JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Changes in Polysaccharide and Protein Composition of Cell Walls in Grape Berry Skin (Cv. Shiraz) during Ripening and Over-Ripening

ANYSIA VICENS, DAVID FOURNAND, PASCALE WILLIAMS, LOUISE SIDHOUM, MICHEL MOUTOUNET, AND THIERRY DOCO*

INRA, UMR 1083, F-34060 Montpellier, France

Polysaccharide modification is the most fundamental factor that affects firmness of fruit during ripening. In grape, because of the lack of information on the modifications occurring in cell wall polysaccharides in skins, but also because this tissue contains large amounts of organoleptic compounds for winemaking, a study was performed on the evolution and extractability of polysaccharides from grape skins of Shiraz cultivar throughout ripening. A HEPES/phenol extraction technique was used to analyze Shiraz grape cell wall material isolated from skins of berries harvested from one to ten weeks after véraison. Total amounts in cell wall polysaccharides remained constant during ripening (4.2 mg/berry). A slight decrease in galactose content of insoluble polysaccharides was observed, as well as a significant de-esterification of methoxylated uronic acids, indicating that some modifications occur in cell wall polysaccharides. The water-soluble fraction represented a very small fraction of the whole polysaccharides, but its amounts increased more than 2-fold between the first and the last sample. Isolated cell walls were also analyzed for their protein composition. Last, hydroalcoholic extractions in model-wine solution were also performed on fresh skins. This extracted fraction was very similar to the water-soluble one, and increased during the entire period. By comparison with polysaccharide modifications described in flesh cell wall in previous works, it can be assumed that the moderate skin polysaccharide degradation highlights the protective role of that tissue.

**KEYWORDS: Grape skin; cell wall; ripening; model extraction; extractability; polysaccharides; proteins**

## INTRODUCTION

Primary plant cell walls consist of a network of cellulose microfibrils tethered to a hemicellulosic polysaccharide matrix. In dicotyledons, hemicellulose is predominantly represented by xyloglucan polymers. This matrix is believed to be the major load-bearing structure in the primary wall. A second domain, more soluble and consisting of pectic polysaccharides, is embedded within the matrix and also in the middle lamella with a function of regulating cell adhesion (*1*, *2*). Other components such as enzymatic and structural proteins, ions and low-molecular-weight compounds are also present in this domain. Pectin is a group of polysaccharides rich in galacturonic acid (GalA) and classified in three types of polymers: the major homogalacturonan polymer, and the two branched side chain polymers, containing rhamnosyl residue, the so-called rhamnogalacturonan I and rhamnogalacturonan II. During fruit ripening, pectins are known to be substantially depolymerized and solubilized (*3*, *4*). A partial degradation of xyloglucan was also observed in the early stage of ripening in persimmon and in tomato (*5*, *6*).

Little is known in grape about the evolution of cell walls in skin, even though many compounds of enological interest (phenolic compounds, aroma precursors) are known to be accumulated in that tissue throughout ripening. The study of Ortega-Regules et al. performed on four different grape varieties has shown a decrease in the quantity of cell wall material in skin and a loss of galactose (*7*). In grape flesh, Nunan et al. (*8*) observed a decrease in type I arabinogalactan content and a solubilization of galacturonan during the development. Additionally, these authors noted an increase in protein content after véraison, especially hydroxyproline-containing proteins. Yakushiji et al. (*9*) also showed a decrease of the molecular masses of pectic and hemicellulosic polysaccharides in the mesocarp at véraison. At this stage, they observed also a decrease of the hemicellulose content while the neutral and acidic sugar contents of water soluble and pectin fraction decreased later during ripening.

The aim of the present paper is to provide information about the specific modifications that occur in the skin cell wall of grape during the ripening and over-ripening period.

## MATERIALS AND METHODS

**Grape Samples.** In 2004, five samples of *Vitis vinifera* var. Shiraz grape were harvested weekly from 1 to 10 weeks after véraison from a vineyard located in Montpellier (France). Berries were calibrated according to their density (i.e., total soluble solids). Density was estimated by flotation of berries in ten different salt solutions (from

* Corresponding author. Tel: 33 (0)4 99 61 26 97. Fax: 33 (0)4 99 61 28 57. E-mail: doco@supagro.inra.fr.

10.1021/jf803416w CCC: $40.75 
Published on Web 02/25/2009

**Table 1.** Physiological Characteristics of Densimetrically Sorted Berries of *Vitis vinifera* L. Cv. Shiraz Grown in Montpellier, France, in the 2003−2004 Season, from One Week to Ten Weeks after Véraison

| samples | weeks after véraison | berry wt (g) | soluble solids (g/L) | titratable acidity (mequiv/L) | pH | potential alcohol (% vol) | sugar to acidity ratio (g/mequiv) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1.89 | 127.3 | 191.0 | 3.07 | 7.56 | 0.67 |
| 2 | 3 | 2.30 | 169.3 | 116.8 | 3.21 | 10.06 | 1.45 |
| 3 | 5 | 2.26 | 193.4 | 97.9 | 3.43 | 11.49 | 1.98 |
| 4 | 7 | 1.96 | 222.4 | 76.5 | 3.67 | 13.21 | 2.92 |
| 5 | 10 | 1.62 | 275.0 | 73.2 | 3.79 | 16.34 | 3.76 |

100 to 190 g/L NaCl) so that the difference in total soluble solids of two consecutive berry batches was about 17 g/L (i.e., 1% vol in potential titratable alcohol). For each date, the class of berries retained after density sorting was not necessarily the major one. For the first weeks after véraison, the less dense classes were selected, and for the last samples, the highest classes were selected, so that the physiological differences between the first (1 week after véraison) and the last sample (10 weeks after véraison) were higher than those that could have been achieved with a nonsorted sampling. A representative sample of 100 sorted berries was squeezed for measurements of pH, total acidity and total soluble solids (using a hand-held refractometer).

A second sample of 100 sorted berries was manually peeled, and skins were immediately frozen in liquid nitrogen, freeze-dried and dry-crushed in liquid nitrogen with a Freezer Mill (Bioblock, Ilkirch, France) for later determination of polysaccharide compound contents. Powders were stored in the dark at +4 °C in the presence of $P_2O_5$.

Finally, a third sample of 90 sorted berries was also manually peeled for model hydroalcoholic extraction on fresh material.

**Extraction in Model Hydroalcoholic Solutions.** Ninety freshly isolated skins were placed in 150 mL of an hydroalcoholic solution containing 12% vol ethanol, 2 g/L potassium hydrogen tartrate and 100 mg/L $SO_2$. All model extraction experiments were performed in duplicate. Flasks were placed under argon and gently stirred at 27 °C for 5 h. At the end of the extraction, residual solid parts were rinsed with a hydroalcoholic solution and immediately frozen in liquid nitrogen to be freeze-dried and dry-crushed with a Freezer Mill. Extraction media were freezed in liquid nitrogen and stored at −80 °C after centrifugation.

**Isolation of Cell Wall Material from the Skin of Grape Berries.** The isolation was performed with a modified procedure (*10*, *11*) of the phenol buffered method described by Huber (*12*). Powders obtained from initial skins were blendered at +4 °C in 45 mL of 40 mM HEPES pH 7.0 for 5 min and then centrifugated for 30 min at 10000*g*. The residue was washed twice with 45 mL of HEPES solution at +4 °C. The supernatants were pooled for the analysis of soluble polysaccharides (see below, Precipitation of Soluble Polysaccharides). The insoluble material obtained was then stirred for 30 min at room temperature in 100 mL of buffered phenol in 0.5 M Tris-HCl pH 7.5. The suspension was centrifuged (14000*g*, for 20 min) and washed again with 100 mL of buffered phenol. After centrifugation (14000*g*, for 20 min), phenol was totally removed by two extractions with 80% ethanol (v/v) followed by two extractions with 80% acetone (v/v). Supernatants were eliminated at each step by centrifugation (3200*g*, for 15 min). The material was resuspended and stirred for 30 min in 40 mL of chloroform:methanol (1:1, v/v) and centrifugated (3200*g*, for 15 min). The cell wall material obtained was dried by successive addition of solvent: absolute ethanol, acetone and diethyl ether (centrifugation after each step, 3200*g*, for 10 min). After 1 h under air, the cell wall material was placed in a vacuum oven for 30 min at 40 °C before storage under $P_2O_5$ in a vacuum desiccator.

**Precipitation of Soluble Polysaccharides.** HEPES-soluble polysaccharides (water-soluble fraction) were precipitated by addition of EtOH at +4 °C to reached a final concentration of 80% EtOH (v/v). The precipitates were washed twice with EtOH. After dissolution in water, the solutions were dialyzed against water for 48 h (spectra/Por 6, 3500 Da, 45 mm). The solutions were then freeze-dried.

Hydroalcoholic-soluble polysaccharides were precipitated similarly with an 80% final concentration of EtOH (v/v) at +4 °C, and then dialyzed and freeze-dried.

**Carbohydrate Analysis.** Neutral sugars were analyzed as their alditol acetate derivatives according to the method of Albersheim et al. (*13*) modified by Blakeney et al. (*14*). The hydrolysis was done in triplicate for cell wall analysis on 5 mg of material with trifluoroacetic acid (2 M) at 120 °C for 75 min. Allose was used as an internal standard. The monosaccharide derivatives were separated by gas chromatography (HP 5890 GC) on an OV 225 column (30 m × 0.32 mm i.d., film thickness of 0.5 μm) using a split injector (ratio 1:10 at 250 °C) and a flame ionization detector. The flow rate of the carrier gas ($H_2$) was 3 mL min $^{-1}$. The oven temperature was 200 °C for 12 min and then raised at 10 °C min$^{-1}$ to 210 °C and maintained for 13 min.

Uronic acids were determined colorimetrically in triplicate by the *m*-hydroxydiphenyl method (*15*). For cell wall analysis, the material was first solubilized in water by action of $H_2SO_4$ as described by Ahmed and Labavitch (*16*). Calibration was made with galacturonic acid (D-(+)-galacturonic acid monohydrate, Fluka).

The degree of methylesterification was determined by saponification of cell wall polysaccharides and using alcohol oxidase enzyme for the determination of the methanol released, as described by Klavons and Bennett (*17*).

**Polysaccharide Linkage Analysis.** Polysaccharides were analyzed according to Hakomori (*18*) by permethylation catalyzed by methyl sulfinyl carbanion in methyl sulfoxide. Cell walls (3 mg) were methylated and then retrieved in 2.5 mL of methanol:chloroform (2:1, vol/vol). This phase was washed five times by addition of water (5 mL), centrifuged (3000*g*, for 3 min) and air-dried. Partially O-methylated polysaccharides were then converted to alditol acetate derivatives (*13*, *14*) with the following modification in the procedure. The methylated polysaccharides were reduced with $NaBD_4$. Partially methylated alditol acetates were separated by gas chromatography−mass spectrometry on a capillary column containing OV-1 phase (30 m × 0.32 mm i.d., 0.25 μm, 100% dimethylsiloxane, Ohio Valley) (temperature programming 135 °C for 10 min, then 1.2 °C/min to 180 °C). GC−MS was performed with a Hewlett-Packard HP-6890 GC coupled to a HP 5973 mass selective detector operated in the EI mode (70 eV, 34.6 μA and *m*/*z* 50 to 650 uma) and under the control of a HP Productivity ChemStation. Coeluted peaks were then separated on a capillary column of OV-225 (0.25 μm, 50% dimethylsiloxane and 50% cyanopropylphenylsiloxane, Ohio Valley).

**Amino Acid Analysis.** Cell wall material (5 mg) was hydrolyzed in 1 mL of chlorhydric acid (6 N) for 24 h at 120 °C. Norleucine was added as an internal standard. After total evaporation under nitrogen, the sample was dissolved twice in water and twice in ethanol 95%. Finally, samples were dissolved in lithium citrate loading buffer 0.20 M pH 2.2 (Biochrom, Cambridge, England) and filtered through a 0.22 μm filter (Millipore Millex-GV). Amino acids were then quantified using a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, England). Due to hydrolysis, asparagine and glutamine were quantified as their acidic form, aspartic acid and glutamic acid. Tryptophan was totally degraded, and cysteine and methionine were randomly transformed.

## RESULTS AND DISCUSSION

**Set of Samples.** Berry weight, total soluble solids, titratable acidity, pH and potential titratable alcohol are given in **Table 1**. Mean weight of sorted berries increased during the first 3 weeks and then gradually decreased, particularly during over-ripening, when berries lost water. This was in agreement with previous studies performed on nonsorted berries (*19*−*21*). The potential titratable alcohol varied from 7.56 up to 16.34% vol, and the soluble solids to acidity ratio varied from 0.67 to 3.76

**Table 2.** Evolution of Insoluble Cell Wall Polysaccharides from Shiraz Skin during Ripening

| | noncellulosic composition (mol %) | | | | | | | | ME[a] | total |
|---|---|---|---|---|---|---|---|---|---|---|
| stage | Rha | Fuc | Ara | Xyl | Man | Gal | Glu | GalA | (%) | (mg/berry) |
| 1 | 2.4 | 1.0 | 12.6 | 5.5 | 2.2 | 9.3 | 3.0 | 64.0 | 51 | 4.07 |
| 2 | 2.4 | 1.0 | 12.0 | 5.2 | 2.2 | 5.5 | 3.1 | 68.6 | 44 | 4.10 |
| 3 | 2.5 | 1.0 | 12.8 | 5.1 | 2.2 | 5.5 | 4.0 | 66.9 | 41 | 4.15 |
| 4 | 2.7 | 0.9 | 12.1 | 5.5 | 2.2 | 4.9 | 4.0 | 67.8 | 39 | 4.41 |
| 5 | 2.6 | 1.0 | 11.1 | 5.1 | 2.2 | 4.7 | 5.0 | 68.4 | 36 | 4.09 |

[a] ME: methylesterification degree (%).

g·mequiv$^{-1}$. The technological maturity corresponded to a potential titratable alcohol of 11.97% vol and to a soluble solids to acidity ratio of 2.36. Therefore, samples 1 to 3 (1 to 5 weeks after véraison) corresponded to a ripening period and samples 4 and 5 (respectively, 7 and 10 weeks after véraison) corresponded to an over-ripening period.

**Evolution of Insoluble Cell Wall Polysaccharides.** The phenol-buffered procedure enables the solubilization of cytoplasmic proteins and the inactivation of cell-wall glycanases. As shown in **Table 2**, the total amount of insoluble material slightly but continuously increased from stage 1 to 4 (from 4.07 to 4.41 mg/berry) before slightly decreasing at stage 5 (4.09 mg/berry). Whatever the ripening stage, the insoluble fraction was characterized by 70% of acidic sugar and 30% of neutral sugars. Acidic sugars were shown to be only galacturonic acid as observed by GC−EIMS analysis of the trimethylsilyl methyl glycoside derivatives (data not shown). The percentage of methylesterification of galacturonic acid (**Table 2**) decreased continuously from 51 to 36% during ripening. This phenomenon was also described in pericarp of tomato, papaya and pear fruit (*22*−*24*). In grape skin, Ortega-Regules et al. observed a decrease of methylesterification in Cabernet Sauvignon, Merlot and Monastrell while no change was observed for Shiraz where the methylesterification degree was about 74% (*7*). In a previous work on pericarp of grape berries, the degree of methylesterification has been shown to decrease from 30% at véraison to less than 20% for ripe berries (*25*). In contrast, in mesocarp, a 58 to 48% decrease was shown to occur during the prevéraison period, the degree of methylesterification then remaining unchanged (*8*).

Neutral sugars were determined by gas chromatography analysis of the alditol acetate derivatives after trifluoroacetic acid hydrolysis. This allowed the quantification of noncellulosic neutral sugars. The noncellulosic neutral sugar content is expressed as a total amount in mg/berry and its composition as a relative molar percentage (mol %, **Table 2**).

Total noncellulosic neutral sugar amount remained constant from stage 1 to stage 5, and the composition was in agreement with previous studies performed on skin grape berries at harvest time (*7*, *26*) with arabinose, galactose and xylose being the main sugars. In Shiraz grape skin, galactose content decreased dramatically from 370 to 220 $\mu$g/berry from stage 1 to stage 2 and then remained unchanged. The release of galactosyl residues from cell walls during ripening has been previously observed in grape mesocarp or whole pericarp (*8*, *27*), as well as in other fruits (*28*). The loss of galactose during fruit ripening is directly related to the degradation of arabinogalactan of highly branched pectic polysaccharides (*29*). In the mesocarp of grape berries, this has been largely attributed to the degradation of the type I arabinogalactan (*8*). General patterns of the other neutral sugars did not change to any extent throughout this period.

Sugar linkages were determined by gas chromatography-electronic impact mass spectrometry (GC−EIMS) of methylated alditol acetate derivatives. The most abundant polysaccharide types found in grape berry cell walls could thus be identified (*8*, *30*, *31*). Estimated relative proportions of the major polysaccharides in cell walls are shown in **Table 3** for the five different stages of ripening. More than 85 mol % of the total sugar linkages were attributed to the different specific polysaccharides. The comparison of the polymer distribution on a mol % basis at the different stages of ripening showed a slight decrease in arabinogalactan type I content from 10% of total polysaccharides in the first stage to 7% in ripe berries. By this technique, on a $\mu$g/berry basis, the galactose content declined dramatically from 423 to 290 $\mu$g/berry. In the same period, arabinogalactan type II also decreased from 5.5 to 3.9% and cellulose increased slightly from 48 to 56% before declining for the ripest berries.

**Table 3.** Evolution of Major Polysaccharides from the Insoluble Material from Shiraz Skin (mol %) during Ripening[a]

| | stage 1 | stage 2 | stage 3 | stage 4 | stage 5 |
|---|---|---|---|---|---|
| xyloglucan | 9.6 | 9.7 | 10.2 | 9.9 | 9.1 |
| cellulose | 41.4 | 44.9 | 47.4 | 48.1 | 45.4 |
| mannan | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 |
| xylan | 9.5 | 10.0 | 7.6 | 7.2 | 9.6 |
| arabinogalactan type I | 10.4 | 8.3 | 7.4 | 7.5 | 7.9 |
| arabinogalactan type II | 4.8 | 3.5 | 4.1 | 3.4 | 3.6 |
| arabinan | 7.5 | 8.5 | 7.8 | 7.9 | 8.1 |
| rhamnogalacturonan | 4.6 | 4.2 | 3.8 | 4.0 | 4.2 |
| total | 89.3 | 90.5 | 89.5 | 89.3 | 89.3 |

[a] Xyloglucan is calculated from the sum of t-Xyl, t-Fuc, 4,6-Glc and one third of its proportion in 4-Glc. The rest of 4-Glc is assumed to be cellulose. The sum of 4,6-Man and t-Man is considered as mannan, and the sum of 5-Ara and 3,5-Ara is considered as arabinan. Arabinogalactan type I was estimated as the sum of 4-Gal and 3,4-Gal plus t-Gal, and arabinogalactan type II as the sum of 3-Gal and 3,6-Gal and an equivalent in t-Ara to the value for 3,6-Gal. 2-Rha and 2,4-Rha were attributed to the rhamnogalacturonan backbone.

**Table 4.** Evolution of Protein Content from the Insoluble Material from Shiraz Skin (mol %) during Ripening

| | amino acid composition (mol %) | | | | |
|---|---|---|---|---|---|
| | stage 1 | stage 2 | stage 3 | stage 4 | stage 5 |
| asp | 7.9 | 7.6 | 7.5 | 7.1 | 7.3 |
| hypro | 1.6 | 3.3 | 3.5 | 5.2 | 4.1 |
| thr | 5.3 | 4.5 | 5.1 | 4.9 | 5.1 |
| ser | 6.0 | 5.9 | 5.9 | 5.6 | 5.6 |
| glu | 10.7 | 10.5 | 10.5 | 10.0 | 10.5 |
| pro | 5.2 | 6.4 | 6.3 | 7.0 | 6.2 |
| gly | 13.1 | 13.8 | 13.2 | 13.0 | 13.6 |
| ala | 9.3 | 8.5 | 8.3 | 7.5 | 8.6 |
| val | 8.3 | 8.0 | 8.0 | 7.5 | 8.3 |
| ile | 5.8 | 5.3 | 5.3 | 5.1 | 5.4 |
| leu | 8.6 | 7.9 | 7.7 | 7.5 | 7.4 |
| tyr | 1.0 | 0.8 | 1.0 | 1.4 | 0.5 |
| phe | 3.7 | 3.6 | 3.5 | 3.5 | 3.0 |
| lys | 7.4 | 8.2 | 8.4 | 8.4 | 8.8 |
| his | 2.0 | 2.4 | 2.4 | 2.7 | 2.3 |
| arg | 4.0 | 3.5 | 3.5 | 3.6 | 3.2 |
| mg/berry | 0.59 | 0.55 | 0.68 | 0.75 | 0.70 |

**Evolution of Insoluble Cell Wall Amino Acid.** The level of cell wall associated proteins increased from 4.70 to 5.76% during the first four stages of ripening, and then decreased at stage 5 (**Table 4**). The amount of proteins raised from 590 to 750 $\mu$g/berry during ripening. After the fourth point (over-ripened berries), the amount of proteins declined to 700 $\mu$g/berry. In quantity, all amino acids increased during the ripening period but the most important evolution concerned hydroxyproline, proline, lysine, tyrosine and histidine. The level of hydroxyproline increased dramatically from 9 to 41 $\mu$g/berry,

**Table 5.** Evolution of Water-Soluble Cell Wall Polysaccharides from Shiraz Skin during Ripening

| | noncellulosic composition (mol %) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| stage | Rha | Fuc | Ara | Xyl | Man | Gal | Glu | GalA | ME[a] (%) | total (mg/berry) |
| 1 | 3.4 | 0.2 | 22.1 | 11.0 | 3.0 | 18.6 | 7.4 | 34.3 | 52 | 0.13 |
| 2 | 3.1 | 0.3 | 23.2 | 7.3 | 2.6 | 23.7 | 9.7 | 30.1 | 57 | 0.16 |
| 3 | 3.0 | 0.2 | 21.6 | 5.7 | 2.3 | 21.0 | 6.5 | 39.7 | 39 | 0.20 |
| 4 | 3.3 | 0.3 | 22.7 | 4.9 | 1.8 | 20.6 | 7.3 | 39.1 | 44 | 0.27 |
| 5 | 3.4 | 0.4 | 22.0 | 4.0 | 2.4 | 18.1 | 8.9 | 40.8 | 38 | 0.35 |

[a] ME: methylesterification degree (%).

**Table 6.** Evolution of Hydroalcoholic-Soluble Cell Wall Polysaccharides from Shiraz Skin during Ripening

| | noncellulosic composition (mol %) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| stage | Rha | Fuc | Ara | Xyl | Man | Gal | Glu | GalA | total (mg/berry) |
| 2 | 2.3 | 0.4 | 23.5 | 8.7 | 4.5 | 36.1 | 15.3 | 9.1 | 0.043 |
| 3 | 2.3 | 0.2 | 27.6 | 6.9 | 3.3 | 37.0 | 15.7 | 7.0 | 0.063 |
| 4 | 2.6 | 0.3 | 20.9 | 3.5 | 2.6 | 27.2 | 11.5 | 31.4 | 0.088 |
| 5 | 2.8 | 0.3 | 26.5 | 3.6 | 2.7 | 30.1 | 15.0 | 18.9 | 0.087 |

in agreement with previous studies performed on mesocarp (*8*). Additionally, these observations were consistent with the expression of genes encoding for P/HRGP (proline and hydroxyproline-rich glycoproteins) after véraison (*32*).

**Evolution of Water-Soluble Cell Wall Polysaccharides.** The noncellulosic water-soluble polysaccharide fraction increased more than 2-fold during the ripening period, indicating a slight but continuous solubilization of cell wall components during the entire period (**Table 5**). This fraction represented a small part of the whole noncellulosic polysaccharides, from 3% at the first stage to 8% for the ripest berries. In contrast with the insoluble fraction, the water-soluble fraction essentially comprised neutral sugars (arabinose and galactose accounting for 62 to 72% of them). Uronic acids represented only 30 to 40% of the total noncellulosic sugars, but their methylesterification degree was similar to that of the insoluble fraction, with a decrease throughout ripening from 52 to 38%.

On a $\mu$g/berry basis, the most spectacular modification concerned the water-soluble galactose content, which increased from 24.8 to 64.8 $\mu$g/berry during ripening (with a more important increase between the first and the second stage), in correlation with the decrease in the insoluble fraction. Such a correlation has already been described in kiwi fruit (*33*). Water-soluble arabinose and rhamnose also increased more than 2.5-fold continuously throughout this period, but the 2-*O*-methyl fucose and the 2-*O*-methyl xylose were not present in this fraction, suggesting that RG-II was not solubilized, in accordance with a previous work performed on mature grape skins of Grenache Blanc (*11*). On a mol % basis, xylose content declined continuously from 11.0 to 4.0 mol % at the over-ripening stage. Nevertheless, on a $\mu$g/berry basis, the amount of water-soluble xylose remained constant (about 10 $\mu$g/berry) over the entire period.

**Extractability of Skin Cell Wall Material in Hydroalcoholic Solution.** To evaluate the amount of skin polysaccharides soluble in a model-wine solution, hydroalcoholic extractions were performed at the four latest stages of ripening (from 3 to 10 weeks after véraison) as described in Materials and Methods.

The total amount of polysaccharides extracted by hydroalcoholic solution represented only a small part of water-soluble cell wall described previously, but in terms of composition those fractions presented some similarities (**Table 6**). On a $\mu$g/berry basis, the amount of total sugars increased 2-fold during the ripening period, from 43.1 to 87.1 $\mu$g/berry, and remained stable during over-ripening. Uronic acids first increased from 9 to 31% and then declined to 19%. On a $\mu$g/berry basis, uronic acids represented 30.3 $\mu$g/berry at the fourth stage and then decline to 18.5 $\mu$g/berry, maybe because of the loss of small polysaccharide fragments during precipitation.

Galactose and arabinose were the most abundant hydroalcoholic-soluble neutral sugars. Arabinose increased from 8.8 to 20.0 $\mu$g/berry, galactose from 16.3 to 27.2 $\mu$g/berry and rhamnose from 0.9 to 2.3 $\mu$g/berry. Xylose presented the same decreasing pattern as in the water-soluble fraction.

**Discussion.** The amount of skin cell wall polysaccharides (4.2 mg/berry) remained constant during grape ripening and over-ripening. The insoluble polysaccharide fraction of noncellulosic cell wall polysaccharides was dominated by the presence of galacturonic acid (65%), followed by arabinose, xylose and galactose. During the first two stages of ripening, an important loss of insoluble galactose occurred in skin. In agreement with observations reported on insoluble polysaccharides from Muscat Gordo Blanco mesocarp (*8*), that loss was mainly attributed to a solubilization of arabinogalactans of type I. In addition, pectins were continuously demethylesterified throughout the period since the degree of methylesterification decreased from 51 to 36%.

On the other hand, the analysis of amino acids of the insoluble fraction showed an accumulation of cell wall proteins during this period. A remarkable increase of hydroxyproline suggests the involvement of hydroxyproline-rich glycoproteins in the cell wall during ripening. As already reported, this proteinic material might participate in the strengthening of cell wall during cell expansion (*34*) or be involved in the phenomenon of end of expansion. In grape skin, such a biosynthesis might also intervene to compensate the moderate polysaccharide solubilization.

Polysaccharide solubilization is known to occur during ripening and swelling of fruits (*3, 35, 33*), and the water-soluble polysaccharides are known to be rich in neutral sugars (*36*). In the present work, the water-soluble fraction represented a small proportion only of total cell wall polysaccharides (3 to 8%). However, this fraction increased more than 2-fold during the ripening period, thus highlighting a moderate cell wall solubilization of the skin tissue. Even though a 2-fold increase was also reported in the mesocarp of Muscat Gordo Blanco grape berries on the same period, the soluble fraction of polysaccharides extracted from that tissue represented between 10 and 23% of the entire cell wall material (*8*), which corresponded to an

increase of 450 μg/berry, compared with a 220 μg/berry increase only for the skin tissue in our study. At an equivalent stage of ripening, the amount of soluble polysaccharides present in Shiraz skin was thus about 270 μg/berry, compared with 920 μg/berry in the mesocarp of Muscat Gordo Blanco (*8*). Similarly, Silacci et al. (*37*) reported an important increase in water-soluble polysaccharides from the mesocarp of Cabernet Sauvignon, with a water-soluble fraction varying from 100 to 700 μg/berry on the same period. These discrepancies between pulp and skin can be due to a pulp to skin weight ratio of about 1:5 for grape, but also to a smaller proportion of water-soluble polysaccharides in skin, as that has been already reported in Grenache Blanc berries harvested at maturity (*11*). In Grenache Blanc, the water-soluble fraction was shown to account for 30 and 13% of the pulp and skin tissues, respectively.

In terms of composition, the water-soluble fraction was characterized by a majority of neutral sugars (mainly arabinose and galactose), at the opposite of the insoluble fraction. Uronic acids, containing galacturonic and glucuronic acids, represented 35% of the water-soluble fraction, with a percentage of methylesterification similar to that observed in the insoluble fraction. In Grenache Blanc grape skin, Vidal et al. reported that uronic acids represented 20% only of the water-soluble fraction (*11*). As described by Robertson et al. (*38*), the composition of water-soluble polysaccharides in grapes can be dependent on the cultivar.

The experiments performed in hydroalcoholic solutions with noncrushed skins led to the same conclusions. The hydroalcoholic-soluble fraction moderately increased during ripening and over-ripening and had a composition quite similar to the water-soluble fraction isolated from crushed skins. Nevertheless it represented a very small part of noncellulosic skin cell wall material (from 1 to 2% only).

In conclusion, continuous but moderate changes were observed in skin cell wall during ripening. These modifications can actually be considered as restrained compared to what is generally described in other fruits or other tissues in grape, particularly the small proportion of water-soluble polysaccharides.

Taken together, these results suggest that skin is a protective tissue, resistant toward solubilization, and whose function is to maintain the whole berry integrity. From a winemaking point of view, these results also make the relationships between skin cell wall degradation and extraction of compounds of interest questionable and suggest that the major part of the polysaccharides present in wines should derive from flesh tissue.

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**Received for review October 31, 2008. Revised manuscript received February 5, 2009. Accepted February 8, 2009.**

JF803416W