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Extractability of Low Molecular Mass Flavanols and Flavonols from Red Grape Skins. Relationship to Cell Wall Composition at Different Ripeness Stages

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ABSTRACT: Flavonol and flavan-3-ol extractabilities from red grape skins were evaluated in Tempranillo grapes harvested at different ripeness stages and with different soluble solid contents within each stage. Flavan-3-ol extractability is related to ripeness stage and also to cell wall composition, mainly to arabinogalactans (AG), mannans, rhamnogalacturonans-I (RG-I), homogalacturonans (HG), xyloglucans (XG), and total polysaccharides content, which are negatively correlated to flavan-3-ol extractability, whereas soluble solid content did not exert any influence on their extraction. Moreover, procyanidin extraction is more strongly related to cell wall composition than prodelphinidin extraction. Flavonol extractability was not influenced by insoluble material contents; although some cell wall components presented a relationship with flavonol extractability, the presence of AG and mannans would decrease total flavonol extractability, whereas protein is positively related to total and major flavonol compounds (i.e., quercetin and myricetin derivatives). The different behaviors observed between those two groups of polyphenol compounds could be due to different tissue and cellular location.

KEYWORDS: *grape skins, flavan-3-ol extractability, flavonol extractability, cell wall*

INTRODUCTION

Polyphenolic compounds in grapes are mainly located in skins and seeds and are released into wines during winemaking. Among polyphenolic compounds present in grape skins are found anthocyanins, flavonols, and flavan-3-ols. With regard to flavan-3-ols or proanthocyanidins, in grape skins both procyanidins and prodelphinidins can be found. All of these polyphenolic compounds are responsible for many of the organoleptic properties of wine, such as color, astringency, or bitterness. Grape phenolic composition and winemaking techniques largely determine the amounts of polyphenolic compounds released into must-wines and, thus, their organoleptic characteristics.

Polyphenolic compound contents in grape skins are known to change during ripening, and it is important to take it into account to choose the optimum harvest moment. Previous studies had reported a decrease in proanthocyanidin contents as ripening progressed[1,2] and an increase in flavonols in the first stages of berry development,[3] mainly related to sun exposure,[4] whereas their contents remain nearly constant in ripe and overripe grapes.[5] It is generally accepted that extractability of phenolic compounds from grape skins increases throughout grape ripening as a consequence of the degradation of the cellular wall by pectolytic enzymes.

Cell walls act as a barrier for the diffusion of the polyphenolic compounds from grapes into must-wine. Changes in grape skin cell walls that occur during ripening are related to the ability of phenolic compounds to be released. These changes involve mainly differences in the methylation of pectins, loosening of the xyloglucan−cellulose network, and an increase in pectin solubility.

Extractability of phenolic compounds from grape skins and also from grape seeds is an important issue that should be taken into account for winemaking processes. Several studies have been done to evaluate the influence of ripening stage on the extraction of phenolic compounds into extraction media. Most of them are focused on anthocyanin extraction and its influence on color.[6,7]

Some of those works have evaluated proanthocyanidin extraction. Fournand and co-workers studied both contents and extraction of proanthocyanidins and anthocyanidins from grape skins at different physiological stages and stated that during a short maceration, the extraction yield of proanthocyanidins remained constant whatever the pulp sugar content.[8] Mattivi and co-workers demonstrated that the amount and structure of extracted proanthocyanidins are related to grape variety.[9] Differences in extraction using various ethanol concentrations have also been evaluated,[6] showing that the higher the ethanol concentration, the higher the proanthocyanidin extraction. Skin characteristics and proanthocyanidin extraction have also been studied; relationships between

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mechanical properties of grape skins and proanthocyanidin extractability were found by Rolle and co-workers,[10] who determined that denser grapes provided higher phenolic extraction and who also stated that thinner skins assisted proanthocyanidin extraction. Recently, Bindon and co-workers[11] found that ripening affects cell wall porosity and its ability to adsorb proanthocyanidins, thus limiting their extraction to maceration media. Ripening also has an influence on the molecular weight of the extracted compounds, which was higher in riper grapes.

Some studies have related ripeness degree to grape skin flavonol composition,[3,4,12,13] but little is known about the influence of ripening on the extraction behavior of these compounds.[5]

The aim of this study was to evaluate the influence of cell wall composition on the extraction of flavan-3-ols and flavonols from red grape skins during ripening. To achieve this goal, red grapes of *Vitis vinifera* L. cv. Tempranillo were harvested at three different ripeness stages (preharvest, harvest, and over-ripened) and three soluble solid contents (22, 24, and 26 °Brix) within each ripeness degree.

MATERIALS AND METHODS

Grape Samples. *V. vinifera* L. cv. Tempranillo red grape samples were collected from a vineyard located in Lleida (Spain), which is under the influence of the Mediterranean climate. The samples were collected at three different ripeness degrees: preharvest (22 days before harvest date), harvest, and over-ripened (20 days after harvest date). In the studied samples notations P, H, and O were used to indicate preharvest, harvest, and over-ripened, 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 solid content (22, 24, and 26 °Brix, respectively). As a result, nine samples were obtained, with three different solid content (22, 24, or 26 °Brix) within each sampling date (P, H, or O). Grape skins were manually separated from the whole grapes, and three subsamples were taken from each sample, one for the exhaustive methanolic extraction, another for the hydroalcoholic extraction, and the third for cell wall isolation and characterization.

Cell Wall Analysis. Cell wall material was isolated from grape skins as the 70% ethanol insoluble residue as previously described.[14] Noncellulosic polysaccharides, pectins' esterification degree, lignin, cellulose, protein, and total phenolic compounds in cell wall material were determined as described in our previous work.[15]

Briefly, noncellulosic polysaccharides (i.e., arabinogalactans, mannans, homogalacturonans, xyloglucans, arabinans, rhamnogalacturonans-I, and rhamnogalacturonans-II) were obtained using an iterative calculation methodology[16] from monosaccharide profiles. Monosaccharides were determined by gas chromatography−mass spectrometry (GC-MS) of their trimethylsilyl ester *O*-methyl glycolsyl derivates (TMS) obtained after acidic methanolysis and derivatization following a modification of the Guadalupe et al. procedure[17]

Pectin esterification degree was determined as described by Femenia et al.[18] using the spectrophotometric measurement as proposed by Blumenkrantz and Asboe-Hansen,[19] but using *o*-hydroxydiphenyl solution[20] and recording the absorbance at 520 nm after 5 min of reaction.

Cellulose was determined as glucose in accordance with Lurie et al.[21] using the phenol method proposed by DuBois et al. for its spectrophotometric determination.[22] Lignin was gravimetrically determined as Klason lignin,[18] following the Saeman procedure.[23]

Flavanol and Flavonol Extractions. *Exhaustive Methanolic Extraction.* Extraction was performed as previously described.[15,24] Briefly, 10 g of grape skins was macerated at 4 °C in methanol containing 0.1% of 12 M HCl until colorless. Methanolic phases were successively pooled, a few milliliters of water was added, and the extract was concentrated under vacuum at 30 °C until methanol was removed and finally made up to 100 mL with ultrapure water.

Hydroalcoholic Extraction. Extraction was performed as previously described.[15,24] Briefly, 10 g of grape skins was macerated in 100 mL of model wine solution (4 g L^{-1} tartaric acid, 12.5% ethanol, adjusted at pH 3.6 with 0.5 M NaOH). The vials were kept at 25 °C and in darkness throughout the experiment. At regular intervals (1, 3, 5, 7, and 9 days) 1500 μL of extraction medium was taken and subjected to chromatographic analyses.

Sample Preparation for Chromatographic Analyses. Samples were prepared as previously described.[25]

For flavonol analysis, the extract was diluted 1:1 with 0.1 M HCl, filtered through 0.45 μm pore size filters, and directly injected into the chromatographic system to determine flavonols as described in the following section.

For the flavan-3-ol analysis, samples were fractionated prior to chromatographic analysis following the procedure described by Gonzalez-Manzano et al.:[26] 1 mL of each extract diluted (1:1) with 0.1 M HCl was eluted through Oasis MCX (Waters Corp., Milford, MA, USA) cartridges previously conditioned with 2 mL of methanol and 2 mL of water. After a washing with 4 mL of ultrapure water, flavan-3-ols and phenolic acids were eluted with 8 mL of methanol, whereas anthocyanins and flavonols were retained in the cartridges. A small volume of water was added to the eluate and concentrated under vacuum at <30 °C until complete elimination of methanol. The aqueous residue was adjusted to a volume of 0.5 mL with ultrapure water, filtered (0.45 μm), and analyzed by HPLC-DAD-MS as described in the following section.

HPLC-DAD-MS Analyses. An Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of an autosampler, a quaternary pump, a vacuum degasser, a thermostated column compartment, and a diode array detector (DAD) and controlled by ChemStation software (version B.04.01; Agilent Technologies) was used for chromatographic analyses. The HPLC system was coupled to a hybrid triple-quadrupole/linear ion trap (QqLIT) mass spectrometer API 3200 QTrap (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V ionization source and controlled by Analyst software (version 1.5; Applied Biosystems) via the DAD cell outlet.

Flavonol analysis was performed as described elsewhere.[27] Briefly, an Aqua C18 column (4.6 mm × 150 mm, 5 μm particle size) thermostated at 35 °C was used. Solvents were (A) 0.1% trifluoroacetic acid and (B) 100% HPLC grade acetonitrile. The elution profile was as follows: isocratic 10% B for 3 min, from 10 to 15% B for 12 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, and from 30 to 35% B for 5 min. The flow rate was 0.5 mL min^{-1} and the injection volume, 100 μL. UV−vis spectra were recorded from 250 to 770 nm with a bandwidth of 2 nm. Flavonols were detected at 360 nm as the preferred wavelength. MS analysis was carried out in positive mode (ESI^+) as described elsewhere:[28] declustering potential (DP), 41 V; entrance potential (EP), 7.5 V; ion spray voltage (IS), 5000 V; ion source gas 1 (GS1), 40 psi; ion source gas 2 (GS2), 50 psi (600 °C); curtain gas (CUR), 20 psi; and collision gas (CAD), set as "high". Both quadrupoles were set at unit resolution. Mass method consisted of three mass experiments: full mass analysis (EMS mode, collision energy (CE) 10 V), MS^2 analysis (EPI mode, CE 25 V), and MS^3 analysis (CE 30 V, excitation energy (AF2) 50 V). Spectra were recorded between m/z 150 and 1100.

Flavan-3-ol chromatographic separation was performed on a reversed-phase column Spherisorb ODS-2 (150 × 4.6 mm, 3 μm) from Waters maintained at 25 °C. Mobile phases A and B were, respectively, 0.25% acetic acid in water and acetonitrile The following linear gradient was used to achieve the chromatographic separation: from 0 to 10% B in 5 min, from 10 to 14.5% B in 35 min, from 14.5 to 19% B in 5 min, from 19 to 55% B in 5 min, hold at 55% B for 5 min, from 55 to 80% B in 5 min, hold at 80% B for 3 min, from 80 to 0% B in 2 min, and hold at 0% B for 5 min. The flow rate and the injection volume were set at 0.5 mL min^{-1} and 100 μL, respectively. UV−vis

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Table 1. Average Values for Cell Wall Components Determined in Grape Skins

	%	μg/mg cell wall material								μg/mg cell wall material	%	μg/mg cell wall material		
	MI[a]	mannans	RG-II[b]	RG-I[c]	HG[d]	XG[e]	AG[f]	arabinans	total PS[g]	lignin	esterification degree	cellulose	protein	polyphenols
P 22	5.20	8.43	11.05	17.29	175.67	52.21	56.36	65.13	386.14	298.97	8.41	31.85	103.73	109.55
P 24	2.90	12.17	16.41	21.68	220.28	75.56	73.58	86.31	505.99	463.92	85.35	34.64	105.11	144.88
P 26	4.50	6.79	12.82	14.40	146.31	52.20	47.26	58.26	338.03	375.00	9.14	33.05	148.07	105.83
H 22	4.50	8.52	17.33	18.90	192.04	68.53	52.04	75.13	432.48	505.05	69.26	43.80	138.04	181.49
H 24	2.00	11.38	16.89	20.06	203.87	91.41	62.19	84.02	489.82	490.00	62.85	36.81	137.79	176.53
H 26	3.40	10.62	16.87	20.11	204.36	90.08	52.66	91.42	486.13	312.50	83.07	39.50	119.88	157.23
O 22	3.80	8.18	18.52	17.76	180.44	62.22	46.92	72.43	406.46	84.21	41.28	38.38	170.43	163.29
O 24	2.50	8.65	14.98	17.59	178.72	55.83	49.84	78.20	403.82	375.00	52.82	40.84	132.82	185.39
O 26	1.30	9.22	16.21	17.90	181.90	65.20	57.02	76.44	423.89	145.83	11.54	43.68	120.92	154.08

[a]MI, total insoluble material. [b]RG-II, type II rhamnogalacturonans. [c]RG-I, type I rhamnogalacturonan. [d]HG, homogalacturonan. [e]XG, xyloglucans. [f]AG, arabinogalactans. [g]Total PS, total polysaccharide content.

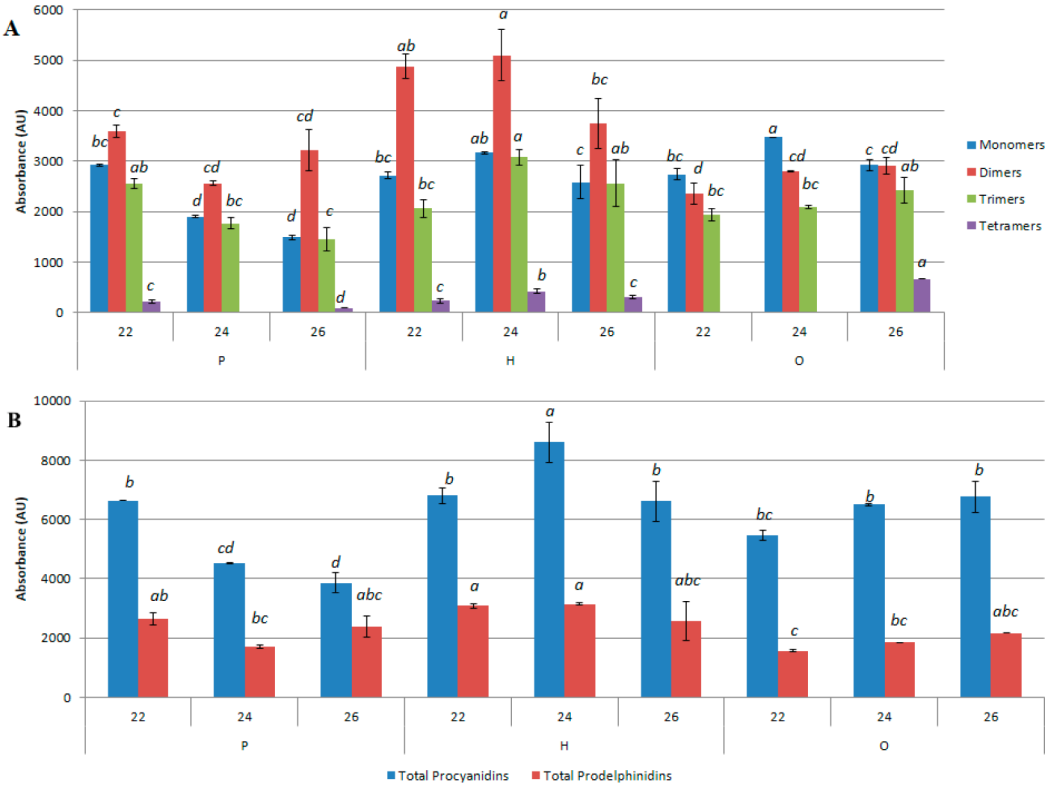

Figure 1. Total proanthocyanidin contents in exhaustive methanolic extracts grouped according to the number of subunits present in the molecule (A) and the structural characteristics (B). Within each group of compounds, different letters indicate statistical differences ($p < 0.05$) between samples by the Tukey HSD test.

spectra were recorded from 200 to 600 nm during acquisition at a selected wavelength of 280 nm.

The mass spectrometer was operated in the negative electrospray ionization (ESI) mode under the following specific conditions: IS, −4500 V; source temperature (TEM), 400 °C; CUR, 20 arbitrary units; GS1, 40 arbitrary units; GS2, 30 arbitrary units; DP, −40 V; EP, −7 V; and CE, −20 eV. Nitrogen (>99.98%) was employed as curtain, ion source, and collision gas. The detection was accomplished in the enhanced MS (EMS) full-scan mode, from m/z 100 to 1700, and in the enhanced product ion (EPI) mode to obtain the corresponding full-scan MS/MS spectra.

Extractabilities. Flavan-3-ol and flavonol extractabilities were calculated as percentages using the ratio between the DAD-HPLC peak areas obtained from hydroalcoholic and exhaustive methanolic extractions, as previously reported for anthocyanin extractability.[24]

Statistical Treatment. Principal components analysis (PCA), an unsupervised pattern recognition method, was used for data analysis. The IBM SPSS 21 for Windows software package (SPSS, Inc., Chicago, IL, USA) was used for data processing.

RESULTS AND DISCUSSION

Cell Wall Composition. Cell wall composition of grape skins harvested at different ripeness stages and with different soluble solid content within each stage has been detailed in our previous work.[15] Results obtained are in agreement with those reported in Ortega-Regules et al.[29] Cell wall composition is summarized in Table 1.

Flavan-3-ol and Flavonol Composition. Extracts obtained after exhaustive extraction of 10 g of grape skins were

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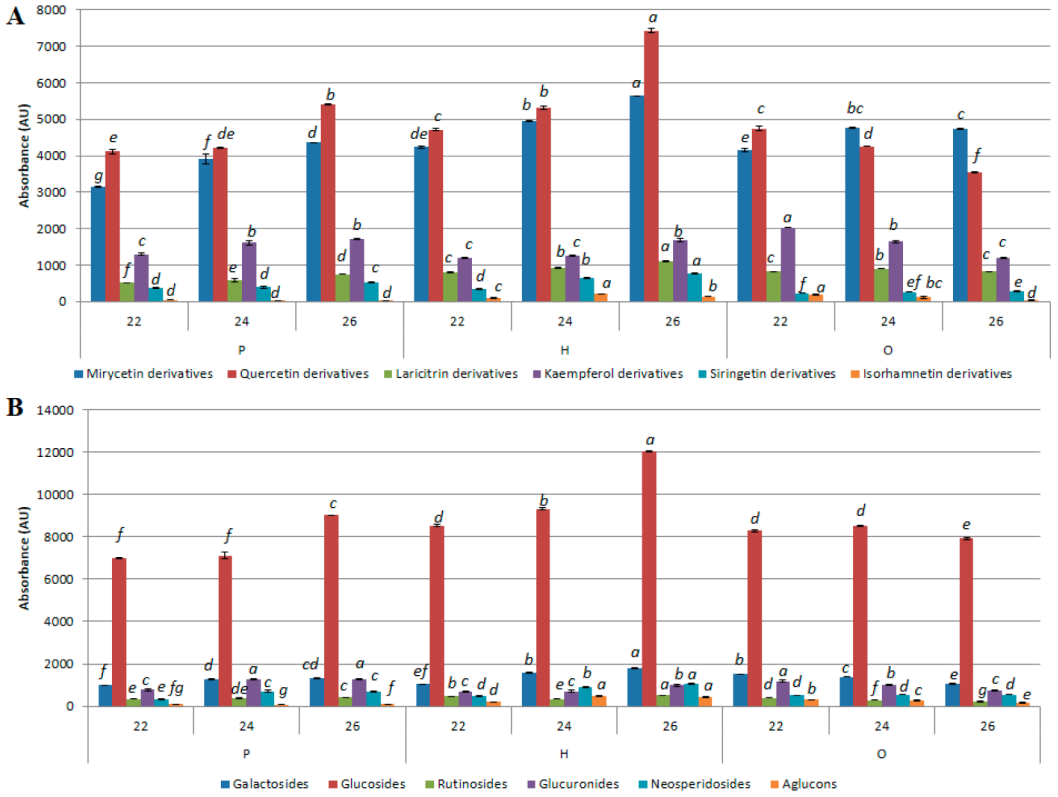

Figure 2. Flavonol total contents in exhaustive methanolic extracts grouped according to flavonol moiety (A) or the sugar moiety (B) present in the molecule. Within each group of compounds, different letters indicate statistical differences ($p < 0.05$) between samples by the Tukey HSD test.

analyzed to ascertain their flavanolic and flavonolic composition.

With regard to polymerization degree, as can be seen in Figure 1A, dimers were the most abundant compounds in preharvest and harvest samples, whereas monomers were, in general, the most abundant ones in over-ripened (O) samples. Trimers represented 20.9−28.4% of the proanthocyanidins quantified in the studied samples, being in all samples the third group in abundance, reaching levels similar to monomers in P 26, H 24, and H 26 samples. Tetramers were the least abundant oligomers in all of the studied samples, showing levels under quantification limits in three of the analyzed samples.

Flavan-3-ol compounds were classified according to their structure in two groups, procyanidins, which only catechin or epicatechin subunits present in their structure, or prodelphinidins, which present one or more gallocatechin or epigallocatechin subunits in their structure. Procyanidin and prodelphinidin contents in grape skins followed the same pattern (Figure 1B), increasing their level from preharvest to harvest and showing a slight decrease in over-ripened (O) samples, although these differences were not significant (Tukey HSD test, $\alpha = 0.05$) for several of the different samples. Despite higher prodelphinidin contents in harvest (H) samples, their percentages were bigger in preharvest (P) samples, varying from 27.5 to 38.3%, than in harvest samples, where they represented 26.8−31.2% of the quantified compounds. O samples presented the lowest percentages of this kind of compound. The abundance of prodelphinidins in red wines has been previously related to good maturity level of the grapes[30] and to the ability to produce high-quality wines.[31]

Up to 17 flavonol compounds were identified and quantified in the studied samples. They included quercetin, myricetin, kaempferol, laricitrin, syringetin, and isorhamnetin derivatives, esterified with different sugar moieties or in their nonglycosylated form. Figure 2A shows flavonol contents grouped as a function of the flavonol moiety present in the molecule for the nine analyzed samples. Flavonol content tended to increase as soluble solid content grew in preharvest and harvest samples, whereas over-ripened samples showed the opposite trend. The highest levels were reached in harvest samples, whereas preharvest and over-ripened samples showed quite lower levels. With regard to the kind of derivative, quercetin and myricetin derivatives were the most abundant compounds in all of the studied samples, as previously reported for Tempranillo grapes.[3,32] Quercetin derivatives were the most abundant found in seven of the nine analyzed samples, which is in agreement with the flavonol profile of the Tempranillo variety.[33] Myricetin derivatives were predominant in the two remaining samples, namely, O 24 and O 26. Kaempferol, laricitrin, syringetin, and isorhamnetin derivatives were also detected and quantified.

With regard to the sugar moiety linked to the flavonol moiety (Figure 2B), the most abundant derivatives were glucosylated compounds, displaying levels from 66.1 to 74.7%. Galactosides and glucuronides were also quite abundant, and small percentages of rutinosides, neosperidosides, and aglucons were also found.

Flavan-3-ol Extractabilities. In previous studies dealing with the average degree of polymerization of wine proanthocyanidins, we have determined in Tempranillo wines values varying from 2.9 to 4.3. That made us focus this extractability study on monomers and oligomers up to four.[26,34]

Up to 22 different flavanols were detected in our samples. Extractability statistical analyses were performed using the

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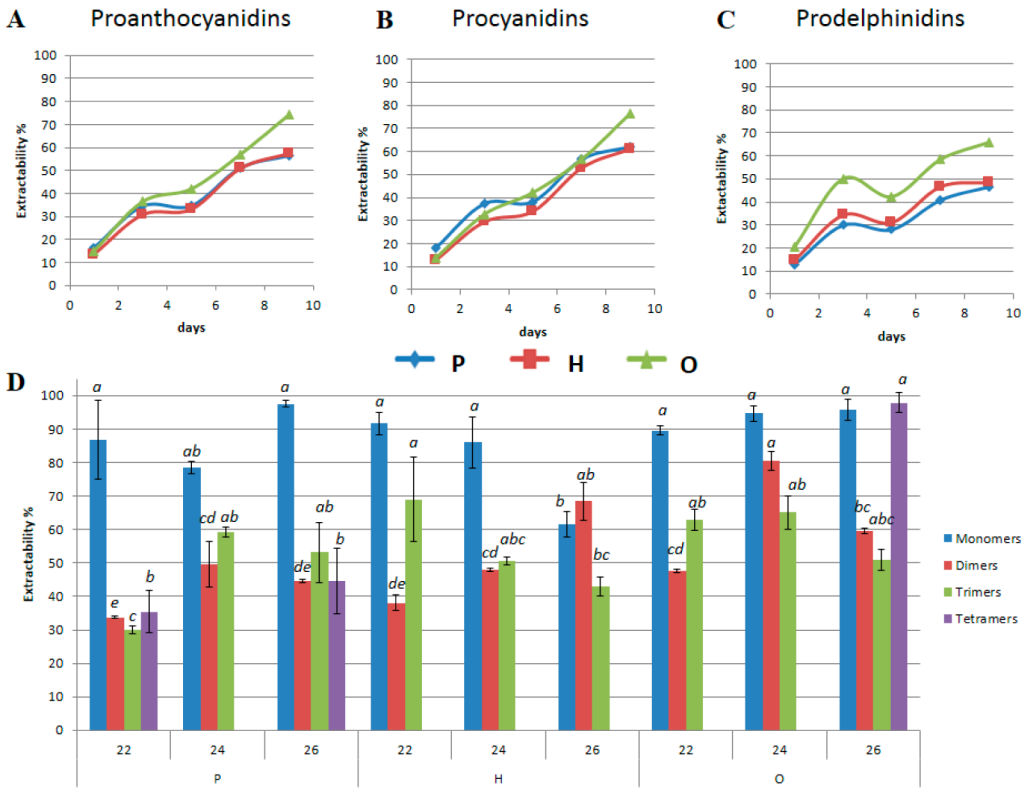

Figure 3. Percentage of extractability of proanthocyanidins (A), procyanidins (B), and prodelphindins (C) in samples with different ripeness degrees and percentage of extractability of flavanols grouped according to the number of subunits present in the molecule (D) on the last day of study. Within each group of compounds, different letters indicate statistical differences ($p < 0.05$) between samples by the Tukey HSD test.

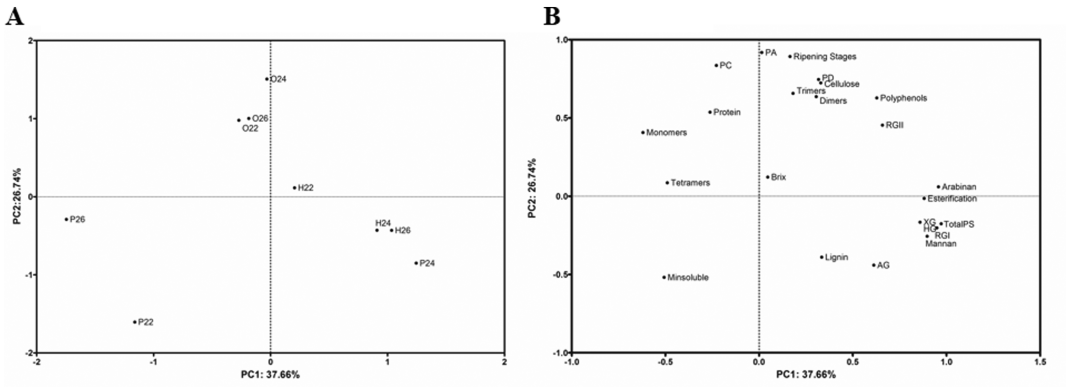

Figure 4. Projection of the samples on the plane defined by the first and second principal components (A) and the corresponding loading plot (B) for proanthocyanidin extractability. Minsoluble, insoluble material; Protein; Polyphenols; Lignin; Cellulose; AG, arabinogalactans; Mannans; HG, homogalacturonans; XG, xyloglucans; Arabinans; RGI, rhamnogalacturonans-I; RGII, rhamnogalacturonans-II; Total PS, total polysaccharide contents; Esterification, degree of esterification of pectins; Brix, soluble solid content; Ripening Stages; Total PA, maximum proanthocyanidin extractability; Total PC, maximum procyanidin extractability; Total PD, maximum prodelphinidin extractability; Monomers, extractability of monomers; Dimers, extractability of dimers; Trimers, extractability of trimers; Tetramers, extractability of tetramers.

compounds grouped as a function of their polymerization degree or their structural characteristics, i.e., procyanidins (polymers of catechin and/or epicatechin) and prodelphinidins (polymers of gallocatechin and/or epigallocatechin).

The maximum values obtained in our study for total proanthocyanidin extractability (Figure 3A) were reached the last day of the study in all samples. Llaudy et al. had observed that the amount of proanthocyanidin extracted to the maceration media from grape skins reached its maximum level after 4−5 days of maceration and then remained stable.[35] In our study, the extraction is extended (10 days), and it could be inferred that it would probably have increased if the maceration had been lengthened. This fact is especially noticeable in over-ripened samples, showing a steeper slope than preharvest and harvest samples, which presented very similar patterns (Figures 3A−C). As can be seen in Figure 3B,C, clear differences between procyanidin and prodelphinidin extractabilities can be observed. Prodelphinidin extractability increases with ripening, whereas this is not so evident for procyanidins. In fact, extractability of procyanidins in O samples is similar to that in H samples and even lower than that in P samples during the first days of the study.

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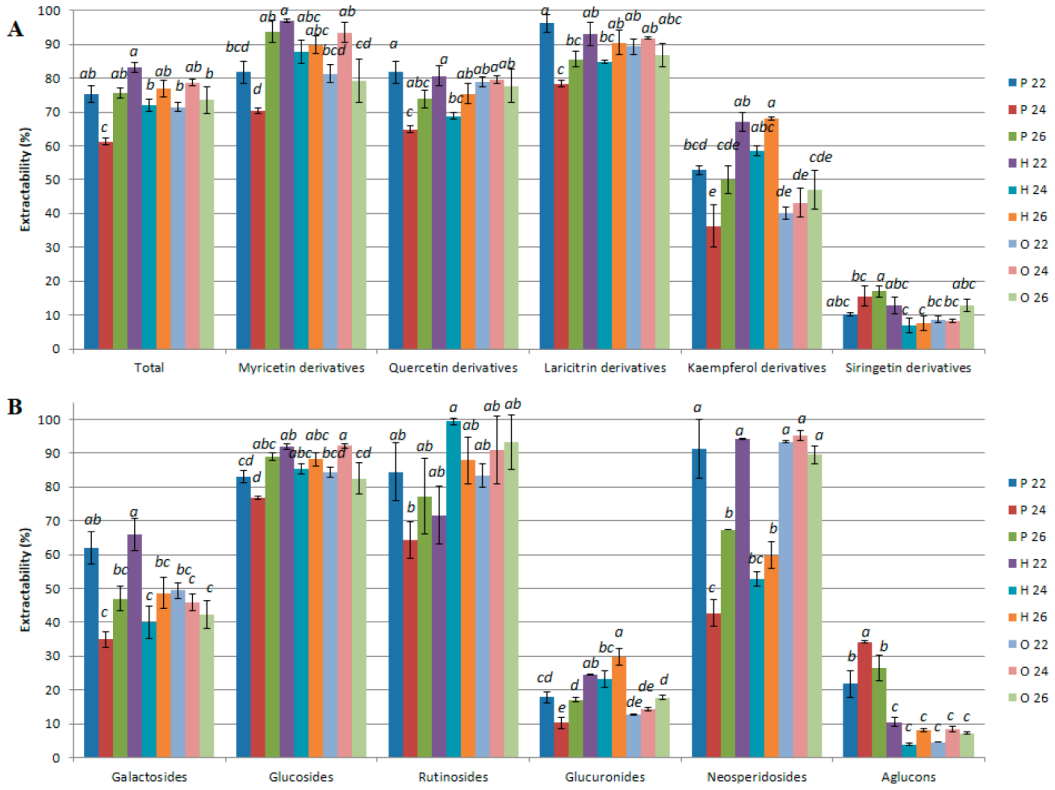

Figure 5. Percentages of extractability of flavonols, on the fifth day of study for the different samples, grouped according to the flavonol moiety (A) and the sugar moiety (B) present in the molecule. Within each group of compounds, different letters indicate statistical differences ($p < 0.05$) between samples by the Tukey HSD test.

Nevertheless, as extraction time increases, the extraction yield of procyanidins in O samples tends to be higher than in H and P samples, indicating that, during ripening, the cell walls become more porous and flavanols would be retained in these pores, reducing the extraction.[11] However, it should be assumed that once the pores are blocked, extractability could be favored due to structural degradation that may occur in over-ripened grapes. This would explain the behavior that we have observed, although further experiments need to be performed to confirm this hypothesis. Furthermore, it seems that the adsorbed flavanol compounds into the pores would consist mainly of procyanidins rather than prodelphinidins. In fact, the extraction of prodelphinidins seems to be faster the first days of the study, although afterward it tends to slow. This could be related to changes in the selectivity pattern of the pore of the cell walls that have been observed as the concentration of flavanols in the medium increases.[11] To our knowledge, no previous studies had evaluated the differences between procyanidin and prodelphinidin extractabilities.

No clear pattern is observed for soluble solid content (data not shown). The absence of a relationship between sugar content and proanthocyanidin extraction in hydroalcoholic media is in good agreement with previously reported studies.[8]

In relation to polymerization degree, as can be seen in Figure 3D, in general monomers were easily extracted in all samples, whereas dimers, trimers, and tetramers had variable behaviors. It is worth noting that tetramers are extracted only in three of the nine samples; this could indicate that the extraction of flavanols becomes more difficult as the polymerization degree increases.

PCA was conducted as unsupervised pattern recognition to observe relationships between proanthocyanidin extractabilities at the last day of the study, when maximum extraction levels were reached, stage of ripening, °Brix, and cell wall composition. The first principal component (PC 1) describes 37.66% of the variability in the data, and the second principal component (PC 2) describes 26.74% of the variability.

As can be seen in Figure 4, stage of ripening showed a clear positive relationship with total proanthocyanidin extractability and also with procyanidin and prodelphinidin extractabilities, whereas soluble solid content did not present any influence on the compound extraction. With regard to cell wall composition, total insoluble material displayed the biggest opposition to proanthocyanidin extractabilities. Some of the components of the cell wall showed a relationship with the proanthocyanidin extractabilities. This indicates that progression of ripening might cause changes in skin cell wall composition that could facilitate the release of proanthocyanidins. Those oppositions are quite stronger for total proanthocyanidins and total procyanidins, whereas they were weaker for prodelphinidin extractability. This behavior reflects a higher tendency of procyanidins to interact with components of cell walls than that of prodelphinidins.

Among cell wall constituents, some polysaccharide families exert an influence on proanthocyanidin total extractability and procyanidin extractability, namely, arabinogalactans (AG), mannans, RG-I, homogalacturonans (HG), xyloglucans (XG), and total polysaccharides content. All of these cell wall components displayed a negative relationship with proantho-cyanidin extractabilities; thus, the higher the content of these compounds in the cell wall, the lower the proanthocyanidin extraction. The opposition to prodelphinidin extractability of those cell wall components is weaker than for procyanidins.

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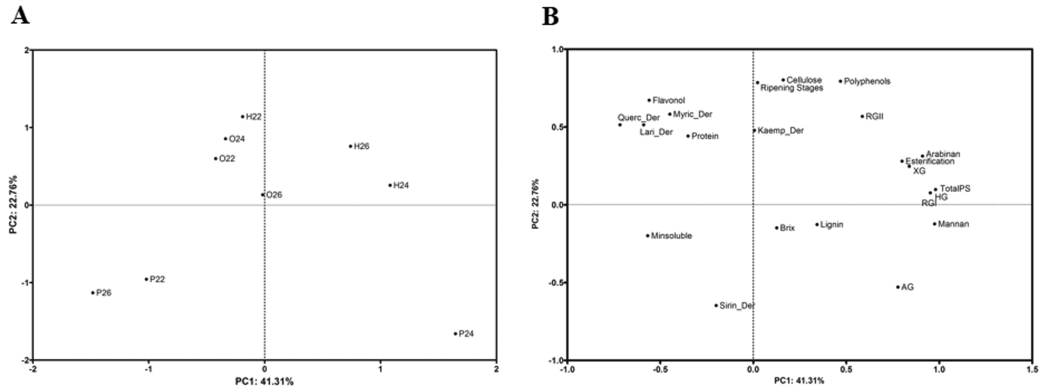

Figure 6. Projection of the samples on the plane defined by the first and second principal components (A) and the corresponding loading plot (B) for flavonol extractability. Minsoluble, insoluble material; Protein; Polyphenols; Lignin; Cellulose; AG, arabinogalactans; Mannans; HG, homogalacturonans; XG, xyloglucans; arabinans; RGI, rhamnogalacturonans-I; RGII, rhamnogalacturonans-II; total PS, total polysaccharide contents; Esterification, degree of esterification of pectins; Brix, soluble solid content; Ripening Stages; TotalFlavonol, maximum flavonol extractability; Querc-der, maximum extractability of quercetin derivatives; Myric-der, maximum extractability of myricitrin derivatives; Lari-der, maximum extractability of laricitrin derivatives; Kaempf-der, maximum extractability of kaempferol derivatives; Sirin-der, maximum extractability of siringitrin derivatives.

Orfila and Knox[36] reported that pit fields contain homogalacturonan. Therefore, a lower content in homogalacturonan is in accordance with the lesser presence of pores and, hence, with an increase in the proanthocyanidin extractability and mainly in procyanidin extractability. This would be in agreement with the negative correlation we have observed. Cellulose showed a strong positive relationship with prodelphinidin extraction and also with procyanidin and total proanthocyanidin extractabilities. Recently, it has been stated that cellulose presents a low affinity for proanthocyanidin binding,[37] whereas the galacturonan rich-fraction of cell wall material, obtained from grape skins, has a high propensity to associate with proanthocyanidins. The above-mentioned results for proanthocyanidin extraction confirm those observations; the higher amount of AG, RG-I, HG, and XG is related to a lesser proanthocyanidin extractability, whereas the higher the cellulose presence, the higher the proanthocyanidin extractability, probably due to the different ability of these polysaccharides to bind proanthocyanidins. With regard to the influence of cell wall composition on the extractability of compounds grouped on the basis of the number of subunits that make up the molecule, the extractability of dimers and trimers of proanthocyanidins displayed an opposition to total insoluble material and it was positively related to the presence of cellulose. The extractability of monomers and tetramers presented high opposition to total polysaccharide content and also to XG, HG, RG-I, and mannans. The above-mentioned results revealed that proanthocyanidin extractability is mainly influenced by harvest date and total insoluble material and to a lesser extent by some cell wall components. The pattern exhibited for proanthocyanidin extractability is quite similar to that previously reported for anthocyaninin extractability.[15]

Flavonol Extractabilities. Flavonol extractabilities reached their maximum at the fifth day of extraction (Figure 5), except for sample P 26, which reached the maximum at the third day of hydroalcoholic extraction, and samples P 24 and O 22, showing their maximum levels at the seventh day of extraction. H 24 extraction shows two maxima, the first one at the fifth day of extraction and the second one at the ninth day, although the percentages of extractability across the two maxima were very similar.

The pattern shown for flavonol extractability points out that flavonol are extracted from grape skins more slowly than anthocyanin compounds.[24] Quercetin, myricetin, and laringetin derivatives were more efficiently extracted than kaempferol or syringetin derivatives (Figure 5A), whereas isorhamnetin derivatives were not extracted in hydroalcoholic media. With regard to the sugar moiety in the molecule (Figure 5B), glucosides, rutinosides, and neosperidosides were more efficiently extracted than galactosides. Glucuronides and aglucons were the less extracted compounds to the hydroalcoholic maceration media.

PCA was conducted to establish relationships between flavonol extractabilities and cell wall composition. PC 1 described 41.31% of the variability observed in the data, and PC 2 described 22.76%. The projection of the samples in the plane defined by PC 1 and PC2 and the obtained loading plot are shown in Figure 6. This analysis revealed that ripening stage or soluble solid content was not related to total flavonol extractability. It showed also the lack of influence of the insoluble material contents on flavonol extractability. Some cell wall components presented a relationship with flavonol extractability; the presence of AG and mannans would decrease total flavonol extractability, whereas protein presence is positively related to total and major flavonol compounds (i.e., quercetin and myricetin derivatives) and to laricitrin derivative extractabilities. A possible explanation for the different behaviors shown for flavonol and other polyphenolic compounds extractabilities (anthocyanins and proanthocyanidins) is the different locations of the diverse polyphenolic compounds in the tissues; flavonol glucosides are located in epidermal cell layers in broad bean leaves,[38] and it is likely that they could be also located in epidermal layers in grape skins, bearing in mind that flavonols are related to UV protection, whereas anthocyanins and proanthocyanidins are located in hypodermal cell layers in grape skins.[39] Moreover, anthocyanins are accumulated inside cell vacuoles,[40] and proanthocyanidins have been found inside vacuoles, linked to proteins in the internal face of tonoplasts and also linked to cell wall polysaccharides.[39] It is possible that the extended release of proanthocyanidins from grape skins to the extraction media observed in this study is related to their different locations; thus, some of them are present as free forms, which would be

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easily released during the first days of maceration, whereas there is also a linked fraction that would need a long maceration period to be loosened. Flavonol cellular location is not clear; some authors have determined the flavonol concentrations inside vacuoles, but other histochemical studies have suggested that they could be in cytoplasm as free forms in flower petals,[41] whereas other authors have pointed out the possibility of association between nucleus structures and flavonol compounds,[38,42] primarily due to the ability of these compounds to avoid cell damage caused by sunlight exposure.

Epidermal cells are covered by an external cuticule layer,[43] composed mainly of waxes, which could act as a barrier for flavonol extraction during winemaking. It is possible that the lack of a relationship between flavonol extraction and insoluble material might be due to the existence of this barrier on the outer surface of the epidermal cell layers, where flavonols are mainly located, whereas this barrier existence would not be related to proanthocyanidin or anthocyanin extraction because those compounds are located in hypodermal cells.

In conclusion, proanthocyanidin and flavonol extractabilities were influenced to some extent by cell wall composition, whereas ripening stage affected only proanthocyanidin extractability. Harvest samples presented the highest contents of both flavonols and flavan-3-ols. The extractions from grape skins were slower than those reported for anthocyanins, reaching their maxima in general on the fifth day for flavonol extraction and on the ninth day for proanthocyanidin extraction. To our knowledge, this is the first time that flavonol extractability throughout ripening has been studied. Flavan-3-ol extractability is increased with ripeness stage, whereas soluble solid contents had a negligible influence. Those differences between flavan-3-ol and flavonol extractabilities could be due to their different tissue and cellular locations in grape skins.

This study points out the importance of the maceration time for phenolic compound extraction during winemaking. This can help in the choice of the optimum maceration time to extract the desired amounts of phenolic compounds, reducing this time to avoid the complete extraction of proanthocyanidins and thus wine astringency or extending this maceration in the case of low phenolic content grapes.

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Notes

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