mL of 36 N H_2SO_4 on an ice bath and stirred for 3 min. The operation was repeated three times. After that 0.5 mL of water was slowly added and the samples were stirred for 10 min. The operation was also repeated three times. The samples were then taken to 10 mL and were assayed for total UA and NS. UA were measured according to Blumenkrantz and Asboe-Hansen (1973). A_{520} readings were evaluated based on a galacturonic acid standard curve. Three independent samples were analyzed for each developmental stage, measurements were done in duplicate and results were expressed as μg of galacturonic acid per mg of AIR. Total sugars were measured by the anthrone method (Yemm and Willis, 1954). A_{620} readings were evaluated based on a glucose standard curve. Measurements for each independent extraction were done in triplicate and were expressed as μg of glucose per mg of AIR.

2.7. Cell wall fractionation

Fractions of different cell wall components were obtained by sequential chemical extraction of the cell wall material (AIR). Approximately 200 mg of AIR residue from each sample was suspended in 15 mL of water and stirred at room temperature for 12 h, then centrifuged at 6,000 x g and 4 °C for 10 min. The supernatant was filtered through glass fiber filters (Whatman GF/C) and the pellet was washed with water. The filtrate and water washings were combined and designated the water-soluble fraction (WSF). The residue was then extracted with 15 mL of 50 mM CDTA, pH 6.5 and for 12 h with stirring. The slurry was centrifuged passed through fiber glass filters, as above, and the pellet was washed with CDTA. The filtrate was collected and designated CDTA-soluble fraction (CSF). The CDTA insoluble pellet was then extracted with 15 mL of 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ at 1 °C for 12 h. After filtration the extraction solution was designated Na_2CO_3-soluble fraction (NSF), it was saved and the pellet was re-extracted with 15 mL of 4 % KOH containing 0.1 % $NaBH_4$ at room temperature for 12 h, with shaking, and the extracted solution was designated as 4 % KOH SF. This fraction was filtered, as above, neutralized with glacial acetic acid, and extensively dialyzed against water. The same procedure was followed to obtain 24 % KOH soluble fraction (24 % KOH SF) by extracting the residue from the 4 % KOH SF with 15 mL of 24 % KOH containing 0.1 % $NaBH_4$. Two independent serial extraction series were

performed for each developmental stage analyzed. Samples were assayed in triplicate for NS and UA as described in section 2.6.

2.8. Size exclusion chromatography

Aliquots of WSF, CSF, NSF, 4 % KOH SF and 24 % KOH SF were dialyzed (Spectrapor, MW cut-off 8 kD) against distilled water for 1 d at 4 °C and lyophilized. Samples from the WSF, CSF and NSF were dissolved in 200 mM NH_4-acetate, pH 5.0, chromatographed on a HW65 (Tosoh Bioscience) size-exclusion column (4 x 30 cm) eluted with 200 mM NH_4-acetate, pH 5.0. Fractions (2.5 mL) were collected at a flow rate of 60 mL h^{-1} and held in a water bath (50 °C for 4 h) to volatilize part of the NH_4OH which can interfere with the UA assay. Measurements for UA and total sugars were done as described above. The size distribution of polymers in 4 % and 24 % KOH SFs was examined by fractionating the extracts on a Sepharose CL-4B column (1.0 x 90 cm) (Pharmacia) and eluting with 0.1 N NaOH. Fractions were collected and aliquots were neutralized with glacial acetic acid prior to assaying for UA and TS as described in section 2.6. The totally included and void volumes were determined with glucose and 5,000,000 MW respectively (Sigma).

2.9. GC-MS analysis

Dried samples from from the WSF, CSF, NSF, 4 % KOH SF and 24 % KOH SF or 2 mg of AIR for total cell wall analysis were hydrolyzed in 2 N trifluoroacetic acid (Albersheim et al., 1967), and converted to alditol acetates (Blakeney et al., 1983) for gas chromatographic analysis of neutral sugar composition. Aliquots of the derivatized samples were injected into a GC fitted with a 30 m x 0.25 mm DB-23 capillary column (J&W Scientific) and a mass selective detector. Temperature in the injector was 250 °C and a linear temperature gradient (initial oven temperature 160 °C, oven increase at 4 °C per min to 250 °C) was used to improve separation. The different alditol acetates were identified based on their MS spectra and also standards containing inositol, rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc) were prepared and neutral sugar amount calculated relative to an inositol internal standard.

2.10. Enzyme assays

Approximately five grams of fruit were grinded in an Ultraturrax with 10 mL of 50 mM HAc-NaAc pH 5.5 containing 10 g L^{-1} polyvinylpolypyrrolidone (PVPP), 1 mM cystein and 1 M NaCl. The homogenates were then stirred at 4 °C for 3 h and centrifuged at 10,000 x g for 20 min. The collected supernatants were dialyzed against 50 mM NaAc pH 5.5 and used for assays of enzymatic activities. For β-galactosidase (β-gal), α-arabinofuranosidase (α-ara), α-rhamnosidase (α-rha) and β-mannosidase (β-man), reaction mixtures containing 200 μL of enzyme extract, 400 μL of 50 mM HAc-NaAc buffer pH 5.5 and 400 μL of 10 mM *p*NP substrates (Sigma) were incubated at 40 °C. Two hundred microlitres aliquots were taken at intervals times, 600 μL of Na_2CO_3 were added and A_{400} was measured in order to determine the linear reaction rate of each enzyme-substrate combination. In the case of acetyl esterase (AE) the reaction mixture contained 700 μL of NaAc buffer pH 5.5, 100 μL of enzyme extract and 200 μL of 30 mM *p*NP-acetate. Samples were incubated at 40 °C and aliquots were taken at different intervals and frozen. When the time-course sampling was finished the samples were thawed, 600 μL of tris propane 0.4 M pH 7.5 were added and absorbance was immediately measured at 400 nm. For pectin methylesterase (PME) five grams of fruit were grinded in an Ultraturrax with 10 mL 1 M NaCl containing 10 g L^{-1} PVPP and 1 mM cystein. The homogenates were then stirred at 4 °C for 3 h and centrifuged at 10,000 x g for 20 min. The supernatants were used to assay the enzymatic activity. The reaction mixture contained 600 μL of 0.15 % w/v pectin (degree of esterification 70 %), 150 μL of 0.01 % bromothymol blue in 0.003 M phosphate buffer pH 7.5 100 μL of water pH 7.5 and 200 μL of enzymatic extract. A_{620} was measured. Polygalacturonase (PG) activity was measured in reaction mixtures containing 400 μL of 50 mM NaAc-HAc buffer pH 5.5, 400 μL of polygalacturonic acid 0.15 % w/v and 200 μL of enzymatic extract. The mixtures were incubated at 40 °C. At different times 200 μL aliquots were taken and 1 mL of sodium borate 1 M was added. Reducing sugars liberated were measured with 2-cyanoacetamide according to Gross (1982). Finally for endoglucanase (EGase) activity the reaction mixtures contained 400 μL of 50 mM NaAc-HAc buffer pH 5.5, 400 μL of 0.2 % (w/v) carboxymethyl-cellulose (CMC) and 200 μL of enzymatic extract. The mixtures were incubated at 40 °C. At different times 200 μL aliquots were taken and 1 mL of sodium borate 1M was added. Reducing sugars were measured as described for PG

activity. In all cases two protein extracts were prepared for each developmental stage analyzed and measurements were made in triplicate. One unit of enzymatic activity was defined as a ΔOD of 0.001 per second and per gram for PME, PG, β-Man, α-rha, α-afa and β-gal and as a ΔOD of 0.1 per gram per second for AE and EGase.

2.11. Statistical analysis

Experiments were performed according to a factorial design. Data were analyzed using ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Cell wall changes accompanying boysenberry *(Rubus hybrid)* fruit development

3.1.1. Fruit weight, pigments, water content, firmness and cell wall yield

Boysenberry fruit was harvested at five developmental stages based on pigment content. Anthocyanin levels increased dramatically towards the end of the developmental process between 100 % R and purple stages (Figure 1 B), while chlorophyll content dropped in early stages of development (Figure 1 C). Fruit weight increased throughout the studied development period, from green to purple fruit (Figure 1 A). This growth pattern is different than that found in other fruits like tomato, in which cell division and rapid cell expansion are evident until full size is reached, before the highly coordinated ripening program commences (Rose and Bennett, 1999). An increase in fruit water content was observed between the green and 25 % R stages and no further changes were found afterwards (Figure 2 A). Interestingly the ethanol-insoluble residue per gram of fresh weight (AIR g^{-1}) did not decrease between the green and 25 % R stage (Figure 2 B). This result together with the fact that water content increased significantly within these stages suggests that a net cell wall synthesis occurred in this period that was marked by no changes in cell wall yield per gram of fruit. Mitcham et al. (1989) reported that cell wall synthesis continues throughout tomato fruit ripening. Later work (Greve and Labavitch, 1991) showed increased incorporation of xylosyl and mannosyl residues into tomato hemicellulosic cell wall fractions of ripening tomato and decreased incorporation of galactosyl residues into chelator-soluble pectins (Greve and Labavitch, 1991). From the 25 % R to the purple stage the AIR g^{-1} was reduced. Although it is possible that newly synthesized wall components were also being deposited during the last half of the sampling period, this shows that total net cell wall loss was a significant process during these later stages. Finally, fruit firmness decreased approximately 50 % between each stage analyzed (Figure 2 C). Firmness measurements are affected by several factors like turgor pressure and tissue architecture. During berry development the mesocarp cells become extensively elongated and it has been suggested that this enlargement could be involved in textural changes (Sexton et al., 1997). However, it has been repeatedly shown that progressive dismantling and modification of cell wall polymers play a main role in the mechanical properties of fruit tissues (Carpita and Gibeaut 1993). Because appreciable fruit softening occurs as fruit progress from the G to 25 % R stages, a period during which cell enlargement also occurs, it is not possible to identify aspects of wall change that are linked to cell growth from those that are involved with fruit softening early in ripening. Wall polymer synthesis and assembly also are occurring in

this period, complicating our ability to identify wall breakdown events and interpret their significance and bringing to mind the possibility that incorporation of new polymers into the wall fabric could contribute to softening, as proposed by Mitcham and colleagues (1989). However, after the 25 % R stage is reached the net pattern of wall change in fruits favors breakdown, allowing some interpretation of aspects of wall polymer metabolism that may support fruit softening.

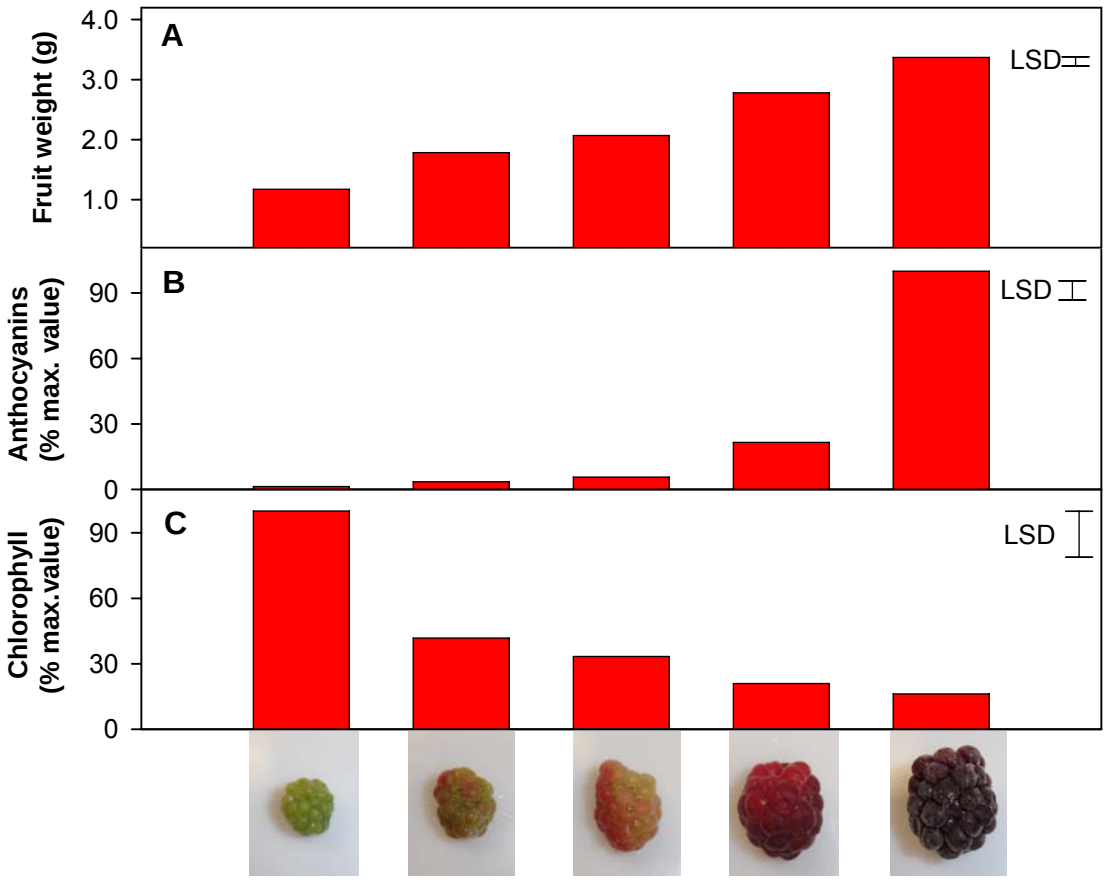


Figure 1: Changes in boysenberry fruit weight (A), anthocyanins (B), and chlorophyll content (C) during development. The least significant difference (LSD) at P = 0.05 is shown.

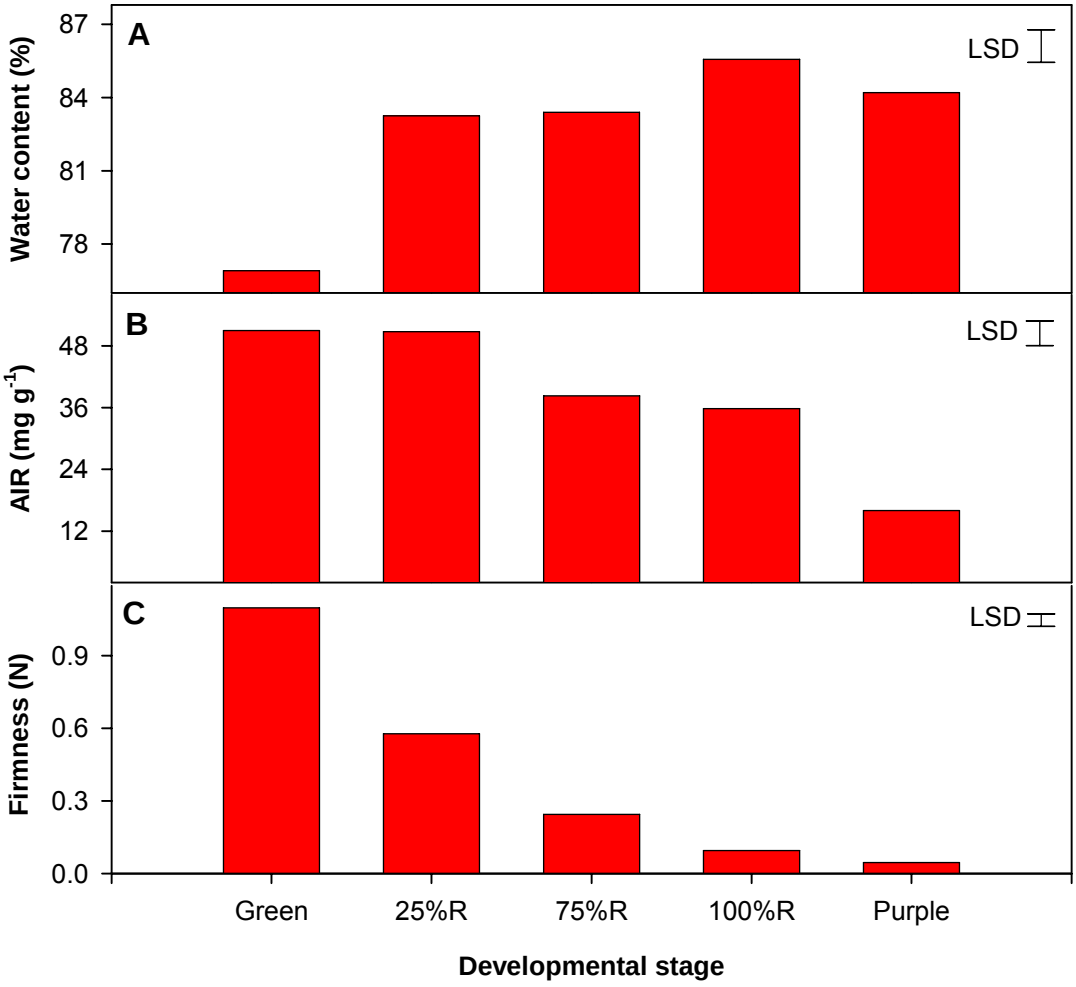


Figure 2: Changes in boysenberry fruit water content (A) cell wall yield (AIR) (B) and firmness (C), during development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The least significant difference (LSD) at P = 0.05 is shown.

3.1.2. Glycan and pectin solubilization

No significant changes in total sugar content were observed in the 4 % KOH SF during boysenberry fruit development (Figure 3 A). In contrast, the total sugar content of the 24 % KOH SF decreased markedly between the 25 % R and 75 % R stages and remained constant thereafter (Figure 3 B). The most abundant hemicellulose in fruits of dicots is xyloglucan (XyG; Rose et al., 1998). The role of XyG in cell wall architecture may be crucial during fruit softening as it can form cross-links with cellulose microfibrils producing a network of interconnecting polymers (Rose and Bennett 1999). In ripening 'Charantais' melon fruits, XyG is depolymerized early in ripening, roughly coincident with the start of fruit softening. In seedling stem tissues (e.g., pea epicotyl segments) the metabolism of cell wall-localized XyG is thought to be the wall-weakening event that allows the initiation of elongation (Labavitch and Ray, 1974). Interestingly in boysenberry the changes in this fraction were concentrated between 25 % R and 75 % R stages of development suggesting a higher role for the metabolism of cross-linking glycans like XyG in the intermediate expansive phase and a lower contribution to late ripening-associated softening where no significant changes occurred. In any case, at least in tomato fruits, XyG metabolism during both fruit expansion and softening is likely influenced by expansin (Brummell and Harpster, 2001). If this is also true for boysenberry, ideas about the relative contribution of XyG change might be better developed by determining whether the fruit has an expansin gene family and then following the expression of different expansin genes as fruits progress through the green, 25 % R and 75 % R stages.

The H_2SO_4-soluble fraction accounted for 20 % of the AIR in green fruit and showed a reduction in early stages of development (from green to 25 % R stages) remaining then without changes up to the purple stage where sugar levels were reduced again (Figure 3 C). This is different from other fruits where no significant changes in the H_2SO_4-soluble fraction have been observed (Ahmed and Labavitch, 1980). This last observation together with the fact that endo-1,4-β-D-glucanase (EGase) is generally not active on crystalline cellulose has led to the proposition that, although important in determining cell wall physical properties, cellulose changes would not have a role in the textural changes observed during fruit ripening. However, as we report in this work on boysenberries, Stewart et al. (2001) also reported extensive cellulose degradation in raspberry during ripening. So it is not possible to rule out that cellulose metabolism especially non crystalline regions could contribute, at least in part, to the textural changes

observed in boysenberry fruit in early and late stages of development. Of course, it is possible that the increase in overall cell wall weight per fruit that is seen in the green and 25 % R stages represents the addition of non-cellulosic polysaccharides to the wall. Thus, the early decrease in wall cellulose content could represent an unchanging cellulose content of walls that are increasing in their pectin and cross-linking glycan contents.

Loosely, ionically and covalently bound pectins were sequentially extracted with water, CDTA and Na_2CO_3. Water-soluble pectins increased during boysenberry fruit development. The clearest increase in these pectins was observed between 25 and 100 % R stages (Figure 4 A). A similar pattern of increase was observed for CDTA-soluble pectins (Figure 4 B). Finally, Na_2CO_3-soluble pectins, representing the greatest part of the fruit wall-associated uronic acid decreased throughout the fruit development (Figure 4C). Pectins are a major component of the plant cell wall and comprise one of the two major coextensive networks in which cellulose microfibrils are embedded (Carpita and Gibeaut, 1993). Increased solubility of pectins during fruit ripening has been shown to occur in an array of fruits (Brummell and Harpster, 2001). Reported ripening-associated increases in tomato water- and CDTA-soluble pectins often are reflected in a decrease in Na_2CO_3-soluble pectins, perhaps suggesting that metabolism of the pectins in this fraction converts them to a water- or CDTA-soluble form (Carrington et al., 1993). Pectin solubility can be affected by several factors. The neutral sugar components of branched pectins play a critical role in cell wall structure, because removal of neutral side chains has been regarded as an essential part of pectin solubilization (Dawson et al., 1992). The calcium cross-linking of HGA domains and borate ester dimerization of RG-II domains also may contribute to the integrity of the pectic network surrounding cellulose (Willats et al., 2001) and possibly also affect pectin solubilization.

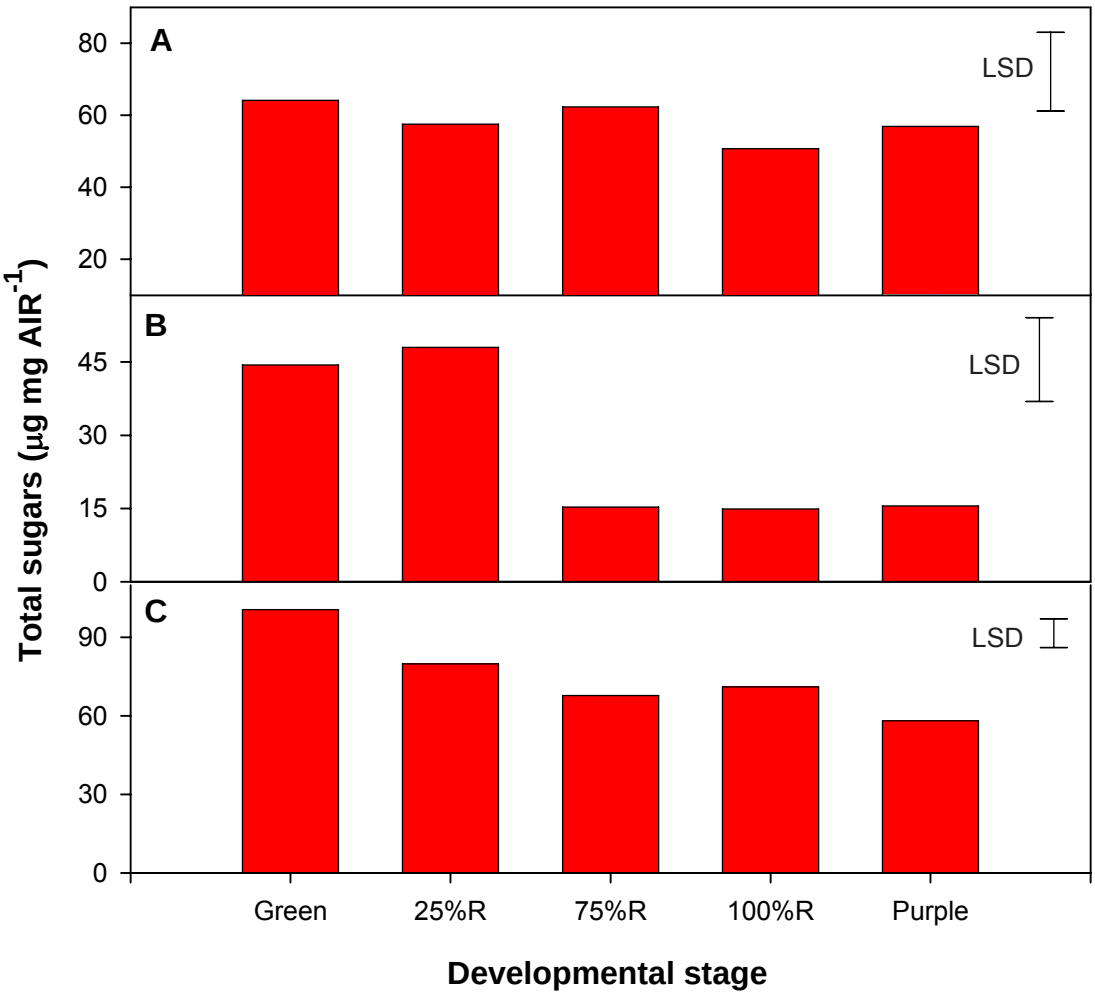


Figure 3: Changes in cellulose and cross-linking glycans throughout boysenberry fruit development. A: 4 % KOH soluble fraction. B: 24 % KOH soluble fraction. C: H_2SO_4 soluble fraction. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The least significant difference (LSD) at P= 0.05 is shown.

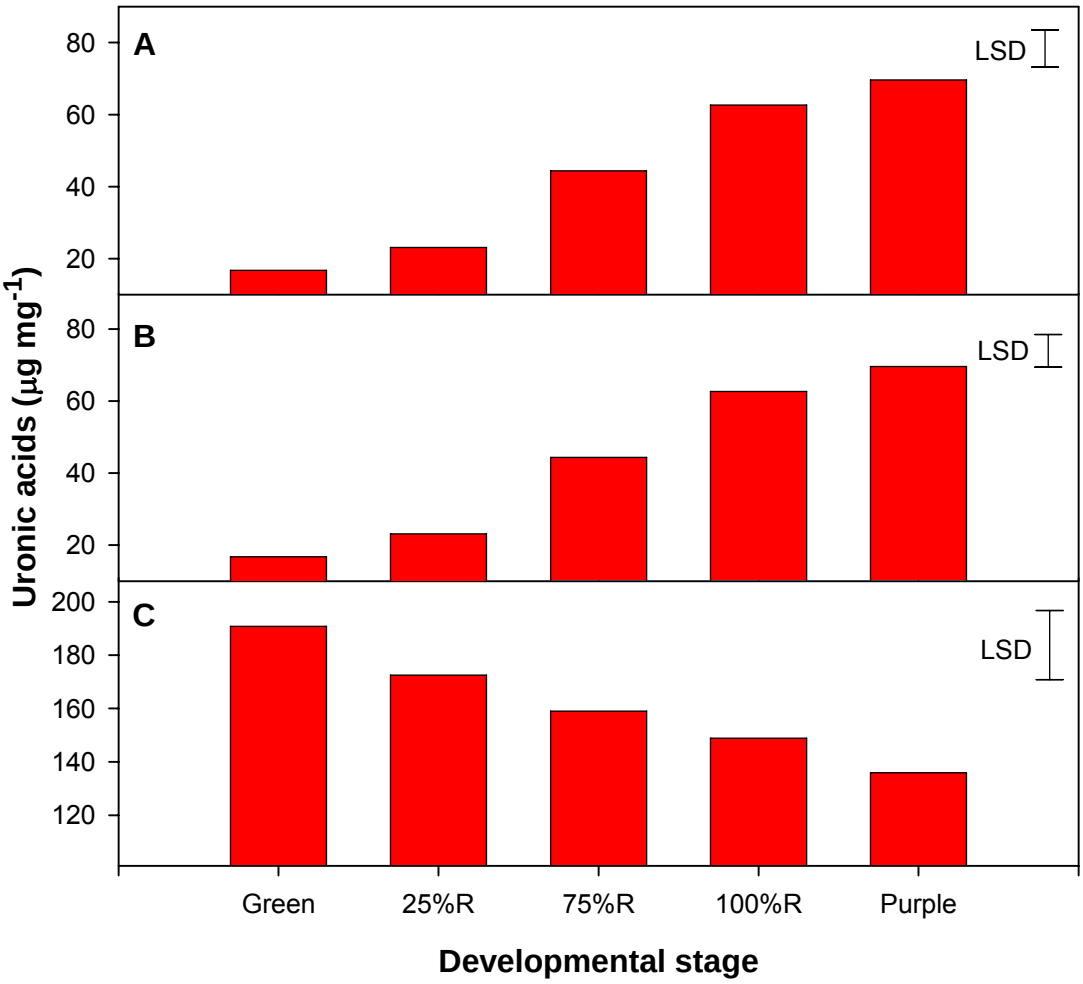


Figure 4: Pectin solubilization throughout boysenberry fruit development. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The least significant difference (LSD) at P= 0.05 is shown.

3.1.3. Cell wall components depolymerization

Fruit softening is accompanied by molecular size changes in different cell wall polymers. In order to determine the nature and timing of this process in boysenberries the fractions enriched in different cell wall components were analyzed by size exclusion chromatography. Depolymerization of cross-linking glycans during development has been reported in several fruits including, tomato (Maclachlan and Brady, 1994), melon (Rose et al., 1998), kiwifruit (Redgwell et al., 1992) and avocado (O'Donoghue and Huber, 1992). In the case of other soft fruits fruit the results reported for strawberries are quite contradictory. Huber (1984) found that the average molecular size of hemicelluloses extracted from strawberry fruit declines dramatically during ripening. However, other work analyzing the changes in cell wall composition in three different strawberry varieties only a slight depolymerization was found, leading these authors to conclude that the impact of hemicellulosic polymer breakdown on strawberry fruit softening was minor (Rosli et al., 2004). In the case of boysenberry, no changes in molecular size were observed in the 4 % KOH SF and 24 % KOH SF during development (data not shown) although a marked increase in the solubilization of these polysaccharides was observed between the 25 % R and 75 % R stages. This increased solubilization without depolymerization suggests that agents that facilitate cell wall loosening, such as expansins (Cosgrove et al., 2002) and/or yieldins (Okamoto-Nakazato et al., 2001), may be operating. In contrast, all pectin fractions showed a dramatic decrease in molecular size during development. Increased depolymerization of the WSF and NSF pectins was apparent at the 100 % R stage (Figure 5 A, C) and the evidence for substantial pectin breakdown in purple stage (over-ripe) fruits was unmistakable. While the size distribution of the CSF pectins remained constant between the green and 100 % R stages, a clear downshift in pectin size was apparent as fruit became over-ripe (Figure 5B). As indicated earlier (Figure 4), higher rates of pectin polymer solubilization were seen in the earlier stages of fruit ripening, when depolymerization was low, showing that polyuronide depolymerization, was not necessary for pectin solubilization in these stages. Studies of pectin metabolism in ripening strawberry fruits have not led to consistent conclusions. Nogata et al. (1993) reported that pectin depolymerization was limited while Rosli et al. (2004) observed clear differences in pectin polymer size, leading them to suggest that strawberry fruit softening was closely linked to pectin metabolism. The report that strawberry fruit with antisense expression of a pectate lyase gene produced ripe fruit with lower cell wall swelling and

pectin solubility, and higher firmness (Jiménez Bermúdez et al., 2002) also supports the conclusion that pectin breakdown and strawberry fruit softening are mechanistically linked. The extensive pectin depolymerization found in late ripening in the case of boysenberry when softening proceeded but without marked changes in the Cel-Hem matrix suggest that pectin size downshift may then be important in softening in terminal stages probably through effects on the integrity of the middle lamella and intercellular adhesion. The conclusion that pectin metabolism is linked to softening in over-ripe fruit is also supported by studies of the ripening of tomato fruits with antisense-suppression of PG gene expression (Hadfield and Bennett, 1998).

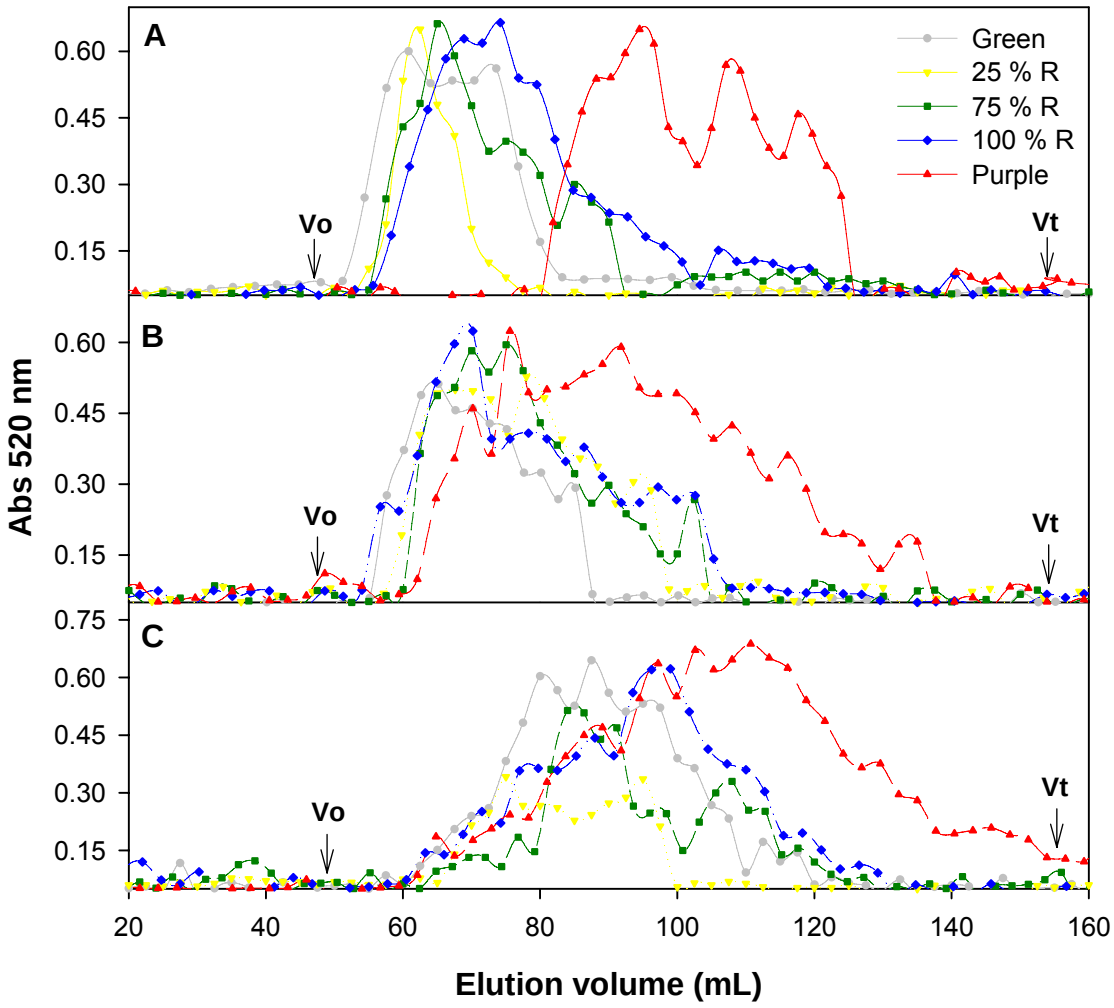


Figure 5: Size exclusion chromatography profiles from pectins throughout boysenberry fruit development, fractionated on HW65. Column fractions (2.5 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Vo, void volume; Vt, total volume. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction.

3.1.4. Cell wall composition

In addition to the depolymerization of certain cell wall components, a characteristic feature of cell wall metabolism in ripening fruit is the loss of neutral sugars from polysaccharides, primarily Gal and Ara from pectin polymers (Redgwell et al., 1997). Preferential loss of Gal moieties during ripening has been reported for several species (Gross and Sams 1984; Koh and Melton 2002). In the case of boysenberry fruit a reduction in arabinose content per gram of AIR was observed with the transition between the 75 % R and 100 % R stages while galactose reduction occurred in the 100 % R to the purple stage transition (Figure 6). Gross and Sams, (1994) found a reduction in both arabinose and galactose in blackberry but in this case the decline in these sugars was found to occur simultaneously. However in their work, three developmental stages were analyzed and this could explain the differences with our findings.

When the proportions of the different sugars in the pectic and 4% KOH soluble fractions were analyzed an increase in arabinose concentration in the pectic fractions was found from the green to the 100 % R (Table 2 and 3). Interestingly, these changes occurred mainly at stages where pectin solubilization but not depolymerization increased. L-arabinans with different degrees of branching at the O-3 and O-2 positions and type I arabino-galactans are attached to the O-4 position of rhamnose residues of RG I, (Brett and Waldron, 1996; Willats et al., 2001). Redgwell et al. (1992) suggested that pectin solubilization may result from the loss of neutral sugars from side chains of rhamnogalacturonans. Such residues may be relevant for the formation of a cohesive pectin matrix, cross-linking pectin molecules to each other, as well as to hemicelluloses and other cell wall components. For instance, in the tobacco (*Nicotiana plumbaginifolia*) mutant *nolac-H14*, RG-Is with low levels of associated arabinans are not retained in the cells walls and middle lamella (Iwai et al., 2001). It could be then hypothesized that the decrease in the highly branched arabinans may be involved then in the increased pectin solubility observed in intermediate developmental stages of boysenberry fruit. Peña and Carpita (2004) found that the loss of the highly branched arabinan presaged the loss of texture in apples. The authors suggested that Ara loss may be a prerequisite to permit other wall components to become susceptible to enzymatic modification. Furthermore, lateral chain removal may also permit RG-I-associated HG to be susceptible to enzymatic hydrolysis, thus influencing the pectin depolymerization observed later in development (Figure 5).

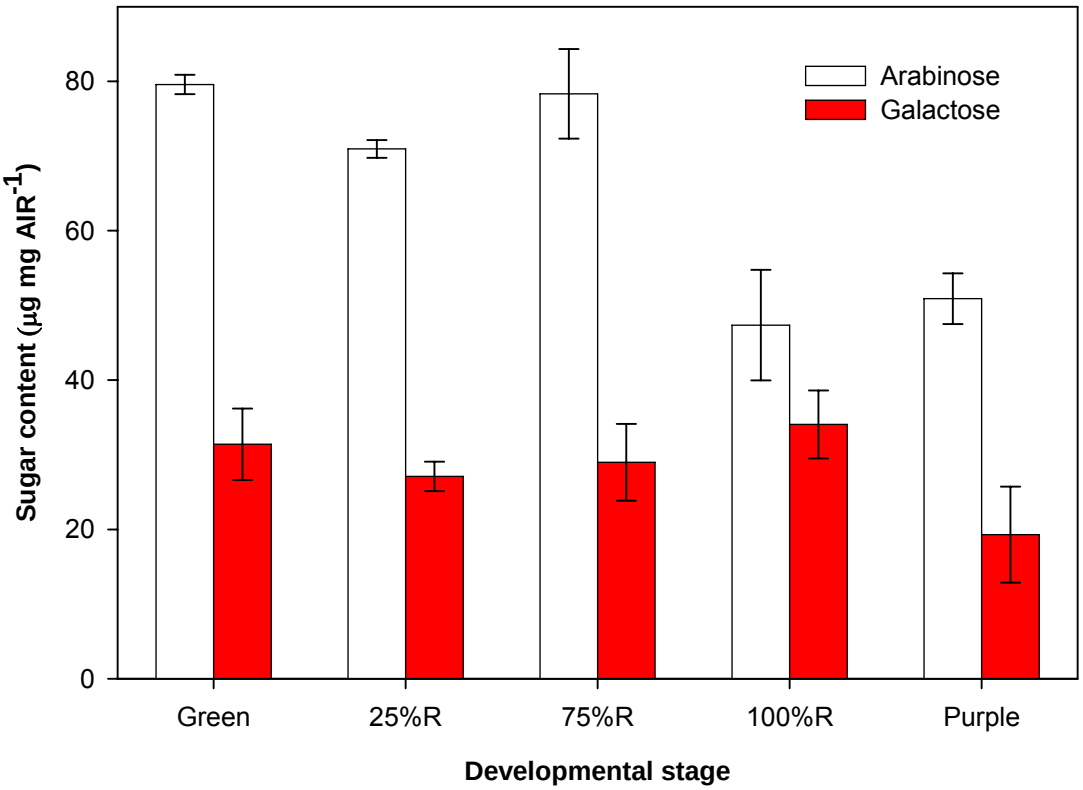


Figure 6: Changes in arabinose and galactose content in boysenberry cell walls during development. Error bars are shown (n= 3).

Table 2: Neutral sugar composition (mol %) of boysenberry water, CDTA and Na_2CO_3 soluble fractions throughout fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color).

Cell wall fraction	Dev. stage	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Water soluble fraction	Green	1.5	0.3	18.4	7.4	5.3	9.3	57.8
	25 % R	1.6	0.7	22.7	9.3	5.0	12.6	48.1
	75 % R	1.3	0.7	35.3	16.5	7.5	20.5	18.2
	100 % R	4.0	0.8	35.2	13.3	5.1	16.5	25.1
	Purple	3.1	0.8	43.8	10.6	4.5	16.5	20.7
CDTA soluble fraction	Green	2.7	0.6	15.2	2.9	4.3	12.0	62.3
	25 % R	2.5	0.6	21.7	6.8	5.3	11.0	52.1
	75 % R	2.3	0.6	24.6	7.2	5.3	13.6	46.4
	100 % R	2.9	0.8	41.0	10.9	5.0	12.9	26.3
	Purple	13.0	0.3	48.4	4.7	3.5	11.2	18.8
Na_2CO_3 soluble fraction	Green	7.4	1.1	40.0	3.9	1.6	11.9	34.0
	25 % R	6.2	0.6	46.5	3.6	1.1	15.5	26.4
	75 % R	8.1	1.1	47.9	3.8	1.2	13.8	24.2
	100 % R	7.6	0.9	49.8	4.3	1.0	15.7	20.7
	Purple	7.7	1.1	51.9	4.0	1.2	13.3	20.8

Table 3: Neutral sugar composition (mol %) of boysenberry 4 and 24 % KOH soluble fractions throughout fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color).

Cell wall fraction	Dev. stage	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
4 % KOH soluble fraction	Green	1.0	0.9	23.5	40.2	3.0	9.7	21.6
	25 % R	1.6	1.2	30.5	37.2	2.8	10.0	16.5
	75 % R	1.8	1.3	28.6	35.3	3.3	11.0	18.6
	100 % R	1.6	0.9	39.9	34.1	2.1	11.0	10.3
	Purple	1.5	0.7	37.8	34.7	2.7	8.9	12.9
24 % KOH soluble fraction	Green	0.4	2.7	14.0	23.1	8.4	11.6	36.8
	25 % R	1.1	4.3	9.9	28.2	6.7	12.5	37.2
	75 % R	0.9	3.1	12.7	26.7	8.3	11.9	36.3
	100 % R	1.1	3.2	9.3	25.6	8.5	13.0	39.2
	Purple	2.1	2.0	10.2	27.2	8.4	10.8	39.5

3.1.5. Cell wall degrading enzymes

Many enzymes capable of catalyzing aspects of cell wall modification and disassembly during fruit development and ripening have been isolated and characterized so far (Fisher and Bennett, 1992). In the case of boysenberry, the developmental activity profiles of the enzymes analyzed do not always show peak activities at the same stage. Lacking a detailed analysis of each of the activities described, including determination of whether multiple isoforms are involved and characterization of the expression of encoding genes, it is impossible to say whether the patterns identified in Table 4, reflect the activity of single proteins or of members of protein families, as well documented for tomato (Brummell and Harpster, 2001). However, in general, the measured activities of the enzymes correlate reasonably closely with a corresponding modification in cell wall polysaccharides with the exception of EGase. Endo-1,4-β-D-glucanases (EGases) are enzymes that hydrolyze internal linkages of 1,4-β-D-glucans. In the cell wall, their natural substrates are presumed to include non-crystalline (amorphous or para-crystalline) regions of cellulose and other non-cellulosic glycans like xyloglucan. In the case of boysenberry, EGase increased during development while no clear depolymerization of hemicelluloses was found. Boysenberries are hybrids between blackberries and raspberries. Although the receptacle does not detach from the drupelets as in raspberries there is still a progressive weakening of the abscission zones. Sexton et al., (1997) found that the surface of the purple ripe receptacles containing the abscission zones had six times more EGase activity per gram of tissue than the inner core and suggested that EGase might be mainly involved in abscission zone weakening. Concerning cell wall esterases, both pectin methyl esterase (PME) and acetyl esterase (AE) were measured. AE did not show significant changes in early development but increased slightly at the Purple stage. PME activity increased from the green to the 100 % R stage and decreased afterwards. Homogalacturonic acid (HG) is an abundant and widespread cell wall component which is synthesized in a highly methyl-esterified state (Doong et al., 1995). Demethylation by PME influences the cleavage of HG by pectinolytic enzymes, such as polygalacturonase and pectate lyase. De esterification of pectic compounds by AE and PME would increase then the availability of preferred substrates for polyuronide depolymeryzing enzymes in late development and maximal depolymerization of all boysenberry pectin fractions was observed between the 100 % R and Purple stages when both pectin esterase activities were at their highest levels. α-L-

arabinofuranosidases are able to hydrolyze terminal, non-reducing arabinofuranosyl residues. In the case of boysenberry an increase of α-ara was found with high enzyme activity correlating closely with a loss in cell wall arabinose, an increased pectin solubilization and a corresponding increase in the proportion of arabinose in the solubilized pectins. Finally both β-gal and PG increased markedly in the late stage of ripening in which a dramatic depolymerization of pectic compounds was observed suggesting that such changes, perhaps in concert with the elevated esterase activities, may be associated with softening in terminal stages.

Table 4: Changes in cell wall degrading enzymes activity throughout boysenberry fruit development. G: large green; 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color); P: purple. The least significant difference (LSD) at P=0.05 is shown.

	Developmental stage					
	Green	**25 % R**	**75 % R**	**100 % R**	**Purple**	**LSD**
Acetylesterase (EAU kg^{-1})	8.9	7.9	7.7	9.9	11.2	1.2
α-arabinofuranosidase (EAU kg^{-1})	10.8	12.4	77.4	102.0	45.0	12.5
β-galactosidase (EAU kg^{-1})	3.1	2.9	3.2	3.3	59.6	9.8
Pectin methylesterase (EAU kg^{-1})	7.1	29.1	104.6	119.9	109.3	26.5
β-(1,4)endoglucanase (EAU kg^{-1})	2.9	5.1	7.3	9.7	10.4	2.1
Polygalacturonase (EAU kg^{-1})	3.4	4.7	7.4	21.3	24.9	8.7
α-rhamnosidase (EAU kg^{-1})	5.5	7.1	21.8	36.0	63.7	17.1
β-mannosidase (EAU kg^{-1})	0.8	0.6	19.9	78.0	188.4	29.6

3.1.6. Model for cell wall changes accompanying boysenberry fruit development

The data suggest that cell wall changes determining fruit growth and softening in boysenberry fruit during development are accompanied by the disassembly of both the Cel-Hem and pectic networks. In the case of the Cel-Hem network a reduction in cellulose content and the initiation of xyloglucan disassembly occurred mainly early in the early expansive phase of development (Figure 7). The changes in cross-linking glycans are characterized by an increase in xyloglucan solubilization without depolymerization suggesting the involvement of proteins able to affect the interactions within the Cel-Hem network but without extensive polymer cleavage. Expansin or xyloglucan-transglycosylase-hydrolase (XTH) might be good candidates for such proteins and both have been implicated in fruit wall metabolism previously. Another candidate, not previously linked to fruit wall metabolism is yieldin (Okamoto-Nazakato, 2002). Previous work also suggested that metabolism of cellulose microfibril-associated xyloglucan may contribute to the initial stages of fruit softening in melon (Rose et al., 1998) and tomato (Maclachlan and Brady, 1994). The overlap of fruit growth and softening in the early stages of boysenberry ripening makes it difficult to propose a specific developmental role for the Cel-Hem network modifications described herein. As development advances, extensive modification of pectin becomes apparent. Such modifications start at intermediate stages and are concomitant with increased α-ara activity and extensive arabinose loss, probably from side chains of RG I As the fruit reaches the 100 % R and purple stages a number of pectin modifying enzymes including β-gal causing a loss of galactose from the cell wall, PME and PG are recruited and extremely high pectin depolymerization occurs. Pectin depolymerization is also a feature of the final stages of wall change in ripening tomatoes and melons, but the polymer size down-shifts in these fruits is not as great as in boysenberry. It is clear that the fruits' content of PG is not a predictor of the extent of pectin depolymerization that will occur. Tomato produces substantial PG (Della Penna et al., 1987), boysenberry produces much less (Table 4) and melon produces very little (Hadfield et al. 1998). Therefore it is important to know the relative content of pectin polymer backbone-cleaving activities like pectate lyase (Jiménez Bermúdez et al., 2002) and specific isoforms of side-chain cleaving activities like β-galactosidase (Smith et al.. 1998) and α-arabinosidase and how these enzymes cooperate with PG in pectin metabolism to accurately model changes in the pectin network that are a common feature of the later stages of ripening in these fruits.

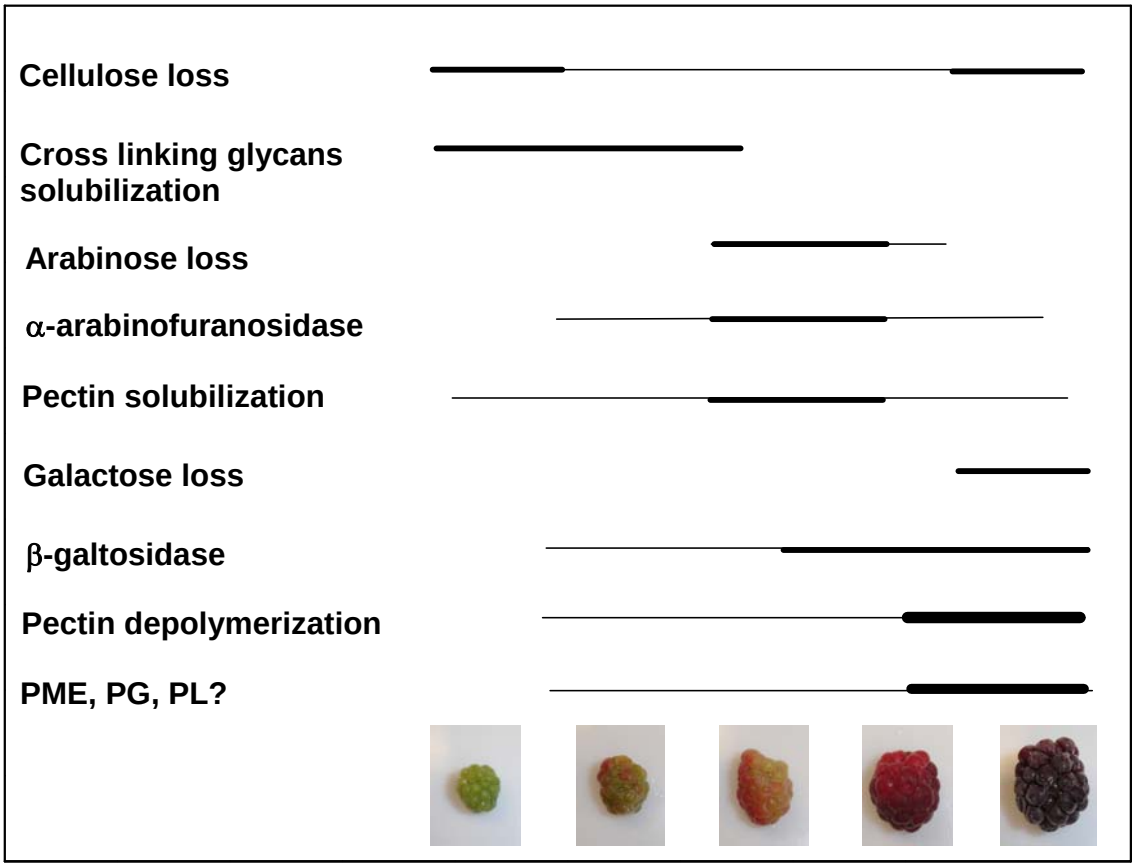
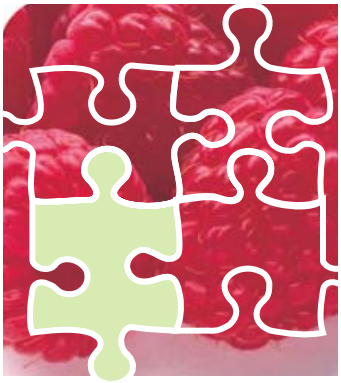


Figure 7: Proposed model for cell wall changes accompanying boysenberry fruit development and softening.

3.2. Cell wall changes accompanying raspberry (*Rubus idaeus*) fruit development



3.2.1 Fruit weight, firmness and cell wall yield

As it was found in the case of boysenberry, raspberry fruit weight also increased throughout development. In this case the enlargement period continued until the 100 % red stage (Figure 8 A). Regarding fruit firmness softening also occurred from the green to the red ripe stages (Figure 8 B). Finally in the case of cell wall yield a clear reduction was found from the green to the 75 % red stage but no changes were observed afterwards (Figure 8 C).

3.2.2. Total cell wall composition

Total cell wall building blocks proportion was measured by GC-MS of the sugar derivatives alditol acetates. Arabinose was found to be the most abundant sugar present in the cell wall (Figure 9 C). This is different from other fruits such as tomato (Gross and Sams, 1984), pepper (Gross and Sams, 1984) and melon (Rose et al., 1998) in which galactose is the predominant sugar and even from previous studies in raspberries cv. Glen Clova and Glen Closen reported that xylose was found to be the most predominant wall component (Stewart et al., 2001) The proportion of the different monosaccharides did not change substantially during development showing clear differences with the results regarding boysenberry in which a clear reduction in arabinose was observed at intermediate stages. Towards the end of the developmental process a reduction in the proportion of galactose was detected which is similar to what has been reported in other fruits and also to the findings in the case of boysenberry. This correlated with an increase in β-gal activity observed from the 100 % R stage. In tomato, a decline in wall galactose and increase in β-gal activity occur much earlier preceding or accompanying the increase in soluble pectins (Gross and Wallner 1979, Kim et al. 1991).

Interestingly the Xyl:Glc ratio was particularly high and it decreased in late ripening. A high ratio Xyl:Glc was also found in other 2 raspberry varieties but the possible explanation for this observation was not discussed (Stewart et al., 2001). Plant cell wall xylose could be present either in xylans which consist of a backbone of β-(1,4)-linked xylose residues associated with side-chains of 4-O-methylglucuronic acid and arabinose which are present in varying amounts (glucuronoxylan and arabinoxylan). Glucuronoxylans are the most abundant hemicellulosic components of wood tissues and grasses cell walls where they play a major role in the organization of lignified cell walls as they can cross link with lignins, particularly in grasses, via feruloylated bonds (Hatfield et al., 1999). However, in the primary cell wall of most dicot species xylose is thought to be associated with hemicelluloces (Albersheim, 1976; Zablackis et al., 1995). Xyloglucan has a backbone composed of 1,4-linked β-D-Glc residues. Up to 75 % of these residues are substituted at O6 xylosyl residues which could be also decorated with galactosyl or galacto-fucosyl chains. Since the ratio Xyl:Glc for xyloglucan based on its structure it is expected to be 3:4 the fact that Xyl was most abundant suggest that xylans might be present in the cell wall of raspberry to account for the remaining xylose. This might also be supported by the increased ratio Xyl:Glc observed in the last stage of development since this change in the proportions of Xyl and Glc could not be accomplished given the structure of xyloglucans.

When both neutral sugars and pectin content were analyzed in the whole cell wall no significant changes were found at the different stages of development analyzed (Figure 9 A, B). This could be a consequence of no changes in these components or due to an equilibrated degradation of both pectic and non-pectic cell wall material leading to a non apparent change in the proportion of the different fractions. However, the fact that no changes in the total level of the wall components does not means that there were no changes in the cell wall structure and/or composition. For instance modification in the polymer size and ramification will profoundly affect the cell wall organization will not be detected in these assays. In order to determine if this was the case a fractionation of the cell wall material was done.

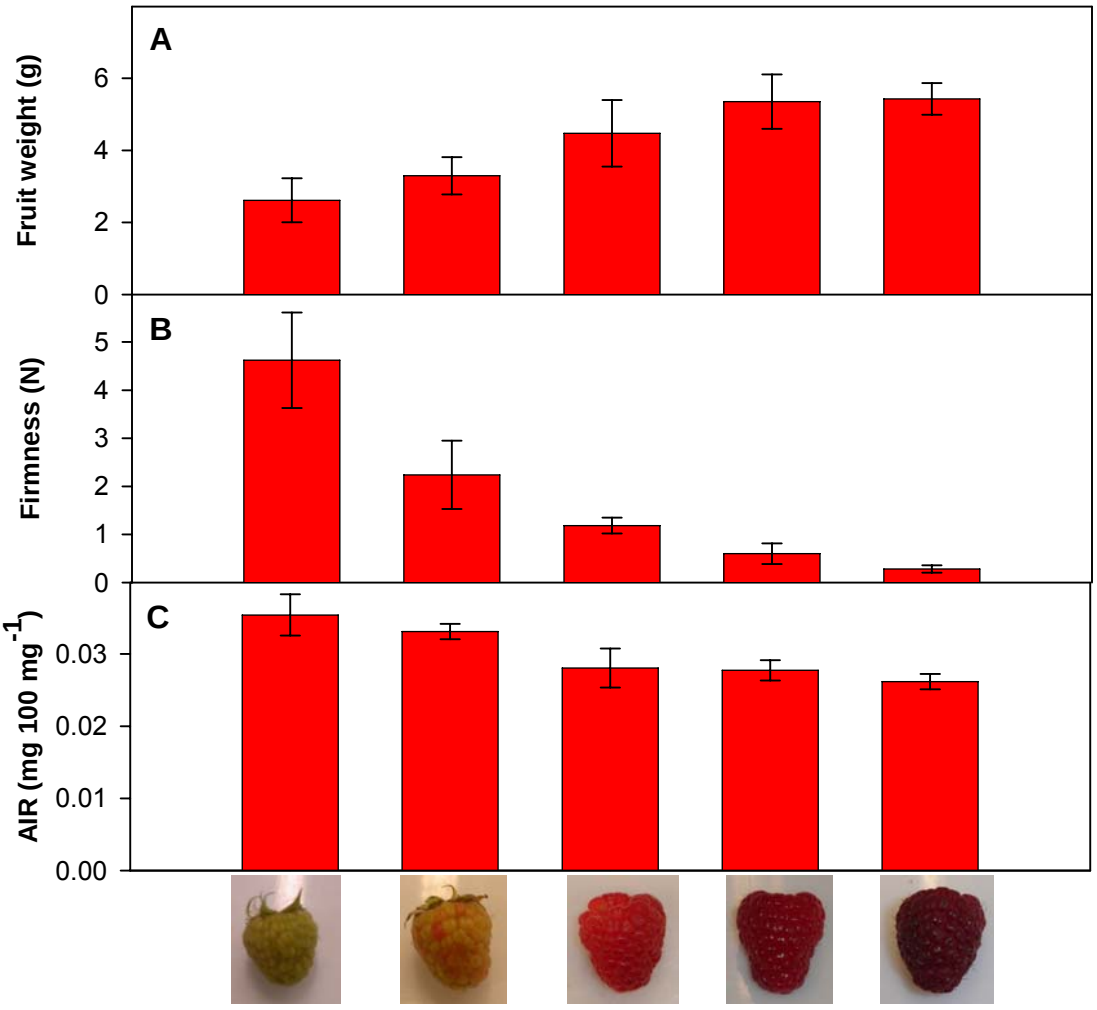


Figure 8: Changes in raspberry fruit weight (A), firmness (B), cell wall yield (AIR) (C) during development. The standard deviation is shown.

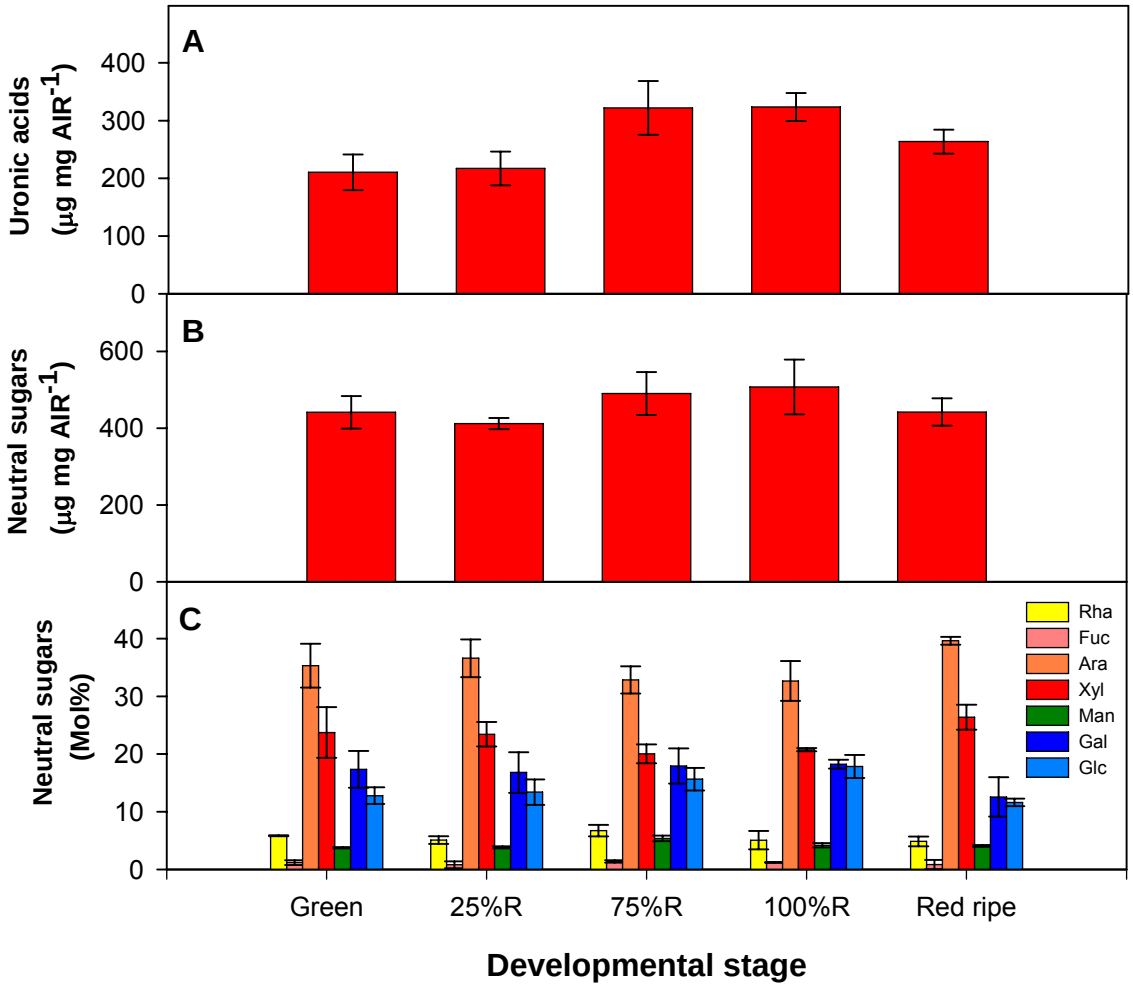


Figure 9: Changes in raspberry total uronic acids (A) neutral sugars (B) and monosaccharide composition (C), during development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color. The standard deviation is shown.

3.2.3. Pectin solubilization depolymerization and composition

Even when the total level of pectins did not change dramatically during development there were clear modifications in the solubility of the pectic compounds in raspberry. In the case of water and CDTA soluble pectins a clear increase was found as fruit development progressed (Figure 10). The changes were mostly concentrated towards the late stage of development differently to boysenberry where a sharp increase in solubilization occurred at intermediate stages associated with cell wall arabinose loss. In the case of Na_2CO_3 soluble pectins as expected an opposite trend was observed decreasing the amount of uronic acids remaining in this fraction at more advanced stages of development. The ratio NS/GA has been used as an approximation to estimate the degree of pectin ramification (Batisse et al., 1996, Rosli et al., 2004). In the case of raspberry the ratio of NS/GA showed a slight increase in the water and CDTA soluble fractions from the green to the 100 % R stage. This could be due to the release of either polyuronids with a higher ramification or neutral sugars containing oligosaccharides from the lateral chains of Na_2CO_3 soluble pectins. However, in the RR stage the water soluble fraction showed a clear reduction in the ratio NS/GA suggesting a decrease in the ramification degree of cell wall pectins at this stage. This is similar to what has been found in other soft fruits such as strawberry where the NS/GA relation decreased throughout ripening (Rosli et al., 2004). When the individual sugar composition of the water and Na_2CO_3 soluble fractions was analyzed by GC-MS, the clearest modification observed was an increase in arabinose proportion in the water soluble fraction concomitant with a reduction in the Na_2CO_3 SF (Figure 11). This is similar to the results found in the case of boysenberry. Pectin solubilization has been associated with a gradual degradation of arabinan side chains (Koh and Melton, 2002). However it is worthy to highlight that in the case of raspberry the correlation between pectin solubilization and arabinose enrichment of the water and CDTA soluble fractions was not as clear. In this case the highest increase in pectin solubilization was concentrated towards the end of ripening instead of at intermediate stages as it was found in boysenberry. Another aspect that was unexpected but that it is consistent with the findings in the case of boysenberry is that the water soluble fraction contained relatively high levels of glucose (Figure 11). Glucose is present in the cell wall in different polysaccharides but mainly in cellulose and hemicelluloses. These polymers are highly insoluble and should be solubilized with alkali and concentrated acid solutions. In some fruits high glucose levels could be a result of starch contamination

during cell wall preparation. However when the cell walls were treated with iodide to detect starch presence the results were negative (not shown) and when the cell walls were treated with amylase prior to the water extraction high levels of glucose were still found. In addition berries are though not to accumulate high starch levels (Souleyre et al., 2004). In the case of strawberry, transient starch accumulation was found very early in the developmental process. At a qualitative level Knee et al. (1977) demonstrated the presence of starch granules in strawberry plastids at 7 days after petal fall, but by day 21 most of these granules had been lost. Darnell and Martin (1988) quantified starch in fruit immediately after anthesis and showed that levels reached a maximum of 40 mg g DW^{-1} at 96 h after pollination and then declined to 20 mg g DW^{-1} at 144 h and even considering these levels of starch the contribution to the total alcohol insoluble residue would be lower than 4%. Another possibility that could account for the high glucose content observed in the water soluble fraction could be contamination with non-wall glucose present in the fruit but this is unlikely because the cell wall material was extensively washed with ethanol during preparation, the water soluble fractions were dialyzed prior to analysis and even if this was the case higher contamination would be expected in late ripening. Then the glucose could be hypothesized to be a glucan that could be associated with cell wall polyuronides. However this will requires further investigation.

In order to determine the modifications in pectin molecular size the different pectic fractions were analyzed by size exclusion chromatography. In the case of the CDTA soluble fraction a slight reduction in pectin size was observed in late ripening (Figure 12). More clear changes were observed in the case of the Na_2CO_3 soluble fraction where the peak eluting at 90 mL was highly reduced in the RR stage. In the case of the water soluble fraction the changes were even more dramatic and an extremely large pectin downshift was detected at the RR stage. The results found then were in general terms similar to those observed in the case of boysenberries and pectin depolymerization seems to be extensive in these fruits and concentrated in late ripening. Previous work looking at raspberry cell wall degrading enzymes showed that PG activity markedly increases in late ripening stages (Ianetta et al., 1999). This suggests that PG might be involved in the dramatic depolymerization observed after the 100 % R stage. However it is interesting to note that no clear correlation between PG activity and relative fruit firmness in 4 different genotypes (Ianetta et al., 1999). Other pectin depolymerizing enzymes such as pectate lyases or their

products of reaction have received much less attention than PGs have also been shown to be present in ripening banana (Dominguez–Puigjaner et al., 1997). A recent report indicated that tomato fruit express genes encoding PL when they ripen (Saladie et al., 2003). Furthermore, the unsaturated oligosaccharides characteristics of PL action were detected in tomato fruit tissues (An et al., 2005). In the case of soft fruits Medina–Escobar et al. (1997) reported the presence of a cDNA highly similar to a pectate lyase and down-regulation of the corresponding gene resulted in higher firmness retention suggesting that they might be crucial in fruit sioftening (Jiménez Bermúdez et al., 2002).

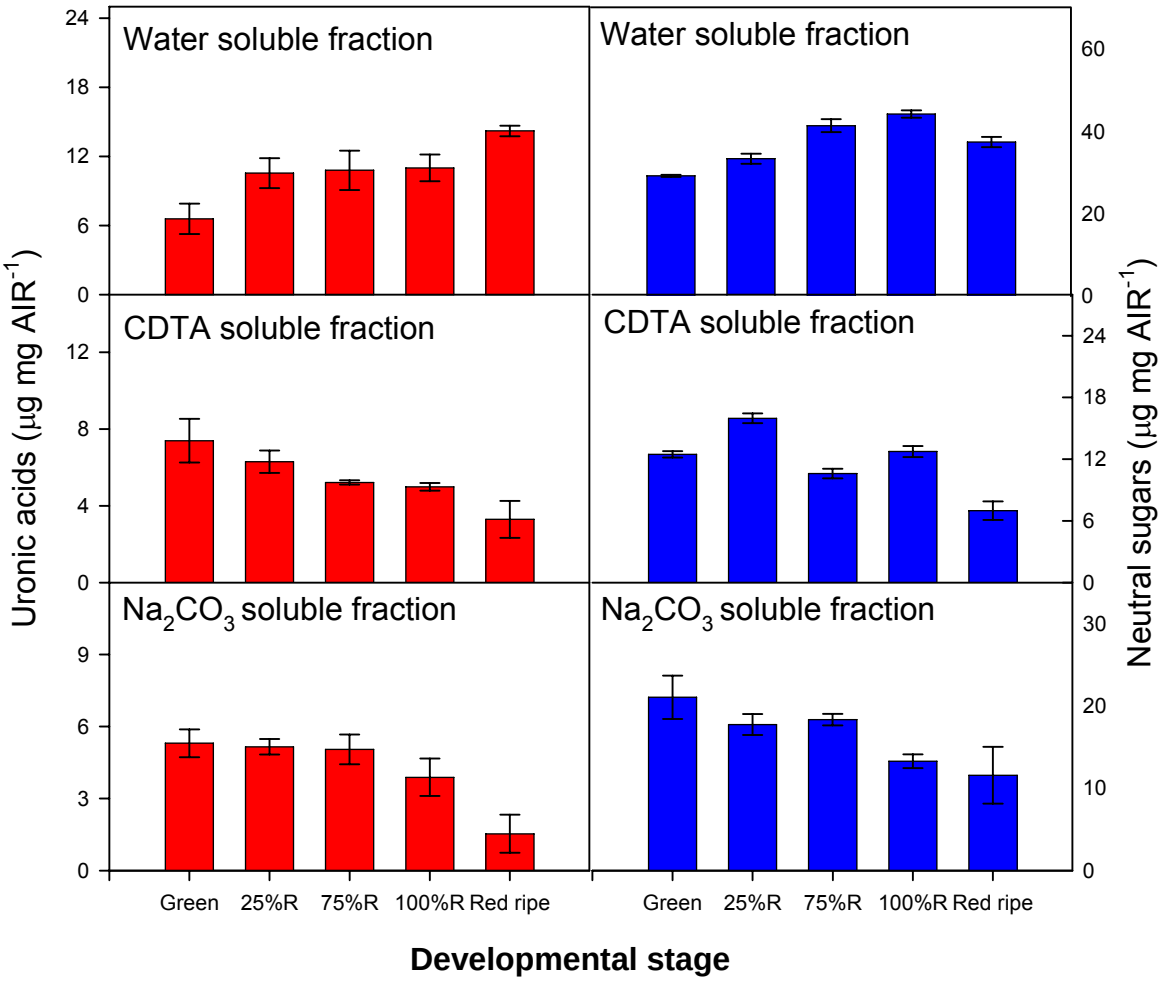


Figure 10: Changes in uronic acids (left panels) and neutral sugars (right panels) in the water (top panel), CDTA (middle panels) and Na_2CO_3 soluble fraction (lower panels) throughout raspberry fruit development. 25 %R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown.

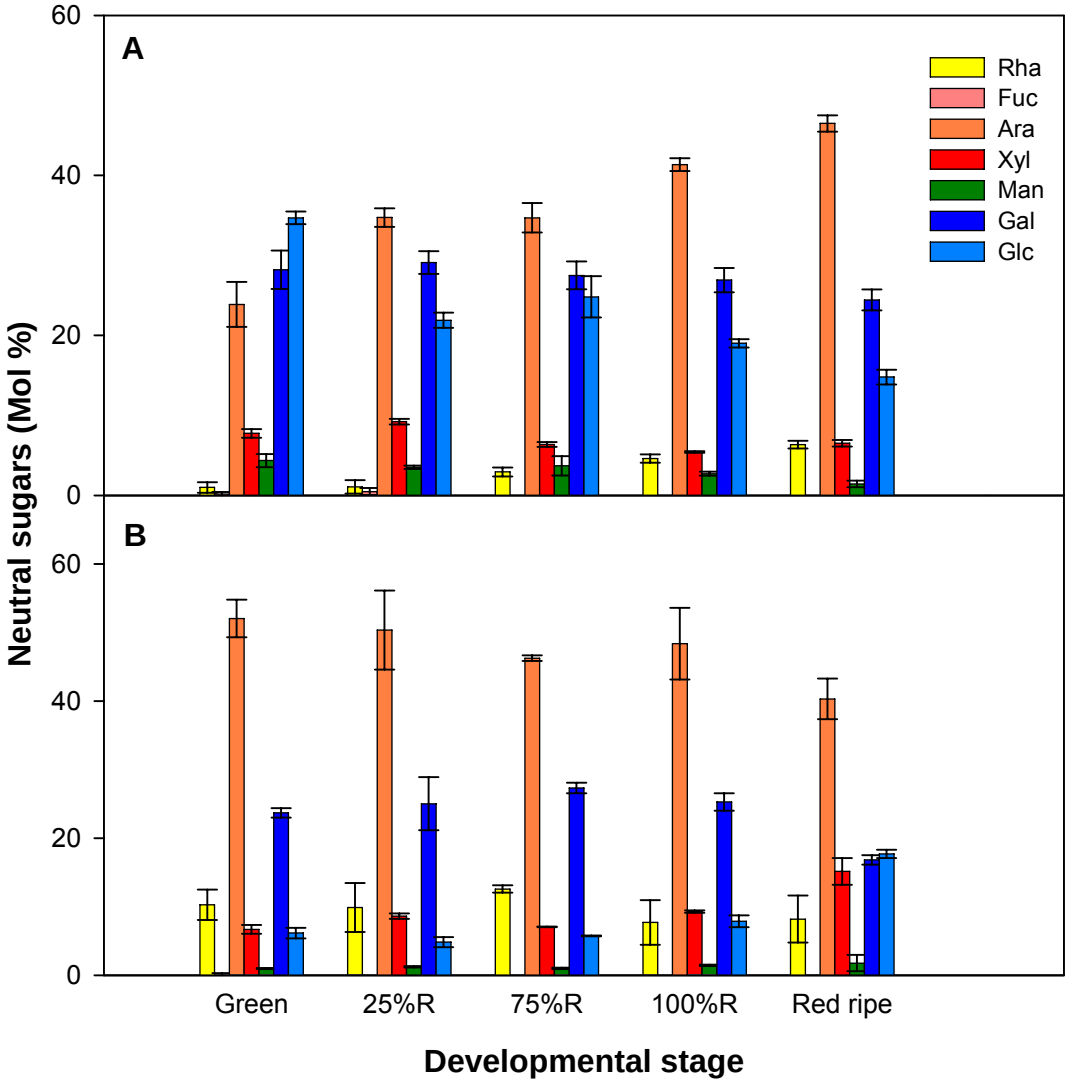


Figure 11: Neutral sugar composition (mol %) of raspberry water (A) and Na_2CO_3 (B) soluble fraction throughout fruit development. 25 %R (25 % surface red color); 75 %R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown.

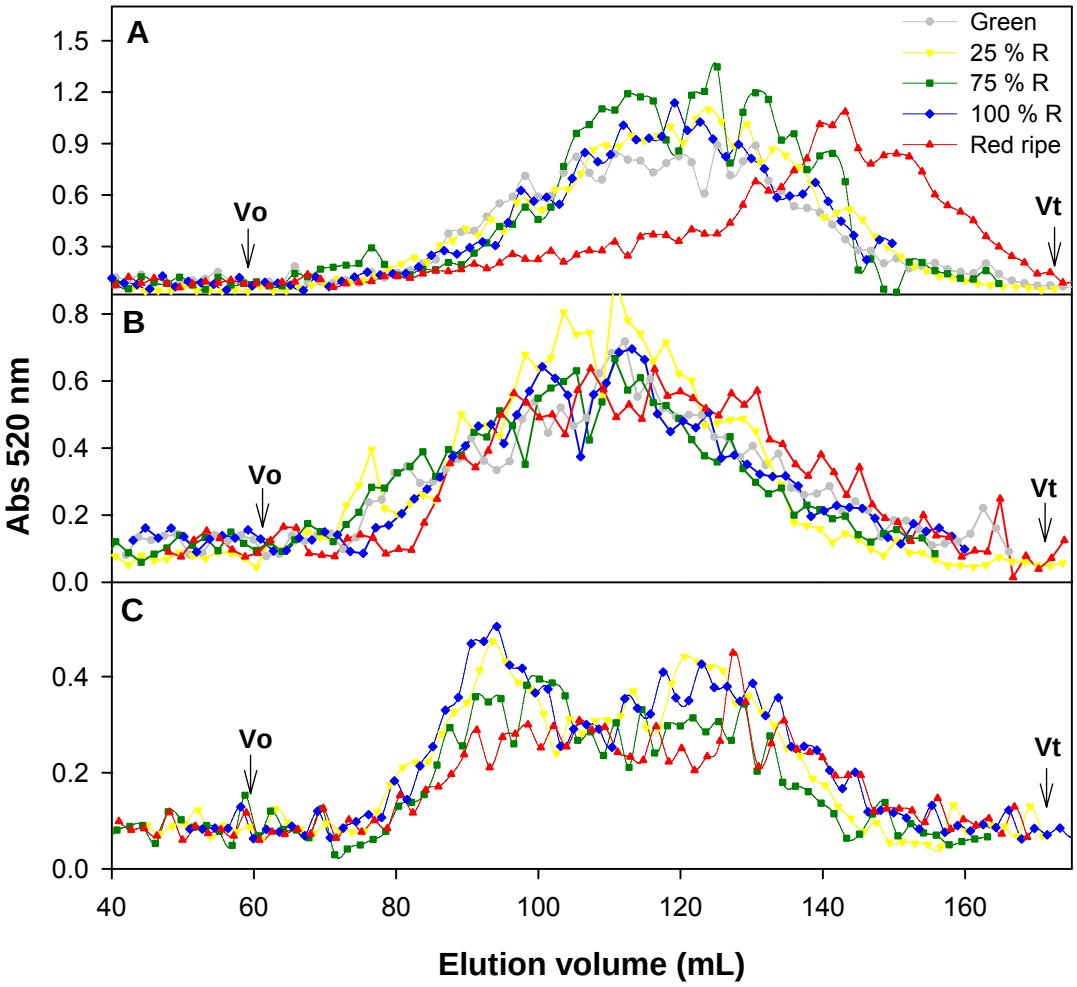


Figure 12: Size exclusion chromatography profiles of pectic polymers throughout raspberry fruit development. The samples were fractionated on HW65. Column fractions (2 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Vo, void volume; Vt, total volume. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction.

3.2.4 Hemicelluloses solubilization, depolymerization and composition

Differently to the findings in the case of pectins, there were no clear changes in the neutral sugar content in the 4 or 24 % KOH soluble fractions (Figure 13). This also differs from observations made in boysenberry where early in development hemicellulose and cellulose levels were reduced.

When the polymer composition was analyzed the main sugars present were xylose and glucose and no significant changes were observed in the 24 % KOH fraction (Figure 14). It is interesting to highlight again that the levels of xylose largely exceeded those of glucose which could not be expected assuming that xyloglugan is the main hemicellulosic polymer in this fraction. This suggests then that xylans could be potentially abundant components of raspberry cell wall. In the case of the 4 % KOH soluble fraction relatively high levels of arabinose and galactose were found suggesting the presence of polyuronides in this fraction probably associated with hemicelluloses. Many cell wall models of dicot species have favor non-covalent interactions between polymers and suggest the existence of a cellulose-xyloglucan network embedded in a matrix of pectic polysaccharides (Carpita and McCann, 2000; Somerville et al., 2004). However recent findings support the existence of xyloglucan–RG-I conjugates in plant cell walls (Popper and Fry, 2005). Putative xylan-pectin complexeshave been reported in ripening tomato fruit (Seymour et al., 1990) and covalent associations between glucuronoxylans and xyloglucan were also found in olive pulp (Coimbra et al., 1995). The resuylts suggest that covalent associations between pectins and hemicelluloses might also exist in the case of raspberry. Similarly to what was observed in the Na_2CO_3 soluble fraction the levels of arabinose and galactose diminished which could probably be associated with a removal of these residues from RGI lateral chains.

The results form the size exclusion chromatography analysis of both 4 and 24 % KOH soluble fraction showed that there were not clear modifications in polymer size throughout development (Figure 15). A reduction in the Cel-Hem matrix components has been described in many fruits (Maclachlan and Brady, 1994; Rose et al., 1998; Brummell et al., 2004). In the case of strawberry early works by Knee et al. (1977) observed that the cell wall became swollen during fruit development and this higher hydration was parallel to change in the neutral sugars of the cell wall fraction as result of a probable degradation of hemicellulose and cellulose. The average molecular size of hemicellulose extracted from strawberry fruit declines dramatically during ripening (Huber 1984). This observed hemicellulose depolymerization

correlates well with a soluble CMCase activity measured in extracts prepared from ripening strawberry fruit (Barnes and Patchett, 1976). Our results show that both boysenberries and raspberries are highly different and do not show extensive changes in the hemicellulosic polymers during development. Conversely the main modifications in the cell wall occur at the level of the pectic polysaccharides.

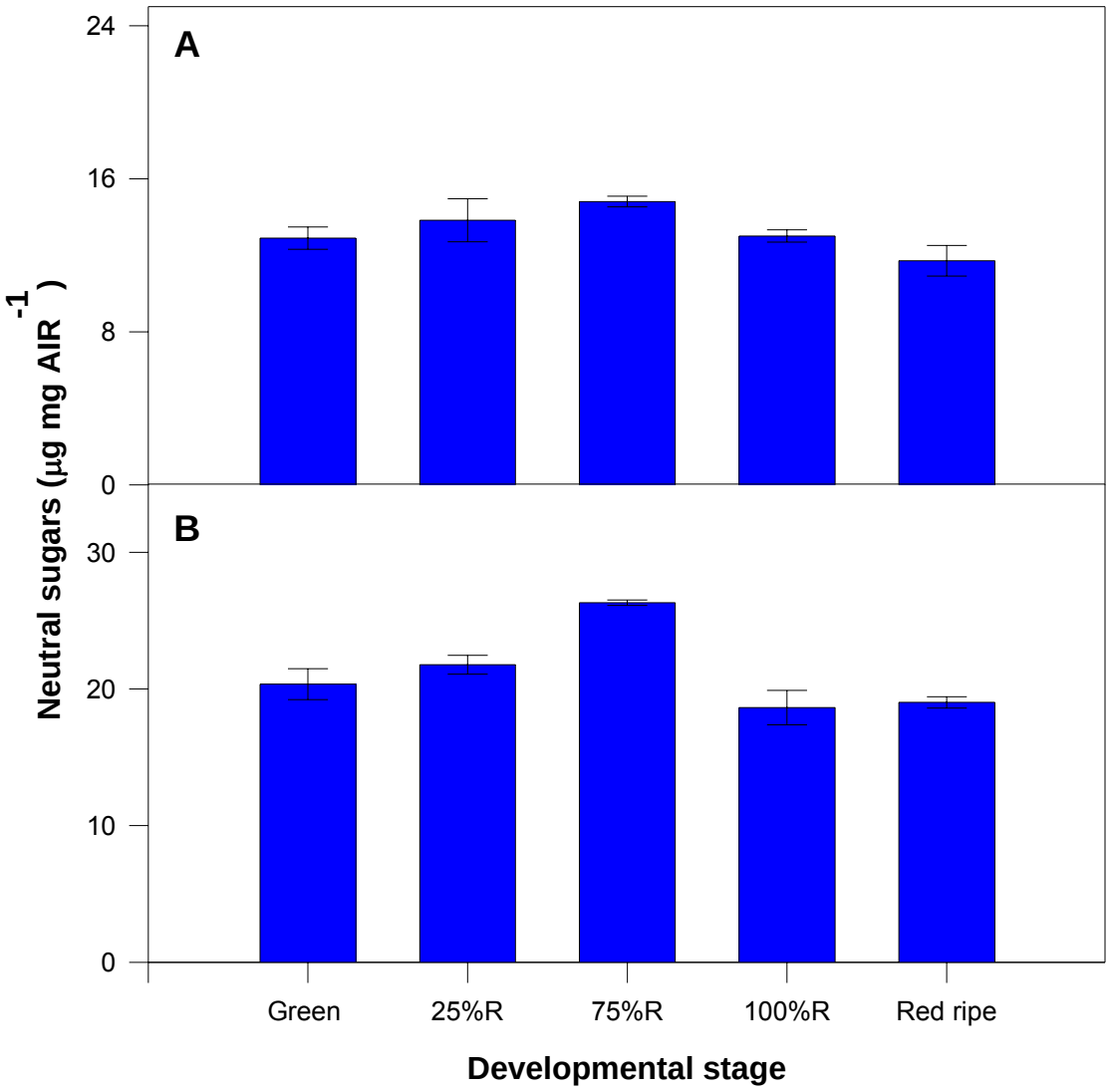


Figure 13: Changes in 4 % (A) and 24 % KOH (B) soluble polymers throughout raspberry fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color).The least significant difference (LSD) at P= 0.05 is shown. The standard deviation is shown.

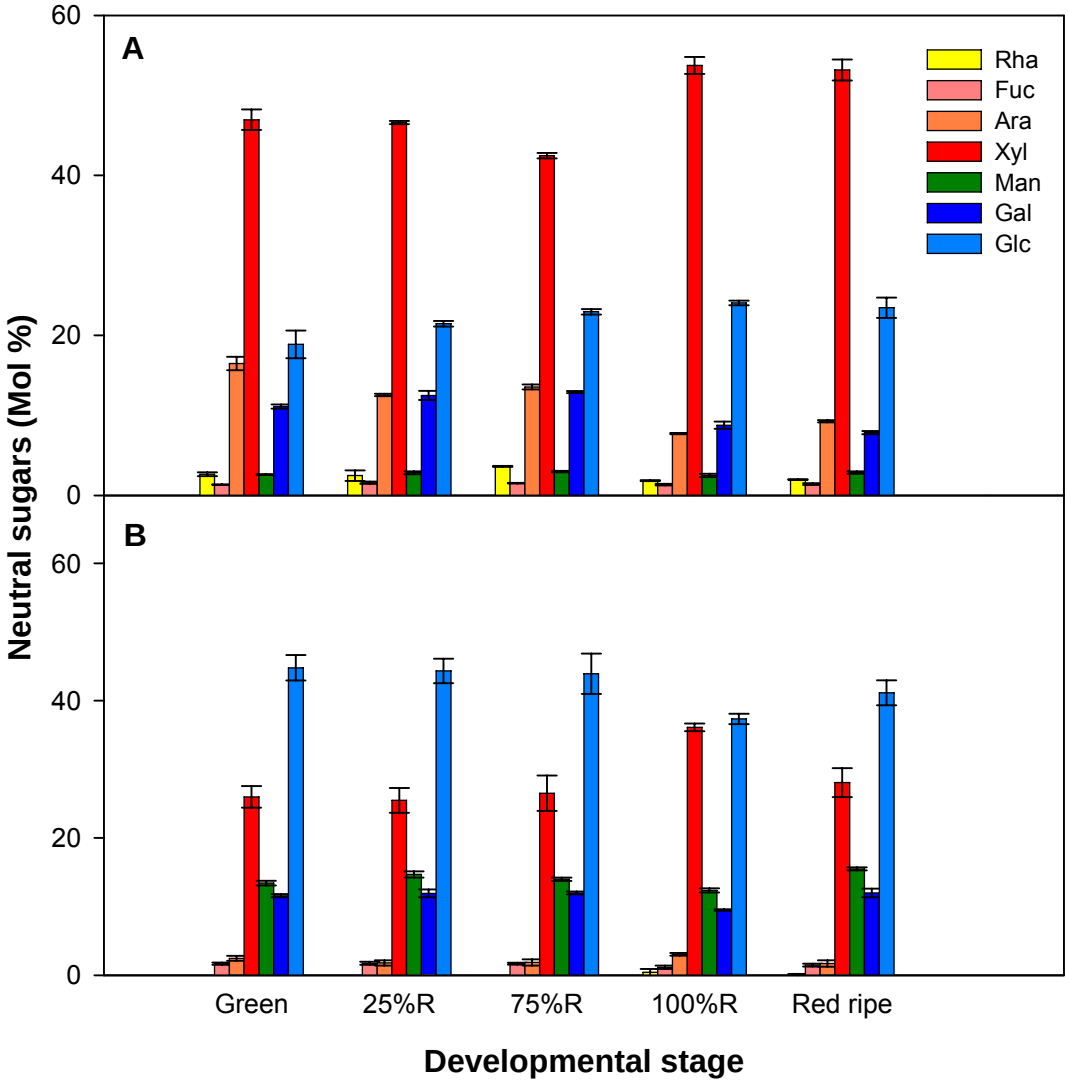


Figure 14: Neutral sugar composition (mol %) of raspberry 4 % KOH (A) 24 % KOH (B) soluble fraction throughout fruit development. 25 % R (25 % surface red color); 75 % R (75 % surface red color); 100 % R (100 % surface red color). The standard deviation is shown

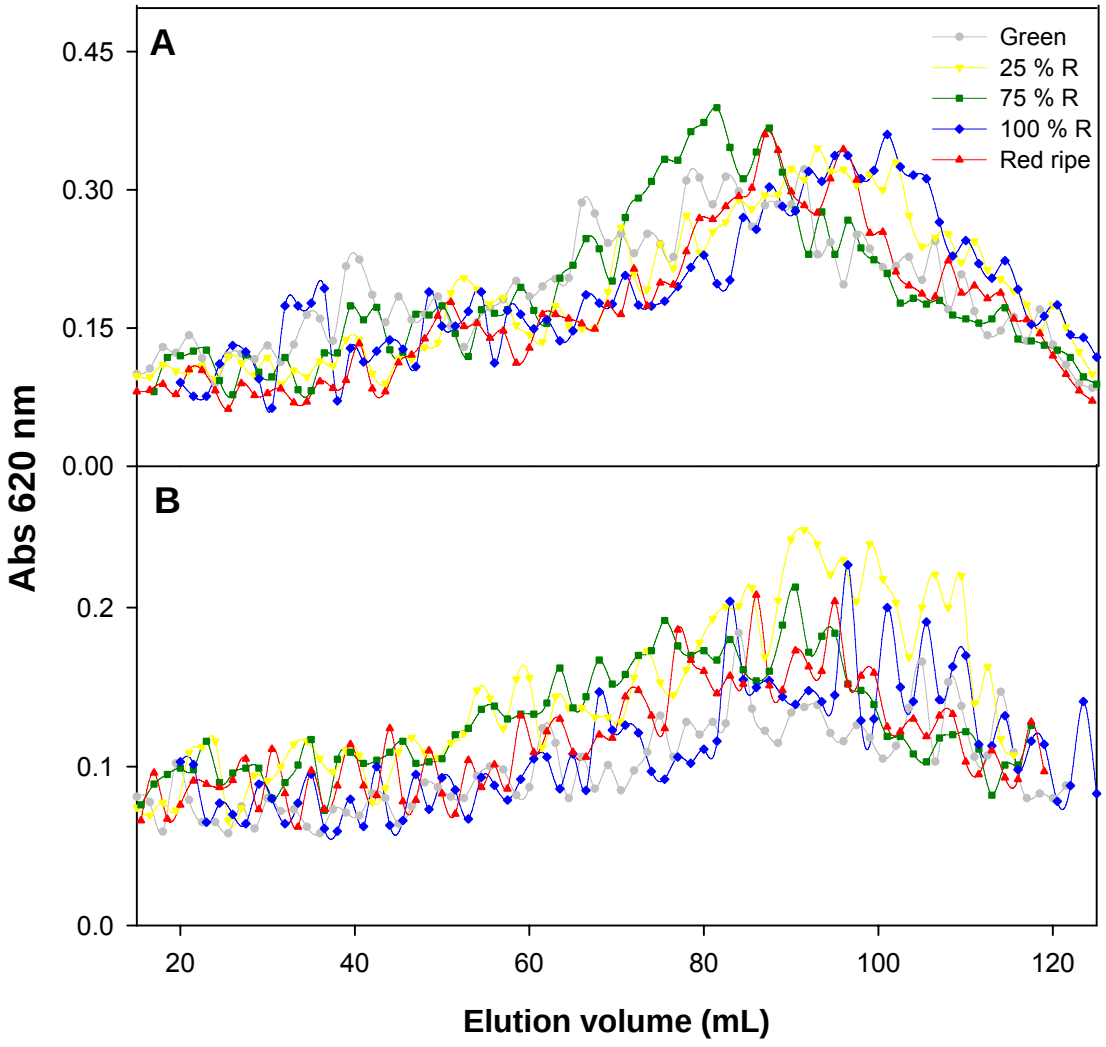


Figure 15: Gel-filtration profiles of 4 % (A) and 24 % KOH (B) soluble polysaccharides derived from five developmental stages of raspberry fruit and fractionated on Sepharose CL-4B. Column fractions (1.5 mL) were assayed for neutral sugars using the the anthrone method (Yemm and Willis, 1954). Vo, void volume; Vt, total volume.

3.2.6. Model for cell wall changes accompanying raspberry development

Overall the modifications observed in raspberry cell wall during development were similar to the results found in boysenberry. Arabinose was the most abundant cell wall sugar followed by galactose. This differs to previous reports in Glen Clova and Glen Closen varieties where xylose was found to be the most predominant wall component (Stewart et al., 2001). Furthermore the Xyl:Glc ratio largely exceded what it would be expected for xyloglucans which are the most abundant hemicelluloses in primary cell walls of dicot species suggesting that xylans might be abundant in raspberry cell wall. Pectic compounds seem to be the cell wall polymers undergoing the most extensive modifications. No clear changes were observed in the Cel-Hem matrix but cell wall polyuronides showed increased solubilization and a dramatic depolymerization in late ripening concomitantly with a large release of arabinose rich polymers to the water soluble fraction. The high glucose content found in the WSF was unexpected could be hypothesized to be related to the presence of glucans associated with cell wall polyuronides but further investigations are required to determine the nature of this observation. Excessive softening is a main difficulty for raspberry growers, handlers and processors. The results found in this work provide a better understanding for the cell wall modifications undergoing in this delicate fruit during development. Form a biotechnological perspective genes involved in pectin matrix disassembly seem to be better candidates to control softening, maintain quality and extend raspberry postharvest life

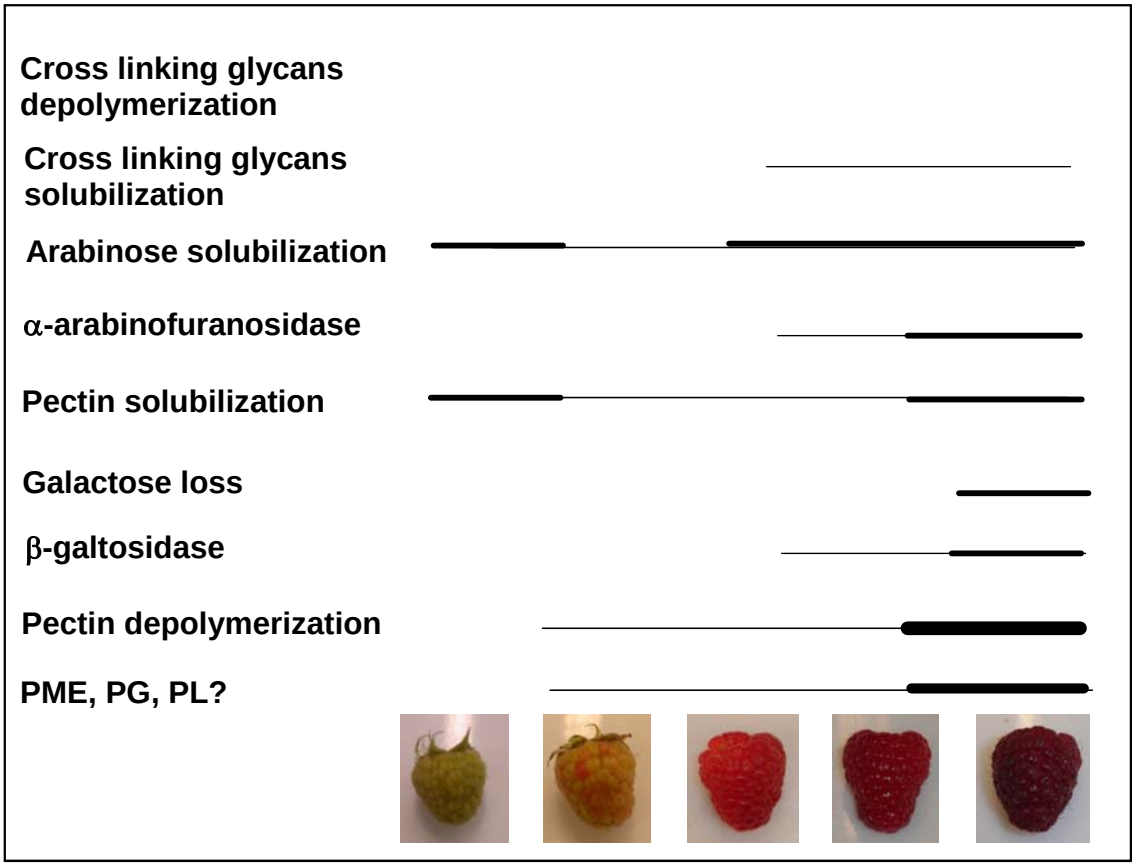
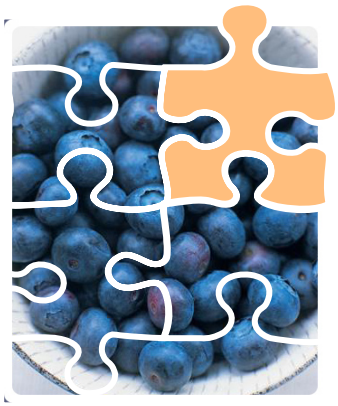


Figure 16: Proposed model for cell wall changes accompanying raspberry fruit development and softening.

3.3. Cell Wall Changes Accompanying Blueberry (*Vaccinium sp.*) Fruit Development



3.3.1 Fruit firmness, cell wall yield and composition

Both fruit firmness and cell wall yield decreased throughout blueberry development (Figure 17 A, B). The highest reduction in fruit firmness was observed from the green to the 25% B stage and was probably associated with fruit enlargement. Interestingly no great differences were observed in firmness between 100 % B and blue ripe fruit (Figure 17A). The total content of cell wall pectins and neutral sugars did not change throughout development either (Figure 18 A, B). Regarding the fruit cell wall sugar composition arabinose and xylose are the most abundant components in blueberry (Figure 18 C). The high levels of arabinose are coincident with the results found in the case of raspberries and boysenberries. However differently to what was observed in boysenberry and to previous reports in other blueberry cultivars no high loss of arabinose (Brummell, 2006) was observed during ripening.

Xyl is not a usually is not very abundant in fruit cell walls. In the primary cell wall of Dicots and non-comelinoid Monocots it is thought to be associated with xyloglucan. In the present work since the cell wall preparations were done with the whole fruit it is not possible to determine whether a particular tissue was enriched in this sugar. Stone cells have been reported to be present in blueberries. Sclereids are dead cells with extremely thick cell walls that make up to 90% of the whole cell volume. The secondary cell wall from these short sclereids might also contribute to the high levels of xylose observed in blueberry fruit. High xylose content was recently reported in bilberry fruit which also belong to the genus *Vaccinium* (Hilz et al., 2005). The bilberry seeds were particularly rich in xylose but the pulp still contained relatively high proportion of this sugar. However the high level of Xyl was concomitant with high proportions of glucose which exceeded the Xyl content either in the peel pulp and seed. This is different to the results obtained in blueberry cv Duke in which xylose content was 6 fold higher than glucose and clearly suggest the the presence of xylans. Further studies would be required to characterize then the nature and origin of the high xylose levels detected in this work in blueberry cell walls. This might valuable because there is currently much interest in the concept of ‘functional foods’. An recent approach is the consumption of food ingredients known as prebiotics. A prebiotic is defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid 1995). Plant cell walls are known to be a potential source of pharmacologically active polysaccharides (Ebringerova and Heinze, 2001). Several oligosaccharides have been reported to possess prebiotic activity (Hayakawa et al., 1990). Recent works have shown that dietary supplementation with xylooligosaccharides and fructooligosaccharides may be beneficial to gastrointestinal health. The effect of xylose containing oligomers was found to be even more effective than that of fructooligosaccharides (Hsu et al., 2004). Xylooligosaccharides may favor the selective growth of *Bifidobacterium* spp, (Rycroft et al., 2001; Palframan et al., 2003) which have important biological effects since they: i) suppress the activity of entero putrefactive and pathogenic intestinal bacteria due to the production of short chain fatty acids and ii) facilitate the absorption of nutrients. This has generated an increased commercial interest in these non-digestible oligosaccharides. The xylan-type polysaccharides are known to occur in several structural

varieties in terrestrial plants (Ebringerova and Heinze, 2001). The richest sources of xylans are represented by many by non edible tissues and species such as woody tissues of dicots and graminaceous monocots (grasses). In grasses, the branched arabinoxylans and mixed-link β-glucans are hypothesized to serve the role that xyloglucans play in dicotyledons (Carpita & Gibeaut 1993). Blueberry besides its known nutraceutical properties due to the high content of antioxidants might then also be a non-processed source of xylooligosaccharides.

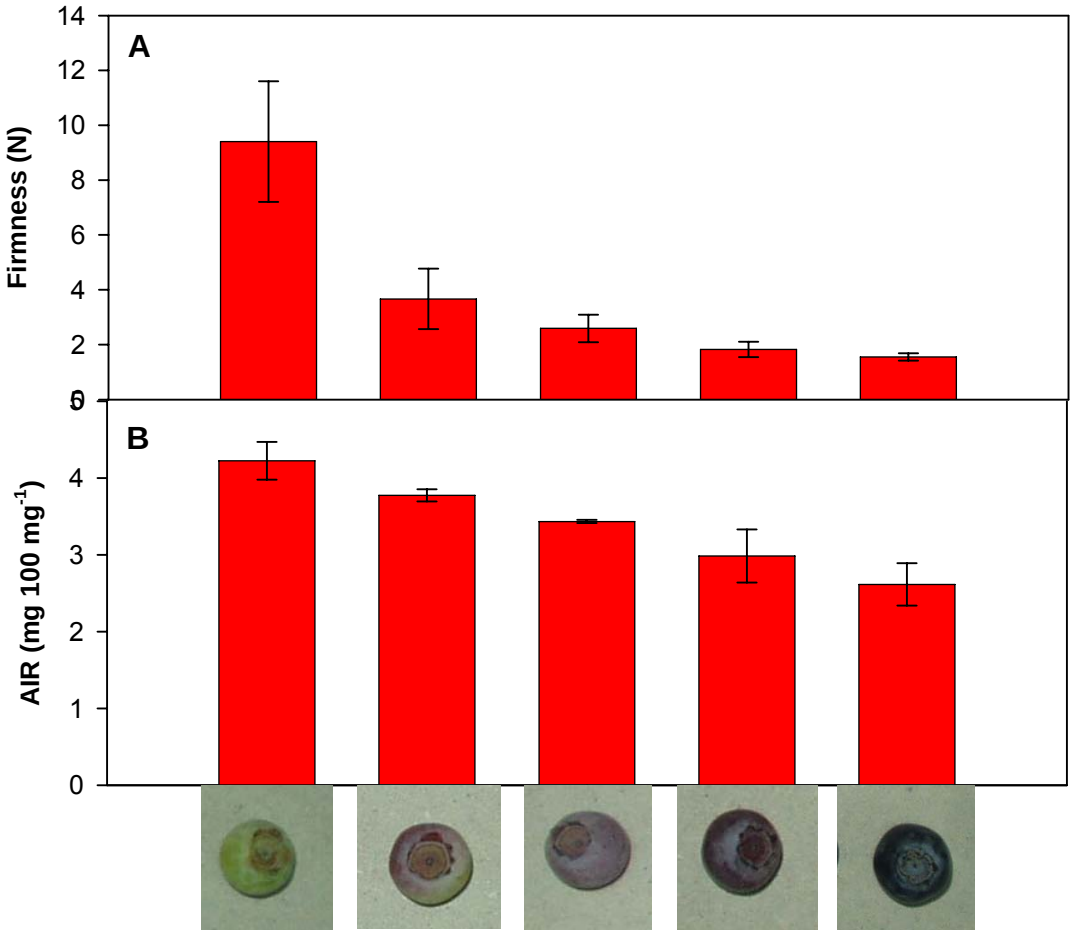


Figure 17: Changes in blueberry fruit firmness (A), and alcohol insoluble residue (B) during development. The standard deviation is shown.

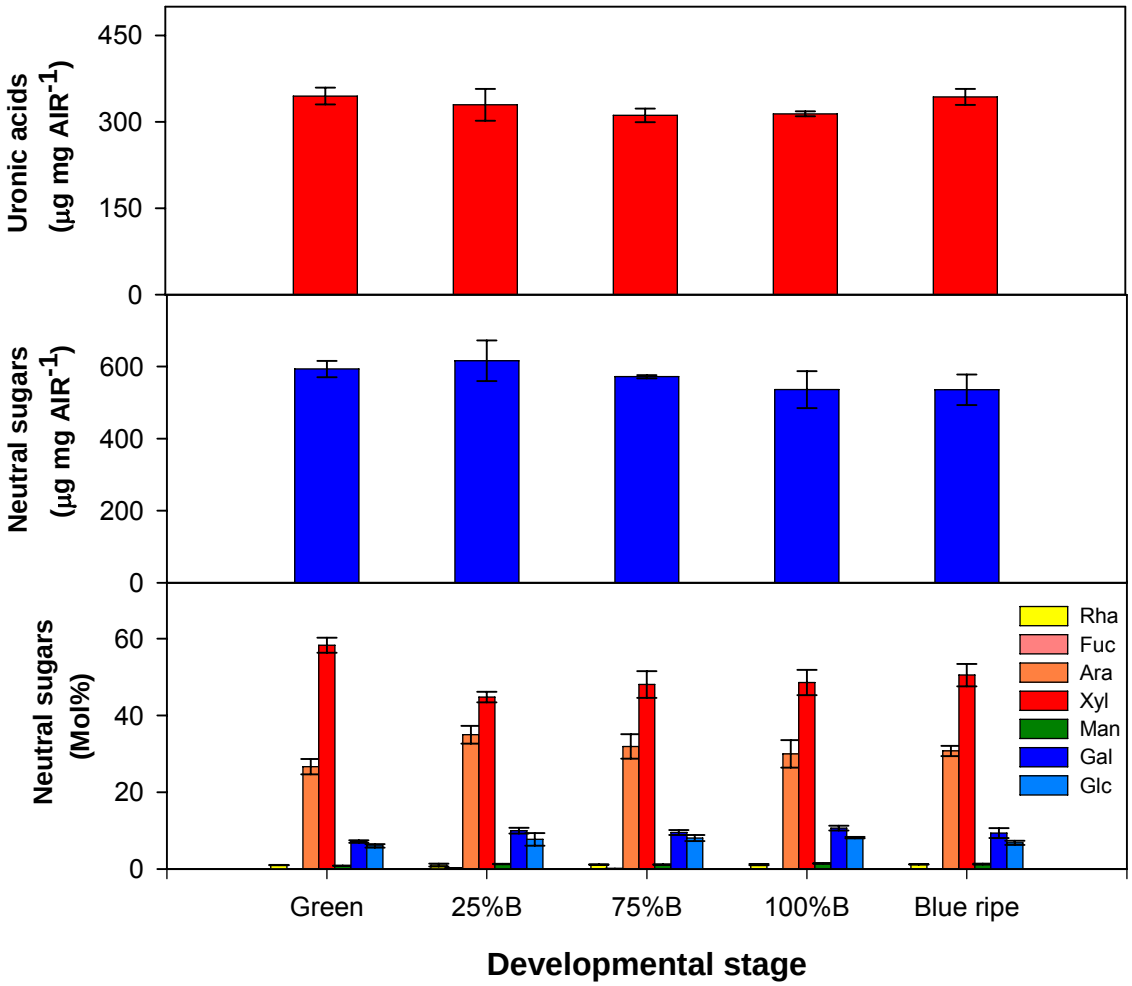


Figure 18: Changes in blueberry fruit uronic acids (A), neutral sugars (B), and cell wall monosaccharide composition (C) during development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

3.3.2. Pectin solubilization depolymerization and composition

Pectin in both the WSF and CDTA SF increased from the green to the 75% B stage (Figure 19). This increased solubilization was associated with a reduction of uronic acids in the NSF. However, after the 75% B stage no further increase in pectin solubilization was found showing a difference with the behavior observed in the case of boysenberries and raspberries. Regarding the sugar composition of the pectic fractions arabinose was found to be the most abundant component (Figure 20). The main changes during development were found in the arabinose and glucose proportions. Arabinose increased in the WSF and was reduced in the NSF while glucose Mol% decreased from the green to the blue ripe stage. These changes were similar to the modifications observed in the case of boysenberry and raspberry. However the results found in the case of pectin depolymerization were highly different from the changes detected in the other 2 berries. Pectin size did not show changes throughout development (Figure 18). The only change observed was a slight downshift in the CDTA fraction at the blue ripe stage but anyway the extent of these changes was much lower than the depolymerization found either in boysenberry and raspberry. The degree of pectin depolymerization has been shown to be variable among fruits (Brummell, 2006). In some cases extensive changes in pectin size are observed such as in avocado and peach. Tomato pectin depolymerization occurs at a lower degree but it is still substantial. Only a few fruits such as peppers have been shown to undergo low changes in molecular size. Early reports also showed a slight modification in CDTA soluble pectins size in strawberry (Huber, 1984. However recent studies found that in some cases HCl soluble pectin depolymerization is substantial. Consequently blueberry seems to be a special case in which softening is observed without clear modifications in pectin molecular size. Highbush blueberry firmness has been improved by postharvest applications of calcium chloride (Hanson et al., 1993) suggesting a role of the pectic matrix in the fruit textural properties. Since the results from the present work show that ripening-associated softening in blueberry occurs without substantial modifications on pectin polymer size, the positive effects of calcium on firmness retention may be caused by a reduced solubilization of pectin due to the formation of calcium bridges or due to an indirect effect on Cel-Hem disassembly but no pectin depolymerization.

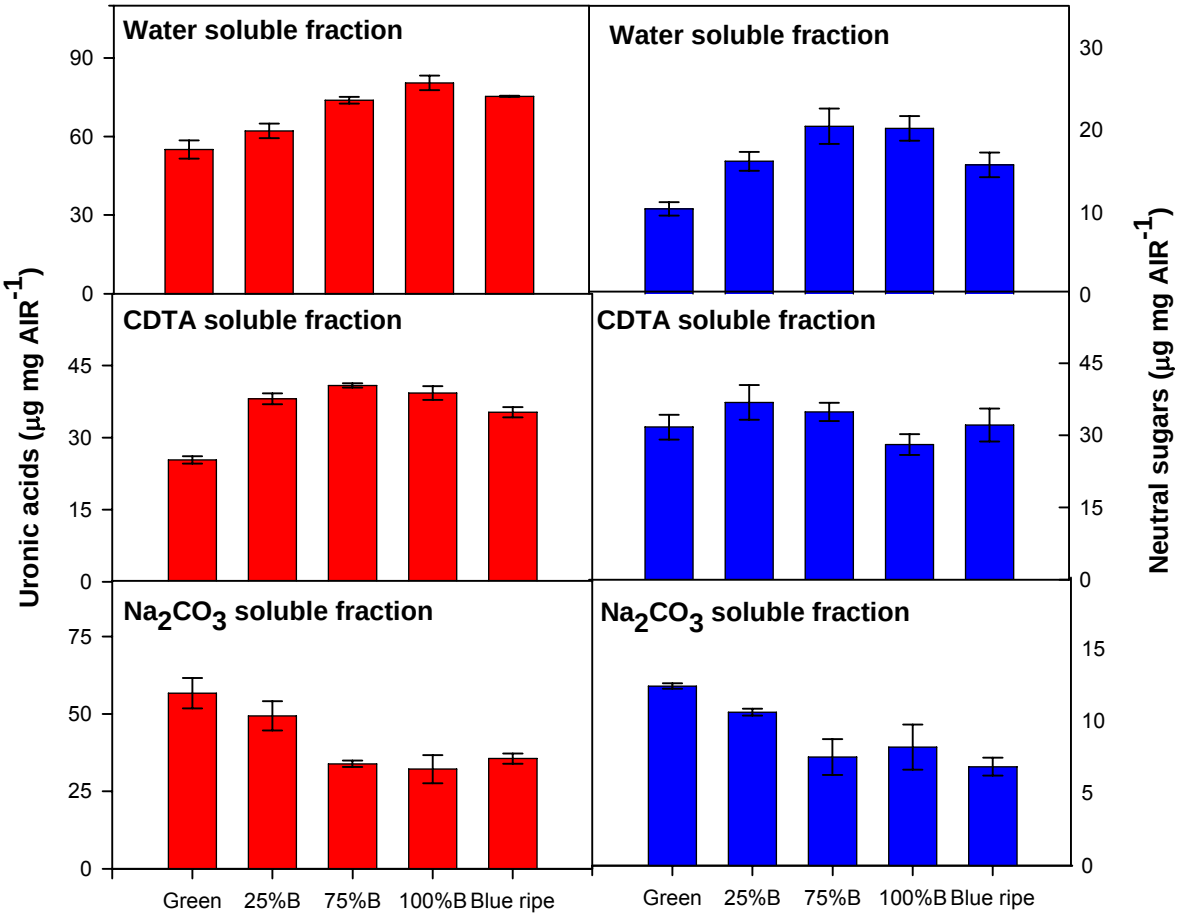


Figure 19: Changes in uronic acids (left panels) and neutral sugars (right panels) in the water (top panel), CDTA (middle panels) and Na_2CO_3 soluble fraction (lower panels) throughout blueberry fruit development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

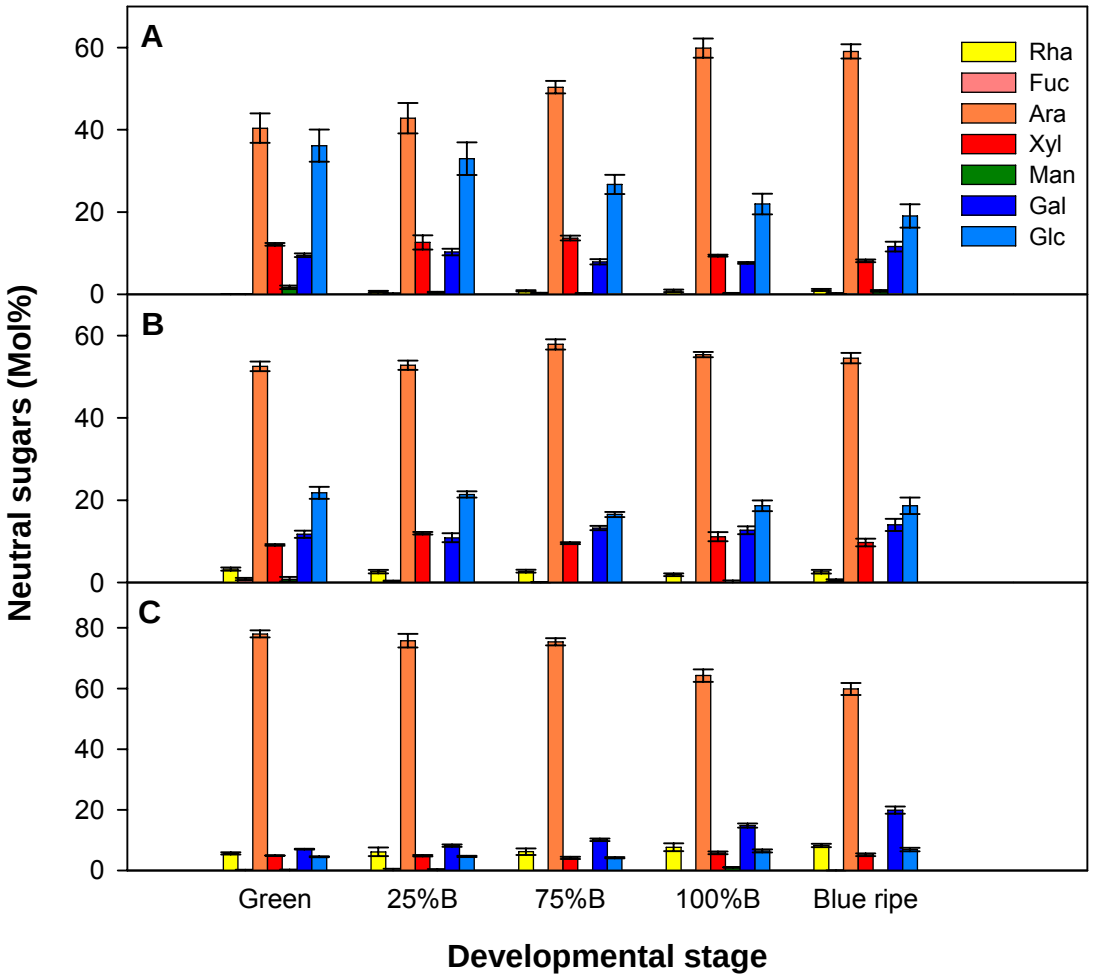


Figure 20: Neutral sugar composition (mol %) of blueberry water (A) CDTA (B) and Na_2CO_3 (C) soluble fraction throughout fruit development. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The standard deviation is shown.

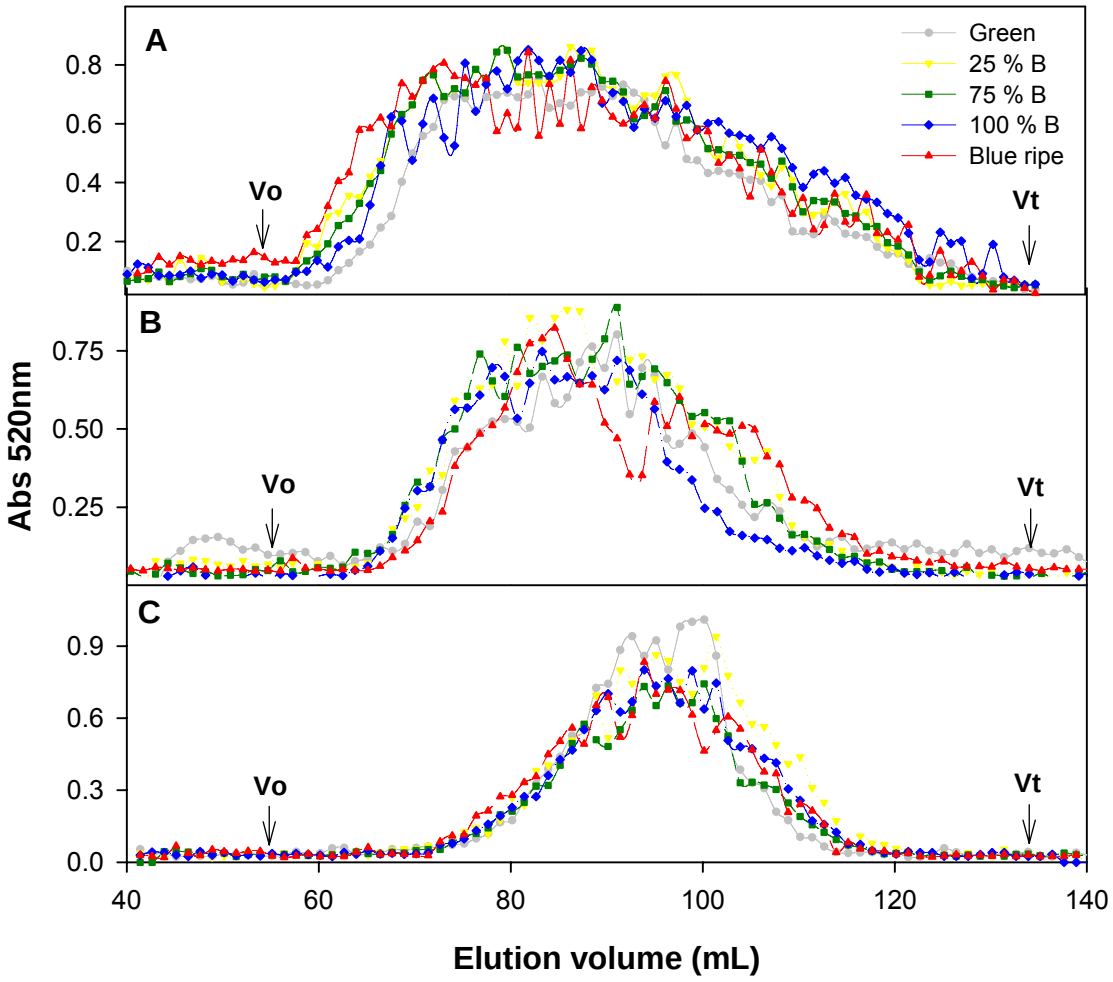


Figure 21: Size exclusion chromatography profiles from pectins throughout blueberry fruit development, fractionated on HW65. Column fractions (2 mL) were assayed for UA content using the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). Vo, void volume; Vt, total volume. A: Water soluble fraction. B: CDTA soluble fraction. C: Na_2CO_3 soluble fraction.

3.3.3. Hemicelluloses solubilization, depolymerization and composition

A reduction in 24 % KOH SF was observed during ripening (Figure 22 B). In the case of the 4 % KOH SF a slight increase was observed during development. The clearest modifications occurred from up to the 100 % B stage and afterwards no changes were observed. This could have been associated with an increased solubility of polymers initially present in the 24 % KOH SF. Regarding the monosaccharide composition of these fractions xylose was highly abundant. In the case of the 4 % KOH SF also high arabinose levels were found. This could be due to the presence of glucouronarabinoxylans which have a backbone of (1→4)β-D-xylan, with side chains of single units of non-reducing terminal α-L-arabinose and α-Dglucuronic acid. The main sugar modification observed in this fraction was a clear reduction in Ara between the 75 and 100 % B stages (Figure 23 A). In the case of the 24 % KOH SF Glc and Xyl were the most abundant components. The Xyl:Glc ratio observed at early stages of development matched with those expected for xyloglucan which has been shown to be the predominant hemicellulosic polymer present in this fraction in many fruits (Brummell, 2006). The main modification observed during development was a decrease in glucose suggesting that the decreased level of neutral sugars detected in late ripening in this fraction was due to the degradation of a glucan (Figure 23 B). The increase in the Xyl:Glc ratio over values of 1 in late stages of development also suggest the presence of xylans also in this fraction.

The loosely bound of matrix glycans, which seems to be particularly rich in arabino-xylans did not show changes in molecular weight profile during ripening (Figure 24 A). This has been observed in many other fruits in which depolymerization of matrix glycans is limited to those tightly bound to cellulose (Brummell and Harpster, 2001). However in the case of the 24 % KOH soluble fractions a clear downshift in molecular size was observed throughout development. From the 25 to the 100 % B stage a virtual disappearance of the peak eluting at 45 mL was observed and afterwards the reduction in polymer size continues but to a lower extent. Depolymerizartion of matrix glycans is thought to be an important contributor to fruit softening (Brummell, 2006) and a reduction in molecular size of these components has been observed in all species tested (Brummell, 2006). Despite of this the depolymerization of cross linking glycans is usually accompanied with reductions also in the pectic matrix componet size. From this view the changes observed in the case ob blueberry cv. Duke seem unique since no changes in the

pectic polymer size were observed throughout development. This also suggest that ripening associated softening in the case of blueberry seem to be more related to modifications in the Hem-Cel matrix than in the pectic matrix in which the changes were much lower. The possibility of pectin matrix affecting softening in overripe stages should not be ruled out. This has been shown in tomatoes in which pectin depolymerization takes place extremely late in ripening. However from a biotechnological perspective earlier softening control might be required and genes involved in Cel-Hem matrix disassembly might be better targets in this fruit.

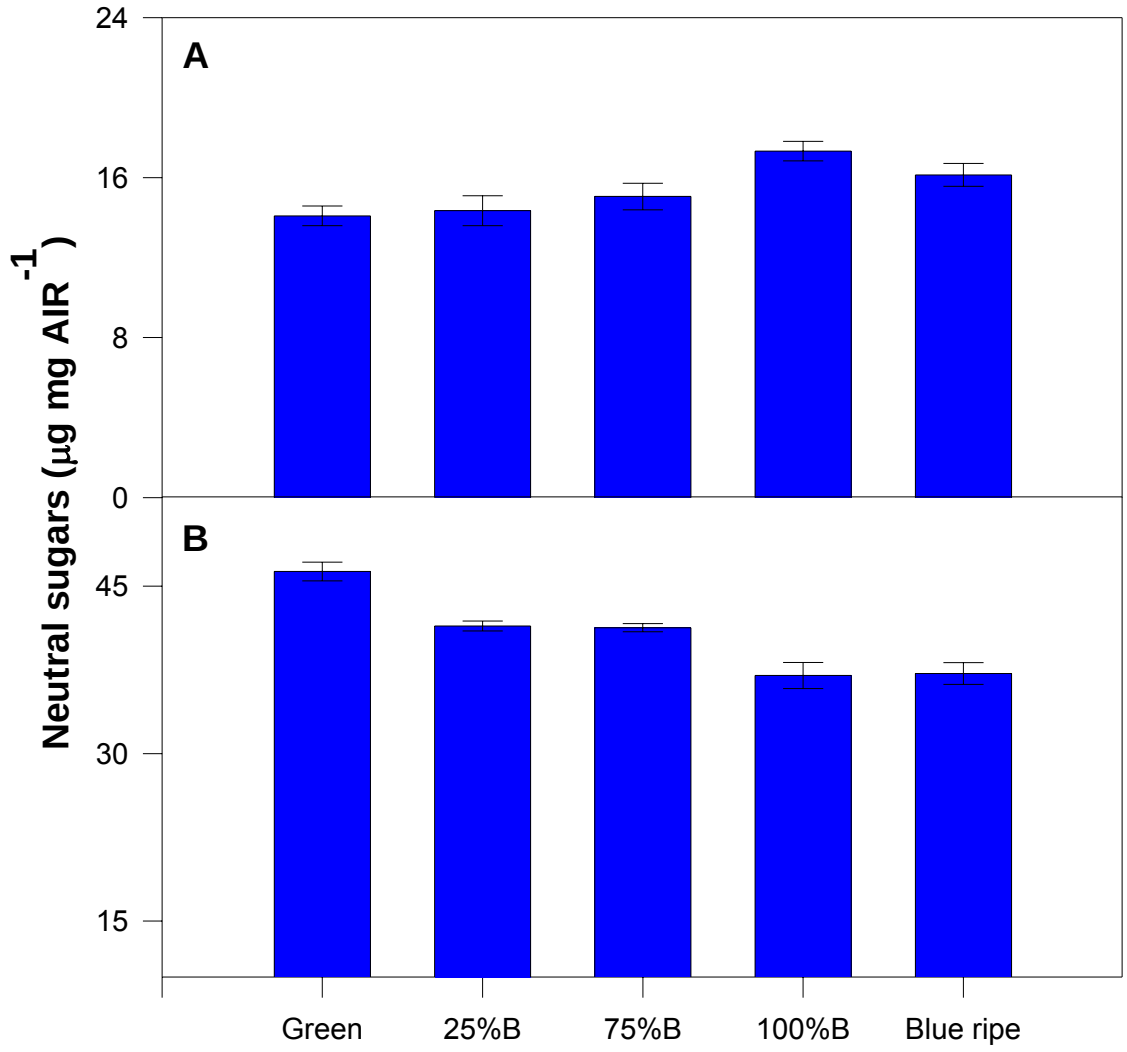


Figure 22: Changes cross-linking glycans throughout blueberry fruit development. A: 4 % KOH soluble fraction. B: 24 % KOH soluble fraction. 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color). The least significant difference (LSD) at P= 0.05 is shown.

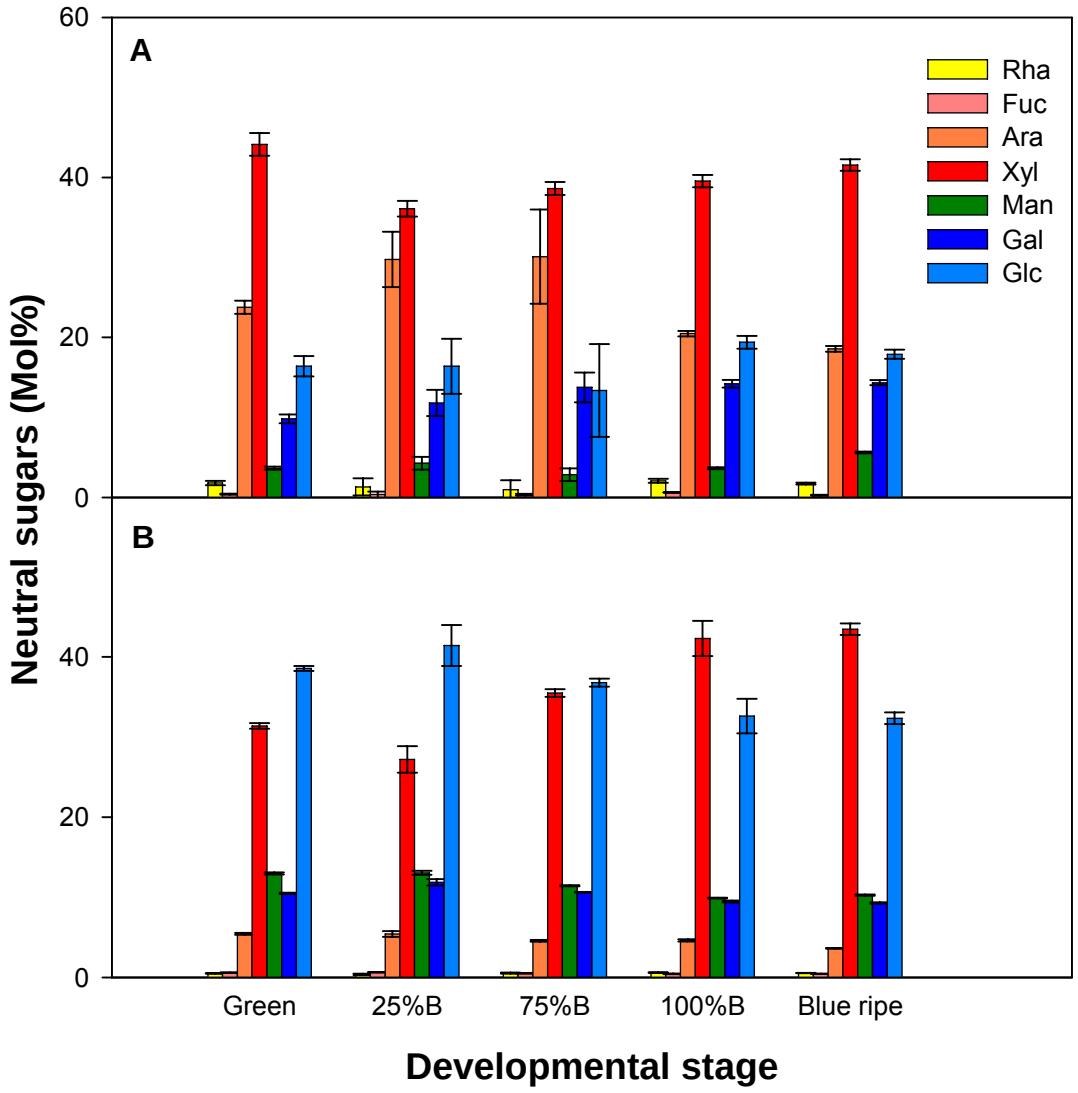


Figure 23: Neutral sugar composition (mol %) of raspberry 4 % KOH (top panel) 24 % KOH soluble fraction throughout fruit development. G: large green; 25 % B (25 % surface blue color); 75 % B (75 % surface blue color); 100 % B (100 % surface blue color); BR: Blue ripe. The standard deviation is shown.

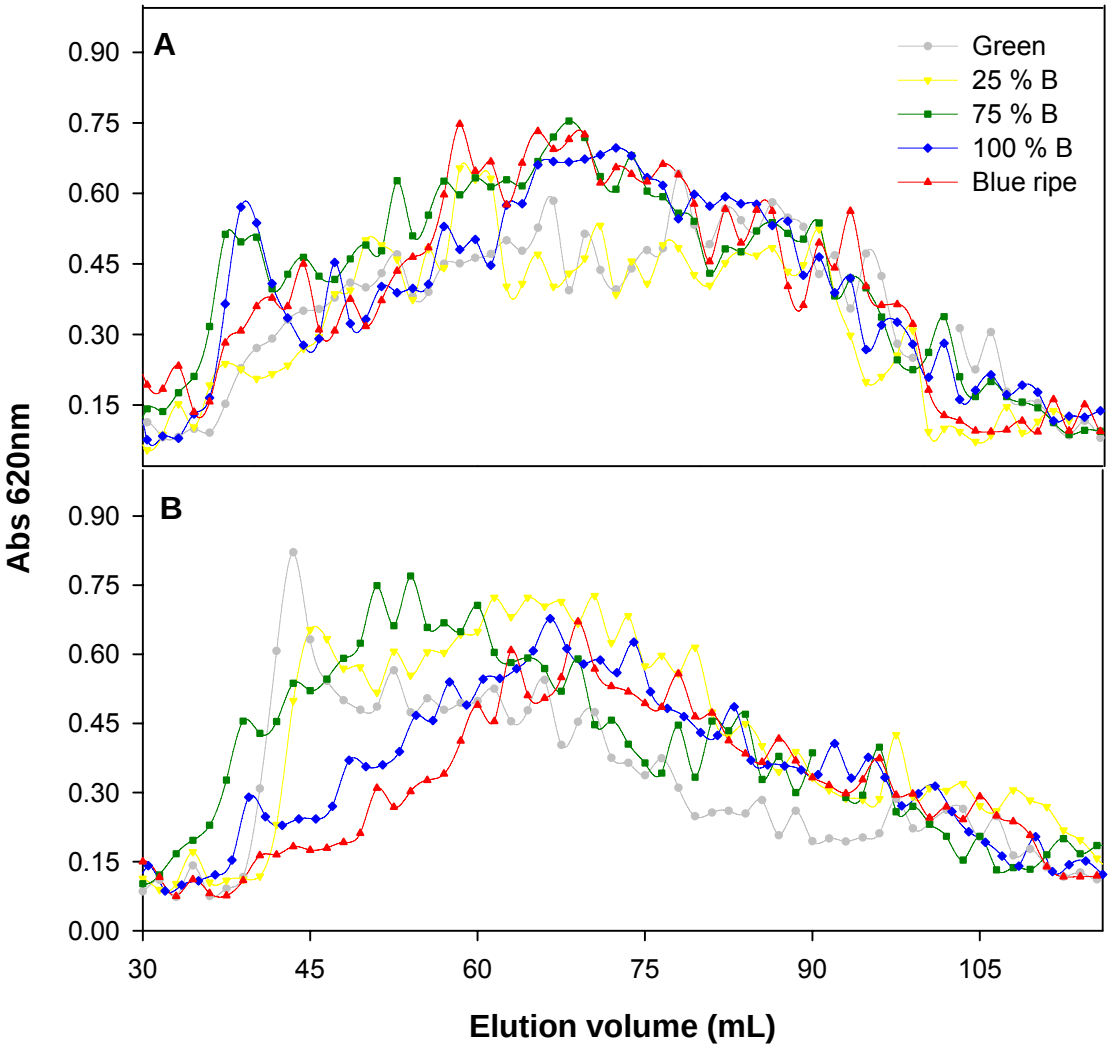


Figure 24: Gel-filtration profiles of 4 % (A) and 24 % (B) KOH-soluble polysaccharides derived from five developmental stages of blueberry fruit and fractionated on Sepharose CL-4B. Column fractions (1.5 mL) were assayed for neutral sugars using the phenol-sulfuric acid method (Dubois et al., 1956). Vo, void volume; Vt, total volume.

3.3.4. Model for cell wall changes during blueberry development

Blueberry firmness is a very important trait in keeping quality, shelf life, and consumer acceptance. Since blueberries ripen very little after harvest, they must be picked at full maturity in order to attain higher flavor and early picking is not a feasible strategy like in other fruits to .have firmer fruit during retail. The increasing conversion from hand to machine harvesting of blueberries by growers with large plantings or co-operatives is another issue that could require varieties with better textural properties. A 20 to 30 % reduction in fruit firmness (20-30%) was caused by machine harvesting. In addition 15 % loss of firmness was observed during grading and sorting (NeSmith et al., 2002). Consequently the generation of firmer fruit varieties might be required. In addition the increasing shipment distances in the fresh blueberry market might also demand the use of fruits with reduced softening to yield longer keeping quality berries (Silva et al., 2005). This work provided some fundamental information for understanding the biochemical basis of cell wall disassembly in blueberry and might be useful for guidance for further works iimed of controlling softening:

1-Xylose is the most abundant sugar in the blueberry cell wall and this is different from what has been reported in other fruits. It would be useful to determine if the xylose-rich polymers are associated with the fruit seeds or with the pulp and the nature of the xylose-containing polysaccharides.

2-The changes associated with blueberry fruit cell wall disassembly are quite different from those reported for other berries studied in this project (boysenberries and raspberries) and also from other fruit models studied to date. The main cell wall modifications observed were:

- an early stage associated with the solubilization of pectin

- a late stage characterized mainly by an increased arabinose solubilization probably from arabino-xylans a reduction in glucan content and downshifts in hemicellulose, but not pectin, molecular size.

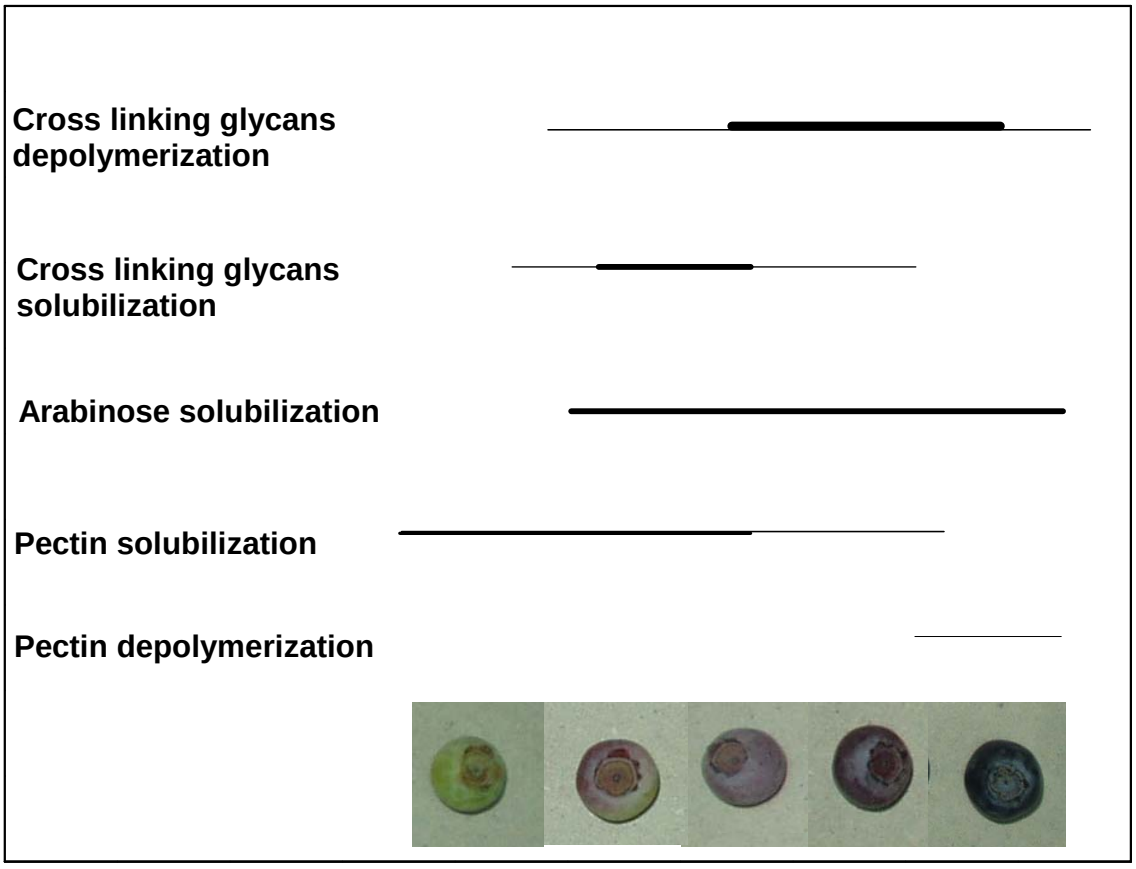


Figure 25: Proposed model for cell wall changes accompanying blueberry fruit development and softening. G: large green; 25 % B (25 % surface blue color); 75 % R (75 % surface blue color); 100 % R (100 % surface blue color); BR: Blue ripe.

4. CONCLUSIONS

Not many studies have been done in fruits analyzing sufficient number of ripening stages and sufficient number of cell wall changes to allow a sequential interrelation of events (Brummell, 2006). Our worked tried to analyze the cell wall modifications in 3 different doft fruits at 5 different ripening stages and aiming to look at most changes cell wall components and proceses (pectiic and hemicelluloses, solubilization, depolymerization and sugar composition). The results provide some basis for understanding the biochemistry of cell wall degradation in boysenberry raspberry and boysenberry, fruits with extremely high cell wall metabolism and high softening rate, with the peculiarity of having overlaps between fruit growth and ripening and for which there have no many prior detailed studies.

The chronology of cell wall modifications in most fruit was recently reviewed (Brummell, 2006). In general cell wall disassembly in boysenberry and raspberry seem to fit in this sequence but the changes found in blueberry seem to be quite different to this general pattern. At a more detailed level there are some other differences in cell wall structure and composition of berry fruits like the high prevalence of arabinans and xylans that would be useful to explore further. Although the cell wall changes associated with fruit development do not proceed in discrete stages and cell wall disassembly results as a consequence of highly regulated changes occurring in a continuum, the results suggest that temporal changes in cell wall degradation in boysenberry includes at least 3 stages: an early stage (Green to 75 % R) associated with cellulose and cross-linking glycan metabolism, an intermediate period characterized by a clear increase in pectin solubilization without depolymerization in which arabinose is lost (75-100 % R), and a final stage characterized mainly by a reduction of galactose and a dramatic increase in pectin depolymerization. In the case of raspberry the similar pattern was observed; no depolymerization of hemicelluloses during development an increase in α-arabinofuranosidase activity consistent with a loss of arabinose from the cell wall and increased solubilization of arabinose-rich polyuronides in later stages and a final dramatic depolymerization of all the pectic fractions at the red ripe stage. In blueberry the temporal sequence of cell wall disassembly included an early stage is associated with the solubilization of pectin while later in development increased arabinose solubilization is followed by a reduction in glucan content and downshift in hemicellulose, but not pectin, molecular size. From a biotechnological perspective enzymes involved in pectin matrix disassembly seem to be the better candidates to affect the late softening of boysenberry and raspberry fruit using genetic intervention. Approaches trying to reduce

pectin depolymerization by affecting PG levels should not disregard the potential functional redundancy that PLs might have in such process. Conversely in the case of blueberry endoglucanases and or expabnsins might be envisioned as candidates to reduce the disassembly of the Hem-Cel matrix. Because all three fruits, raspberry, blueberry, boysenberry pectins are enriched in arabinose relative to galactose, in contrast to tomato and melon pectins, strategies considering α-ara as a potential target to reduce late softening by directly affecting pectin solubilization and indirectly pectin depolymerization and or cross linking glycans disassembly should also consider the potential negative side effects of altering arabinan metabolism in a fruit in which enlargement occurs simultaneously with ripening.

5. APPENDIX I:

"RECENT FINDINGS IN PLANT CELL WALL STRUCTURE AND METABOLISM: FUTURE CHALLENGES AND POTENTIAL IMPLICATIONS FOR SOFTENING"

ABSTRACT

One of the main problems limiting the postharvest life of horticultural products is excessive softening. The textural properties of produce are associated with modifications in cell wall metabolism that affect wall structure and composition. Recent findings have increased the breadth of our understanding of cell wall metabolism. This article discusses future challenges and potential implications of softening. Work in the last decade has changed the way we visualize the cell wall. In terms of cell wall structure, observations showing the existence of covalent interactions between the walls's pectic and hemicellulose-cellulose microfibril networks have challenged some features of earlier cell wall models. There is also clear evidence showing that the borate ester cross-linking of the pectic compound rhamnogalacturonan II (RG-II) may influence plant growth and cell wall physical properties. It would be useful to evaluate the potential role of RG-II metabolism in fruit growth and ripening-associated softening. Another striking observation relates to wall plasticity, the way the wall's polymer composition can be varied and still maintain homeostasis. Understanding the extent to which cell walls could be modified without negative phenotypic side effects would probably contribute to the generation of alternative strategies for genetic manipulation of softening. A great deal is known about ripening-related changes in wall-modifying enzyme activities, as well as the developmental changes in the expression of the genes that encode these proteins. However, the biochemical characterization (eg, determination of *in vivo* substrates, specific steric considerations that influence enzyme-substrate interactions, etc.) is still incomplete. Furthermore, investigations to identify wall-modifying activities that are known influence wall polymer integrity during plant cell growth, interactions with pathogens, etc. (eg, pectin acetylesterases, rhamnogalacturonases, yieldins and lipid transfer proteins) have received little emphasis in studies of fruit growth and ripening. The genetic engineering of fruits to delay softening via down-regulation of individual genes encoding wall-modifying enzymes has given limited success in some cases. However, approaches yielding the modified expression of combinations of genes could be useful for altering cell wall disassembly more dramatically or overcoming functional redundancy; eg, polygalacturonase and pectate lyase, enzymes that use different mechanisms for cleaving the same homogalacturonan pectin target. Finally, most of the strategies evaluated to date have focused on reducing or altering cell wall degradation. An alternative

approach that could have value in modifying wall behavior would be a focus on modifying cell wall synthesis in order to generate custom-designed cell walls that could have desired functional properties.

Abbreviations

Hem-Cel Hemicellulose–Cellulose Network

PG Polygalacturonase

RG-II Rhamnogalacturonan II

eiF-5A Eukaryotic Translation Initiation Factor-5A

1. INTRODUCTION

Fruit ripening is a highly regulated process involving changes in fruit appearance, shape, size, color, flavor, aroma and texture (Giovannoni 2001; White, 2000). Controlled softening is a desired change in fruits from the consumers' perspective. However, excessive loss of firmness is a major problem in postharvest technology of horticultural crops (Fisher and Bennett, 1991). Reduction of firm texture affects fruit quality and postharvest storage (Peña and Carpita, 2004), reducing consumer acceptability, limiting transportation (Brummell and Harpster, 2001) and, perhaps also increasing decay due to reduced strength of the cell wall, a main barrier against tissue colonization by plant pathogens (Vorwerk et al., 2004). Fruit firmness is affected by several factors. For example, it may change due to altered hydrostatic pressure (turgor) within fruit cells (Shackel et al., 1991; King et al., 2000; Salentijn et al., 2003). Membrane damage and dehydration and mesocarp cell enlargement could be involved in textural changes in some fruits (Sexton et al., 1997; Waldron et al., 2003). However, fruit textural changes are thought to be, at least in part, a consequence of changes in the composition and architecture of the cell wall (Brummell and Harpster, 2001). Plant cell walls are highly complex, dynamic and organized structures composed of polysaccharides, proteins and phenolic compounds, as well as some ions (Carpita and Gibeaut, 1993). They perform a diversity of functions during the life of the plant, such as determining the direction of growth and cell shape, counterbalancing the osmotic pressure, regulating diffusion of material through the apoplast, serving as a source of biologically active signalling molecules, protecting the cell against pathogens and providing mechanical support (Brett and Waldron, 1996; Carpita

and McCann, 2000). In the last few years, there have been several new insights in plant cell wall structure and disassembly. General features of these findings, potential implications for fruit softening control, and directions for further research to increase our understanding of cell wall structure and disassembly are discussed.

2. THE ROLE OF RG II AS A MODULATOR OF CELL WALL PROPERTIES

A detailed description of the plant cell wall structure and composition has been reviewed elsewhere (Brummell and Harpster, 2001, Carpita and McCann, 2000). The classification of the composition of wall polymers is based, historically, on chemical structure and means of extraction (Selvendran et al., 1985). There are three major categories of wall polysaccharides: cellulose, hemicelluloses and pectins. The pectic polysaccharides of the primary cell walls of all land plants are among the most structurally complex macromolecules occurring in nature (Willats et al., 2001; Ridley et al., 2001). Pectins include a mixture of polysaccharides with varying degrees of complexity. They are characterized as being rich in D-galacturonic acid and are particularly abundant in, but not limited to the middle lamella. The most studied pectins are homogalacturonan and rhamnogalacturonans I and II (RG-II). In particular, RG-II has been structurally and functionally characterised in the last few years, giving rise to new and interesting ideas about the relationship of wall polysaccharides and their metabolism to cell wall functions. RG-II is a structurally complex pectic polysaccharide containing 12 different glycoses (O'Neil et al., 2004). It was identified in 1978 as a minor component of sycamore cell walls and has been shown to be present in the primary walls of all vascular plants examined to date (O'Neil et al., 2004; Matsunaga et al., 2004). Other studies reported a correlation between the boron requirement of plants and the pectin content of the cell wall (Hu et al., 1996). However, until recently, the molecular basis for this correlation was not known. Kobayashi et al. (1996) reported that borate can interact with RG-II, cross-linking two polymeric RG-II monomers to form a dimer in the primary walls of angiosperms. The functional implications of making or removing these complexes have been shown to be more exciting than the structural and chemical characterization of the RG-II cell wall component. Direct evidence showed that changes in the mechanical properties of the wall result from reduced cross-linking of RG-II (Fleischer et al., 1998). Boron deficiency in pumpkin plants resulted in a substantial reduction in growth (O'Neil et al.,

2001). The tissues of boron-deficient plants are often brittle and do not expand normally. Thickening of cell walls correlates with the increased RG-II monomer:dimer ratio that results when cell walls develop under borate-deficient conditions (Ishii et al., 2001). The results of these studies suggest that a primary function of borate is to covalently cross link wall pectin and that this cross linking is required for controlling the size exclusion limit of the wall (hence protein mobility in the apoplast), the mechanical properties of the primary wall and turgor-driven wall expansion (O'Neil et al., 2001, 2004; Ishii and Matsunaga, 1996; Ishii et al., 1999). These advances in our knowledge of the of RG-II structure, combined with studies in molecular biology and plant nutrition and the availability of mutant plants, have begun to provide insight into the relationship between the structure and biological functions of this cell wall pectic polysaccharide. However, not much attention has been focused on understanding the potential role of RG-II metabolism during fruit growth and ripening-associated softening.

3. NEW EVIDENCE OF CELL WALL POLYMER INTERACTIONS

While the information about structural features of the different cell wall components is quite well established, it has been much more difficult to determine how these components are associated with each other in a mature wall. The organization and interactions of wall components are not known with certainty. Based on the biochemical information of the different cell wall components, their proportions, their ease of extraction with different treatments, cell wall biophysical properties, and microscopy studies, several plant cell wall models have been proposed (Carpita and Gibeaut, 1993; Keegstra et al., 1973; Cosgrove et al., 2001; Thompson, 2005). Such models are useful to provide a framework for understanding the cell wall architecture. Some models of primary cell walls of dicotyledons emphasize non-covalent interactions between polymers and suggest the existence of a cellulose-xyloglucan network embedded in a matrix of pectic polysaccharides (Carpita and McCann, 2000; Somerville et al., 2004). Hemicelluloses are thought to interact through hydrogen bonds with the cellulose, coating and connecting adjacent microfibrils producing a network of interconnecting polymers (Rose and Bennett, 1999). This hemicellulose-cellulose network (Hem-Cel) is embedded in a matrix in which various types of non-covalent cross-links between pectins involving calcium ion hydrogen bonds, and hydrophobic interactions have been described (Voragen et al., 1995). The occurrence of covalent xyloglucan–pectin linkages

within the dicot cell wall was first proposed in the model by Keegstra et al. (1973), but direct evidence about these interactions remained elusive. Recent findings support the existence of xyloglucan–RG-I conjugates, which are widespread in the cell walls of angiosperms (Popper and Fry, 2005). Research involving *in vitro* studies have provided no evidence for molecularly intimate (ie, covalent) associations between cellulose and pectins (Chanliaud and Gidley, 1999). However, other authors have proposed that neutral sugar side chains of pectins could bind to cellulose (Oechslin et al., 2003; Vignon et al., 2004). Recently Zykwinska et al. (2005) provided evidence of molecular interactions between cellulose microfibrils and pectins, presumably through the arabinan or galactan side chains. This study supports the existence of structural interactions between pectin and the Hem-Cel matrices, which have been considered to be quite independent in many cell wall models. A clearer understanding of these structural interactions would be of value in interpreting the observations of studies in which reverse genetic approaches for modifying cell wall metabolism in ripening fruit have produced unexpected results (Brummell and Harpster, 2001). For example, Brummell et al. (1999) reported that reduced expression of *Exp 1* (encoding tomato fruit ripening related expansin) that was thought to be involved in the relaxation of the Hem-Cel matrix substantially reduced the breakdown of pectin polysaccharides as ripening proceeded. Over-expression of polygalacturonase (PG) also affected Hem-Cel matrix degradation (Vicente, unpublished results). These observations have two corollaries that should be considered:

• Cell wall disassembly should be viewed as a cooperative process involving the modification of structurally different but interacting polymers.

• It is important to follow changes in all cell wall polymeric components in studies in which transgenic modification targets expression of a gene encoding a protein whose action is thought to be specifically and narrowly understood. Understanding the nature and significance of these interactions from a practical perspective would be useful in defining new searches for candidates and strategies to delay softening.

4. MOVING FROM CELL WALL MODELS TO REAL CELL WALL ARCHITECTURE AND METABOLISM

Plant cell wall models are extremely useful representations of cell wall architecture. However it is important to recognize that that the composition and arrangement of the cell wall components differ between species, cultivars, tissues and even regions within an individual cell (Carpita and McCann, 2000). Novel techniques of 'seeing the wall' could be extremely useful for overcoming these limitations of general wall models. Several technical advances to accelerate the analysis of wall structure have been made recently. One promising technique includes the use of Fourier-transform infrared spectroscopy, which has been used to screen populations of mutants for cell wall phenotypes (McCann et al., 2001; Mouliie et al., 2003). Chemometric analyses of infrared spectra, obtained from cell walls isolated from mutant lines, have identified wall preparations with altered compositions and architectures (McCann et al., 2001). The analysis can also be used to describe changes in polysaccharide distribution across tissues. Concerning proteome analysis, experimental tools and computational prediction is expanding the catalogue of wall localized proteins and providing the basis for further exploration of the 'plant wall compartment' (Lee et al., 2005). Techniques for global analysis of 'glycomes' would also be useful. Willats et al. (2002) reported the development of a new slide surface coated with a range of carbohydrate epitope-recognizing antibodies that is capable of immobilizing glycan structures without modification, allowing the development of comprehensive carbohydrate microarrays. Enzymatic fingerprinting is another rapid means of analyzing a specific cell wall polysaccharide component. Digestion of the complex mixture of wall polysaccharides with a specific polysaccharide hydrolase produces a signature of product oligosaccharides that could be then determined by matrix assisted laser desorption ionisation time of flight mass spectrometry analysis (Lerouxel et al., 2002; Pauly et al., 2001). Recently, Barton et al. (2005) showed that carbohydrate gel electrophoresis is a technique well suited for screening quantitative or structural alterations in specific polysaccharides. Finally, the use of antibodies coupled to fluorescent markers, has been useful for determining tissue-specific distributions of particular cell components (Knox, 1997; Willats et al., 2000). Using these techniques, it has been shown that methyl-esterified pectin is present throughout the walls of parenchyma cells, that the galactan side-chains of RGI are next to the

plasma membrane, but excluded from the middle lamella region that sticks two cells together, and that the calcium cross-linked junction zones are localized to the cell corners (McCann et al., 2001).

5. UNDERSTANDING CELL WALL PLASTICITY

Several reports have shown relatively high plasticity in cell wall polymer composition. Cell walls of tobacco under osmotic stress have shown decreased levels of cellulose and higher proportions of hemicelluloses, but no changes in the pectic fractions (Iraki et al., 1989). His et al. (2001) analyzed the changes in cell wall composition in a *kor* mutant lacking a membrane bound β-1,4-endoglucanase. The mutants had lower levels of cellulose but, interestingly, had enrichment of pectic polysaccharides in excess of the increase expected simply due to cellulose reduction; presumably a compensatory mechanism. These findings may reflect an adaptation of the cell to compensate for reduced cell wall strength by increasing the pectin network, suggesting that feedback mechanisms translate changes in the cell wall composition or even strength into modifications of cell wall polysaccharide synthesis, assembly or processing. This plasticity, implying efforts of plant cells to maintain homeostasis by varying cell wall composition, may determine organs able to have normal functions (eg, fruit growth) even with some differences in cell wall composition. However, the dismantling of such distinct walls would probably require different processes (or at least different degrees, timing or spatial distribution of wall catabolism). Very little is known about the metabolic interconnections that account for this plasticity and help to predict the extent to which cell walls could be modified without negative phenotypic side effects. An understanding of this would probably contribute in the generation of alternatives to manipulate softening.

6. FURTHER BIOCHEMICAL CHARACTERIZATION OF MANY CELL WALL DEGRADING AGENTS

Fruit development requires a fine and concerted balance between cell wall synthesis and degradation, generating a structure that can fulfill the requirements of a growing fruit in terms of extensibility (Cosgrove et al., 2002), and finally lead to the dramatic disassembly of the structure accompanying the ripening process (Rose et al., 2004). The pattern and biochemical basis of this ‘organized disorganization’ varies depending on the species or the variety considered. Different enzymes and loosening agents contribute to the changes in cell wall architecture (Fisher and Bennett, 1991;

Giovannoni, 2004). A detailed review about changes associated with fruit ripening has been published recently (Lashbrook, 2005). Within a single species; each wall-modifying activity includes protein isoforms, some of which are active at different stages of fruit growth and others that are specifically associated with ripening. The most characterized and studied cell wall degrading proteins in fruits have been recently reviewed by Owino et al. (2005) and include PGs (Hadfield and Bennett, 1998), pectin methylesterases (Tieman and Handa, 1994; Tieman et al., 1992), β-galactosidases (Smith et al., 1998; Smith and Gross, 2000, 2002), endo-β-1,4-D-glucanases (Brummell et al., 1999; Llop-Tous et al., 1999), expansins (Brummell et al., 1999; Rose et al., 2000) and to a lesser extent mannanases (Bewley et al., 2000), xylosidases (Martínez et al., 2004; Itai et al., 1999), α-arabinofuranosidases (Saha, 2000; Sozzi et al., 2002; Tateishi et al., 2005), pectin and pectate lyases Dominguez–Puigjaner et al., 1997; Medina–Escobar et al., 1997; Jiménez–Bermúdez et al., 2002), and xyloglucan transglycosylase hydrolases (Percy et al., 1996; Redgwell et al., 1993). In some cases, much is known about ripening-related changes in enzyme activity and gene expression in different species, but the biochemical characterization of many cell wall degrading enzymes is still incomplete. For instance, the *in vivo* substrates of many enzymes are unknown and only a few investigations have tried to address this kind of question (Maclachlan and Brady, 1994). Furthermore, with a few exceptions, the specific steric considerations governing requirements, substrate-enzyme interactions and subsequent catalysis are largely uncharacterized. An exception is PG whose main substrate is homogalacturonan, which is secreted in a highly esterified fashion and methyl group removal markedly increases PG activity. Efforts in this direction will be helpful to understand the significance of cell wall degrading agents *in muro*.

7. STUDIES ON NEW OR ALMOST UNCHARACTERIZED AGENTS AFFECTING WALL DEGRADATION

Many proteins are known to catalyse aspects of polysaccharide synthesis and disassembly. For example, *Arabidopsis* contains 379 glycoside hydrolases (Henrissat et al., 2001). While several studies have been devoted to understanding the roles of certain cell wall degrading proteins in fruit softening, other agents affecting wall degradation have received much less attention, perhaps because of the difficulty assaying them *in vitro*.

Acetyl esterases: In addition to methyl esters in pectic compounds, O-acetylation can occur at C2 and C3 of galacturonosyl residues. Acetylated homogalacturonan appears to be particularly abundant in sugar beet roots and potato tubers (Ishii, 1997; Pauly et al., 2000). Xylans can be also acetylated. Acetylation, like methyl esterification, is thought to occur during biosynthesis and before deposition in the cell wall. As with pectin methylesterification, the degree of acetylation modulates the properties of pectin molecules, including influencing their degradation and contributing to the structural complexity of the pectin network (Mc Neil et al., 1984). Acetylesterase (EC 3.1.1.6), which specifically deacetylates cell wall carbohydrate polymers (Vercauteren et al., 2002), have been studied mainly in microorganisms (Searle–van Leeuwen et al., 1992). These enzymes have been identified in *Arabidopsis* (Vercauteren et al., 2002), and purified from mungbean (Bordenave and Goldberg, 1995) and orange (Williamson, 1991). Pectin acetylesterases catalyse the deacetylation of esterified pectin. Deacetylation of pectin, diminishing the pectin backbone hydrophobicity, increases its solubility in water (Dea and Madden, 1986). Removal of these esters from the pectin backbone also makes the polysaccharide more accessible to pectin-degrading enzymes, such as pectate lyases. For example, pectate lyase secreted by *Erwinia sp.* cleaves only those galacturonic acid residues that are not acetyl-esterified (Davis et al., 1984; Shvchik et al., 1997).

Rhamnogalacturonan hydrolase: Rhamnogalacturonan hydrolase cleaves the bond between the alternating galacturonic acid and rhamnose residues in rhamnogalacturonan-I (Mutter et al., 1998). Activity of rhamnogalacturonan hydrolase has been found in apples, grapes and tomatoes (Gross et al., 1995), but not many studies have analysed the characteristics and potential significance of these enzymes in cell wall modification.

Yieldin: A protein named yieldin, isolated from the cell wall of growing hypocotyls has been demonstrated to regulate plant cell wall strength (Okamoto–Nakazato et al., 2001; Okamoto–Nakazato, 2000). Yieldin was found to be present in the highest concentration in the apical pre-elongation region of hypocotyls and decreased toward the elongated mature base, indicating that yieldin disappeared with the cessation of cell elongation. It would be particularly interesting to determine the potential role of these proteins especially in fruits in which expansion occurs until late stages of development and overlaps with ripening.

Lipid transfer proteins: Lipid transfer proteins are small, basic proteins with eight Cys residues at conserved positions (Kader, 1996). In *Arabidopsis thaliana*, for example, 71 putative lipid transfer proteins with highly divergent sequences have been identified (Beisson et al., 2003). Lipid transfer proteins (identification based on structural homologies with members of the lipid transfer protein family) have been recently identified as a type of cell wall-loosening protein that does not share any homology with expansins (Nieuwland et al., 2005). The authors have hypothesized that lipid transfer protein s are associated with hydrophobic wall compounds, causing non hydrolytic disruption of the cell wall and subsequently facilitating wall extension. Their potential involvement in fruit expansion has not been explored yet.

Deoxyhypusine synthase: Deoxyhypusine synthase catalyses the first of two sequential reactions that result in the conversion of a conserved Lys residues in inactive eukaryotic translation initiation factor-5A (eIF-5A) to the unusual amino acid, hypusine (Park, 1989; Park et al., 1993). Hypusinated eIF-5A appears to facilitate protein synthesis, translocating specific subsets of mRNAs from the nucleus to the cytoplasm for translation (Bevec and Hauber, 1997). Wang et al. (2005) suppressed deoxyhypusine synthase (deoxyhypusine synthase; EC 2.5.1.46) in tomato. There were no discernible differences in phenotype between wild-type plants and some transgenic lines during growth and development; fruit from both plants ripened normally under postharvest storage conditions, but loss of tissue integrity in senescing tomato was delayed.

8. ALTERNATIVE BIOTECHNOLOGICAL STRATEGIES FOR CONTROLLING FRUIT SOFTENING

A major practical goal of plant cell wall research is to generate plants with genetically defined variations in composition and architecture to permit assessment of modifications on wall properties and plant development (Carpita et al., 2001). Transgenic plants modified in the expression of cell wall modifying proteins have been used to investigate the role of particular activities in fruit softening during ripening, and in the manufacture of processed fruit products (Brummell and Harpster, 2001). In most cases, individual genes have been targeted. Co-operative enzyme action might also be essential for the modification of other polysaccharide matrices or networks during cell wall disassembly (Rose and

Bennett, 1999). Consequently, approaches affecting combinations of genes would be useful to influence cell wall disassembly more dramatically or to overcome functional redundancy. Most of the strategies tested so far have focused on reducing or altering cell wall degradation. Plants need an enormous biosynthetic machinery to synthesise wall polysaccharides and then assemble them into a cell wall in the apoplast. In Arabidopsis, 415 putative glycosyl transferase genes have been identified to date on the basis of their gene sequences (http://afmb.cnrs-mrs.fr/CAZY/). The isolation, characterization and mapping of wall mutants, together with biochemical approaches, have led to significant advances in our understanding of wall polysaccharide synthesis at a molecular level (Scheible and Pauly, 2004). An alternative approach for modifying the firmness of ripe fruit would be to focus on modifying cell wall synthesis by selected alterations in wall biosynthetic enzymes in order to generate fruits with custom-designed textural characteristics.

9. CONCLUSIONS

Great progress has been achieved in our understanding about cell wall structure, composition and metabolism. Many interesting questions remain about how cell wall disassembly and fruit softening can be altered without negative side effects. Answers to these questions will be of considerable value in developing alternative approaches to control fruit softening.

6. APPENDIX II:

"CONTROL OF POSTHARVEST SPOILAGE IN SOFT FRUIT"

Purpose of the review: The incidence of disease is a significant postharvest problem in soft fruit. This paper discusses traditional methods and emerging technologies used to control this problem.

Findings: Currently the most common approaches taken to reduce postharvest spoilage of soft fruit are based on controlling the rate of fruit ripening and pathogen growth using low temperature and modified atmosphere storage. Other strategies such as irradiation, short heat treatments, application of chemicals (calcium, 1-methylcyclopropene and nitric oxide), plant defense response elicitors, natural compounds and the use of antagonists have been evaluated. Physical methods such as heat treatments and irradiation are promising methods for extending soft fruit shelf-life. Biological control and treatments that could elicit the activation of fruit natural responses have also shown encouraging results and seem to be feasible especially where application of fungicides is restricted. However, there are still many aspects that should be understood before these are adopted on a commercial scale. The rapid advances in metabolic engineering of plants may open another field of research with possibilities for reducing fruit susceptibility. Rapid cooling and low temperature storage (0ºC, 90–95 % relative humidity) are crucial for reducing soft fruit spoilage, but the best approach seems to require the use of several integrated preharvest and postharvest strategies.

Directions for future research: Although there are some emerging technologies that could be useful to complement the benefits of low temperature storage and modified atmospheres, we must evaluate their feasibility and consider their limitations on a commercial scale. Testing the effectiveness of new techniques in low temperature storage conditions must be carried out to find alternatives that could improve currently used methods. Further research is also needed to understand aspects of fruit-pathogen interaction by examining, for example, pathogen virulence factors and the regulation of natural fruit-defense strategies. This information would be extremely useful in the selection of candidate genes for breeding, biotechnological approaches and to develop effective and consistent methods based on the activation of fruit natural defense.

Abbreviations: CA, Controlled Atmosphere ; HT, Heat Treatment; NO, Nitric Oxide; MA, Modified Atmosphere; MCP, 1-Methylcyclopropene; PG, endo-Polygalacturonase; PGIPs , Polygalacturonase-inhibiting Proteins ; UV-C, Ultraviolet-C

1. INTRODUCTION

Soft fruit are very important, both for fresh consumption and for the production of sauces, jams, jellies, yogurts, frozen derivatives and other delicacies. Strawberries, blueberries, raspberries, blackberries and their hybrids are usually included in this group (Manning, 1993). Although the species differ by their systematic position and by the specific organs that form the edible portions, several factors make the grouping of soft fruit practical. These fruit have edible fleshy structures with attractive shape, flavor and color, are characterized by a high softening rate (Jiménez Bermúdez et al., 2002) and accumulate anthocyanins, which give their characteristic blue or red color (Manning, 1993). Soft fruit are generally considered non-climacteric (Abeles and Takeda, 1990; Lipe, 1978; Kader, 1992), showing low ethylene production (0.1–1μl C_2H_4 kg^{-1} h^{-1} at 20 °C) and low sensitivity (Burdon and Sexton, 1993; Walsh et al., 1983; Perkins–Veazie and Nonnecke, 1992; Perkins–Veazie et al., 2000). Soft fruits usually have high economic value but short postharvest life. Two major factors that determine their high perishability are the extremely high rate of softening and the incidence of postharvest diseases (Salunkhe and Desai, 1984). This article reviews traditional and emerging technologies used to control soft fruit spoilage.

2. SPOILAGE IN SOFT FRUIT

The incidence of diseases is a major factor limiting the postharvest life of soft fruit (Maas, 1984). Decay is caused by the pathogens *Botrytis cinerea*, *Rhizopus stolonifer*, *Mucor mucedo* and *Colletotrichum acutatum*. *B. cinerea* and *Rhizopus stolonifer* are most frequently associated with infections during postharvest storage and retail (Elad, 2004), and their control is discussed in more detail in this review.

2.1. Gray mould rot (*Botrytis cinerea* rot)

B. cinerea is a polyphagus pathogen that infects over 200 plant species and can cause significant problems during postharvest storage; losses from *B. cinerea* are several billion US dollars per year (Jarvis, 1980; Maude, 1980). Infections are favored in high relative humidity and low ventilation, and can be particularly problematic during spring and autumn when the conditions for pathogen development are favorable (Elad et al., 2004). With some exceptions, *Botrytis* attacks mainly soft, damaged, senescent or

dead tissues (Agrios, 1999). The infection process frequently starts in the flowers and can be in a quiescent form, but symptoms of aggressive infection and tissue decomposition are commonly observed in ripe fruit Bristow et al., 1986; Paulus, 1990). The rotting sites are usually observed in the basal region of the fruit and are associated with the presence of infected petals or stamens that have adhered to the calyx or parts of the fruit. The presence of wounds and lesions caused by insects and other pathogens also facilitates rot development (Agrios, 1999; Olías et al., 1998). The symptoms begin as small yellowish spots that expand rapidly, forming brown irregular soft regions that are eventually covered with white mycelia and grey spores. By expansion, the rotting can cover the whole fruit. Dissemination of the disease is promoted by the large number of conidia from the infected fruit (Jarvis, 1980). In recent years there has been substantial progress in our understanding of Botrytis biology and pathology. A detailed analysis of these aspects is available from Elad et al. (2004).

2.2. Rhizopus rot

Rhizopus stolonifer spores are usually airborne and thus can be disseminated easily. The fungus penetrates ripe fruit primarily through wounds. Infected fruit have discolored regions that gradually become light brown. Eventually the regions coalesce and the cellular contents are exuded. Finally, the fruit may be covered by a white mycelium with long sporangiophores ending in black sporangia (Maas, 1984). Although rotting by *Rhizopus* is distributed widely, its prevalence has been reduced because storage below 5ºC limits pathogen growth and sporulation (Mitchell et al., 1996).

3. STRATEGIES TO CONTROL SPOILAGE

There are several strategies that can be used to control spoilage in soft fruit.

3.1. Inoculum reduction

The main strategy for reducing spoilage in soft fruit is to minimize the amount of inoculum. Consequently, the effective control of postharvest diseases starts in the field. Since postharvest fungicides are not used on strawberries, preharvest disease control is crucial (Mitcham, 1996). Cultural practices are extremely important for *Botrytis* control. Some of these include using plants with good

sanitary quality, removing fruit and other organs showing disease symptoms and controlling water condensation on the fruit surface. The use of drip-tape irrigation instead of overhead sprinkler irrigation systems could also be beneficial because canopy relative humidity will not be excessive. Avoiding excessive plant density (Legard et al., 2000) and excessive fertilization (especially with nitrogen), and using mulch to prevent fruit contact with the soil, also contributes to reduced *Botrytis* incidence.

Fungicide sprays can be an important component of an integrated control program to reduce disease development and prevent rotting. Unlike the situation with other diseases, a good fungicide program is not a guarantee of *Botrytis* control when conditions favor its development (Daugaard, 1999). Benzimidazole fungicides such as benomyl and thiophanate methyl have been demonstrated to control Botrytis. More recently, the dicarboximide fungicides (vinclozolin and iprodione) were introduced and the control of the pathogen was improved. However, as with the benzimidazole fungicides, resistance to these fungicides also has developed (Spotts and Cervantes, 1986; Washington et al., 1992). Weekly applications, beginning when the blossoms open through bloom, have been shown to be effective in controlling *Botrytis* fruit rot on strawberry, reducing disease incidence by more than 41% compared with the untreated control (Blacharski et al., 2001; Mertely et al., 2002). Captan is a fungicide frequently used for fruit rots, one of few that can be used close to harvest. As new, effective fungicides become available it must be taken into consideration that they should be used in rotation with other existing products in order to reduce the possibility of resistance development.

3.2. Fruit harvest and handling

Harvesting operations are of significant importance in any postharvest disease-control program. Most postharvest pathogens penetrate the host tissues through wounds. Consequently all operations should minimize contamination, bruises, wounds and physical damage (Kader, 1992). Fruit harvested early during ripening will have a lower susceptibility to pathogens, but flavor and aroma will be compromised since sugar accumulation takes place in the later stages of ripening (Manning, 1993). Wet fruit are especially susceptible to pathogen attack therefore; harvest operations should be carried out when the fruit do not have free water on their surface. Harvesting when the temperature is not excessively high will be useful in reducing the retained field heat that must be removed during cooling

operations. Only clean and disinfected containers without sharp or abrasive surfaces should be used. At harvest, infected berries may be soft and leaky, or may have masses of fungal spores growing on them. Pickers should be equipped with receptacles for discarded fruit to minimize contamination. Raspberries and blackberries are very difficult to grade without causing some skin abrasion and consequent loss of quality therefore, field grading is preferred for these fruits. Reducing the time between harvesting and transportation and the availability of proper roads and transportation vehicles is also important for minimizing fruit damage. Another preventive action is to eliminate tissue residues and infected fruit from the packinghouse. Pathogens are transported to packinghouses with the fruit. Over time the air, water and equipment will be contaminated, so proper sanitation is required to minimize fruit decay. Finally, training and supervision are critical. Incentives to harvest with care and monitoring of harvested trays are extremely useful sources of information for detecting problems (Mitcham, 1996).

3.3. Cooling and storage conditions

The importance of temperature management in maintaining fruit quality is well recognized (Kader, 1992) and is particularly relevant for soft fruit (Mitchell, 1992). For many years horticultural products have been cooled by room cooling. This method is generally sufficient to keep produce at a low temperature once the product has been initially cooled, but it often does not remove field heat rapidly enough to maintain the quality of highly perishable crops (Thompson et al., 2002). Forced-air cooling is an adequate method to cool soft fruit. This method can significantly reduce the metabolic activity of the fruit, delay softening and thus reduce decay susceptibility. Cooling time can be 4–10 times faster than room cooling depending on the airflow rate. Other methods such as hydro-cooling or vacuum cooling could potentially damage the fruit and increase water loss, promoting pathogen attack (Anderson et al., 2004).

Forced air-cooling is accomplished by exposing packages of produce in a cooling room to higher air pressure on one side than on the other. This pressure difference forces the cool air through the packages, removing heat from the fruit (Thompson et al., 1998, 2002). Because air is forced through the produce packages by pressure differences on the opposing sides, it is necessary to fill the containers properly and stack them to minimize voids and openings that allow the air to pass around rather than through the packages, reducing cooling efficiency. Sufficient open space should be provided in the sides

and bottom of packages to ensure adequate air movement through the containers, so that 5–8 % of the lateral surface and 3–5 % of the bottom are open. For strawberry, blueberry, blackberry and raspberry, 0ºC is an adequate temperature (Kader, 1992). Moving air tends to remove water from the surface of produce, causing wilting, shrinkage and general loss of quality and value, so in addition to controlling air temperature and airflow, humidity should be maintained at nearly 100 %. Delayed cooling has negative impacts on soft fruit quality (Mitcham and Mitchell, 2002). Nunes et al. (1995) found that a 6-hour delay in the start of the cooling process resulted in 50 % higher water loss and increased shriveling. Cooling delays also caused greater firmness loss and reductions in ascorbic acid, soluble solids and acids (Nunes et al., 1995).

Once the products have been cooled, the appropriate temperature for storage is 0–1ºC (Hardenburg et al., 1986; Mitcham, 1996). Under these conditions the postharvest life of raspberries and blackberries is 3–5 days, 7–10 days for strawberries and 2 weeks for blueberries. These products are highly susceptible to water loss so quality is rapidly reduced if they are not stored at a relative humidity near 85–95 % (Mitchell, 1996).

3.4. Controlled and modified atmospheres

Modified/controlled atmospheres (MA/CA) are used as supplements to temperature management to extend the postharvest life of fresh products (Kader, 1992) sdies have shown that the use of CAs and MAs can be useful for controlling spoilage in soft fruit (Kader, 1991; Li and Kader, 1989; Smith and Skog, 1992).

3.4.1. CO_2 modification: High-CO_2 treatments reduce postharvest rots caused by fungal pathogens by inhibiting some pathogen metabolic functions. Furthermore, CO_2 can slow fruit ripening. The concentration of CO_2 inhibiting mycelium growth varies with fungal species. In the early work by Brown (Brown, 1922) highest inhibition of fungal growth and spore germination in *B. cinerea* and other phytopathogenic fungi was obtained by keeping the temperature at the lowest level and CO_2 at the highest concentration tolerated by the plant product. Soft fruit are relatively tolerant to high CO_2 partial pressures (Watkins et al., 1999). CO_2-enriched atmospheres (10–20 % in air) are used to extend the

postharvest life of strawberries (Holcroft and Kader, 1999a, 1999b). CO_2 treatments reduce respiration, delay softening, or even increase firmness in strawberry fruit (Smith and Skog, 1992). An increase in CO_2-induced firmness occurs in most cultivars (Watkins et al., 1999) and is influenced by berry maturity (Goto et al., 1995). Harker et al. (2000) suggested that the mechanism for CO_2-induced firmness enhancement in strawberry could be due to changes in the pH of the apoplast. Such changes in pH may promote the precipitation of soluble pectin and thus enhance cell-to-cell cross-linking in strawberry fruit. Therefore, the suppressive activity of CO_2 on *Botrytis* rot results from the combined effect of a direct fungistatic activity on the pathogen (determined by delayed conidia germination and germ-tube elongation and lower mycelium growth, which prevents the spread of the disease), and an indirect effect on the host, by delaying senescence and preserving natural resistance in the fruit (Bertolini et al., 2003). The use of CA and MA has also been shown to be effective for reducing the incidence of *B. cinerea* and slowing raspberry and blueberry metabolism (Kim et al., 1995; Haffner et al., 2002; Joles et al., 1994; Smittle et al., 1988; Ceponis and Capellini, 1985). Kim et al. (1995) found that optimal storage conditions were 17–18 % CO_2 and 9 % O_2. In the case of raspberries, storage in CAs (10 % O_2 and 15 % CO_2) significantly suppressed fruit rotting (Haffner et al., 2002).

Although many benefits could be attained by the use of CA and MA, when fruit are exposed to concentrations of CO_2 beyond 20 %, or extremely low levels of O_2, undesirable negative effects such as increases in acetaldehyde, ethanol and ethyl acetate occur (Ke et al., 1993; Larsen and Watkins, 1995b). The sensitivity of strawberries to CO_2 with the accumulation of undesirable metabolic products depends on the cultivar (Watkins et al., 1999). An increase in acetic acid in strawberries under elevated CO_2 has also been reported (Larsen and Watkins 1995a). Furthermore, excessive CO_2 can produce changes in strawberry fruit such as external color change from red to red-purple and a reduction in red color in internal tissues (Kader, 1986; Holcroft and Kader 1999a, 1999b).

3.4.2. O_2 modification: Reducing O_2 level to 1 % when fruit was stored at 5 °C was also beneficial for reducing decay and softening without detrimental effects on other quality attributes (Ke et al., 1991). The effect of the application of superatmospheric oxygen on fruit quality and decay was tested by Wszelaki

and Mitcham (2000). In this case storage with 60–100 % O_2 reduced decay, but also increased the accumulation of metabolites that negatively affected fruit organoleptic characteristics.

3.4.3. Carbon monoxide: Fungistatic effects of carbon monoxide, without negative effect on quality, have been also been demonstrated in strawberry (El-Kazzaz et al., 1983). Hertog et al. (1999) generated a model to predict decay by *B. cinerea* in strawberry fruit as affected by different atmospheric conditions and temperatures. The proposed model was able to explain 83 % of the experimental results found in different fruit batches. Although the fruit-pathogen interaction should be more complex than the proposed model, this type of approach would be useful to adapt in package and transport conditions, in order to meet specific product demands. Further development of models integrating the effect of environmental factors, not only on spoilage but also on other aspects of fruit quality accounting for consumer acceptability, would be extremely useful.

3.5. Irradiation

Irradiation has been used to delay ripening-associated processes and control pathogens or insects. Different types of radiation have been tested in soft fruit.

3.5.1. Ionizing radiation: Ionizing radiation has been used to sterilize or kill insects and microbial pests. Boysenberries and raspberries can tolerate up to around 1 kGy. Strawberries on the other hand can tolerate 2–4 kGy, depending on variety. Irradiation has been tested on strawberries for decay control with mixed success. Doses needed for adequate decay control result in excessive berry softening.

3.5.2. Ultraviolet irradiation: Pre-storage applications of ultraviolet-C (UV-C) light (0.5–4.5 kJ m^{-2}) have been used to control storage rots in strawberry (Baka et al., 1999; Pan et al., 2004; Marquenie et al., 2002, 2003a). Treatments with 9.2 kJ m^{-2} of UV-C light, before refrigerated storage, were shown to be effective for boysenberries (Vicente et al., 2004). The mode of action of this treatment could be related, at least in part, to the germicidal effects of the UV-C radiation. Pan et al. (2004) reported that UV-C treatment (4.6 kJ m^{-2}) delayed *B. cinerea* germination but did not affect the germination of *Rhizopus* conidia. However, the combination of UV-C with heat treatment delayed the spore germination of both

fungi and allowed better control of spoilage in strawberry (Pan et al., 2004). Nigro et al. (2000) and Marquenie et al. (2003a) inoculated *B. cinerea* spores on UV-C treated strawberries and found a reduction of decay, suggesting that fruit-defensive responses could have been activated by the treatments as it has been reported for other fruits (Ben-Yehoshua et al., 1992, 1995). Phenylalanine ammonia-lyase activity increased after UV-C irradiation (Nigro et al., 2000). The activation of this enzyme could play a key role in the biosynthesis of phenolic compounds, many of which have antifungal activity (Yamamoto et al., 2000) and contribute to disease resistance of UV-C treated fruit. Reduction of strawberry fruit softening by UV-C application has been reported (Baka et al., 1999) and a reduction in cell-wall disassembly could also reduce the tissue colonization by fungal pathogens.

3.6. Heat treatments

Several reports have described the beneficial effects of short postharvest heat treatments (HTs) in fruits and vegetables (Lurie, 1998). Couey and Follstad (1966) effectively controlled strawberry fruit decay by heating with moist air at 44ºC. García et al. (1995, 1996b) reported that submersion in water at 45ºC for 15 minutes reduced postharvest losses, maintained firmness and improved key factors involved in flavour. However, other research (Pan et al., 2004) reported no benefits due to hot water treatments. Using hot air at 45ºC for 3 hours, Civello et al. (1997) found that softening was delayed and the incidence of postharvest diseases was significantly reduced. The beneficial effects of HTs were also observed when they were combined with traditional refrigerated storage at 0ºC (Vicente et al., 2002). The benefits of HT can be enhanced by performing the treatment in the presence of a film with reduced CO_2 permeability, which creates a MA (Vicente et al., 2003). Primarily, HTs cause delayed fruit ripening and consequently a reduction in pathogen susceptibility and softening. The HT directly affected fungal attack by reducing *R. stolonifer* and *B. cinerea* conidia germination. Indirect defense responses mediated by changes in the fruit ripening program could also be modulated by HT (Schirra et al., 2000). Increases in polyphenol oxidase activity and reduction in cell--wall degradation in the fruit may impair tissue colonization. The treatments increased the enzymatic and non-enzymatic antioxidative defenses which protect the fruit against reactive oxygen species. It is also interesting to mention that the level of salicylic acid, which has been associated with defense-response signaling in plants, is also increased by HT (Vicente, 2004). Lurie et al. (1997)

suggested that the induction of a peroxidase could be involved in reduced susceptibility to *B. cinerea* in heat-treated fruit. HT (45ºC, 1 hour) has been also shown to be effective for reducing damage and decay in boysenberry (Vicente et al., 2004).

3.7. Calcium treatments

Calcium applications have been used to reduce softening and decay in fruits and vegetables (Poovaiah, 1986). For strawberries, foliar applications of $CaCl_2$ have been reported to delay ripening and mold development (Chéour et al., 1990, 1991; Wójcik and Lewandowski, 2003). The treatments could delay cell-wall degradation and consequently reduce tissue colonization by pathogens. Lara et al. (2004) found that strawberries infiltrated with $CaCl_2$ retained higher levels of ionically-bound pectin that could contribute to the maintenance of cell-wall integrity. However, others have shown that $CaCl_2$ did not provide protection against gray mould in strawberry (Erincik et al., 1998). The efficiency of $CaCl_2$ sprays for improving fruit quality and strawberry shelf-life depends mainly on the rate of Ca^{2+} uptake of exogenous Ca^{2+} by fruit tissues and on the chemical and physical properties of the spray solution (Swietlik and Faust, 1984). The low mobility of calcium in plants may explain the inconsistency in the case of foliar applications since the translocation into the fruit could be low. Adding Tween 20 as an adjuvant could increase the efficiency of $CaCl_2$ sprays (Wójcik and Lewandowski, 2003). Postharvest dips in 1 % $CaCl_2$ solution were shown to be effective for controlling strawberry postharvest decay and maintaining firmness (García et al., 1996a). Calcium applications have also been shown to be useful for maintaining firmness in highbush blueberry (Hanson et al., 1993) and for delaying ripening and reducing decay in raspberry (Montealegre and Valdés, 1993).

3.8. Nitric oxide

The production of nitric oxide (NO) by plants has been known for some time (Leshem, 1996), and its involvement in regulation of plant growth (Gouvea et al., 1997) and development has been demonstrated (Lamattina et al., 2003). The effects of NO are not limited to whole plants, and fruits and vegetables can display some responses that could be useful from a postharvest perspective. Wills and Kim, (1995) reported that applications of NO (5–10μL L^{-1}) for 2 hours prior to storage could extend

strawberry postharvest life by 50 % when the fruit was stored at 5ºC. One practical difficulty of these treatments is that NO is rapidly oxidized to NO_2 in the presence of oxygen.

3.9. Chitosan

Chitosan is a polycationic β-1,4-linked-D-glucosamine polymer (El Ghaouth, et al., 1991a). Several studies have shown that this compound inhibits development of soft fruit pathogenic fungi (El Ghaouth, et al., 1991b, 1992a, 1992b). Dips in a chitosan solution protected strawberry fruit from decay as effectively as dipping them in fungicide (El Ghaouth, et al., 1997). Preharvest sprays have been used to control initial infection in the field and to obtain fruits free of infection (Aharoni and Barkai–Golan, 1987). The mode of action of chitosan is not fully elucidated. The application of a chitosan solution may plants to respond more rapidly to pathogen attack by stimulating chitinase and glucanase production (Benhamou, 1996). Induction of phytoalexins has also been reported (Hahn, 1996).

3.10. Biological control

The search for alternatives to chemical strategies for the control of postharvest diseases has increased recently, with the aim of developing approaches that may be less harmful to human health and the environment. Biological control is a promising alternative to fungicide application (Wisniewski and Wilson, 1992). A number of microorganisms that effectively control postharvest pathogens have been identified. Peng and Sutton (Peng and Sutton, 1991) found that isolates of *Trichoderma viride* and *Gliocladium roseum* were highly suppressive to *B. cinerea* in all studies, and were at least as effective as standard captan sprays. Several fungi also suppressed sporulation of *B. cinerea* when applied to strawberry leaves colonized by the pathogen. Yeasts appear to be the most promising biocontrol agents. Treatments with *Candida albidus* during strawberry flower bloom reduced the incidence of gray mould on ripe fruits after harvest by 21–33 % (Helbig, 2002). Treatments with *Metschnikowia fructicola* reduced fruit rots by 56–69 % (Karabulut et al., 2004b). The yeast reported suppressed postharvest incidence of fruit rot significantly better than fenhexamid (Karabulut et al., 2004b). The effectiveness of the yeast was increased when formulation substances (alginate, xanthan and cellulose) were added to the cell suspension (Helbig et al., 2002). Treatments with *Trichoderma* significantly controlled strawberry fruit

artificially inoculated with *B. cinerea* but were ineffective against latent infections (Pratella and Mari, 1993). Lima et al. (1997) showed that *Candida oleophila* and *Aureobasidium pullulans* were effective antagonists of *B. cinerea*. The mode of action of these agents is diverse, but includes competition for nutrients, direct interaction with the pathogens or induction of fruit defense responses.

3.11. Ozone treatments

Ozone is a very reactive oxidizing agent with a short half-life. It has been used for a long time, and was suggested as a generally recognized as safe sanitizer or disinfectant in foods (Graham et al., 1997). It could be an interesting alternative for use in organic processing (including postharvest handling). The mechanism of action of ozone is associated with its high oxidative properties. It can react by direct oxidation of compounds or can oxidize compounds through the hydroxyl free radicals that are produced during ozone decomposition. This could be useful for controlling pathogens. Furthermore, ozone could affect the fruit metabolism and under certain circumstances, the induced stress reactions may enhance plant tolerance of a second stress (Sandermann 1996; Sandermann et al., 1998). However, the commodities could be damaged if the treatments are excessive. Several publications on ozone phytotoxicity are available (Graham et al., 1997; Lima et al., 1997) so the doses and times of exposure for postharvest operations should be selected to avoid produce damage.

Barth et al. (1995) showed that ozone treatments could reduce decay in blackberries. Perez et al. (1999) found that ozone treatments of strawberries (0.35 mL L^{-1}) were effective to partially control decay. However, when fruit was stored for one week no differences were found between treated and untreated fruit. Furthermore, a reduction in flavor and aroma compounds was found in response to the treatments. Additional investigations assaying appropriate treatment conditions for each commodity will be useful to exploit the potential benefits of these types of treatments.

3.12. 1-Methylcyclopropene

Recently 1-methylcyclopropene (1-MCP) has been added to the list of options for extending the shelf-life and quality of plant products (Blankenship and Dole, 2003). The compound is thought to tightly bind ethylene receptors so that ethylene cannot bind and elicit action (Sisler and Serek, 1997). Although most

soft fruit are non-climacteric and do not show marked stimulation of ripening processes in response to ethylene, exposure to this compound increases strawberry deterioration during marketing (Wills and Kim, 1995). Jiang et al. (2001) found that 1-MCP reduced softening and color change in strawberries. However, others reported different results, depending on the 1-MCP concentrations and the ripening stage of the fruit. Ku et al. (1999) found that beneficial effects were observed from treatment with low MCP concentrations, while high concentrations increased disease incidence. Bower et al. (2003) concluded that 1-MCP treatment is unlikely to be a cost-effective method for extending storage life of strawberries. 1-MCP may be less effective for other soft fruit, like boysenberries. The results suggest that the benefits of applying 1-MCP in soft fruit would be much less significant than in other fruits and vegetables.

3.13. Other methods tested

Several other methods have been investigated for controlling spoilage of soft fruit.

3.13.1. Ethanol: Ethanol treatments have been shown to be effective for reducing decay in fruits and vegetables (Feliciano et al., 1992; Karabulut et al., 2004a; Litcher et al., 2003; Yuen et al., 1995). In the case of strawberry, ethanol sprays (50 % v/v) applied 1 hour before harvest reduced the incidence of *B. cinerea* (Karabulut et al., 2004a).

3.13.2. Benzoic acids: Plant phenolics have been shown to be active against fungi (Lattanzio et al., 1996). The mode of action of these compounds could be associated with their antioxidant activity or with their inhibition of fungal extracellular enzymes required for pathogenesis (Lattanzio et al., 1996; Scalbert, 1991; Elad, 1992). A marked reduction in decay was observed in strawberries treated with 0.01 M 2,5-dimethoxybenzoic acid (Lattanzio et al., 1996).

3.13.3. Natural volatiles: It has been shown that some natural volatile compounds have antimicrobial properties (Archbold et al., 1997; Vaughn et al., 1993). Incorporation of the appropriate concentration of these compounds in the storage atmosphere could be useful to help control postharvest pathogens.

Archbold et al. (1997) found that hexenal, 1-hexanol, 2-hexen-1-ol, 6-nonenal, 3-nonen-2-one and methyl salicylate could control *Botrytis* in strawberry and blackberry, without causing phytotoxic effects. However, these treatments may have little effect when fruit is stored at low temperatures (Wang, 2003).

3.13.4. Acetaldehyde: Tests of this compound, which inhibits pathogen growth, have shown contradictory results. Furthermore, fruit sensory properties could be adversely affected (Pessis and Avissar, 1990).

3.13.5. Sodium bicarbonate: This compound is used as antimicrobial agent in the food industry and has been reported to be effective in controlling postharvest diseases in some fruits (Fallil et al., 1997; Karabulut et al., 2004a). The applications of 1 % sodium bicarbonate sprays in organically grown strawberry fruit 1 hour before harvest produced a slight reduction in postharvest diseases, but the efficacy of the treatments was not consistent in different experiments (Karabulut et al., 2004a).

3.14. Plant breeding and genetic engineering

Traditional plant breeding programs and the rapidly growing biotechnology sector have the potential to contribute to the reduction of postharvest decay in soft fruit. However, this requires the identification of traits that could reduce susceptibility without affecting fruit yield and quality or have a negative impact in the environment. Biotechnological approaches should be particularly useful for identifying key genes and their products that influence susceptibility; this information can be used either in conventional breeding or genetic engineering programs. Efficient genetic transformation protocols have been developed for strawberry using *Agrobacterium tumefaciens* (Nehra et al., 1990). Recently, a GalUR promoter from strawberry has been characterized (Agius et al., 2005). The promoter is fruit-specific and increases during the ripening and reaches very high level in mature red fruits making it interesting for biotechnological studies and genetic engineering. Changing the level of expression of different candidate genes could be explored to control the incidence of postharvest diseases.

-Genes involved in cell wall degradation: Jiménez Bermúdez et al. (2002) showed that transgenic plants that incorporate an antisense sequence of a strawberry pectate lyase gene, under the control of the 35S promoter, maintained firmness. This result indicates that this gene is an excellent candidate for biotechnological improvement of fruit softening and that could potentially influence the susceptibility to decay.

-Increase in antifungal compounds: Manipulation of phenylpropanoid metabolism has been shown to be effective in reducing pathogen attack in some plant species (Shadle et al., 2003; He et al., 2000) and it could be interesting to explore this alternative in soft fruit.

-Protease inhibitors: A gene coding for a phytocystatinin (cystein protease inhibitor) from strawberry fruit recently was characterized (Martinez et al., 2005). The protein expressed in *E. coli* inhibited *B. cinerea* growth in vitro making Cyf1 a potential gene for disease control (Martinez et al., 2005). It would be interesting to assess the effect of changing the protein levels on decay incidence *in vivo*.

-Polygalacturonase: Fungal endo-polygalacturonases (PGs) have important functions during the early stages of plant pathogenesis. They hydrolyse pectic components of the cell walls facilitating the colonization of plant tissues (Agüero et al., 2005). PG-inhibiting proteins (PGIPs) are plant cell-wall proteins that specifically inhibit fungal PGs. The inhibition of fungal PGs by PGIPs suggests that PGIPs have a role in plant resistance to fungal infections and this has been observed in transgenic plants expressing PGIPs. Heterologous expression of PGIP in transgenic tomato plants improved their tolerance to *B. cinerea*, manifested by a decrease in symptom development; i.e., slowed expansion of lesions and associated tissue maceration (Powell et al., 2000).

3.15. Increase of natural defense responses: Many fruit metabolites may act as natural disease resistance compounds in fruits and vegetables (Terry et al., 2004; Di Venere et al., 1998). These compounds can modulate pathogen quiescence mechanisms and provide new options in the control of postharvest decay and in the reduction of pesticide use (Prusky, 1996). Physical–chemical and biological

elicitors could activate natural defensive mechanisms (Prusky et al., 1996). For instance it has been shown that acibenzolar (benzo[1,2,3]thiadiazole-7-carbothioic-S-acid) app lied as a preharvest spray can suppress postharvest diseases (Terry and Joyce, 2004; Huang et al., 2000; Willingham et al., 2002) by activating natural defense responses. The level of proanthocyanidins has also been reported to be associated with resistance to *B. cinerea* (Hébert et al., 2002). Saks et al. (1996) found that a 2-hour treatment with fluorescent light was useful to reduce "white shoulders" in strawberry. Of importance, is the authors' observation that fungal attack was also delayed, probably due to the light-induced accumulation of fungal inhibitor(s). Since the direct measurement of many of these defensive compounds is inapplicable in a commercial scale, developing models based on simple, fast and non-destructive measurements that could be used to predict fruit or decay susceptibility would be extremely useful from a practical perspective. Consistent with this idea is the work of Schouten et al. (2002) who used color measurements of fruit at harvest and a color/proanthocyanadin development model to predict the keeping quality of strawberry batches as limited by Botrytis. Using this strategy, they were able to account for 90 % of the variability in the grey mould susceptibility of fruit samples. Although additional research is needed to assess the validity of these models under different *Botrytis* pressures and cultural practices and for different varieties, these kinds of studies could potentially assist decision making related to postharvest practices. Perhaps similar models, based on simple analyses of other fruit components potentially involved in or correlated with decreased fruit susceptibility, could be proposed and tested. This, in turn, could provide a basis for testing field or postharvest manipulations of plants/fruits with the intent of managing fruit pathogen susceptibility.

4. CONCLUDING REMARKS AND FUTURE PROSPECTS

In all cases, proper temperature management is essential to control soft fruit spoilage, but no single method is completely effective. A variety of methods have been shown to reduce fruit spoilage including UV-C irradiation, short heat treatments, calcium sprays, use of biological control agents and application of elicitors of plant natural defense responses. Many of these approaches have not been extensively tested on a commercial scale, so further research is required to evaluate their feasibility under these conditions. Testing the effectiveness of new techniques in low temperature storage conditions is

necessary to find alternatives that could improve the currently used methods. Biotechnology also could play a key role in elucidating the mechanisms underlying fruit-pathogen interactions, identifying genes involved in pathogenesis and defense, and assisting conventional breeding or genetic engineering programs to generate fruit less susceptible to fungal attack. Using several integrated strategies, starting in the preharvest environment and continuing after harvest seem to be the best approach to maintain quality and control soft fruit spoilage.

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