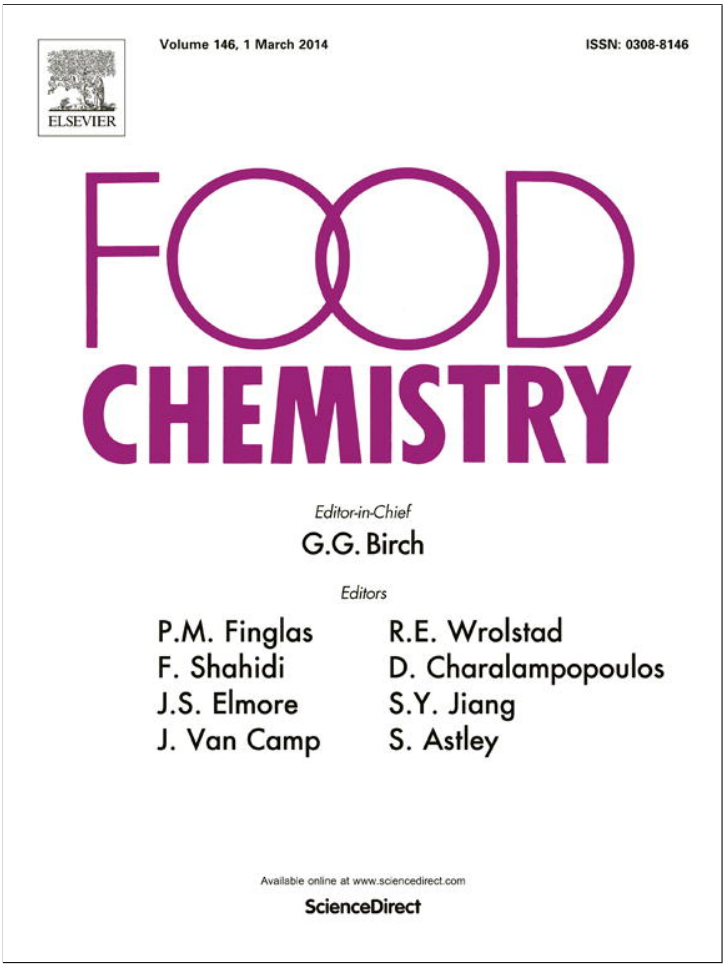

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Relationship between skin cell wall composition and anthocyanin extractability of *Vitis vinifera* L. cv. Tempranillo at different grape ripeness degree

José Miguel Hernández-Hierro [a,1], Natalia Quijada-Morín [a], Leticia Martínez-Lapuente [b], Zenaida Guadalupe [b], Belén Ayestarán [b], Julián C. Rivas-Gonzalo [a], M. Teresa Escribano-Bailón [a,*]

[a] Grupo de Investigación en Polifenoles, Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, E-37007 Salamanca, Spain
[b] Instituto de Ciencias de la Vid y del Vino, Universidad de La Rioja, 26006 Logroño, Spain

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ABSTRACT

The relationship between cell wall composition and extractability of anthocyanins from red grape skins was assessed in Tempranillo grape samples harvested at three stages of ripening (pre-harvest, harvest and over-ripening) and three different contents of soluble solids (22, 24 and 26 °Brix) within each stage. Cell wall material was isolated and analysed in order to determine cellulose, lignin, non-cellulosic polysaccharides, protein, total polyphenols index and the degree of esterification of pectins. Results showed the influence of ripeness degree and contents of soluble solids on cell wall composition. Furthermore, principal components analysis was applied to the obtained data set in order to establish relationships between cell wall composition and extractability of anthocyanins. Total insoluble material exhibits the biggest opposition to anthocyanin extraction, while the highest amounts of cellulose, rhamnogalacturonans-II and polyphenols were positively correlated with anthocyanin extraction. Moreover, multiple linear regression was performed to assess the influence of the cell wall composition on the extraction of anthocyanin compounds. A model connecting cell wall composition and anthocyanin extractabilities was built, explaining 96.2% of the observed variability.

1. Introduction

Phenols are a large and complex group of secondary metabolites naturally present in grapes which contribute directly or indirectly to the characteristics and quality of red wine (Sun, Pinto, Leandro, Ricardo-Da-Silva, & Spranger, 1999). Among them, anthocyanins are responsible for the colour of red wines and their interactions with other phenolic compounds are of crucial importance to determine the colour changes observed during ageing (Boulton, 2001). Anthocyanins, which are located inside the vacuoles of grape skins cells (with the exception of tenturier *Vitis* varieties, which also have anthocyanins inside the grape pulp cells) are released from grape skins during the maceration/fermentation processes (Canals, Llaudy, Valls, Canals, & Zamora, 2005; Fournand et al., 2006). Cell wall provides mechanical strength, maintains cell shape and plays an important role in intercellular communication and transport and in cell protection against pathogens. It is therefore important to characterise the cell wall composition during ripening in order to better understand the relationship between its composition and release of anthocyanins. Regarding cell wall composition of grape skins, it is commonly accepted that its degradation by pectolytic enzymes increases the extractability of anthocyanins from grape skins during ripening. Moreover, differences on contents of polysaccharides based on galactose and arabinose, together with changes on the cellulose content and degree of methylation of pectins might also be responsible for the different extractabilities of the aforementioned phenolic compounds (Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2006). Physical properties of grape skins like berry skin hardness, berry skin thickness, number of cell layers, cell wall thickness; which are linked to the anthocyanin extractability, also depend on cell wall composition (Río-Segade, Giacosa, Gerbi, & Rolle, 2011; Torchio, Cagnasso, Gerbi, & Rolle, 2010).

Grape variety has also shown a great influence on extractability of anthocyanins among Galician cultivars (Sousón, Brancellao, Ferrol, Merenzao, Mouratón and Mencía) (Río-Segade, Soto Vázquez, & Díaz Losada, 2008). This varietal influence on anthocyanins extraction has been previously described for Cabernet Sauvignon, Merlot, Shiraz and Monastrell cultivars (Romero-Cascales, Ortega-Regules, Lopez-Roca, Fernandez-Fernandez, & Gomez-Plaza, 2005). At each ripening stage grapes present heterogeneity which influence their

* Corresponding author. Tel.: +34 923 294 537; fax: +34 923 294 515.
E-mail address: escriban@usal.es (M.T. Escribano-Bailón).
[1] Current address: Food Colour & Quality Laboratory, Department of Nutrition & Food Science, Universidad de Sevilla, Facultad de Farmacia, 41012 Sevilla, Spain.

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composition and quality and therefore it should be also considered (Kontoudakis et al., 2011).

Our previous work has revealed the influence of ripeness degree and soluble solids contents on the amounts of anthocyanins. Furthermore, it has been also demonstrated that differences in the anthocyanin extractabilities were highly influenced by the ripeness degree and also, but in a lesser extent, by the soluble solids contents (Hernández-Hierro, Quijada-Morín, Rivas-Gonzalo, & Escribano-Bailón, 2012). The same extractability pattern has been previously observed using different grape cultivars (Romero-Cascales et al., 2005; Río-Segade et al., 2008). Nevertheless, anthocyanin extractability has also been studied throughout ripening using *Vitis vinifera* L. cv. Shiraz and it remained constant whatever the physiological stage of berries (Fournand et al., 2006).

The aim of this study was to determine the relationship between cell wall composition and extractability of anthocyanins from red grape skins during ripening. For this purpose three different ripeness stages (pre-harvest, harvest and over-ripening) and three soluble solids contents (22, 24 and 26 °Brix) within each stage were taken into account using *Vitis vinifera* L. cv. Tempranillo which is the most often used variety to produce quality red wines in Spain.

2. Material and methods

2.1. Grape samples

Vitis vinifera L. cv. Tempranillo red grape samples were collected from a vineyard located in Lleida (Spain) which is under the influence of Mediterranean climate. The samples were collected at three different ripeness degrees: pre-harvest (22 days before harvest), harvest and over-ripening (20 days after harvest). In the studied samples notations **P**, **H** and **O** were used to indicate pre-harvest, harvest and over-ripening respectively. Thirty clusters were collected at each stage and their grapes were sorted according to their density. The density of grapes was estimated by flotation of berries in different NaCl solutions (130–150, 150–170, and 170–190 g L^{-1}) corresponding to different solids contents (22, 24 and 26 °Brix, respectively). As a result, grapes of different soluble solids content (i.e 22, 24 and 26 °Brix) were obtained within each sampling date (i.e P, H and O), providing the nine grape samples used in this study. Grape skins were separated manually from the whole grapes and three subsamples were taken from each sample, one for the exhaustive methanolic extraction another one for the hydroalcoholic extraction, and the last one for cell wall isolation and the subsequent characterisation analysis.

2.2. Anthocyanin extractabilities

Anthocyanin extractabilities previously obtained as described elsewhere in Hernández-Hierro et al. were used. Briefly, 10 g of grape skins were macerated at 4 °C in acid methanol until colourless of skins and another aliquot of 10 g of grape skins were macerated in model wine solution (4 g L^{-1} tartaric acid, 12.5% ethanol, adjusted at pH 3.6 with NaOH 0.5 M) for different periods of time. Anthocyanin extractability was calculated as percentage using the ratio between hydroalcoholic and exhaustive methanolic extractions both determined by Rapid Resolution Liquid Chromatography (RRLC).

The RRLC analysis conditions were carried out in accordance with Hernández-Hierro et al. Anthocyanins were identified according to their spectroscopic and chromatographic features which had been previously acquired in our laboratory and the quantification was carried from the peak areas (Hernández-Hierro et al., 2012).

2.3. Isolation of cell wall material

Cell wall material was isolated from grape skins as the 70% ethanol residue following Apolinar-Valiente et al. (Apolinar-Valiente, Romero-Cascales, López-Roca, Gómez-Plaza, & Ros-García, 2010). Briefly, 10 g of grape skins were suspended in 15 mL of boiling water for 5 min and after that were homogenized using a Polytron (Glen Mills Inc., Clifton, NJ, USA) during 2 min. Forty milliliters of 96% ethanol were added and the mixture was placed into an ultrasound bath for 15 min at ambient temperature and then into a bath for 30 min at 40 °C. The raw alcohol insoluble solids were separated by decantation and extracted using 80 mL of 70% ethanol. The abovementioned mixture was placed into an ultrasound bath for 15 min at ambient temperature and then into a bath for 30 min at 40 °C. An aliquot from the liquid phase was taken for soluble sugar analysis (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The washing procedure with fresh 70% ethanol was repeated several times until the test indicated no sugars in the 70% ethanol phase. Then, the alcohol insoluble cell wall material was washed twice with 80 mL of 96% ethanol and once with 80 mL acetone, and finally dried overnight under an air stream at 20 °C.

The isolated insoluble cell wall material was ground to obtain a homogeneous powder, weighed and used in the subsequent analysis in order to ascertain its composition in each case.

2.3.1. Non-cellulosic polysaccharides

Non-cellulosic polysaccharides (i.e. arabinogalactans, mannans, homogalacturonans, xyloglucans, arabinans, rhamnogalacturonans-I and rhamnogalacturonans-II) were obtained using an iterative calculation methodology (Arnous & Meyer, 2009) from monosaccharide profiles. Monosaccharides were determined by gas chromatography–mass spectrometry (GC–MS) of their trimethylsilyl-ester *O*-methyl glycolsyl-derivades (TMS) obtained after acidic methanolyisis and derivatisation following a modification of Guadalupe et al. procedure (Guadalupe, Martínez-Pinilla, Garrido, Carrillo, & Ayestarán, 2012). Five milligrams of cell wall material were treated with 1 mL of the methanolysis reagent (MeOH containing HCl 0.5 M), which was obtained adding acetyl choride (140 μL) to 4 mL of dry methanol, in order to hydrolyse neutral and acidic monosaccharides to their corresponding methyl glycosides. The reaction was conducted under nitrogen atmosphere at 80 °C for 16 h and thereafter the excess of reagent was removed using a stream of nitrogen gas. The conversion of the methyl glycosides to their trimethylsilyl (TMS) derivatives was performed adding 1 mL of pyridine: hexamethyldisilazane: trimethylchlorosilane (10:2:1 v/v) to the dried material. The reaction was carried out at 80 °C for 30 min and after that the reagent was removed using a stream of nitrogen gas. TMS derivatives were extracted with 0.5 mL of hexane and then centrifuged. An aliquot of 0.2 mL of the supernatant was taken and a solution (12.5 μL) of derivatized myo-inositol was then added as internal standard for the GC–MS determination. GC–MS was performed with 5 μL of this solution.

The GC–MS analysis conditions were carried out in accordance with Guadalupe et al. (2012). Different standard carbohydrates were also converted to their corresponding TMS derivatives and analysed by GC–MS in order to obtain patterns for identification taking into account their MS fragmentation patterns (Doco, O'Neill, & Pellerin, 2001) and to obtain the standard calibration curves (Guadalupe et al., 2012).

2.3.2. Esterification of pectins

Cell wall material was studied using FT-IR spectroscopy in order to observe the presence of esterified groups on its composition which might indicate the esterification of pectins (Femenia, Sánchez, Simal, & Rosselló, 1998). A Perkin-Elmer FT-1730

(Perkin-Elmer Inc, Waltham, Massachusetts, USA) was used, KBr disk were prepared using 2 mg of cell wall material and spectral measurements were made over the 2000–900 cm^{-1} wavenumber range. Spectra were recorded at intervals of 2 cm^{-1} with 12 scans being performed for each sample.

The degree of esterification of pectins (i.e. the percentage of total uronic acids that are esterified) was also quantitatively determined. Two aliquots (2 mg each one) of cell wall material were taken, one for its reduction and the other one without any reduction as reference. Esterified pectins, but not de-esterified pectins, were reduced to galactose using 1 mL of sodium borohydride (10 mg mL^{-1}) in 50% ethanol overnight (Femenia et al., 1998; Lurie, Levin, Greve, & Labavitch, 1994). Then uronic acid contents were determined in both aliquots following a spectrophotometric method as described by Blumenkrantz and Asboe-Hansen (1973), but using *o*-hydroxydiphenyl solution (Segarra, Lao, López-Tamames, & De La Torre-Boronat, 1995) and recording the absorbance at 520 nm after 5 min of reaction. The difference in uronic acids content between reduced and non reduced aliquots was recorded as the percentage of esterification of pectins.

2.3.3. Cellulose and lignin

Cellulose was determined as glucose in accordance with Lurie et al. (1994) using the phenol method proposed by DuBois et al. for its spectrophotometric determination (DuBois et al., 1956). Briefly, 2 mg of cell wall material were first extracted with 400 µl of 2 M trifluoroacetic acid (120 °C, 2 h) and then the insoluble material was dissolved in 1 mL of 12 M H_2SO_4 and examined for glucose using the phenol method (DuBois et al., 1956).

Lignin was gravimetrically determined as Klason lignin. Ten milligrams of cell wall material were hydrolysed in 12 M H_2SO_4 at room temperature for 3 h and then in 1 M H_2SO_4 at 100 °C for 2.5 h following the Saeman procedure (Saeman, Moore, Mitchell, & Millett, 1954). After that process, insoluble material was recovered by filtration (sinter 2) and washed with hot water until absence of acid. Finally, the residue was drying at 105 °C overnight and weighed as Klason lignin (Femenia et al., 1998).

2.3.4. Protein and phenolic compounds

Proteins and total phenolic compounds of cell wall were determined by Bradford (1976) and Folin–Ciocalteu (Singleton & Rossi, 1965) methods, respectively. Ten micrograms of the isolated cell wall material were dissolved with 1 mL of 1 M NaOH (100 °C, 10 min) and centrifuged (1300 rpm, 5 min) to remove the insoluble material prior to the analysis. Two aliquots of 10 µL were taken from each sample, one protein analysis and the other one for phenolic compounds analysis. Protein and total phenolic were expressed as bovine serum albumin (BSA) and gallic acid (GA) (Sigma–Aldrich, St. Louis, MO, USA) equivalents, which were respectively used as standards for the above mentioned analysis (Ortega-Regules et al., 2006).

2.3.5. Chemometric analysis

Unsupervised methods are applied to observe patterns in the data indicating relationships between samples and/or between variables (Brereton, 2003). The unsupervised pattern recognition method used for data analysis was principal components analysis (PCA), which was applied to the correlation matrix of the original variables.

Backward stepwise multiple linear regression (MLR) was performed in order to develop a model for anthocyanin extraction using cell wall composition.

The SPSS 13.0 for Windows software package (SPSS, Inc., Chicago, IL) was used for data processing.

3. Results and discussion

3.1. Anthocyanin extractabilities

Table 1shows the anthocyanin extractabilities that have been previously obtained as described elsewhere in Hernández-Hierro et al. (2012). Our previous work has demonstrated that the maximum percentages of anthocyanin extraction were reached at the third day of hydrolacoholic maceration of grape skins and these values were used for the subsequent data analysis in this study. This pattern has also been previously observed by Canals et al. (2005). It is also noteworthy that anthocyanin extractabilities were highly influenced by the ripeness degree and in a lesser extent by the soluble solids contents. Generally, non-acylated anthocyanins were better extracted than the acylated anthocyanins (Hernández-Hierro et al., 2012) which is consistent with previous studies (Fournand et al., 2006).

3.2. Cell wall material composition

The percentage of cell wall material relative to the grape skin weight, obtained in samples of 22, 24 and 26 °Brix at preharvest, harvest and over-ripening stages are presented in Fig. 1a. In Fig. 1b, mean values at preharvest, harvest and over-ripening are presented. An important decrease in the insoluble material was observed during ripening. The percentage of cell wall material decreased from 4.2% at pre-harvest to 3.3% and 2.5%, harvest and over-ripening, respectively (Fig. 1b). This behaviour is associated with berry softening and skin loosening (Huang, Huang, & Wang, 2005) and it is in agreement with those previously reported for other cultivars (Ortega-Regules, Ros-Garcia, Bautista-Ortin, Lopez-Roca, & Gomez-Plaza, 2008). Nonetheless, it has also been reported that during ripening cell wall material slightly but continuously increased before showing a small decrease for Shiraz (Vicens et al., 2009).

Arabinose, rhamnose, fucose, xylose, mannose, glucose, galactose, galacturonic acid, glucuronic acid, apiose, aceric acid, 2-*O*-methylfucose, 2-*O*-methylxylose, and Kdo (3-deoxy-D-manno-octulosonic acid) were determined by GC–MS analyses. The percentages of the different monosaccharides remained almost constant in the studied samples (Fig. 2a). The biggest percentual variations were observed in galactose and glucose, and in both cases the differences were lower than 7% between the sample with highest and lowest percentages of each compound. It is noteworthy that the sample with the highest percentage of galactose (P22) showed the lowest percentage of glucose, and the sample with the highest glucose percentage (H26) also presented the lowest galactose percentage. This behaviour, previously observed by Ortega-Regules et al, suggests that there may exist a relationship between these compounds proportions (Ortega-Regules et al., 2008). Cell wall enzymes, which are mainly hydrolases, are involved in the hydrolysis of cell wall compounds and act in concert. It may be speculated that those related to the degradation of compounds that contain hemicellulosic glucose and galactose act

Table 1
Percentage of extraction of non-acylated and acylated anthocyanins on the third day of hydroalcoholic maceration.

		Pre-harvest	Harvest	Over-ripening
22 °Brix	Non acylated	58.9	55.1	66.3
	Acylated	54.5	52.4	52.9
24° Brix	Non acylated	51.5	81.7	54.9
	Acylated	42.2	58.9	61.4
26 °Brix	Non acylated	42.8	54.3	77.2
	Acylated	30.7	51.1	68.0

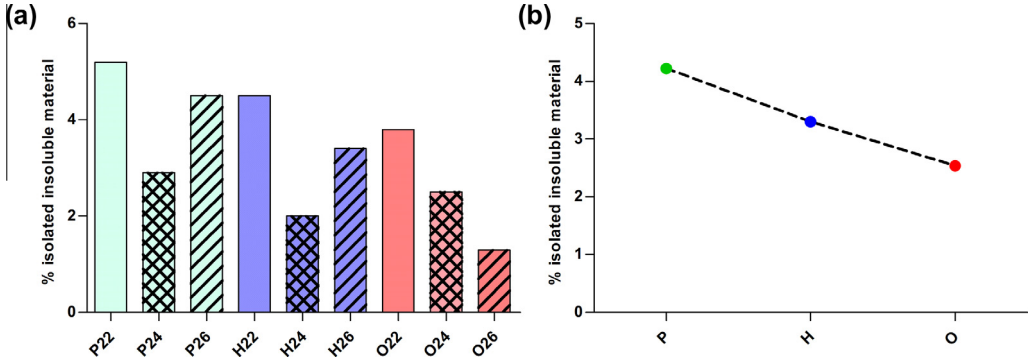

Fig. 1. Percentages of cell wall material, isolated as the 70% ethanol residue, from the skin of grapes cv. Tempranillo during ripening. (1a) P22: samples of 22° Brix collected at preharvest, P24: samples of 24° Brix collected at preharvest, P26: samples of 26° Brix collected at preharvest. H22: samples of 22° Brix collected at harvest, H24: samples of 24° Brix collected at harvest, H26: samples of 26° Brix collected at harvest. O22: samples of 22° Brix collected at over-ripening, O24: samples of 24° Brix collected at over-ripening, O26: samples of 26° Brix collected at over-ripening. (1b) Mean values from the samples collected at preharvest (P), harvest (H) and over-ripening (O).

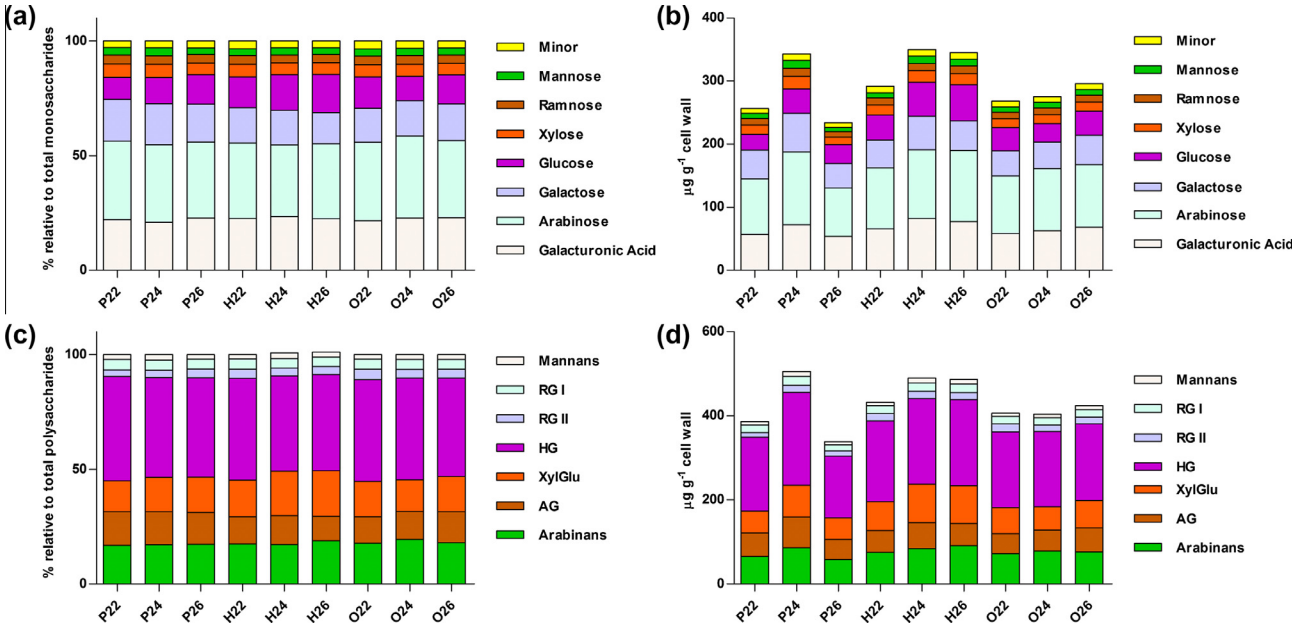

Fig. 2. Percentages (a) and contents (b) of the different monosaccharides during ripening. Minoritary monosaccharides contents match to the sum of the amounts of glucuronic acid, apiose, aceric acid, 2-*O*-methylfucose, 2-*O*-methylxylose, and Kdo. Percentages (c) and contents (d) of the different polysaccharides during ripening. Man (mannan); RGI (rhamnogalacturonans-I); RGII (rhamnogalacturonans-II); HG (homogalacturonans); XylGlu (xyloglucans); AG (arabinogalactans); Arab (arabinans).

on a common metabolic pathway that inversely regulates each activity.

During ripening, the amounts of almost all the quantified monosaccharides showed a slight increase and then a decrease in over-ripening samples (Fig. 2b). Aceric acid and 2-*O*-methylfucose presented a weak increase during ripening, while galactose showed a decrease. Galactose behaviour has been previously described in Shiraz grapes (Vicens et al., 2009), in Cabernet Sauvignon, Merlot, Syrah, and Monastrel grapes (Ortega-Regules et al., 2008), and it is related to the degradation of arabinogalactans and the initiation of fruit softening. All monosaccharides contents presented an increase followed by a decrease in pre-harvest samples as the content in soluble solids is raised, while harvest and over-ripening samples did not show a homogeneous pattern when the soluble solid content is increased.

Arabinogalactans, mannans, homogalacturonans, xyloglucans, arabinans, rhamnogalacturonans-I and rhamnogalacturonans-II were obtained using the iterative calculation (Arnous & Meyer, 2009) from monosaccharide profiles. The percentages of the different polysaccharides were nearly constant in all samples which indicate conservation of structures (Fig. 2c). It is noteworthy that homogalacturonans were the dominant polysaccharides from grape skin cell wall material as previously reported. Xyloglucans were the predominant hemicelluloses (Arnous & Meyer, 2009; Vidal, Williams, O'Neill, & Pellerin, 2001). An increase in their proportions is detected in pre-harvest and harvest samples as the soluble solids content is increased. Harvest samples showed the maximum levels, and a decrease is observed in over-ripening samples, whatever the soluble solids content. Regarding polysaccharides contents evolution during ripening in cell wall material, an increase followed by a decrease is observed for all the polysaccharides families except for arabinogalactans, which reached their maximum value in pre-harvest samples and then showed a continuous decrease (Fig. 2d). As for the medium values for each soluble solids content it is noteworthy that the amount of all polysaccharides presented an increase ensued by a decline as the soluble solids content is arose. The same behaviour could be observed for pre-harvest samples while for harvest and over-ripening samples no clear pattern was found.

Homogalacturonans and rhamnogalacturonans I presented highly correlated patterns in all samples, both in amount and proportion, being homogalacturonans levels around tenfold rhamnogalacturonan I levels. It has been proposed that homogalacturonan could be a side chain of rhamnogalacturonan I (Vincken et al., 2003) and the existence of linkages between them has been proved (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007).

The presence or absence of esterification was assessed by the evaluation of the FT-IR spectra. Carboxylic acid present a characteristic C–O stretching near 1430 cm^{-1} and esters C=O bonds produce stretching bands that are observed near 1700 cm^{-1} (Femenia et al., 1998). FT-IR spectra of cell wall material showed a carboxylic acid stretch at 1446 cm^{-1}. An ester band at 1740 cm^{-1}, which indicated the occurrence of esterified pectins, was also evident in all samples (Fig. 3a).

Once the esterification of pectins had been qualitatively assessed, the degree of esterification of pectins was also quantitatively determined. The samples could be classified as low and high esterification pectins since they presented degrees of esterification lower and higher than 50%, respectively (Guillotin, Van Loey, Boulenguer, Schols, & Voragen, 2007). These values ranged from 8.4% to 85.3% but there was not a clear pattern for the degree of esterification of pectins during ripening (Fig. 3b). It may be speculated that heterogeneity of esterases contents, not only during ripening but also at the same maturation stage, may cause variations in the esterification degrees. Nonetheless, further work should be made in order to confirm this tentative hypothesis. On the other hand, a decrease of the degree of esterification has been previously reported for other cultivars (Monastrell and Cabernet Sauvignon). It is also noteworthy that no clear variation in the degree of esterification has been also described for Shiraz (Ortega-Regules et al., 2008). These changes in pectin methylation levels seem to be cultivar specific since no common patterns could be found across different varieties.

Fig. 4 shows the evolution of lignin, cellulose, protein and polyphenols in cell wall material during ripening. Lignin presented a slight increase and then an important decrease during ripening but there was not a clear trend (Fig. 4a). These values ranged from 84 to 505 μg mg^{-1} cell wall and constituted on average nearly 30% by weight of cell wall material. These relatively high amounts of lignin are in agreement with those previously described which classified skin cell wall material as lignin-rich biomass material (Arnous & Meyer, 2009). The amounts of cellulose were lower than those relatively high concentrations previously reported (Ortega-Regules et al., 2008) although there was not a clear pattern during ripening a slight increase could be observed from pre-harvest to harvest, and then the values remain nearly constant in over-ripening samples (Fig. 4a). A scarce increase was observed in the case of protein content during ripening (Fig. 4b) whereas polyphenols content presented an increase and then a slight decrease (Fig. 4b). These patterns during ripening have been also reported for other cultivars (Ortega-Regules et al., 2008). It has been proposed that the increase in protein content compensates the partial loss of wall structural polysaccharides as ripening draws on and contributes to maintain cell wall integrity in the skins of ripening grape berries. (Huang et al., 2005). As previously observed (Vicens et al., 2009), changes in grape skins cell wall composition during ripening are continuous but moderate, and occur in lesser extent than in other fruits or tissues.

3.3. Relationship between skin cell wall composition and anthocyanin extractability

PCA was applied to the correlation matrix constructed from the extraction percentages of acylated and non-acylated anthocyanins, ripeness degree, soluble solids content and cell wall composition (i.e insoluble cell wall material, protein, polyphenols, lignin, cellulose, arabinogalactans, mannan homogalacturonans, xyloglucans, arabinans, rhamnogalacturonans-I, rhamnogalacturonans-II and the degree of esterification of pectins) obtained from each sample. Fig. 5 shows the loading plot of the PCA analysis. The first principal component (PC 1) describes 43.23% of the variability in the data and the second (PC 2) describes 24.44%. Total insoluble material exhibits the biggest opposition to both anthocyanin extractions, slightly higher for non-acylated anthocyanin extraction, while the highest amounts of cellulose, rhamnogalacturonans-II (RG-II) and polyphenols are positively correlated with anthocyanin extraction. Polyphenols, cellulose and RG – II are within the total insoluble cell wall material, which presented the biggest opposition. It is inferred that qualitative composition within cell wall could pave the way for the anthocyanin extraction. Although the amount of cell wall is a crucial factor, its molecular structure also plays an important role. The ripeness degree is also positively correlated with higher extraction levels as stated previously (Hernández-Hierro et al., 2012). This analysis also reveals the existence of more slightly oppositions to anthocyanin extraction for the rest of the studied parameters (i.e. the remaining polysaccharides, protein, lignin, soluble solids content and degree of esterification of pectins). It is also noteworthy that almost all polysaccharides present the same trend in the PCA loading plot with the exception of RG II, which is the most branched polysaccharide. HG and RG I presented the exact same trend in this analysis, which supports the existence of a strong relationship between these two polysaccharide types.

The positive relationship between polyphenols content in cell wall and anthocyanin extraction may suggest the existence of copigmentation processes between polyphenols present in cell wall material and anthocyanins while the extraction is taking place, so they could be able to shift the balance of the extraction process, assisting it (Boulton, 2001).

MLR was performed to assess the influence of the cell wall composition on the extraction of anthocyanin compounds. Total anthocyanin extraction percentage was selected as dependent variable, whereas all the quantified monosaccharides amounts, protein, polyphenols, cellulose, lignin, pectins esterification level and total

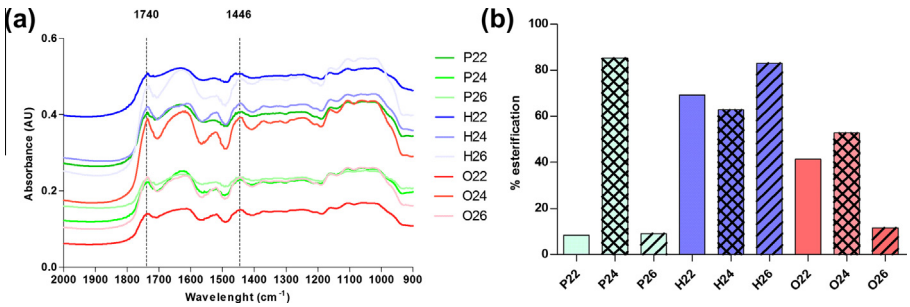

Fig. 3. Qualitative (FT-IR) (a) and quantitative (b) determination of the degree of esterification of pectins in cell wall material during ripening.

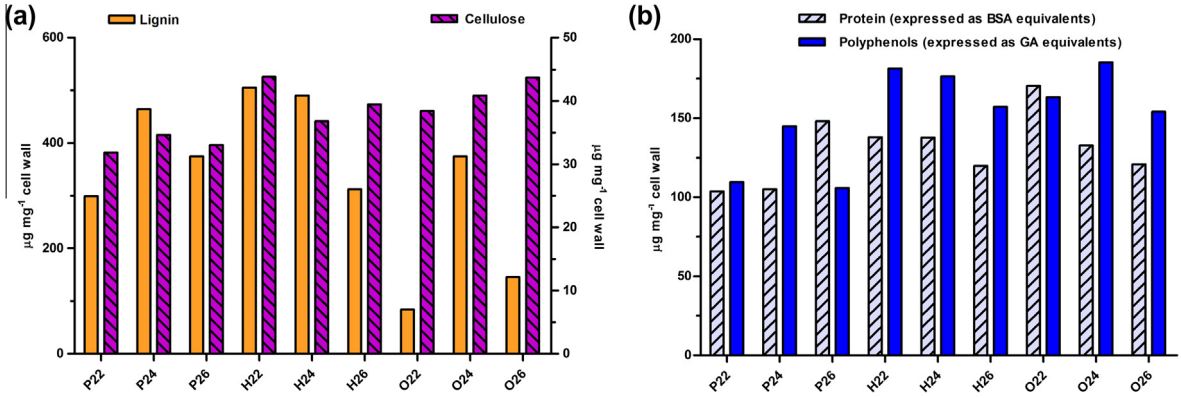

Fig. 4. Contents of lignin, cellulose (a), protein and polyphenols (b) in cell wall material during ripening.

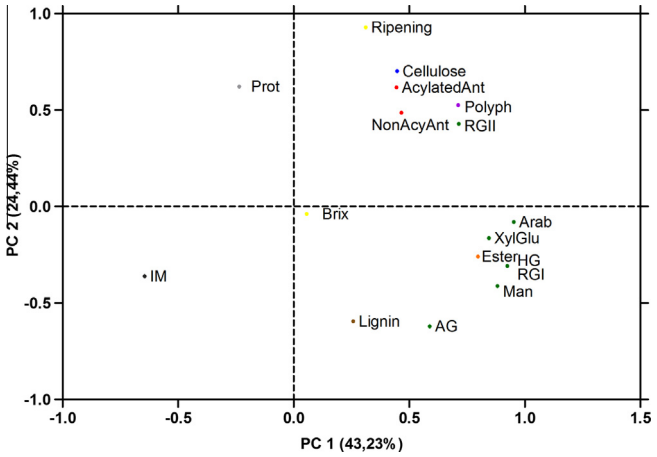

Fig. 5. Loading plot of the principal components analysis. IM (insoluble material); Prot (protein); Polyph (polyphenols); Lignin; Cellulose; AG (arabinogalactans); Man (mannan); HG (homogalacturonans); XylGlu (xyloglucans); Arab (arabinans); RGI (rhamnogalacturonans-I); RGII (rhamnogalacturonans-II); ester (degree of esterification of pectins); AcylatedAnt (acylated anthocyanins); NonAcyAnt (non-acylated anthocyanins), Brix (soluble solids content) and Ripening (ripeness degree).

insoluble material were used as independent variables. MLR was conducted applying a backward-stepwise strategy, which involves starting with all considered variables and removing the least significant one at each step of the process. The model is refitted after each step including only the most significant variables. Parameters such as R^2 (multiple correlation coefficient), B (non-standardized regression coefficients) and β (standardized regression coefficients) were obtained.

Among all the variables considered, eight of them were considered statistically significant ($p < 0.05$) in the fitted final model which presents the following adjusted equation:

$$\begin{aligned}\text{Total anthocyanin extraction} = {} & 73.1867 \\ & + 1.47262^{*}[\text{Galacturonic acid}] \\ & - 1.23289^{*}[\text{Glucose}] \\ & - 17.1139^{*}[\text{Rhamnose}] \\ & + 7.43013^{*}[\text{Mannose}] \\ & + 41.8535^{*}[\text{Apiose}] \\ & - 96.604^{*}[2-O-\text{methylxylose}] \\ & + 36.5243^{*}[\text{Glucuronic acid}] \\ & - 0.0684884[\text{Lignin}]\end{aligned}$$

The coefficient of determination ($R^2 = 0.962$) indicated that the proposed model explains the 96.2% of the variability of the anthocyanins total extraction, which supposed a good fit to the data.

The β parameter is a better estimation about the contribution of each variable to the model than B parameter. Rhamnose ($\beta = -1.590$), glucose ($\beta = -0.993$), lignin ($\beta = -0.7310$ and apiose ($\beta = -0.582$) showed a negative relationship between their amounts in cell wall material and anthocyanin extractabilities, while glucuronic acid ($\beta = 1.081$), galacturonic acid ($\beta = 1.076$), mannose ($\beta = 0.944$) and 2-*O*-methylxylose ($\beta = 0.598$) exhibited a positive relationship. Rhamnose displayed the highest contribution to the model, followed by glucuronic and galacturonic acids. Glucose and mannose showed similar scale contributions, but with opposite trends. Lignin showed a slightly smaller negative contribution whereas apiose and 2-*O*-methylxylose presented the lowest contributions to the model.

Previously, Ortega-Regules et al. (2006) proposed an equation that correlated the extractability index and cell wall composition. It is noteworthy that several variables that they found statistically significant were also significant in the aforesaid equation, and the shared independent variables present the same trend in both cases. In our case, high levels of galacturonic acid, mannose, apiose and glucuronic acid would enhance the extraction of anthocyanins, while glucose, rhamnose, 2-*O*-methylxylose and lignin would prevent anthocyanin extraction from skins.

Grape skin is a protective tissue whose function is to maintain the whole berry integrity and acts as a protection against external factors. It has been previously reported that thickness or density of the aforementioned skin cell wall might influence anthocyanins extraction since it is a barrier for the compounds located inside the cells. The number of cell layers in grape skin and their morphology might also influence the amounts of cell wall material and consequently anthocyanin extraction (Ortega-Regules et al., 2006; Ortega-Regules et al., 2008).

4. Conclusions

Results showed the influence of ripeness degree and contents of soluble solids on cell wall composition. Generally, the most important factor was the ripeness degree. Most of the studied compounds showed an increase followed by a decrease as ripening progressed, (i.e. almost all polysaccharides, lignin, cellulose, polyphenols), with the exception of arabinogalactans, that reached their maximum level in pre-harvest samples and then showed a continuous decrease, and protein content, that tend to increase during ripening. Furthermore, total insoluble material exhibits the biggest opposition to anthocyanin extraction, while the highest amounts of cellulose, rhamnogalacturonans-II and polyphenols

within the cell wall composition were positively correlated with anthocyanin extraction.

A model connecting cell wall composition and anthocyanin extractabilities was built, explaining 96.2% of the observed variability. Nonetheless, a comprehensive study should be made in order to evaluate factors, such as different grape varieties, vintages and production areas.

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