

Compositional Changes in Cell Wall Polysaccharides from Five Sweet Cherry (*Prunus avium* L.) Cultivars during On-Tree Ripening

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Supporting Information

ABSTRACT: Excessive softening is a major cause of postharvest deterioration during transportation and storage of fresh cherries. In continuing our studies to identify the factors determining the textural differences between sweet cherry fruit genotypes, we evaluated the solubilization, depolymerization, and monosaccharide composition of pectin and hemicelluloses from five sweet cherry cultivars ('Chelan', 'Sumele', 'Brooks', 'Sunburst', and 'Regina') with contrasting firmness and cracking susceptibility at two developmental stages (immature and ripe). In contrast to what is usually shown in most fruits, cherry softening could occur in some cultivars without marked increases in water-soluble pectin. Although polyuronide and hemicellulose depolymerization was observed in the water-soluble and dilute-alkali-soluble fractions, only moderate association occurs between initial polymer size and cultivar firmness. In all the genotypes the Na₂CO₃-soluble polysaccharides (NSF) represented the most abundant and dynamic wall fraction during ripening. Firm cultivars showed upon ripening a lower neutral sugars/uronic acid ratio in the NSF, suggesting that they have a lower proportion of highly branched polyuronides. The similar molar ratios of arabinose plus galactose to rhamnose [(Ara+Gal)/Rha] suggest that the cultivars differed in their relative proportion of homogalacturonan (HG) and rhamnogalacturonan I (RG-I) rather than in the size of the RG side chains; with greater proportions of HG in firmer cherries. Ultraviolet matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was useful to identify the depolymerization patterns of weakly bound pectins, but gave less accurate results on ionically bound pectins, and was unable to find any pattern on covalently bound pectins.

KEYWORDS: pectin, hemicellulose, softening, development, MALDI-TOF

■ INTRODUCTION

Cherry fruit exports to Asia and other emerging markets have rapidly grown in recent years.^{1,2} Long distance transportation has increased the demand for fruit with higher storage capacity.³ Size, soluble solids content (SSC), and fruit and stem color are the most relevant quality attributes in cherry.⁴ However, excessive softening is a common cause of postharvest losses, especially under long-term storage, and could increase the susceptibility to postharvest rots, thus boosting deterioration.^{5,6} In this globalized market context, fruit texture has become more important.⁷ Cherry fruit grow and accumulate sugar until late developmental stages; thus, anticipated harvests yielding firmer fruit are not recommended as they would compromise production and flavor. Consequently, the fruit is picked when fully ripe, and tight postharvest handling is needed in order to prevent deterioration.⁶

Ripe cherry cultivars show large variations in texture.^{8–11} These properties have been associated with the composition and metabolism of the cell wall composite.^{12,13} During

development, the solubility of polyuronides increases as rhamnogalacturonan I (RG-I) side chains are cleaved.^{9,13} Early studies comparing cherries with contrasting firmness suggested that firm cultivars may synthesize wall materials until more advanced developmental stages than soft genotypes.^{8,14} At the ultrastructural level, the middle lamella of crisp cultivars showed higher integrity compared to soft genotypes.^{11,12} However, the biochemical changes that may explain the textural differences between cherry cultivars remain obscure, with variable results reported. Soft cherries have been related with low degree of polymerization of pectins,^{11,12} though firmness loss has been shown to occur without marked changes in polyuronide size, and with low activity of major known polyuronide depolymerizing enzymes such as endo-polygalac-

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turonase.^{15,16} We have previously found that the general pattern and extent of uronic acid solubilization and depolymerization showed high similarity in firm and soft cherries.⁹ However, soft cherries had lower total cell wall content (alcohol-insoluble residue, AIR) and higher branching of tightly bound pectins than firm fruit. Unfortunately, almost all studies looking at cherry genotypes with contrasting firmness have compared a single firm cultivar to a soft genotype, making it difficult to determine whether or not the changes observed are valid in broader contexts. In order to explore further this, we have extended the chemical study by analyzing the changes in cell wall polysaccharides on five cherry cultivars ('Sunburst', 'Regina', 'Chelan', 'Sumele', and 'Brooks') at two developmental stages (immature and ripe). Pectin evaluations were complemented by their analysis by ultraviolet matrix-assisted laser desorption/ionization time-of-flight (UV-MALDI-TOF) mass spectrometry.

MATERIALS AND METHODS

Plant Material. Cherry fruit cultivars 'Chelan', 'Brooks', 'Sumele', 'Sunburst', and 'Regina' were randomly picked from trees located in the Río Negro Upper Valley, Argentina (39° 01' 32'' S, 67° 44' 22'' W, 242 m above sea level). Samples of about 1.5 kg of each genotype were collected in 2009 at two different developmental stages, namely immature (I) ('Sumele', 45 days after anthesis (DAA); 'Regina' and 'Chelan', 47 DAA; 'Brooks' and 'Sunburst', 49 DAA) and ripe (R) ('Sumele', 73 DAA; 'Brooks' and 'Chelan', 75 DAA; 'Sunburst', 84 DAA; 'Regina', 90 DAA).

Firmness, Soluble Solids Content, Titratable Acidity, Surface Color, Dry Matter, and Cracking Index. Firmness was measured at 20 °C for each cultivar on 20 randomly selected fruits, by measuring the force required to compress the fruit over the equatorial zone (distance, 0.5 mm; speed, 50 mm/s) using a Durofel texture analyzer (Güss FTA, Strand, South Africa). Given the average diameter of the fruit (25–27 mm), this test yielded only a small compression (ca. 2%). For soluble solids measurements, juice samples obtained by squeezing ten cherries were evaluated with a hand-held temperature-compensated refractometer (Atago Co., Tokyo, Japan). Measurements were done in triplicate. Titratable acidity (TA) was determined by titrating a 10 mL juice sample (out of 20 cherries) with 0.1 mol/L NaOH to an end point of pH 8.2, and expressed as g malic acid/100 g juice (% w/w). Three replicates per condition were evaluated. Fruit surface color measurements were taken with a CR-300 chroma meter (Minolta, Osaka, Japan) using D65 illuminant lighting conditions, in 20 cherries per cultivar. Dry matter was determined on ten cherries by drying in an oven at 45–55 °C until constant weight (ca. 7 d). The cracking index (CI) was determined after leaving 50 cherries per condition in deionized water at 20 °C. After 2, 4, and 6 h, the fruit was checked for wounds or exocarp/skin leaks, and those unaffected were submerged again. The index was calculated as

$$CI = \frac{5n_{2h} + 3n_{4h} + n_{6h}}{250} \times 100$$

where n_{xh} is the number of affected fruit after x hours.¹⁷

Alcohol Insoluble Residue (AIR). After removal of the core and peduncle, the pulp was treated as described by Ponce et al.¹⁸ in order to obtain the AIR. Briefly, about 100 g of pulp were put in cool 80% ethanol (4 mL/g fruit) and blended in an Omni Mixer homogenizer (Omni International, Kennesaw, GA). The homogenate was boiled for 30 min, cooled, and filtered through glass filter paper (Whatman GF/C). The retentate was thoroughly washed with 95% (v/v) ethanol. The solids were then resuspended in a mixture of chloroform:methanol (1:1, 3 mL/g fruit), stirred for 15 min, and filtered. The retentate was washed with the same solvent mixture. The insoluble material was washed with acetone, yielding the crude cell wall extract (AIR). The AIR was air-dried in a hood and in a vacuum desiccator overnight and weighed.

Cell Wall Fractionation. AIR fractionation was performed as previously described¹⁸ with minor modifications. Briefly, 1 g of AIR was stirred for 4 h at room temperature with 100 mL of 0.02% (w/v) thimerosal aqueous solution and filtered. The suspension was filtered, and the filtrate was saved and designated as water-soluble fraction (WSF). Sequential extraction of the pellet with 0.05 mol/L *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) in 0.05 M NaOAc/HOAc buffer, pH 6, containing 0.02% (w/v) thimerosal (24 h), 0.1 mol/L Na₂CO₃ containing 0.02 mol/L NaBH₄ (24 h), 1 mol/L KOH containing 0.02 mol/L NaBH₄ (24 h), and 4 mol/L KOH containing 0.02 mol/L NaBH₄ (24 h), yielded the CDTA-soluble fraction (CSF), Na₂CO₃-soluble fraction (NSF), and 1 and 4 mol/L KOH-soluble fractions (1KSF and 4KSF), respectively. The supernatants were recovered after centrifugation at 13100g at 6 °C for 40 min. In the case of the KOH-soluble fractions, pH was adjusted to 5 with glacial acetic acid. All fractions were dialyzed (molecular weight (MW) cutoff 6000–8000 Da) against tap water for 2 days and against distilled water for 1 day at 4 °C. The fractions were freeze-dried and stored until analysis.

Uronic Acids, Total Carbohydrate, and Neutral Sugar Measurements. Uronic acids (UAs) were quantitated with the *m*-hydroxybiphenyl method¹⁹ using galacturonic acid as standard, and expressed as anhydro units. Total carbohydrates were determined by the phenol-H₂SO₄ method²⁰ using glucose as standard. The proportion of neutral sugars (NSs) was determined after subtracting the UA content from that of total carbohydrates. For this purpose, the phenol-H₂SO₄ reaction was also carried out with a galacturonic acid standard, which showed an absorbance ratio of 0.28 against the same glucose weight.¹⁸

Size-Exclusion Chromatography (SEC). To examine the size distributions of polymers in CSF and NSF, ca. 3 mg of lyophilized samples from each fraction were dissolved in 0.8 mL of 0.4 mg/mL imidazole, to which 0.2 mL of 1 mol/L ammonium acetate (pH 5) was added. The samples were centrifuged, and the supernatants were chromatographed on a low-pressure SEC by employing a 300 mm × 9 mm i.d. Sepharose CL-2B column (Sigma Chemical Co., St. Louis, MO). Samples were eluted at room temperature with 0.2 mol/L ammonium acetate, pH 5. Fractions were collected, and aliquots were assayed for total carbohydrates.²⁰ Samples from the WSF, 1KSF, and 4KSF were dissolved in 0.1 mol/L NaOH, cleaned up by centrifugation, and chromatographed on a 300 mm × 9 mm i.d. Sepharose CL-6B column (Sigma Chemical Co., St. Louis, MO) eluted at room temperature with 0.1 mol/L NaOH. Fractions were collected, and aliquots were assayed for total carbohydrates.²⁰

Neutral Sugar Composition. Each fraction (ca. 3 mg) was hydrolyzed with 1 mL of 2 mol/L trifluoroacetic acid (TFA) for 90 min at 120 °C in closed-cap vials. The TFA was eliminated by evaporation, and the resulting monosaccharides were reduced to alditols using NaBH₄, converted to alditol acetates, and subsequently analyzed using a Hewlett-Packard 5890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) fitted with a capillary column (30 m × 0.25 mm i.d. × 0.20 μm, SP-2330, Supelco Inc., Bellefonte, PA) and equipped with a flame ionization detector (FID) operated at 240 °C. The injector temperature was 240 °C, and the oven temperature was kept isothermally at 220 °C. Nitrogen was used as the carrier gas at a head pressure of 1.05 bar. Samples were injected with a split ratio of 80:1. *Myo*-inositol was used as the internal standard, and the different alditol acetates were identified by comparison with authentic standards. The percentage of the different monosaccharides was calculated by considering that the integrated FID responses are proportional to the MW of the alditol acetates.

UV-MALDI-TOF Mass Spectrometry. Solutions of pectin samples (1 mg/mL) were prepared in Milli-Q water with the aid of sonication at 20 kHz (3 × 20 min). Two different matrices were used: nor-harmane (9-*H*-pyrido-[3,4*b*]indole) and gentisic acid (2,5-dihydroxybenzoic acid). The stock solutions of the matrices were prepared by dissolving 2 mg of the selected compound in 1 mL of methanol (HPLC-grade)/water (75:25, v/v). In order to prepare the analyte–matrix sample the thin-film layer method (sandwich method²¹) was used. Typically 0.5 μL of the matrix solution was

Table 1. Soluble Solids Content (SSC), Acidity, Weight, Lightness (L^*), Firmness, and Cracking Index of Ripe ‘Sunburst’, ‘Brooks’, ‘Chelan’, ‘Sumele’, and ‘Regina’ Cherries^a

cultivar	SSC (% w/w)	acidity (% w/w)	weight (g)	L^*	firmness (g/mm)	cracking index
Sunburst	22 ± 3 a	1.29 ± 0.03 a	7.8 ± 0.9 c	24.3 ± 0.04 e	415 ± 2 e	68 ± 2 b
Brooks	20 ± 1 ab	0.92 ± 0.01 c	9.2 ± 0.8 b	31.9 ± 0.02 a	440 ± 2 d	98 ± 5 a
Chelan	20 ± 2 ab	0.98 ± 0.05 b	7.9 ± 1.1 c	29.6 ± 0.04 b	475 ± 1 c	15 ± 2 d
Sumele	19 ± 1 b	0.96 ± 0.04 b	8.0 ± 1.1 c	28.6 ± 0.03 d	492 ± 3 b	71 ± 4 b
Regina	20 ± 2 ab	1.01 ± 0.06 b	10.3 ± 1.0 a	28.8 ± 0.01 c	561 ± 1 a	34 ± 3 c

^aDifferent letters within a column indicate differences based on a Tukey test at a level of significance of $P < 0.05$. Values are expressed as means ± SD ($n = 50$ for cracking index, 30 for weight, 20 for firmness and lightness, 3 for SSC and acidity).

placed on the sample probe tip and air-dried at room temperature. Subsequently, 0.5 μ L of the analyte solution were placed on the sample probe tip, covering the matrix and partially dissolving it, and air-dried. Then, two additional portions (0.5 μ L) of matrix solution were deposited on the same sample probe tip, producing a partial dissolution of the previously deposited thin-film, and air-dried. The matrix-to-analyte ratio was 3:1 (v/v), and the matrix and analyte solution loading sequence was (i) matrix, (ii) analyte, (iii) matrix, and (iv) matrix. Comparative experiments were also conducted preparing the analyte–matrix sample by the mixture method. The pre-prepared mixture was done mixing the matrix and analyte solutions in 3:1 (v/v) and 1:1 (v/v) ratios. Three portions (0.5 μ L) of the mixture were successively loaded on the probe, and air-dried as described above. Similar results were obtained with both sample preparation methods. Spectra were recorded on a Bruker Ultraflex II TOF/TOF, controlled by the FlexControl 3.0 software (Bruker Daltonics, Bremen, Germany). Desorption/ionization was performed using a frequency-tripled Nd:YAG laser emitting at 355 nm with a 100 Hz shot frequency. All mass spectra were taken in the positive linear and reflectron modes. Experiments were performed first using the full range setting for laser firing position in order to select the optimal position for data collection, and second fixing the laser firing position in the sample sweet spots. The laser power was adjusted to obtain high signal-to-noise ratio while ensuring minimal fragmentation of the parent ions, and each mass spectrum was generated by averaging 500 laser pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively. MTP plate steel with 384 circular spots, 3.5 mm diameter, was used (Bruker, Bremen, Germany).

Statistical Analysis. Statistical significance was determined by one-way ANOVA with the Stratgraphic software package (Manugistics Inc., Rockville, MD). The model assumptions of homogeneity of variance and normality were probed by means of Levene’s and Shapiro-Wilk’s tests, respectively. When these assumptions were not satisfied, data were transformed into ranks for further analysis. When a significant F -value was found, treatment means were compared using the Tukey’s studentized range test ($P < 0.05$).

RESULTS AND DISCUSSION

Fruit Size, Soluble Solids Content, Acidity, Color, Firmness, Dry Matter, and Cracking Susceptibility. Quality attributes of ripe fruit of the five varieties are shown in Table 1. ‘Brooks’, ‘Sumele’, ‘Chelan’, and ‘Regina’ showed similar levels of soluble solids (19–20% w/w) and acidity (0.92–1.01% w/w) upon ripening. ‘Sunburst’ presented slightly higher SSC (22% w/w) and titratable acidity (1.29% w/w). ‘Regina’ and ‘Brooks’ yielded fruit having higher weight (Table 1) and size (ca. 27 mm), whereas the remaining three cultivars produced the lighter and smaller cherries (ca. 25 mm). Fruit lightness (L^*) was highest in ‘Brooks’, with minimum values in ‘Sunburst’. The dry matter content varied between 21 and 23% with no association to the cultivar firmness.

Firmness showed a large inter-cultivar variation. ‘Regina’ cherries were the firmest followed by ‘Sumele’, ‘Chelan’, and ‘Brooks’. Soft cherries were produced by ‘Sunburst’. The

genotypes with the extreme firmness values were studied previously, on another batch.¹⁰ Although prior work on strawberry showed that for a given cultivar, larger fruit tended to be softer,²² in the current study no correlation was found between fruit size and firmness. This indicates that fruit size is not a major factor contributing to the genotypic differences in cherry texture. The cultivars also showed large differences in their cracking susceptibility. ‘Chelan’ fruit manifested relatively low cracking incidence, as opposed to ‘Brooks’ which was highly susceptible. Firmness as measured in compression tests was not related to the susceptibility of the fruit to cracking. The variations in cracking incidence and severity may be more highly associated with differences in cuticle thickness and composition.²³ Variations in the cell wall composition yielding changes in tissue physical properties under tension may be also important.

Cell Wall Yield and Fractionation. To further characterize the wall composition of the cultivars with contrasting firmness, the cell walls (AIR) were obtained after treatment of the fruit tissue with ethanol and chloroform/methanol, and further dehydration with acetone. The AIR was later fractionated into five different fractions, corresponding to pectins which are loosely bound (WSF), ionically bound (CSF), covalently bound (NSF), and loosely bound (1KSF) and strongly bound (4KSF) cross-linking glycans (hemicelluloses). Their yields for the five cultivars and two ripening stages are shown in Figure 1. The overall yield of non-cellulosic biopolymers from AIR was above 50% for immature cherries, and usually much less (<40%, with the exception of ‘Sumele’) for ripe cherries. The yields of the cell wall preparations dropped during ripening in all cultivars (Figure 1). No association between the accumulated yield of the different cell wall fractions and cultivar firmness was found at the immature or ripe stages, differing from previous reports.⁹

Growth and ripening change the architecture of fruit cell wall.²⁴ Although soluble polysaccharides usually accumulate during ripening⁸ and some studies have associated softening of cherry with high levels of water-soluble polysaccharides, this does not seem to be a general feature, but rather a cultivar-dependent response. In fact, in the present study the yield of WSF increased with ripeness in ‘Sunburst’ and ‘Regina’, whereas no marked variation was detected in ‘Brooks’ and ‘Sumele’. Moreover, a decrease in the WSF yield was found in ‘Chelan’. It is also worth noting that the fraction of loosely bound polysaccharides represented for all cultivars (even at the ripe stage) less than 5% of the total wall non-cellulosic polysaccharides.

Tightly bound pectins (NSF) comprised the most abundant cell wall fraction in the five cultivars regardless of the ripening stage. The drop in polysaccharide yield accompanying ripening was in all cases due to the decrease of the NSF (Figure 1). A

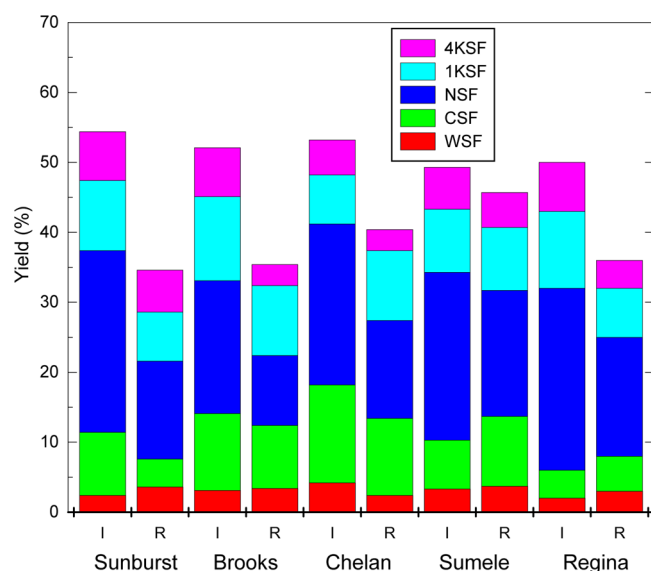


Figure 1. Accumulated yields of the water-, CDTA-, Na_2CO_3 -, 1 M KOH-, and 4 M KOH-soluble fractions (WSF, CSF, NSF, 1KSF, 4KSF) of immature (I) and ripe (R) 'Brooks', 'Chelan', 'Sumele', 'Sunburst', and 'Regina' cherry fruit. Data represent an average of two independent weight measurements.

similar trend was previously observed on cherries,^{8,9} grapes,²⁵ raspberries,²⁶ among other fruit species.²⁷

Depolymerization of Pectins and Cross-linking Glycans. The MW distribution of all the isolated samples was determined by SEC, using Sepharose CL-6B for fractions WSF, 1KSF, and 4KSF, and Sepharose CL-2B for CSF and NSF. The WSF of immature cherries showed a wide profile of MWs in most cultivars. Depolymerization of pectins with ripening is highly variable among fruit species. Pectin downshifts were very limited in strawberry²⁸ and apple,²⁹ moderate in tomato³⁰ and peach,³¹ but extensive in avocado.³² Even within a single

species, large variations have been reported depending on the cultivar.³³ In the present work a clear shift of the WSF profile toward lower MWs was observed as ripening proceeded. Firm 'Regina' cherries showed little or no depolymerization. In contrast, a downshift trend was observed in the WSF of the remaining cultivars. No association between firmness and pectin size was observed in these four genotypes indicating that the extent of fruit softening is not directly dependent on the degree of polymerization of loosely bound polyuronides.

The CSF showed a broad distribution of pectin MWs from early development, with no major changes occurring during ripening. A slight loss of high-MW polysaccharides was evident in soft 'Sunburst' fruit. The highly abundant NSF showed some unexpected features considering the traditional direct association that has been made between depolymerization and softening in several fruits. Indeed, the firmest genotype showed a lower degree of polymerization than soft 'Sunburst' cherries. Thus, the intermolecular interactions between polymers or their preferential distribution within the cell wall may be more relevant than their individual polymerization degree. No marked depolymerization was observed in this fraction as ripening progressed with the exception of relatively firm 'Sumele' fruit.

As occurs in blueberry,³⁴ and in contrast to raspberry²⁶ depolymerization of matrix glycans was related to ripening. For both loosely and tightly bound cross-linking glycans, soft 'Sunburst' cherries showed a greater reduction in polymer size than firm 'Regina' fruit. However, it is important to note that the firmest cultivar ('Regina') presented unripe cross-linking glycans with a relatively low MW. The 1KSF profile was characterized by a second, sharper peak with relatively high MW upon ripening, already observed for prunes and in previous work on cherries.^{9,18} Overall, results from low-pressure SEC of pectin and hemicelluloses suggest that cherries show some depolymerization of WSF and 1KSF. However, with a few exceptions, the degree of polymerization of non-cellulosic

Table 2. Neutral Sugar Composition (mol/100 mol) of the Water-, CDTA-, and Na_2CO_3 -Soluble Fractions in Immature (I) and Ripe (R) 'Sunburst', 'Brooks', 'Chelan', 'Sumele', and 'Regina' Cherries^a

fraction	cultivar	monosaccharide													
		Rha		Fuc		Ara		Xyl		Man		Gal		Glc	
		I	R	I	R	I	R	I	R	I	R	I	R	I	R
WSF	Sunburst	8	10	—	1	31	40	11	7	12	9	26	18	12	15
	Brooks	8	7	—	1	40	40	6	7	9	10	27	20	10	15
	Chelan	9	10	—	1	44	30	8	8	10	11	20	23	9	17
	Sumele	9	8	1	1	44	35	5	7	10	11	24	24	7	15
	Regina	6	9	1	1	31	36	12	5	12	12	23	22	13	16
CSF	Sunburst	13	12	3	1	64	66	5	3	3	3	10	11	2	3
	Brooks	12	11	1	1	59	80	3	1	4	1	17	4	5	2
	Chelan	11	12	1	1	73	70	1	2	tr	1	13	10	1	4
	Sumele	10	14	1	2	53	70	6	3	8	1	19	9	3	2
	Regina	9	12	1	1	64	67	4	4	6	2	11	10	6	4
NSF	Sunburst	8	8	tr	tr	69	84	—	2	—	tr	23	5	—	1
	Brooks	10	8	tr	tr	75	81	1	1	—	tr	13	7	tr	2
	Chelan	10	7	tr	1	76	83	1	2	tr	tr	12	5	1	2
	Sumele	10	8	1	1	70	77	2	3	tr	1	16	9	1	1
	Regina	10	8	tr	tr	66	78	1	2	tr	tr	19	8	3	3

^aThe average value is indicated ($n = 2-3$).

Table 3. Neutral Sugar Composition (mol/100 mol) of the 1 M KOH- and 4 M KOH-Soluble Fractions of Immature (I) and Ripe (R) ‘Sunburst’, ‘Brooks’, ‘Chelan’, ‘Sumele’, and ‘Regina’ Cherries^a

		monosaccharide													
fraction	cultivar	Rha		Fuc		Ara		Xyl		Man		Gal		Glc	
		I	R	I	R	I	R	I	R	I	R	I	R	I	R
1KSF	Sunburst	5	4	1	1	52	55	21	16	2	1	11	11	8	11
	Brooks	5	4	1	1	50	60	17	12	4	4	12	7	11	11
	Chelan	5	4	1	1	46	57	23	15	4	4	11	9	11	10
	Sumele	3	4	1	1	57	59	22	16	3	4	8	8	7	8
	Regina	3	4	tr.	1	56	56	24	13	1	5	9	12	7	9
4KSF	Sunburst	4	5	3	2	34	36	24	18	6	5	15	20	14	14
	Brooks	3	4	2	2	36	33	21	22	5	4	13	11	20	24
	Chelan	4	5	2	1	29	30	23	20	7	8	11	13	25	24
	Sumele	3	6	2	2	37	37	23	17	5	4	10	9	19	24
	Regina	4	6	2	2	27	38	26	14	5	9	10	13	26	18

^aThe average value is indicated ($n = 2-3$).

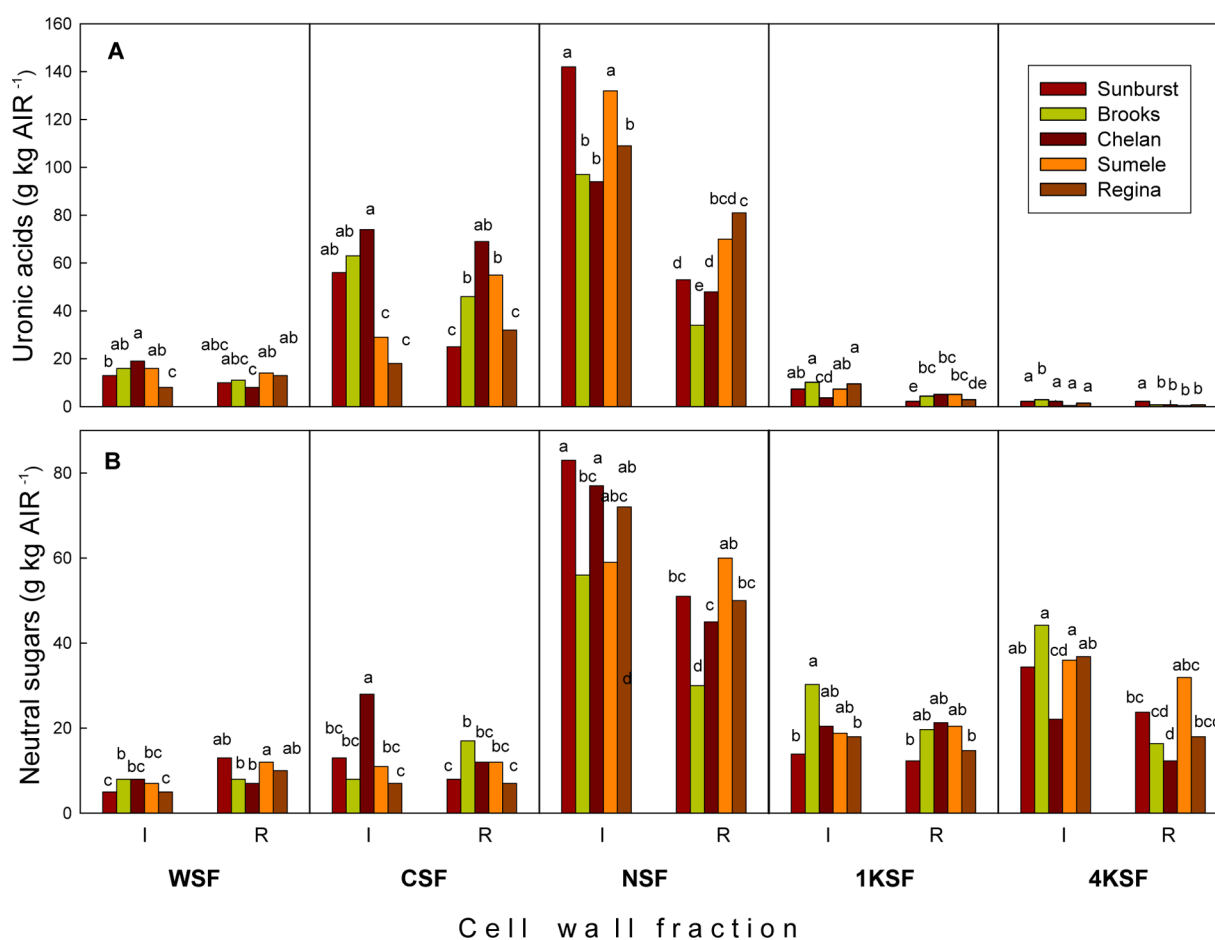


Figure 2. Uronic acids (A) and neutral sugars (B) in the water- (WSF), CDTA- (CSF), Na_2CO_3 - (NSF), 1 M KOH- (1KSF), and 4 M KOH-soluble fractions (4KSF) of immature (I) or ripe (R) ‘Brooks’, ‘Chelan’, ‘Sumele’, ‘Sunburst’, and ‘Regina’ cherries during development. Different letters indicate significant differences between cultivars and stages based on a Tukey test at a level of significance of $P \leq 0.05$.

polysaccharides at immature stages and its variation during ripening do not correlate with genotype firmness.

Polysaccharide Neutral Sugar Composition. The NS compositions of the different cultivars were surprisingly similar. Arabinose was the most abundant monosaccharide in all pectic fractions and cultivars (Table 2). Arabinose represented 31% of total NSs in the WSF. Its level increased to ca. 60% in the CSF

and 65–76% in the NSF. In pear, arabinose was rather lost,³⁵ but in cherries this sugar increases its proportion with ripening. Galactose and rhamnose were also present in substantial amounts, indicating the presence of RG-I in all three pectic fractions and cultivars. Xylose, glucose, and mannose only appeared in considerable levels in the WSF, whereas the most abundant pectic NSF fraction only showed low proportions of

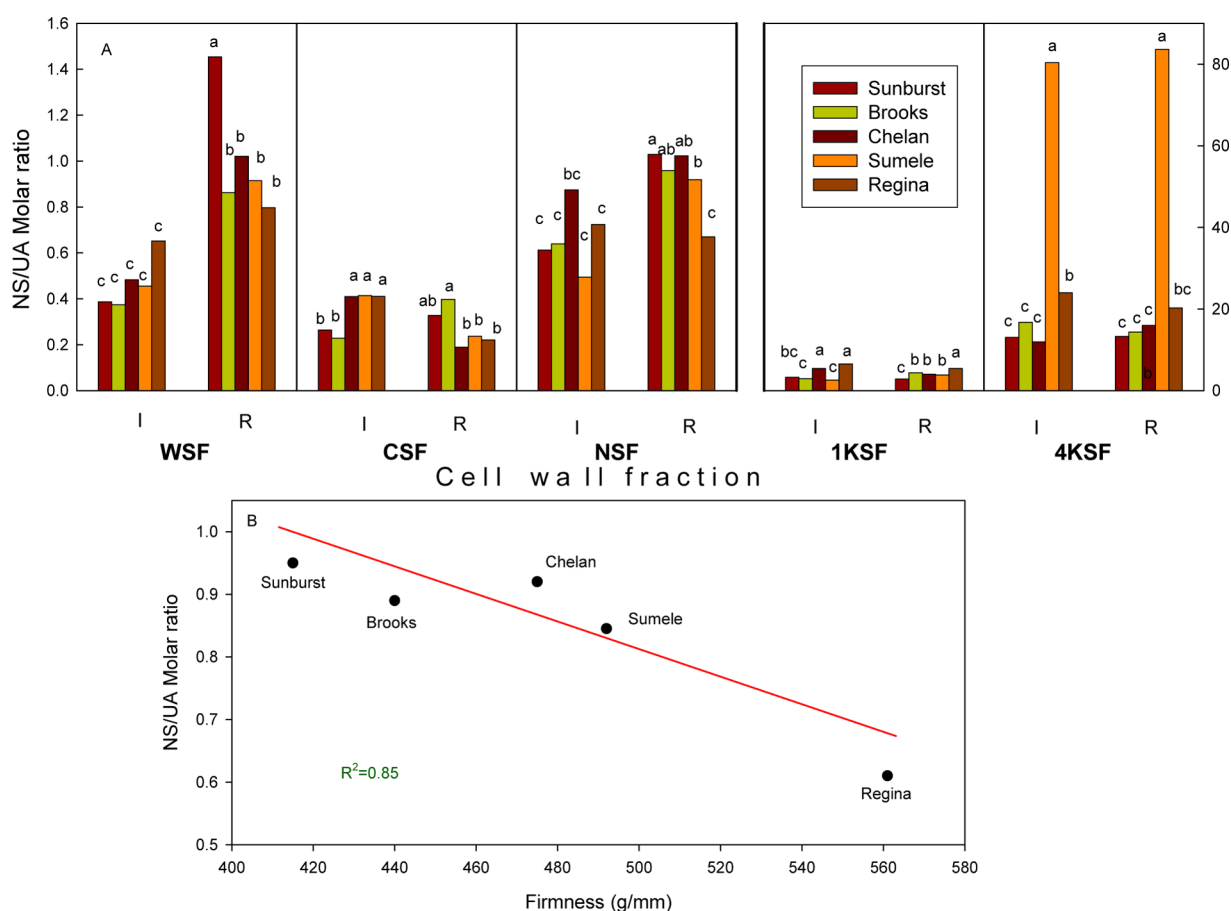


Figure 3. (A) Neutral sugars-to-uronic acids molar ratios calculated for the water- (WSF), CDTA- (CSF), Na_2CO_3 - (NSF), 1 M KOH- (1KSF), and 4 M KOH-soluble fractions (4KSF) of immature (I) and ripe (R) 'Brooks', 'Chelan', 'Sumele', 'Sunburst', and 'Regina' cherry fruit during development. Different letters indicate differences based on a Tukey test at a level of significance of $P \leq 0.05$. Note the different scale for the two last figures. (B) Association between the NS/UA ratio and firmness from the NSF of ripe 'Brooks', 'Chelan', 'Sumele', 'Sunburst', and 'Regina' cherries.

these residues (Table 2). The proportion of arabinose in the WSF varied with ripening in a different fashion depending on the cultivar. This fraction also showed some increase in glucose proportion at the ripe stage.

No marked enrichment or drop of a specific NS was observed during ripening in the ionically bound pectins (CSF), regardless of the cultivar. The UA/rhamnose ratio may be indicative of the relative proportion of RG-I to homogalacturonan (HG) in the pectic fractions, with higher values being expected in fractions enriched in HG. The NSF showed lower UA/rhamnose ratios (10–20) than the other pectic fractions, suggesting a higher prevalence of RG-I. The proportion of arabinose in this fraction increased in all the cultivars during ripening, with a corresponding reduction in galactose. This points out to a preferential cleavage of galactan side chains from RG-I, or from an arabinose enrichment originated in material associated with cross-linking glycans or cellulose. Loss of galactose is important in some species but not in others.^{35–38}

Although hemicelluloses are usually considered to be predominant in the KOH-soluble fractions, arabinose still represented 50% of total NS in the 1KSF. The residual pectins present in the cross-linking glycan fraction 1KSF show considerably lower ratios (UA/rhamnose = 4–12) than those of the pectin-rich fractions. The ratio is even lower (<2) for the 4KSF, suggesting that these residual pectins are RG-I-rich and that tight association through side-chain interactions exists between hemicelluloses and RG-I regardless of the cultivar.^{39,40}

(Table 3). Xylose was the second most abundant sugar, but its proportion (ca. 20%) dropped upon ripening. The presence of xylose in levels exceeding those of glucose, suggests that besides some xyloglucan, xylans may be present. This may also contribute to the relatively high arabinose levels which could be, at least in part, in the form of arabinoxylans.

Arabinose proportion was, as expected, lower in the 4KSF, but, differently from other fruit species, it still represented the most abundant NS, closely followed by xylose and glucose. As for the 1KSF, the level of xylose tended to decrease with ripening. Noteworthy, the presence of small but constant levels of rhamnose and UA suggests that some RG-I still remains in this fraction, possibly entangled to the cellulose-xyloglucan/arabinoxylan network.

Pectin and Hemicellulose Solubilization. At the immature stage UAs were ca. 2- and 7-fold higher in the NSF than in the CSF and WSF (Figure 2A). The UA of the tightly bound pectin showed a marked decrease during ripening. This has also been observed in other fruits.²⁷ The drop of UA in the NSF did not occur with a concomitant accumulation in the water or CDTA-soluble fractions, suggesting that it was caused by the action of exo-acting galacturonate hydrolases yielding free galacturonic acid, which may be possibly lost in the ethanol washings of the AIR. At the ripe stage, firmer 'Regina' cherries maintained higher levels of tightly bound UA (Figure 2A). However, the absolute level of Na_2CO_3 -soluble polyuronides showed no high association with

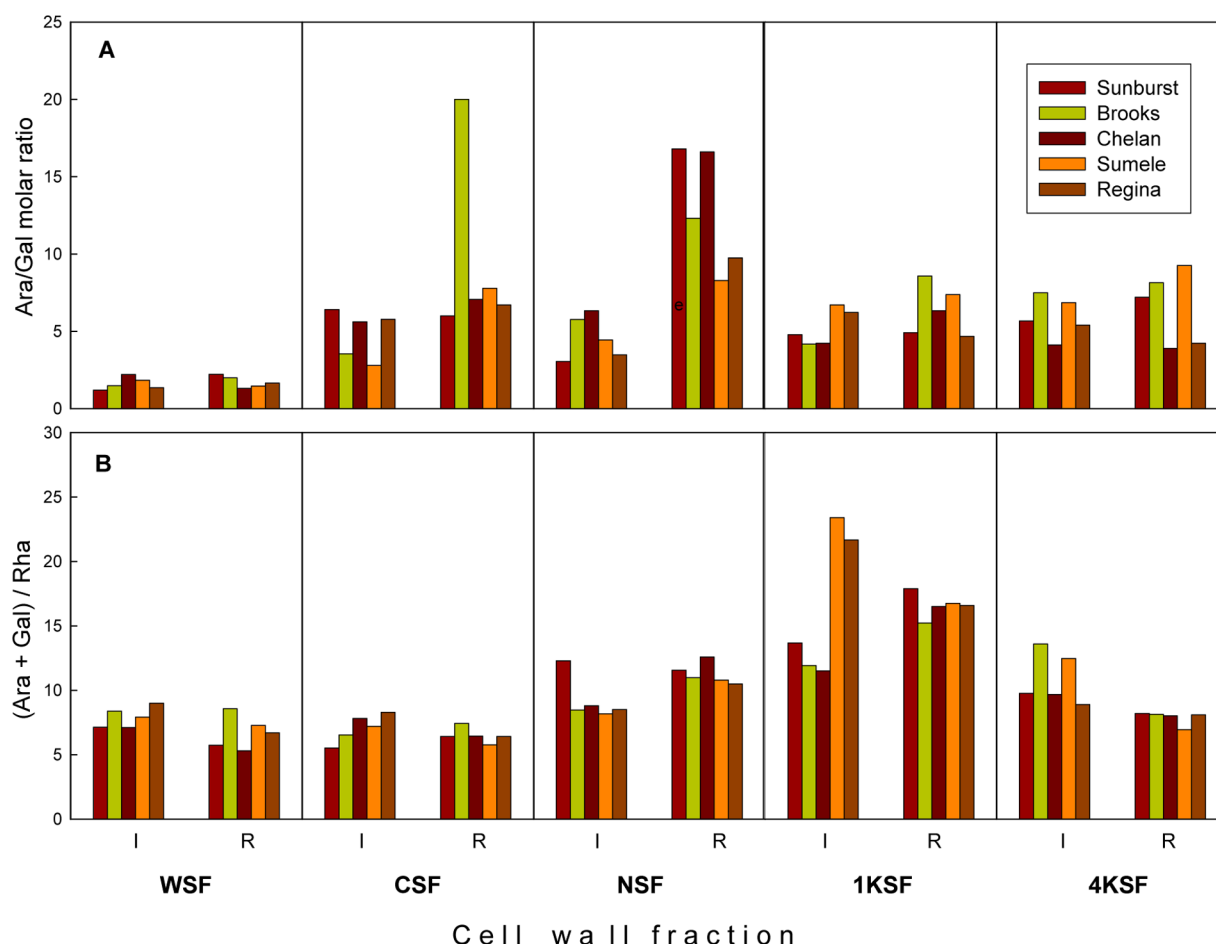


Figure 4. (A) Ara/Gal and (B) (Ara+Gal)/Rha molar ratios calculated for the water- (WSF), CDTA- (CSF), Na_2CO_3 - (NSF), 1 M KOH- (1KSF), and 4 M KOH-soluble fractions (4KSF) of immature (I) and ripe (R) 'Brooks', 'Chelan', 'Sumele', 'Sunburst', and 'Regina' cherry fruit during development. Different letters indicate differences based on a Tukey test at a level of significance of $P \leq 0.05$.

cultivar firmness. For the CSF, a more erratic behavior of the UA content changes with ripening was observed; they increased with ripening in 'Sumele' and diminished in 'Sunburst'. The strong interaction between pectic and hemicellulosic matrices by covalent linkages^{39,40} was also evident in cherry by the fact that alkali-soluble fractions still contained considerable amounts of UA. These polyuronides showed a progressive loss between early ripening stages and commercial maturity.

Analysis of the fate of the NSs on ripening (Figure 2B) showed small quantitative variations for WSF, an erratic behavior for CSF, but a decreasing tendency in the remaining fractions. The sum of NSs diminished clearly on ripening for all cultivars excluding 'Sumele', where it remained unchanged. The decrease ranged from 28% in 'Regina' and 'Sunburst' to 37% in 'Chelan' and 'Brooks'.

Relationship between Cell-Wall Polysaccharides. By considering the neutral sugars-to-uronic acids molar ratio (NS/UA) (Figure 3A), an outline of the proportion of branching on RG-I can be assessed on pectic fractions, as well as the hemicellulose/pectin ratios on cross-linking glycan fractions. Pectin fractions WSF, CSF, and NSF were enriched in UAs, and thus gave relatively low NS/UA ratios (0.2–1.4). The glycan fractions 4KSF gave high values (12–84), whereas intermediate levels were observed in the 1KSF (3–6). No major variations in the NS/UA molar ratio with ripening were observed for the cross-linking glycan fractions 1KSF and 4KSF.

However, some inter-cultivar differences could be depicted (Figure 3A). For WSF, a significant increase in the NS/UA ratio with ripening was observed (Figure 3A) in all varieties. A lower but still significant increase was also found in the NSF fraction of three varieties. On the other hand, the CSF fractions showed the lowest ratio among the pectic fractions (Figure 3A), suggesting that they are HG-rich. Only a small variation with ripening was observed in this fraction. For the most abundant NSF, a high negative correlation ($R^2 = -0.85$) was found between the NS/UA ratio and cultivar firmness (Figure 3B). Firm cherries presented pectic polymers with lower NS/UA ratio, suggesting that the branching of polyuronides or the proportion of branched RG-I to HG may contribute to the differences in firmness between cultivars.

The Ara/Gal molar ratio allows an estimate of the relative importance of these monosaccharides in RG-I side chains. These ratios (Figure 4A) were always well above 1. The lowest ratios were found in the WSF (i.e., the most galactose-rich RG-I). For the CSF, the arabinose/galactose ratios for immature fruit were at or below 5, but they increased for ripe fruit, especially in 'Brooks'. The NSF showed a clear increase in this ratio upon ripening for all cultivars. This suggests that during ripening, higher loss of galactan or arabinogalactan chains occurs as compared to those of arabinans, regardless of the cultivar. However, since cherries show overlap between growth and ripening, *de novo* deposition of polysaccharides may occur

providing more complexity to the interpretation of the results. The Ara/Gal ratios in the cross-linking glycan fractions (Figure 4A) showed a more or less constant value, close to 5, regardless of the ripening stage, and changes were more dependent upon the variety.

As these Ara and Gal moieties are mostly part of the side chains of RG-I, their ratios relative to Rha estimate the degree of branching on RG-I. These results are shown in Figure 4B. A small decrease of these ratios upon ripening was observed in WSF and CSF, but for NSF these ratios became larger and increased further at the ripe stage. Ripe fruit showed (Ara + Gal)/Rha ratios in these fractions close to 11–12, regardless of the cultivar. This suggests that the variation in NS/UA ratio of the tightly bound pectin was more likely due to different proportions of HG and RG-I. The independent presence of HG and RG has been questioned by some authors. Vincken et al.⁴¹ proposed a molecular brush model in which HG, (arabino) galactan, and arabinan occur as side chains of RG-I. Considering this model and the results found herein, firm cherry fruit would have a brush structure richer in HG side chains than soft cultivars. Overall, our results suggest that branching of polyuronide occurs early in development, and that the proportion of branched RG-I to HG may contribute to the differences in firmness between cherry cultivars. Studies evaluating differences in the levels of enzymes involved in pectin biosynthesis (e.g., rhamnosyl, galactosyl and arabinosyl transferases) could be of great interest for further work.

Few studies have analyzed changes in turgor pressure during fruit ripening, but early reports suggested that turgor pressure may be also an important contributor to fruit texture.⁴² Recent work⁴³ has shown that turgor pressure drops during cherry fruit development. Thus, the potential role of differences in turgor pressure on cultivar texture could not be ruled out.

UV-MALDI-TOF Mass Spectrometry of Pectins. Determination of changes in polymer composition attracts the interest of researchers in the field of plant cell wall carbohydrates synthesis and degradation. Even with only a limited number of monosaccharides used as building blocks, variations in sequence, linkage, branching, side-chain distribution, and functional group modifications result in a wide structural diversity. No single analytical method alone is capable of resolving carbohydrate structures, especially when present in complex mixtures. Mass spectrometry is a selective and powerful technique to identify and obtain structural information and quick profiles on compounds present in complex mixtures.^{44,45} Since it requires only small amounts of sample, it is an excellent tool for researchers interested in detecting changes in composition of complex plant carbohydrates. Nevertheless, detection of native non-derivatized polysaccharides by UV-MALDI MS is still a challenge due to the shortage of adequate matrices.^{44–49} So far, there have been only a few studies reported for detection of native polysaccharides with MW > 40 000 Da^{44,45,49–51} In the current work, our challenge was to observe if the association of this pattern of depolymerization with firmness and development among cultivars can be foreseen in a different fashion using UV-MALDI-MS than that found by traditional size exclusion chromatography.

In the current work, we have found that 2,5-dihydroxybenzoic acid⁴⁶ and 9H-pyrido[3,4b]indole (nor-harmane)⁴⁸ are good matrices for MALDI-MS analysis of native polysaccharides with high MWs and broad mass ranges (m/z 40 000–160 000).

In order to further characterize the cell wall changes occurring on development and to identify putative factors associated with firmness variation in cherry fruit genotypes, we performed a detailed evaluation of the UV-MALDI-TOF mass spectra of the different pectin fractions WSF, CSF, and NSF for the five cultivars. Experiments were conducted with two different matrices, nor-harmane and gentisic acid, in order to get complementary information, and in five independent experiments. In this analysis, the intensity of the signals, the signal-to-noise ratio (S/N), and the number of signals with intensities higher than a given threshold ($S/N > 2$) in all the m/z region analyzed were taken into account. Although each mass spectrum appears as a bidimensional image (intensity vs m/z), the number of “true” signals in each m/z region yields additional data. These three variables (intensity, number of signals, and m/z) were taken into account for the comparative analysis of all the extracts studied. In the range m/z 80 000–200 000, a large number of pectin signals originated in the WSF fractions from immature fruit were very conspicuous by this technique, showing clear spectra. The CSF gave much poorer spectra with very low S/N, whereas for the NSF almost no signals were observed in this m/z range. As an example, the results obtained for cv. Regina using nor-harmane as matrix are shown in Figure 5. From ripe cherries, WSF showed poor

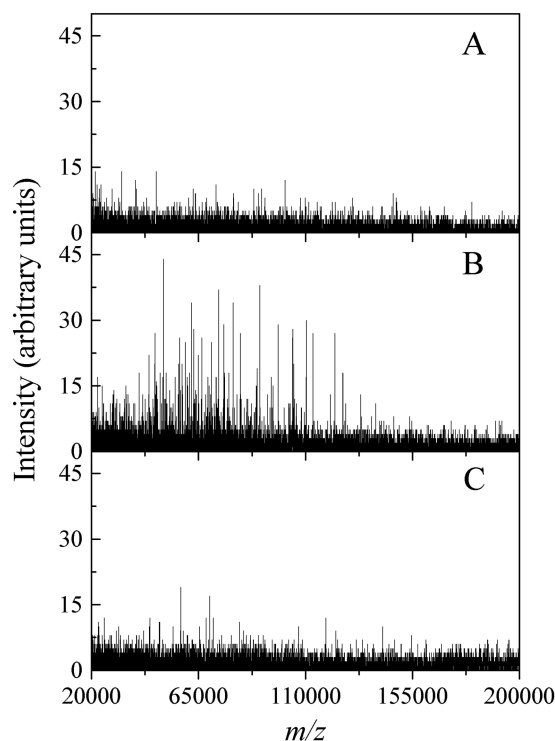


Figure 5. UV-MALDI-MS analysis of ‘Regina’ fractions in positive ion mode using nor-harmane as matrix. Characteristic profiles obtained for WSF from ripe fruit (A), WSF from immature fruit (B), and CSF from immature fruit (C).

spectra in the range m/z 20 000–150 000 (no signals with intensity >15 arbitrary units and $S/N > 2$) (Figure 5A). In contrast, WSF of immature cherries showed clear profiles with abundant high-intensity ion signals (Figure 5B) in the same m/z range. Poor spectra with very few signals at the m/z range 40 000–80 000 with the stated intensity and S/N were obtained when the corresponding CSF was analyzed (Figure

5C). The differences observed in the spectra agree with the facts that complex mixtures of polysaccharides were analyzed and with the possibility that ion source decomposition occurs.^{44,45} Similar characteristic profiles were obtained for immature WSF from the other four varieties studied.

In this work, we have focused on the evaluation of the changes in cell wall polysaccharides of five cherry cultivars ('Chelan', 'Sumele', 'Brooks', 'Sunburst', and 'Regina') with contrasting firmness at two developmental stages, showing that, contrary to what is usually shown in most fruits, cherry softening could occur in some cultivars without marked increases in water-soluble pectin. The degree of pectin or hemicellulose depolymerization or the solubilization of polyuronides is not directly associated with fruit firmness. The tightly bound polyuronides represented the most abundant cell wall fraction in all cultivars. A high negative correlation was found between the NS-to-UA ratio in this fraction and tissue texture, causing firm cultivars to exhibit lower NS/UA than soft cherries. The similar molar ratio of arabinose and galactose relative to rhamnose [(Ara+Gal)/Rha] suggests that the cultivars differed in their relative proportion of homogalacturonan and rhamnogalacturonan I rather than in the size of RG side chains, with greater proportions of HG in firmer cherries. Finally, we showed that UV-MALDI-TOF mass spectrometry may be a powerful analytical approach to complement traditional methods used to study polysaccharides contributing to improve our understanding of cell wall catabolism, as the mass spectra allowed immature to be distinguished from ripe fruits. WSF of immature cherries showed clear profiles with abundant ion signals in the range m/z 20 000–150 000, but poor spectra with very few signals in the range m/z 40 000–80 000 were obtained when CSF was analyzed. On the contrary, from ripe cherries WSF showed poor spectra with either matrix at the former m/z region.

■ ASSOCIATED CONTENT

■ Supporting Information

NS composition of the fractions expressed in g/kg AIR; SEC profiles of all the fractions; and UV-MALDI-MS analysis of selected fractions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Notes

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■ ABBREVIATIONS USED

AIR, alcohol-insoluble residue; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; CSF, CDTA-soluble fraction; DAA, days after anthesis; HG, homogalacturonan; 1KSF, 1 M KOH-soluble fraction; 4KSF, 4 M KOH-soluble fraction;

NS, neutral sugar; NSF, Na₂CO₃-soluble fraction; RG-I, rhamnogalacturonan I; SEC, size-exclusion chromatography; S/N, signal-to-noise ratio; SSC, soluble solids content; TFA, trifluoroacetic acid; UA, uronic acid; WSF, water-soluble fraction

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