JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 11333–11339 11333
DOI:10.1021/jf102265c

Effect of Different Enological Practices on Skin and Seed Proanthocyanidins in Three Varietal Wines

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Proanthocyanidins are important for wine quality since they participate in astringency, bitterness and color. Given the localization of proanthocyanidins in the berry (skin and seeds), different methods have been developed that help to modulate the release of these phenolic compounds. In this study, the effect of two low prefermentative temperature techniques (cold soak and must freezing with dry ice) and the use of macerating enzymes has been studied during the vinification of three different varietal wines (Monastrell, Syrah and Cabernet Sauvignon) to assess their influence on wine proanthocyanidin concentration and composition. Syrah wines showed the lowest proanthocyanidin content, together with the lowest mDP and the highest percentage of galloylation in its proanthocyanidins. Monastrell and Cabernet Sauvignon wines showed similar proanthocyanidin concentration. The application of the low temperature prefermentative maceration (cold soak) was the most effective treatment, increasing the proanthocyanidin concentration in Monastrell and Cabernet Sauvignon wines although neither of the treatments had any effect on Syrah wines. As regards the effect of the different treatments on the proanthocyanidin composition, the results seem to indicate that the observed increases were mainly due to an increase in seed proanthocyanidins, even in the case of cold soak treatments, which occur in the absence of ethanol, suggesting that ethanol is not so crucial in the extraction of seed proanthocyanidins.

KEYWORDS: Proanthocyanidins; wine; grape; enological practices

INTRODUCTION

Proanthocyanins in grape berry are located in the skin and seeds, and their composition and concentration vary depending upon the tissue of origin (*1*, *2*). Seeds contain a higher concentration of proanthocyanidins than skin and a higher proportion of galloylated procyanidins (*3*, *4*), whereas skin contains prodelphinidins (*3*, *5*). In addition, skin proanthocyanidins have been shown to have a higher mean degree of polymerization (mDP) than seed proanthocyanidins (*6*, *7*).

In the seeds, they are located in the seed coat (*8*, *9*). In the skins, these molecules are synthesized in the cytoplasm and stored in the vacuole. They can be associated with cell walls (*10*). Amrani and Glories (*11*) observed that the organization of proanthocyanidins varied according to their localization; aggregated structures were always present near the cell wall, while proanthocyanidins in the vacuoles became condensed.

Proanthocyanidins are important for wine quality since they participate in astringency, bitterness and color. A direct link between polymeric proanthocyanidins present in wine and perceived astringency has not yet been shown conclusively to date, although numerous papers suggest that they are responsible for the perception of astringent sensations (*12*, *13*). As regards color, they participate in the stabilization of wine color through the formation of new compounds such as those formed by direct link of anthocyanins and proanthocyanidins, ethyl linked anthocyanin−proanthocyanidin adducts and flavanyl pyranoanthocyanins (*14*).

The extraction of skin proanthocyanidins during the fermentative maceration process, as it has been described for anthocyanins, which also located in the vacuoles of skin cells, requires the cell walls to be broken to allow their vacuole contents to be extracted, or to diffuse into the wine. It is, therefore, essentially a diffusion process, and the rate and extent of extraction is influenced by the skin proanthocyanidin concentration, the composition of berry cell walls, that clearly affects the extractability (*15*), and the processing methods. On the other hand, it has been generally accepted that the extraction of proanthocyanidins of the seeds needs longer maceration time, because the lipids present in the seed must be eliminated first (*16*), which is normally done by the increasing concentrations of ethanol.

As regards processing methods, and given the localization of anthocyanins and proanthocyanidins in the berry skin, different methods have been developed that help to rupture skin cell walls and facilitate the release of phenolic compounds (*17*).

One of these methods is the use of macerating enzymes. Such enzymes have been widely studied for their effect on wine

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Published on Web 10/07/2010

anthocyanins and color characteristics, although with conflicting results (*18*−*22*), but less attention has been paid to the effect of these macerating enzymes on wine proanthocyanidins although some results could be found. In this way, they have been studied using a precipitation method (*23*, *24*) or the vanillin assay after fractionation of wine proanthocyanidin (*22*), any increase described usually being attributed to a greater degree of skin degradation. As regards composition, only Ducasse et al. (*25*) described the concentration and composition of proanthocyanidins in Merlot wines treated with maceration enzymes.

Other methods proposed to help to liberate skin phenolic compounds are the use of low temperatures before fermentation (cold soak or must freezing). Low temperature prefermentation or cold soak technique is designed to improve the extraction of pigments, proanthocyanidins and aromas from grape skins to the wine. During cold maceration treatment, the must is held at a low temperature, usually 10−15 °C, for several days before fermentation starts. Phenolic compound extraction takes place in the absence of ethanol because these low maceration temperatures prevent yeasts from starting the fermentation process. Again, attention has been mostly paid to anthocyanins and color characteristics although some of the studies have given data on proanthocyanidin concentration (*24*). However, as regards composition, only Koyama et al. (*26*) compared the composition of the proanthocyanidins of cold soak wines and a control wine. Peyrot des Gayons and Kennedy (*27*) also studied the extraction of proanthocyanidins using cold soak maceration but without comparing it with a control vinification.

Must freezing, mainly using dry ice, has also been proposed as an interesting technique. This causes the berry cells to burst, breaking cell membranes. Freezing increases the volume of intracellular liquids, disrupting the membranes and providing an easy exit for phenolic compounds. Freezing may also break the proanthocyanidin containing cell of seeds, increasing extractability. Couasnon (*28*) used dry ice to freeze Merlot must and compared the results with control wines; those produced after must freezing were found to contain 52% more proanthocyanidin and 50% more anthocyanins, but no details on proanthocyanin composition were given. Similar results were obtained with Cabernet Sauvignon and Cabernet Franc (*28*). Alvarez et al. (*29*) also studied the effect of must freezing on the concentration of proanthocyanidins of Monastrell wines, describing a slight increase in proanthocyanidin concentration with this technique.

In this study, the effect of two low prefermentative temperature techniques (cold soak and must freezing with dry ice) and the use of macerating enzymes were studied in three different varietal wines (Monastrell, Syrah and Cabernet Sauvignon) to assess their influence on wine proanthocyanidin concentration and composition.

MATERIALS AND METHODS

Grapes from *Vitis vinifera* L. cvs Monastrell, Cabernet Sauvignon and Syrah were harvested in 2009 from a commercial vineyard in Jumilla (SE Spain). For grape analysis, berry sampling was done choosing 40 vines per variety. Groups of five to six berries from different parts of the cluster and from different clusters on the same vine were sampled randomly. Berry samples (ca. 600 g) collected from all vines were divided in three subsamples for triplicate analysis. For the vinifications, grapes were carefully harvested in 20 kg boxes and transported to the winery. The chemical composition of the grapes at the moment of harvest is shown in **Table 1**.

Vinifications. Two low temperature prefermentation treatments were studied: cold soak at 10 °C and must freezing with dry ice. Also, a control vinification was carried out together with another vinification where a commercial enzyme was used. All vinifications were made by triplicate in 100 L stainless steel tanks using 90 kg of grapes. For all vinifications, after the grapes had been crushed and destemmed, sodium metabisulfite was added (8 g of SO_2/100 kg of grapes).

Table 1. Physicochemical Characteristics of the Grapes at the Moment of Harvest[a]

	°Brix	titratable acidity (g/L)	pH	tartaric acid (g/L)	malic acid (g/L)
Monastrell	28.2 a	4.2 a	3.6 a	5.3 a	0.9 a
Cabernet S.	28.2 a	4.5 a	3.8 b	5.3 a	2.5 b
Syrah	29.0 b	5.6 b	3.9 b	5.4 a	4.1 c

[a] Different letters within the same column indicate significant differences ($P < 0.05$).

Treatment 1. Must Freezing with Dry Ice (*DIW*). The dry ice was added directly into the tank, mixing it with the crushed grapes. 100 kg of dry ice was used for each tank, which kept the must frozen for two days.

Treatment 2. Cold Soak (*CSW*). Tanks containing the crushed grapes were introduced into a refrigeration camera at 10 °C for 10 days. After that, the tanks were returned to the winery.

Treatment 3. Vinification with a Commercial Enzyme (*EW*). The enzyme (Enozym Vintage, Agrovin, Spain) was added to the crushed grapes at a dose of 5 g/100 kg of grape.

Treatment 4. Control Vinification (*CW*). For all the treatments and before alcoholic fermentation started, total acidity was corrected to 5.5 g/L and selected yeasts were added (Levuline GALA, Oenofrane, France, 10 g of dry yeast/100 kg of grapes). All the vinifications were conducted at 25 ± 1 °C. Throughout the fermentative pomace contact period (10 days in all cases), the cap was punched down twice a day and the temperature and must density were recorded. At the end of this period, the wines were pressed at 1.5 bar in a 75 L tank membrane press. Free-run and press wines were combined and stored at room temperature. Malolactic fermentation occurred spontaneously, and samples were collected when no malic acid was detected, using for this determination an enzymatic test kit from Tecnología Difusión Ibérica, S.L. (Spain).

Physicochemical Determinations in Grapes. The optimum ripeness stage was determinated for each treatment and variety according to the phenolic and chemical composition of the grapes. Grape analysis involved the traditional flesh measurements carried out with a NIR system (Spectrum One FT-IR Spectrometer, PerkinElmer LLC, CT) (*30*).

Proanthocyanidin Extraction and Analysis in Grapes and Wines. The seeds and skins of 10 berries were separated from the mesocarp and rinsed with distilled−deionized water. Whole seeds and skins, previously ground to a powder with liquid nitrogen, were extracted separately in covered Erlenmeyer flasks with 10 mL of 2:1 acetone/water at room temperature for 24 h on an orbital shaker at 200 rpm. To minimize proanthocyanidin oxidation, solutions were spurged with nitrogen and the extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35 °C to remove acetone, and then lyophilized to a dry powder. This powder was redissolved in 1 mL of methanol in a volumetric flask.

Skin and seed proanthocyanidins were determined according to the method described by Kennedy and Jones (*31*) with some modifications, as follows. A solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid, was prepared (phloroglucinolysis reagent). 100 μL of methanolic extract was reacted with 100 μL of phloroglucinolysis reagent (1:1) in a water bath for 20 min at 50 °C and then combined with 2 volumes of 200 mM aqueous sodium acetate to stop the reaction.

For wines, the samples were prepared by an optimization of the method described by Pastor del Río et al. (*32*). 5 mL of wine was evaporated in a centrivap concentrator (Labconco, USA), redissolved in 3 mL of water and then passed through a C18-SPE column (1 g, Waters, Milford, MA), previously activated with 10 mL of methanol followed by 20 mL of water. The cartridge was washed with 20 mL of water, and compounds of interest were eluted with 10 mL of methanol, evaporated, and then dissolved in 1 mL of methanol. Phloroglucinolysis was then carried out as described above.

HPLC analysis followed the conditions described by Ducasse et al. (*25*). The HPLC apparatus was a Waters 2695 system (Waters, Milford, MA) equipped with an autosampler system, a Waters 2996 photodiode array

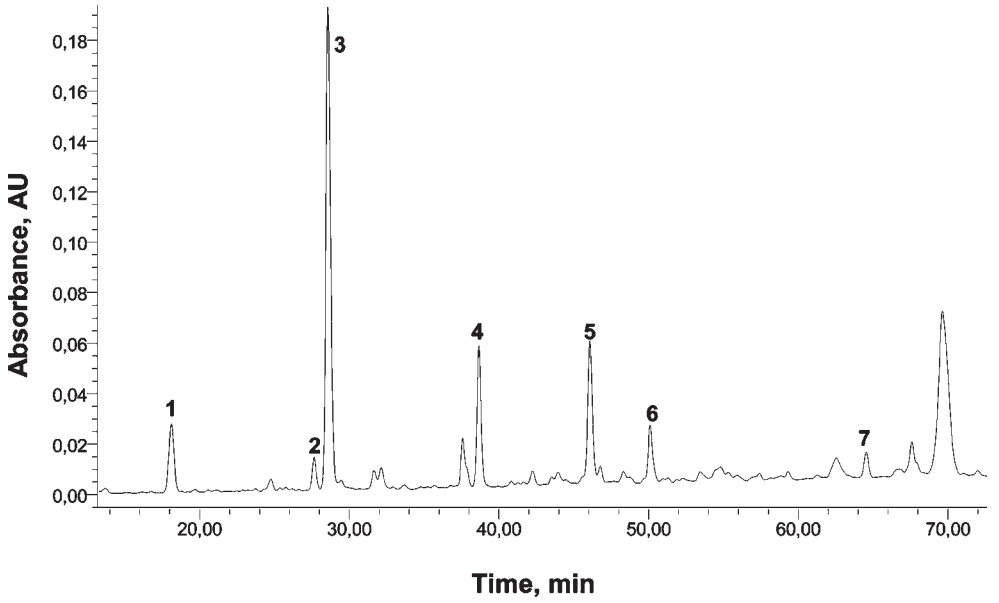

Figure 1. HPLC chromatogram of proanthocyanidin cleavage product of a Cabernet Sauvignon wine following acid catalysis in the presence of phloroglucinol. Compounds **1**, **2**, **3**, and **5** are the phloroglucinol adducts of the (−)-epigallocatechin, (+)-catechin, (−)-epicatechin and (−)-epicatechin gallate respectively, and compounds **4**, **6**, and **7** are the (+)-catechin, (−)-epicatechin and (−)-epicatechin gallate respectively.

detector. Samples (10 μL injection volume) were injected on an Atlantis dC18 column (250 × 4.6 mm, 5 μm packing) protected with a guard column of the same material (20 mm × 4.6 mm, 5 μm packing) (Waters, Milford, MA). The elution conditions were as follows: 0.8 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (98:2, v/v), and solvent B, acetonitrile/solvent A (80:20 v/v). Elution began with 0% B for 5 min, linear gradient from 0 to 10% B in 30 min and gradient from 10 to 20% in 30 min, followed by washing and re-equilibration of the column.

Proanthocyanidin cleavage products (**Figure 1**) were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard. These analyses allowed determination of the total proanthocyanidin content, the apparent mean degree of polymerization (mDP) and the percentage of each constitutive unit. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) divided by the sum of all flavan-3-ol monomers (in moles).

Statistical Data Treatment. Significant differences among wines and for each variable were assessed by analysis of variance (ANOVA). LSD test was used to separate the means ($P < 0.05$) when the ANOVA test was significant. This analysis, together with a Cluster analysis, was conducted using Statgraphics 5.0 Plus.

RESULTS AND DISCUSSION

Grape Skin and Seed Concentration and Composition in the Three Varieties. Grapes were harvested with a very similar sugar content, although differences could be observed in titratable acidity, with Syrah grapes showing higher acidity (mainly due to higher malic acid content) but similar pH (**Table 1**).

The concentration and composition of skin proanthocyanidins in the three varieties are shown in **Table 2**. As regards skin proanthocyanidin concentration, Syrah grapes showed the lowest concentration of skin proanthocyanidins expressed as μg/g skin or mg/kg of grapes, with Monastrell presenting the highest values (2-fold those of Syrah) and Cabernet Sauvignon presenting intermediate values. The quantitative results of Syrah grapes are very similar to that reported by Hernandez-Jimenez et al. (*33*) for grapes grown in the same area, although this was not the case for Monastrell, the grapes in this study presenting higher proanthocyanidin concentration. Both vintage and vineyard localization have been observed to have an effect in other studies (*34*−*36*), great interannual differences being reported. Cabernet Sauvignon values were lower than those reported by Cosme et al. (*37*).

Table 2. Mean Values and Standard Deviations of Concentration and Composition of Skin Proanthocyanidins in the Berries of the Three Studied Varieties Expressed as μg/g Skin, μg/Berry and mg/kg of Grapes[a]

	Monastrell	Cabernet Sauvignon	Syrah
total PAs			
μg/g	7747 ± 891 c	5299.6 ± 700 b	3443.8 ± 441 a
μg/berry	1387 ± 117 c	706.5 ± 110 b	414.4 ± 49 a
mg/kg	721 ± 84 b	667.8 ± 63 b	282.0 ± 40 a
mDP	14.1 ± 0.1 b	26.6 ± 0.7 c	8.5 ± 1.0 a
% G	1.4 ± 0.1 a	1.6 ± 0.2 a	6.6 ± 0.3 b
% yield	53.8 ± 1.2 c	33.9 ± 2.3 b	20,1 ± 1.1 a
% tCat	4.1 ± 0.1 a	3.6 ± 0.2 a	9.4 ± 1.1 b
% tECat	2.3 ± 0.1 b	0.0 ± 0.0 a	2.5 ± 0.3 b
% extCat	1.0 ± 0.0 b	1.6 ± 0.0 c	0.0 ± 0.0 a
% extECat	63.7 ± 3.5 c	43.8 ± 1.1 a	58.5 ± 0.9 b
% extEGCat	27.5 ± 2.3 a	49.4 ± 1.7 b	23.0 ± 2.4 a
% extECatG	1.4 ± 0.1 a	1.6 ± 0.0 a	6.6 ± 0.6 b

[a] Abbreviations: total PAs, total proanthocyanidins; mDP, mean degree of polymerization; % G, percentage of galloylation; % yield, percentage of recovery yield resultant from conversion of phenolic polymer to known proanthocyanidin subunits; % tCat, percentage of terminal (+)-catechin; % tECat, percentage of terminal (−)-epicatechin; % extCat, percentage of extension (+)-catechin; % extECat, percentage of extension (−)-epicatechin; % extEGCat, percentage of extension (−)-epigallocatechin; % extECatG, percentage of extension (−)-epicatechin gallate. Different letters within the same row indicate significant differences ($P < 0.05$).

Also, differences were found in the mDP, with Cabernet Sauvignon presenting the highest values. The values observed for the Syrah and Cabernet Sauvignon skin proanthocyanidins were generally lower than those reported by others (*31*, *37*, *38*). For example, Cosme et al. (*37*) found an mDP of 27 in Syrah skins and 41.7 in Cabernet Sauvignon skin proanthocyanidins and Bogs et al. (*39*) between 25 and 40 in Syrah skins, both higher than our reported values. Also the values found in this study for Monastrell grape skins were lower than that reported by Hernandez-Jimenez et al. (*33*)

As regards skin proanthocyanidin composition, the main constituent of skin terminal subunits in Syrah was catechin, similar to the data reported by Downey et al. (*38*). Cabernet Sauvignon did not present terminal (−)-epicatechin. Extension (−)-epicatechin was the most abundant subunit in Syrah and Monastrell, consistent with others (*31*, *33*, *38*) and extension

Table 3. Mean Values and Standard Deviations of Concentration and Composition of Seed Proanthocyanidins in the Berries of the Three Studied Varieties Expressed as μg/g Seeds, μg/Berry and mg/kg of Grapes[a]

	Monastrell	Cabernet S.	Syrah
total PAs			
μg/g	38121 ± 3992 a	35658 ± 5814 a	32591 ± 826 a
μg/berry	3023 ± 584 b	2214 ± 236 a	2979 ± 164 b
mg/kg	1562 ± 258 a	2105 ± 272 b	2012 ± 23 b
mDP	6.8 ± 0.2 b	6.1 ± 0.3 ab	5.5 ± 0.1 a
% G	15.2 ± 0.4 b	12.4 ± 0.6 a	17.5 ± 0.2 c
% yield	94.4 ± 2.3 a	97.4 ± 1.6 a	97.3 ± 1.3 a
% tCat	6.0 ± 1.0 a	6.4 ± 1.0 a	6.1 ± 0.3 a
% tECat	5.8 ± 0.2 a	7.0 ± 0.3 b	7.1 ± 0.2 b
% tECatG	3.1 ± 0.1 a	3.1 ± 0.1 a	5.0 ± 0.1 b
% extCat	10.0 ± 0.6 b	6.3 ± 0.7 a	6.5 ± 0.1 a
% extECat	63.1 ± 1.2 a	67.8 ± 1.5 b	62.8 ± 0.2 a
% extECatG	12.1 ± 0.2 b	9.3 ± 0.4 a	12.5 ± 0.3 b

[a] Abbreviations: total PAs, total proanthocyanidins; mDP, mean degree of polymerization; % G, percentage of galloylation; % yield, percentage of recovery yield resultant from conversion of phenolic polymer to known proanthocyanidin subunits; % tCat, percentage of terminal (+)-catechin; % tECat, percentage of terminal (−)-epicatechin; % tECcatG, percentage of terminal (−)-epicatechin gallate; % extCat, percentage of extension (+)-catechin; % extECat, percentage of extension (−)-epicatechin; % extECatG, percentage of extension (−)-epicatechin gallate. Different letters within the same row indicate significant differences ($P < 0.05$).

(−)-epigalocatechin in Cabernet Sauvignon skins. Syrah grape skins presented the highest percentage of galloylation (6.6%), while Monastrell and Cabernet Sauvignon showed lower and similar values. Souquet et al. (*5*) found values of galloylation in grape skins of 3−6%, and they stated that this percentage was independent of the skin proanthocyanidins' mDP. In contrast to this, Hernández-Jiménez et al. (*33*) observed a positive correlation between mDP and the percentage of galloylation.

Grape seeds had a higher proanthocyanidin concentration than grape skin (**Table 3**). When expressed as μg/g of seed, the quantities found in the three varieties were similar, ranging from 32591 μg/g in Syrah to 38121 μg/g in Monastrell. However, when expressed as mg/kg of fresh berries, Cabernet Sauvignon and Syrah showed very similar values and higher than Monastrell. The results found by Hernandez-Jiménez et al. (*33*) for Monastrell and Syrah were very similar. In other studies of Syrah, Harbeston et al. (*40*) reported 1.4 mg/berry for seed while Ristic et al. (*41*) reported 1.61−1.93 mg/berry of seed proanthocyanidins in Syrah, values lower than those reported in this study.

With regard to seed proanthocyanidin composition, the major difference between skin and seed proanthocyanidins was the absence of (−)-epigallocatechin and a higher percentage of (−)-epicatechin-3-*O*-gallate. Monastrell presented the highest mDP, while Syrah presented the lowest mDP and the highest percentage of galloylation. These values are in the range described by Prieur et al. (*4*), that is, percentages of galloylation between 13 and 29 and mDP between 2.3 and 15.1.

(+)-Catechin and (−)-epicatechin were the major terminal subunits. Cabernet Sauvignon and Syrah showed the highest values of (−)-epicatechin and Syrah of (−)-epicatechin-3-*O*-gallate. As regards extension subunits, (−)-epicatechin clearly dominated, Cabernet Sauvignon showing the highest value. Monastrell and Syrah presented the major percentage of (+)-catechin and (−)-epicatechin-3-*O*-gallate. Mattivi et al. (*42*) reported the higher values of (−)-epicatechin for Syrah and lower for Cabernet Sauvignon. Downey et al. (*38*) found a similar percentage of (−)-epicatechin and major values of (−)-epicatechin-3-*O*-gallate.

Proanthocyanidin Composition in the Three Single-Variety Control Wines. Comparing the three control wines (**Tables 4**, **5** and **6**),

Table 4. Mean Values and Standard Deviations of Concentration and Composition of Monastrell Wine Proanthocyanidins as Affected by the Different Enological Treatments[a]

	CW	CSW	EW	DIW
total PAs (mg/L)	835 ± 85 a	1111 ± 22 b	926 ± 111 ab	927 ± 83 ab
mDP	6.2 ± 0.2 a	6.4 ± 0.3 ab	6.8 ± 0.0 ab	6.9 ± 0.2 b
% G	2.6 ± 0.2 b	3.3 ± 0.4 c	2.6 ± 0.0 b	2.0 ± 0.1 a
% yield	37.5 ± 2.7 a	36.2 ± 2.5 a	34.8 ± 2.3 a	35.6 ± 1.1 a
% tCat	10.4 ± 0.4 a	9.7 ± 0.4 a	9.9 ± 0.1 a	9.8 ± 0.1 a
% tECat	4.3 ± 0.4 b	3.6 ± 0.2 ab	3.5 ± 0.1 a	3.7 ± 0.3 ab
% tECcatG	0.2 ± 0.0 a	0.5 ± 0.1 b	0.2 ± 0.0 a	0.2 ± 0.0 a
% extCat	2.0 ± 0.0 a	2.4 ± 0.2 b	1.8 ± 0.2 a	1.8 ± 0.1 a
% extECat	60.5 ± 0.2 a	60.8 ± 0.1 a	61.2 ± 0.8 ab	62.5 ± 0.8 b
% extEGCat	20.2 ± 0.6 a	20.2 ± 1.2 a	21.0 ± 1.0 a	20.2 ± 0.9 a
% extECatG	2.4 ± 0.2 bc	2.8 ± 0.3 c	2.3 ± 0.1 b	1.8 ± 0.1 a

[a] Abbreviations: total PAs, total proanthocyanidins; mDP, mean degree of polymerization; % G, percentage of galloylation; % yield, percentage of recovery yield resultant from conversion of phenolic polymer to known proanthocyanidin subunits; % tCat, percentage of terminal (+)-catechin; % tECat, percentage of terminal (−)-epicatechin; % tECcatG, percentage of terminal (−)-epicatechin gallate; % extCat, percentage of extension (+)-catechin; % extECat, percentage of extension (−)-epicatechin; % extEGCat, percentage of extension (−)-epigallocatechin; % extECatG, percentage of extension (−)-epicatechin gallate; CW, control wine; CSW, cold soak macerated wine; EW, enzyme-treated wine; DIW, dry ice-macerated wine. Different letters within the same row indicate significant differences ($P < 0.05$).

Table 5. Mean Values and Standard Deviations of Concentration and Composition of Cabernet Sauvignon Wine Proanthocyanidins as Affected by the Different Enological Treatments[a]

	CW	CSW	EW	DIW
total PAs (mg/L)	845 ± 45 a	957 ± 14 b	938 ± 56 b	820 ± 55 a
mDP	6.6 ± 0.2 c	5.2 ± 0.2 a	6.1 ± 0.2 b	5.5 ± 0.1 a
% G	3.6 ± 0.1 a	4.5 ± 0.0 c	4.2 ± 0.1 b	4.1 ± 0.1 b
% yield	29.6 ± 2.7 a	32.5 ± 0.9 a	30.9 ± 0.2 a	30.5 ± 2.1 a
% tCat	9.8 ± 0.2 a	11.6 ± 0.3 b	10.2 ± 0.4 a	11.6 ± 0.1 b
% tECat	4.8 ± 0.3 a	6.8 ± 0.2 d	5.6 ± 0.2 b	6.2 ± 0.1 c
% tECcatG	0.5 ± 0.1 a	0.7 ± 0.0 c	0.6 ± 0.0 b	0.5 ± 0.1 a
% extCat	2.2 ± 0.1 a	2.7 ± 0.1 c	2.5 ± 0.1 b	2.7 ± 0.0 c
% extECat	43.9 ± 1.6 a	48.5 ± 0.5 bc	47.0 ± 0.8 b	49.5 ± 0.3 c
% extEGCat	35.8 ± 1.4 c	25.9 ± 0.8 a	30.6 ± 1.3 b	25.9 ± 0.7 a
% extECatG	3.1 ± 0.0 a	3.8 ± 0.0 b	3.6 ± 0.0 b	3.6 ± 0.1 b

[a] Abbreviations: total PAs, total proanthocyanidins; mDP, mean degree of polymerization; % G, percentage of galloylation; % yield, percentage of recovery yield resultant from conversion of phenolic polymer to known proanthocyanidin subunits; % tCat, percentage of terminal (+)-catechin; % tECat, percentage of terminal (−)-epicatechin; % tECcatG, percentage of terminal (−)-epicatechin gallate; % extCat, percentage of extension (+)-catechin; % extECat, percentage of extension (−)-epicatechin; % extEGCat, percentage of extension (−)-epigallocatechin; % extECatG, percentage of extension (−)-epicatechin gallate; CW, control wine; CSW, cold soak macerated wine; EW, enzyme-treated wine; DIW, dry ice-macerated wine. Different letters within the same row indicate significant differences ($P < 0.05$).

Syrah wine showed the lowest proanthocyanidin content, together with the lowest mDP and the highest percentage of galloylation in its proanthocyanidins. Monastrell and Cabernet Sauvignon wines resulted more similar in their proanthocyanidin concentration. Adams and Scholz (*43*) found an average proanthocyanidin concentration of 484 mg/L in Syrah wines, which is lower than that reported here, although they used a precipitation method to quantify proanthocyanidins. The study of Romero-Cascales et al. (*23*), also with a precipitation method, reported very similar values of Cabernet Sauvignon and Syrah wine proanthocyanidins and lower values for Monastrell. Whatever the case, it should not be forgotten that year-by-year variability can be very high. In Cabernet Sauvignon wine, for example, Cosme et al. (*37*) reported proanthocyanidin values that ranged from 289 to 776 mg/L in three different years.

Table 6. Mean Values and Standard Deviations of Concentration and Composition of Syrah Wine Proanthocyanidins as Affected by the Different Enological Treatments[a]

	CW	CSW	EW	DIW
total PAs (mg/L)	592 ± 87 a	616 ± 47 a	467 ± 51 a	446 ± 72 a
mDP	3.0 ± 0.1 a	2.6 ± 0.1 a	3.0 ± 0.1 a	2.6 ± 0.2 a
% G	5.0 ± 0.1 a	5.6 ± 0.2 b	5.5 ± 0.2 b	5.3 ± 0.2 b
% yield	28.7 ± 0.7 a	33.1 ± 1.7 b	28.5 ± 2.2 a	27.5 ± 1.8 a
% tCat	16.5 ± 0.3 a	17.2 ± 0.7 a	16.4 ± 0.9 a	18.4 ± 2.0 a
% tECat	16.1 ± 1.0 a	20.3 ± 1.4 b	16.1 ± 0.5 a	20.1 ± 2.0 b
% tECcatG	0.6 ± 0.1 a	1.0 ± 0.1 b	0.7 ± 0.1 a	0.7 ± 0.1 a
% extCat	3.0 ± 0.0 a	3.4 ± 0.1 b	2.9 ± 0.0 a	2.9 ± 0.0 a
% extECat	46.9 ± 0.3 a	43.6 ± 1.5 a	45.4 ± 2.3 a	39.9 ± 5.0 a
% extEGCat	12.5 ± 1.2 b	10.0 ± 0.8 a	13.7 ± 0.8 b	13.5 ± 0.9 b
% extECatG	4.5 ± 0.2 a	4.6 ± 0.4 a	4.8 ± 0.2 a	4.6 ± 0.1 a

[a] Abbreviations: total PAs, total proanthocyanidins; mDP, mean degree of polymerization; % G, percentage of galloylation; % yield, percentage of recovery yield resultant from conversion of phenolic polymer to known proanthocyanidin subunits; % tCat, percentage of terminal (+)-catechin; % tECat, percentage of terminal (−)-epicatechin; % tECcatG, percentage of terminal (−)-epicatechin gallate; % extCat, percentage of extension (+)-catechin; % extECat, percentage of extension (−)-epicatechin; % extEGCat, percentage of extension (−)-epigallocatechin; % extECatG, percentage of extension (−)-epicatechin gallate; CW, control wine; CSW, cold soak macerated wine; EW, enzyme-treated wine; DIW, dry ice-macerated wine. Different letters within the same row indicate significant differences ($P < 0.05$).

The percentages of (−)-epigallocatechin, which can only come from the skins, were highest in Cabernet Sauvignon wine and lowest in Syrah wine, as also found in the grapes. Terminal (+)-catechin predominated in Monastrell and Cabernet Sauvignon wines and the same percentage of terminal (+)-catechin and terminal (−)-epicatechin was found in Syrah wine. (−)-Epicatechin was the predominant extension subunit in the three wines.

Peyrot des Gachon and Kennedy (*27*) stated that, given that the extension subunit composition was invariant with extraction time, and that the seed and skin extension subunits composition vary considerably, it is possible to determine the percentage of seed and skin proanthocyanidins extracted into the wine by comparing the proportional proanthocyanidin extension subunit composition in wine relative to the proportional extension subunit composition in the corresponding grapes. Thus, by measuring the relative molar amount of extension (−)-epigallocatechin and extension (−)-epicatechin it should be possible to determine the relative proportion of seed and skin proanthocyanidins in wine. According to that, the percentage of skin proanthocyanidins was 73.4%, 72.4% and 54.34% of the total Monastrell, Cabernet and Syrah wine proanthocyanidins, respectively. Comparing with the results of Peyrot des Gachons and Kennedy (*27*) in Pinot Noir wines, after 8 days of fermentation, they found that 56.6% of wine proanthocyanidins came from the skins while Cerpa-Calderon and Kennedy (*45*) determined that, after nine days of maceration, 73% of a Merlot wine proanthocyanidins came from the skins. Sampaio et al. (*46*) found an extraction of skin and seed proanthocyanidins of 41.7 and 57.6% respectively in finished wines of Pinot Noir made in a commercial winery. These results showed that skin proanthocyanidins may account for more than 50% of the wine proanthocyanidins.

Also, the theoretical proportion of skin and seed proanthocyanidin extraction can be calculated from the grape data and the calculated percentages of the contribution of skin and seed proanthocyanidins to the total composition of the wine proanthocyanidins. Considering that the yield of wine in our press is 0.65 L/kg, the potential concentration for skin and seed proanthocyanidins was 1109 and 2404 mg/L for Monastrell grapes, 1027 and 3239 mg/L for Cabermet Sauvignon grapes and 434 and 3095 mg/L for Syrah grapes. Given the quantities

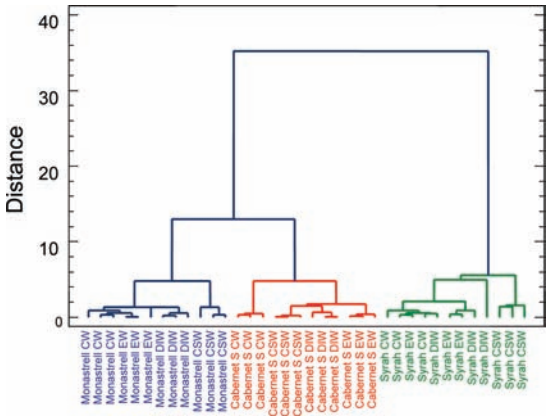

Figure 2. Dendogram obtained from the cluster analysis.

found in our control wines, the calculated yield in the wines was 55.3% and 9.2% from the skins and seeds of Monastrell grapes, 59.6 and 7.18% from the skins and seeds of Cabernet Sauvignon and 74.1 and 8.7% from the skins and seeds of Syrah grapes. Cerpa-Calderon and Kennedy (*45*), comparing with the theoretical maximum extraction, found lower levels of proanthocyanidin extracted from skins and higher levels extracted from seeds than those reported in our study.

Effect of the Enological Treatments on Wine Proanthocyanidins. When the results of the different practices were compared in Monastrell wines (**Table 4**), the proanthocyanidin concentration was greatest when cold soak was used (an increase of 33% in the proanthocyanidin concentration), and no effect of the enzyme or the dry ice treatment was observed. In Cabernet Sauvignon wines (**Table 5**), the proanthocyanidin content was maximum when cold soak was used, with a total increase of 13.2%, although the enzyme treatment also significantly increased the proanthocyanidin content. Álvarez et al. (*29*) also found a positive effect of low temperature prefermentative maceration in the concentration and polymerization of proanthocyanidins and in the stability of Monastrell wine color. These authors also stated that the phenolic concentration was not related to the duration of the treatments since the results did not improve when prefermentative maceration time was increased but the effect was more evident when grapes were not completely mature.

No significantly different increases in proanthocyanidin content were found in Syrah treated wines (**Table 6**). In the same way, Gil-Muñoz et al. (*24*) did not observe an increase in the proanthocyanidin content in Syrah wines when different low temperature prefermentative treatments were used.

As regards the proanthocyanidin composition, the different treatments did not modify the percentages of (−)-epigallocatechin in Monastrell wines and resulted in a decrease in Cabernet Sauvignon and Syrah wines. Very little differences were found in mDP in Monastrell and Syrah treated wines (an increase only being observed in the dry ice treatment in Monastrell wines) while the same parameter decreased in the treated Cabernet Sauvignon wines, compared with the control wine. The percentage of galloylation increased when cold soak prefermentative maceration was used for all the wines (although the increase was not significant in Syrah wines). It might be expected that, with the application of these low temperature techniques, which are supposed to help the physical degradation of skin cell walls, the concentration of skin proanthocyanidins would increase in the wines, but our results did not show this clearly. The lack of increase of the percentage of (−)-epigallocatechin, the stabilization or decrease of the mDP value and the increase in the percentage of galloylation indicate that the proanthocyanidin

increase, when detected, seems to be mainly due to an increase in seed proanthocyanidins. These results also indicated that, even when the low temperature maceration treatments occurred in the absence of ethanol, seed proanthocyanidins increased, suggesting that ethanol is not so crucial in the extraction of seed proanthocyanidins or that cold soak treatment affects seed structure, facilitating proanthocyanidin extraction during alcoholic fermentation.

Our results also showed that the effect of the maceration enzyme had only limited effect on proanthocyanidin concentration and composition, which does not agree with Ducasse et al. (*25*), who found a higher proanthocyanidin content in Merlot wines treated with enzymes, higher mDP and a higher percentage of (−)-epigallocatechin, indicating that this treatment favored the extraction of proanthocyanidins of higher molecular weight as a consequence of a larger enzymatic skin cell wall degradation.

To check whether the different treatments modified the wine proanthocyanidin profile to an extent that enabled the wines to be grouped according to the enological treatment used, a cluster analysis was conducted using all the studied variables (**Figure 2**). This statistical analysis is an unsupervised method for pattern recognition, where the samples were clustered without prior knowledge of their belonging to any variety or enological treatment. Distance, that measures the similarity or dissimilarity between the different samples, was calculated using square Euclidean distances, and an average linkage method algorithm was used to group the samples. The analysis showed that wine samples were grouped according to grape variety and not to enological treatment, Syrah wines being very different from Monastrell and Cabernet Sauvignon wines. Although for confirmation of these results, the experiment should be repeated at least one more year, these results are similar to those reported by Harbertson et al. (*44*) when studying the proanthocyanidins of commercial wines (therefore elaborated under different enological conditions) from five grape varieties, three continents, and three countries. These authors reported that wines made from different grape varieties could be differentiated from each other based on the average proanthocyanidin concentration, although sometimes there was some overlap.

In conclusion, the three different grape varieties presented a different proanthocyanidin profile and these differences were maintained in the respective wines. The different treatments only led to small differences in the quantitative and qualitative wine proanthocyanidin profile, the largest differences being observed in the cold soak vinification. The results also indicated that the increase in proanthocyanidin concentration, when detected, was mainly due to an increase in seed proanthocyanidins, even in the case of low temperature maceration treatments, that occurred in the absence of ethanol, suggesting that ethanol may not be so crucial in the extraction of seed proanthocyanidins.

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Received for review June 13, 2010. Revised manuscript received September 13, 2010. Accepted September 22, 2010. This work was made possible by financial assistance of the Ministerio de Ciencia e Innovación, Project AGL2009-12503.