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Proanthocyanidin Composition and Antioxidant Potential of the Stem Winemaking Byproducts from 10 Different Grape Varieties (*Vitis vinifera* L.)

María Reyes González-Centeno,[†] Michael Jourdes,[§] Antoni Femenia,[†] Susana Simal,*[,†] Carmen Rosselló,[†] and Pierre-Louis Teissedre[§]

[†]Department of Chemistry, University of the Balearic Islands, Crta Valldemossa, Km 7.5, 07122 Palma de Mallorca, Spain

[§]Unité de Recherche Œnologie, EA 4577, USC 1366-INRA, Institut de Sciences de la Vigne et du Vin, Université Bordeaux Segalen, 210 Chemin de Leysotte, 33882 Villenave d'Ornon Cedex, France

ABSTRACT: Stem byproducts from 10 different grape (*Vitis vinifera* L.) varieties were evaluated in terms of their total phenolic and total proanthocyanidin contents, flavan-3-ol and proanthocyanidin profiles, and antioxidant capacity measured by ABTS, CUPRAC, FRAP, and ORAC assays, with a view to the recovery of their natural bioactive compounds. Stems from Callet, Syrah, Premsal Blanc, Parellada, and Manto Negro varieties yielded the highest total phenolic and total proanthocyanidin contents and showed the greatest antioxidant capacities, whereas Chardonnay and Merlot stems presented the lowest values. Varieties differed significantly ($p < 0.05$) with regard to both the phenolic composition and antioxidant capacity of their stems. However, no significant differences ($p > 0.05$) were observed when stems from red and white varieties were considered separately. For the 10 grape varieties investigated, this is the first study presenting a detailed description of their stem flavan-3-ol composition determined by HPLC-UV-fluo. All of the analyses confirmed the stem byproducts as a potential polyphenol-rich source, especially promising in the case of the Callet variety.

KEYWORDS: *grape stems, winery byproducts, proanthocyanidins, antioxidant capacity, total phenolic content, mean degree of polymerization*

INTRODUCTION

It is well-known that moderate wine consumption plays an important role in protection against certain human diseases and dysfunctions, mainly due to the beneficial effects of its natural bioactive compounds, which have potential health-promoting and disease-protective qualities.[1] Most of these components, or at least their precursors, come directly from the vineyard and remain in significant concentrations in the two main wine-making byproducts, stems and grape pomaces. Stems may preserve their original phenolic profile almost intact, because they are usually directly discarded.[2] Thus, they are recognized as rich sources of interesting plant secondary metabolites.[3] The main reason is the high value of these biocompounds and their promising applications in the cosmetic, pharmaceutical, and food industries, as an alternative to synthetic substances commonly used in these fields, which are increasingly being rejected by consumers concerned about their healthiness.[4]

According to the ninth General Assembly of the International Organization of Vine and Wine (Porto, 2011), with a global production of 65 million tons in 2009, grapes are the main fruit crop in the world, around 80% being used for wine production. Considering these data and the fact that the winemaking process generates large amounts of solid waste, which might account for >30% (w/w) of the grapes used,[5] the residues derived from the wine industry exceeded 15 million tons in 2009. As this constitutes an important environmental problem, there is a continuous and growing pressure to develop new exploitation strategies for these underutilized resources, to reassimilate them into the food cycle, taking advantage of their interesting potential. To date, the most common revaluation of these inexpensive and easily available agricultural byproducts is the production of antioxidant and/or dietary fiber concentrates in the form of value-added ingredients for food supplementation[6,7] or their utilization to make fertilizers.[8] For this second purpose, extraction of polyphenols is required before further treatment, due to their phytotoxicity and antimicrobial effects during the composting process. Thus, the main potential uses and applications of wine waste involve the recovery and reuse of their phenolic constituents, which would represent a significant step forward in maintaining environmental balance and supporting a sustainable agricultural production.

There is a great diversity of grape varieties, each one characterized by different contents and profiles of phenolic compounds, the antioxidant capacity and health-promoting properties of which could significantly differ from one to another. In this regard, different studies have been carried out in recent years to evaluate the quantity and/or quality of the phenolic constituents in the winery residues. Most of these studies focused on grape pomace byproduct, above all from the Cabernet Sauvignon grape variety, whereas information about stem byproducts is rather scarce,[5] despite they also contain an important amount of polyphenols. In fact, there is no detailed description of stems' flavan-3-ol composition previously

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published in the literature for any of the grape varieties investigated in the present research. Thus far, only Sun et al.[9] have reported the flavan-3-ol profile in stems from the Tinta Miúda grape variety. More recently, Anastasiadi et al.[10] have assessed the content of some bioactive flavan-3-ols and the antioxidant potential of the stem extracts from six native Greek grape varieties. In any case, there are also very few studies about the total antioxidant capacity of stem byproducts, which only use a single method instead of a combination of different assays to give a global vision of their antioxidant properties.

From economic and environmental points of view, to exploit these underutilized winemaking byproducts more efficiently and extensively, it is important to know their qualitative and quantitative phenolic composition. Thus, the aim of the present study was to characterize the phenolic fraction and the antioxidant potential of the stem byproducts of 10 different cultivars of *Vitis vinifera* (Cabernet Sauvignon, Callet, Chardonnay, Macabeu, Manto Negro, Merlot, Parellada, Premsal blanc, Syrah, and Tempranillo), to identify and compare their interesting properties to be used in the agrifood industry as functional ingredients.

MATERIALS AND METHODS

Chemicals. Copper(II) chloride dihydrate, ammonium acetate, potassium peroxodisulfate, hydrochloric acid, ethyl alcohol, iron(III) chloride hexahydrate, sodium acetate 3-hydrate, glacial acetic acid, Folin−Ciocalteu reagent, and gallic acid were purchased from Scharlau (Barcelona, Spain). 2,4,6-Tri-(2-pyridyl)-*s*-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Acros Organics (Morris Plains, NJ, USA). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Biochemica (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dodecahydrate, fluorescein, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), neocuproine (2,9-dimethyl-1,10-phenanthroline), phloroglucinol, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin (EGC), (−)-epicatechin-3-*O*-gallate (ECG), procyanidin B1 [(−)-epicatechin-(4β-8)-(+)-catechin], and procyanidin B2 [(−)-epicatechin-(4β-8)-(−)-epicatechin] were supplied from Sigma-Aldrich (Saint Quentin Fallavier, France). Acetonitrile (HPLC grade), formic acid (HPLC grade), methanol (HPLC grade), glacial acetic acid (HPLC grade), L-ascorbic acid, and sodium acetate were purchased from Prolabo-VWR (Fontenays/Bois, France). Procyanidin B3 [(+)-catechin-(4α-8)-(+)-catechin], procyanidin B4 [(+)-catechin-(4α-8)-(−)-epicatechin], and trimer (C1) [(−)-epicatechin-(4β-8)-(−)-epicatechin-(4β-8)-(−)-epicatechin] were obtained from Polyphenols Biotech (Villenave d'Ornon, France).

Samples. The raw material consisted of stem byproducts from six red (Cabernet Sauvignon, Callet, Merlot, Manto Negro, Syrah, and Tempranillo) and four white (Chardonnay, Macabeu, Parellada, and Prebsal blanc) grape varieties (*Vitis vinifera* L.) and was provided by the Pere Seda S.L. winery (Mallorca, Balearic Islands, Spain) from the 2009 vintage. Callet, Manto Negro, and Premsal blanc varieties are indigenous to the Balearic Islands, and the Parellada variety is native to Catalonia, whereas the others are well-known and widely distributed elsewhere. For a better comparison and to mitigate the influence of external factors, all samples were from the same geographical area and vintage. In all cases, the samples were obtained the day of grape harvest after the destemming process and stored vacuum-packed at −80 °C until analysis.

Polyphenol Extraction Procedure. Each stem sample was first lyophilized and mechanically ground with a ceramic laboratory mortar. Phenolic compounds were extracted from the obtained powder, by using an ASE 350 Accelerated Solvent Extraction System equipped with a solvent controller (Dionex Corp., Sunnyvale, CA, USA). The ASE experimental conditions were static time set at 4 min, pressure set at 1500 psi, and temperature set at 40 °C, with a heating period of 5 min. A N_2 flush was used to prevent oxidation during extraction. Nearly 10 g of each sample was loaded into the extraction cell with a cellulose paper filter at the bottom of the cell and submitted to eight solid/liquid extractions by acetone/water (80:20, v/v) followed by three more with MeOH/water (60:40, v/v). All extracts were combined and evaporated under reduced pressure. The obtained solid residue was redissolved in 30 mL of water prior to lyophilization and finally stored, as a dry powder, under dark conditions until analysis. All samples were extracted in duplicate.

Determination of Total Phenolic Content. Total soluble polyphenols were spectrophotometrically measured in accordance with the Folin−Ciocalteu method,[11] using 96-well microplates. Stock solutions (4 mg/10 mL) of the stem extracts were prepared in EtOH/H_2O (25:75, v/v). For the measurement, 184 μL of distilled water was placed in each well, adding 24 μL of the sample solution followed by 12 μL of the Folin−Ciocalteu reagent and 30 μL of the 20% (w/v) Na_2CO_3 solution. The microplates were placed in a microplate spectrophotometer (MultiSkan Spectrum, Thermo Scientific) to incubate the mixture for 1 h under dark conditions at 25 °C and then to measure the absorbance at 765 nm. Gallic acid (0−24 ppm) was used as a standard for calibration, and the phenolic content results were expressed as milligrams of gallic acid per 100 g of stem sample (on a dry matter basis). Each result value was a mean of six determinations.

Determination of Total Proanthocyanidins. Total proanthocyanidin content was estimated spectrophotometrically through the Bate−Smith reaction, which is based on the transformation of proanthocyanidins in colored anthocyanidins by heating at 100 °C in acid conditions.[12] In separate test tubes, control and hydrolysis, 2 mL of stock solutions of the extract samples (0.5 mg/mL for all the stem extracts), 1 mL of distilled water, and 3 mL of HCl 37% were added. Control tubes were allowed to stand at room temperature under dark conditions, whereas hydrolysis tubes were capped and heated at 100 °C for 30 min. Then, after the tubes had been cooled in an ice bath for 10 min, 0.5 mL of EtOH 96% was added to stop the reaction. Absorbance difference between hydrolysis and control tubes was measured at 550 nm with a Varian Cary 300 Bio UV−vis spectrophotometer, using distilled water as a blank sample. Each determination was performed in triplicate, and the results were expressed in milligrams of proanthocyanidins per 100 g of stem sample (dm).

HPLC Analysis of Monomeric and Oligomeric Flavan-3-ols. Stem extracts were solubilized in methanol at a concentration of 10 mg/mL. Monomeric and oligomeric flavan-3-ol analysis was performed on a Thermo-Finnigan Surveyor HPLC system consisting of an UV−vis detector (Surveyor PDA Plus), a fluorescence detector (Surveyor FL Plus Detector), an autosampler (Surveyor autosampler Plus), and a quaternary pump (Surveyor MS pump Plus). UV−vis and fluorescence data treatment were controlled by Xcalibur and ChromQuest 4.2 software, respectively.

These analyses were carried out on a reversed-phase LiChrospher 100 RP18 (250 mm × 4 mm, 5 μm) column. The mobile phases were 1% (v/v) aqueous formic acid (solvent A) and 1% (v/v) formic acid in acetonitrile (solvent B). The binary elution system was as follows: 8% B at initial time, a linear gradient from 8 to 18% B in 21 min, from 18 to 100% B in 1 min, 100% B for 2 min to wash the column, a linear gradient from 100 to 8% B in 1 min and then 8% B for 6 min to re-equilibrate the system before the next injection. Flow rate was set at 1 mL/min, UV−vis detection wavelength at 280 nm, and fluorescence detection at 280 and 320 nm, respectively, for excitation and emission wavelengths. Identification and quantification of the main peaks were carried out using external standard calibration (flavan-3-ol monomers (+)-catechin (C), and (−)-epicatechin (EC); oligomers B1, B2, B3, B4, and C1). Results were expressed as milligrams per 100 g of stem sample (dm). All flavan-3-ol analyses were performed in duplicate.

HPLC Analysis of Mean Degree of Polymerization (mDP). The proanthocyanidin mDP values were estimated by phloroglucinolysis.[13] Briefly, 200 μL of methanolic solutions of the stem extracts (10 mg/mL) reacted with 200 μL of the phloroglucinol reagent (solution of 0.1 N HCl in methanol, containing 50 g/L phloroglucinol and 10 g/

L ascorbic acid) at 50 °C for 20 min and was then mixed with 1 mL of 40 mM aqueous sodium acetate to stop the reaction. The equipment used for this analysis was a Thermo-Accela HPLC system consisting of an UV−vis detector (Accela PDA Detector), an autosampler (Accela autosampler), and a quaternary pump (Accela 600 pump) and controlled by Xcalibur data treatment software. Separation was performed on a reversed-phase Waters XTerra RP18 (100 mm × 4.6 mm, 3.5 μm) column, by applying a binary gradient with mobile phases containing 1% (v/v) aqueous acetic acid (solvent A) and MeOH (solvent B), at a flow rate of 1 mL/min. The elution conditions were as follows: 5% B for 25 min, from 5 to 20% B in 20 min, from 20 to 32% B in 15 min, and from 32 to 100% B in 2 min. The column was then washed with 100% B for 5 min and re-equilibrated with 5% B for 3 min until the next injection. All mDP analyses were performed in duplicate. Apparent mDP values were calculated as the ratio between the total number of released subunits and the number of terminal ones.[14]

Evaluation of the Antioxidant Capacity. Plant-derived polyphenols are well-known for their antioxidant capacity, which is not a single reaction but includes multiple and a wide range of mechanisms. As no single method is able to assess them all, it is usually recommended to use more than one technique to determine the antioxidant capacity of the samples.[15] Thus, four different antioxidant capacity assays were used: the spectrophotometric ABTS, CUPRAC, and FRAP assays, which are based on electron transfer, and the fluorometric ORAC assay, which is based on hydrogen transfer. In all cases, an automated microplate reader was used: a MultiSkan Spectrum (Thermo Scientific) for the first three analyses and a FLUOstar Optima (BMG LabTech) for the fourth one. As for the total phenolics assessment, for the antioxidant capacity spectrophotometric methods, solutions of the stem extracts (4 mg/10 mL) were prepared in EtOH/H_2O (25:75, v/v). More diluted stock solutions of the sample extracts (20 mg/1L) were prepared in 75 mM phosphate buffer (pH 7.4) for the ORAC measurement. The difference in absorbance between a final reading and the reagent blank reading was correlated with Trolox stardard curves in all assays. Because the moisture level of each stem sample was quite different, antioxidant capacity was reported on a dry weight basis to enhance comparison with the literature. Thus, the results were expressed as milligrams of Trolox per gram of stem sample (dm). Each result value was a mean of six determinations.

ABTS Assay. This method was performed as described by Re et al.,[16] but applied in 96-well microplates. Briefly, the ABTS radical cation ($ABTS^{\bullet+}$) was prepared by the reaction of equivalent volumes (1:1) of both aqueous solutions of 7 mM ABTS and 2.45 mM potassium persulfate. This stock solution was allowed to react for 12−16 h at room temperature (~23 °C) in the dark and used within the two following days stored in the same thermal and light conditions. At the moment of the analysis 8 mL of the ABTS solution was diluted with EtOH/H_2O (25:75, v/v) in a 100 mL volumetric flask to obtain an absorbance of 1.00 ± 0.02 unit at 734 nm. In a 96-well microplate, stem extract solutions and ABTS reagent (190 μL in each well) were prewarmed at 25 °C for 20 min. Then, a reagent blank reading was taken at a wavelength of 734 nm. The reaction was carried out by adding 10 μL of the stem extract solution to each well. After 3 min of shaking, the mixture was incubated at the same temperature for a 30 min period, and then the absorbance decrease was measured at the same wavelength. Trolox standard solutions were prepared at a concentration ranging from 0 to 0.8 mM (R^2 = 0.995), by using EtOH/H_2O (25:75, v/v) as a solvent.

CUPRAC Assay. The cupric reducing antioxidant capacity of the sample extracts was based on a modified version of the experimental procedure described by Apak et al.,[17] to fit in 96-well microplates. CUPRAC reagent was prepared just before the analysis by reacting equal volumes (1:1:1) of 10 mM Cu(II) aqueous solution, 7.5 mM neocuproine in EtOH 96% freshly prepared, and ammonium acetate buffer (1 M, pH 7). In a 96-well microplate, stem extract solution and 190 μL of CUPRAC reagent for each determination were incubated under the same conditions as the ABTS assay. After the initial absorbance had been read at 450 nm, 10 μL of the stem extract solution was added to each well. After 3 min of shaking, the mixture was incubated at 25 °C for 30 min, and then the absorbance increase was measured at the same wavelength. Trolox standard curve was linear between 0 and 1.3 mM (R^2 = 0.996).

FRAP Assay. The ferric reducing antioxidant power assay was carried out according to the method of Benzie et al.[18] with some modifications to fit in 96-well microplates. The fresh working FRAP reagent was prepared by mixing a 0.01 M TPTZ solution in 0.04 M HCl, a 0.02 M $FeCl_3 \cdot 6H_2O$ aqueous solution, and acetate buffer (pH 3.6, 3.1 g of sodium acetate and 16 mL of acetic acid glacial per liter of buffer solution) at a ratio of 1:1:10. All of these solutions were prepared on the day of analysis, except for the buffer and hydrochloric solutions. For the measurement of the antioxidant activity by the FRAP method, the protocol and experimental conditions were exactly the same as those reported for the ABTS and CUPRAC assays. However, the increase in absorbance was measured at 593 nm and the Trolox calibration curve was obtained using concentrations from 0 to 1.6 mM (R^2 = 0.996).

ORAC Assay. The oxygen radical absorbance capacity analysis was applied by using 96-well fluorescence microplates.[19] The reaction was carried out in phosphate buffer (75 mM, pH 7.4). In this order, 30 μL of the stem extract solution, 180 μL of fluorescein (117 nM final concentration), and 90 μL of AAPH (40 mM) were added to each well. The mixture was shaken and allowed to stand for 1.5 h at 37 °C. Fluorescence was recorded every minute during this period at excitation and emission wavelengths of 485 and 530 nm, respectively. Simultaneously on the same microplate, a blank sample (phosphate buffer replaced the sample) and Trolox calibration solutions (1−40 μM) were also performed (R^2 = 0.983). The area under the curve (AUC) was calculated for each extract sample by integrating their relative fluorescence curves. By subtracting the AUC of the blank, the net AUC of the stem extracts was calculated and correlated with Trolox concentrations.

Statistical Analysis. The study of the variability among grape varieties in the polyphenol content and antioxidant properties of their stems was performed by the statistical package R version 2.14.2 (R Foundation for Statistical Computing, Wien, Austria). All experimental results were reported as mean values with their corresponding standard deviations. Correlation between variables and regression analysis were also assessed. Normality and homocedasticity of the data were evaluated for all parameters, by using the Shapiro−Wilk test and Levene's test, respectively. When populations were distributed normally and presented homogeneity in variance, the parametric ANOVA and Tukey tests were used to evaluate the existence and degree of significant differences. These statistical analyses were substituted, respectively, by the nonparametric Kruskal−Wallis and pairwise-Wilcox (with BH adjustment) tests, if populations were not distributed normally and/or presented heterogeneity in variance. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Polyphenol Extraction Yields. Comparison of the reported data in the literature of vinification byproducts is quite difficult, largely because of the utilization of different reference units, such as either fresh or dry matter basis or either extract or sample matter basis. To enhance comparisons with future studies, equivalence factors of polyphenol extraction yields are given in Figure 1, as grams of extract per 100 g of dry matter (dm) and fresh weight (fw) of stem samples. As observed, there were significant differences ($p < 0.05$) in the polyphenol extraction yields depending on the grape variety. Nevertheless, as previously suggested in the literature,[1] it is important to point out that these extraction yields are not completely related to either the phenolic content or antioxidant activity of the samples, because of the possible presence of nonphenolic compounds and/or inactive polyphenols that may influence the expected values.

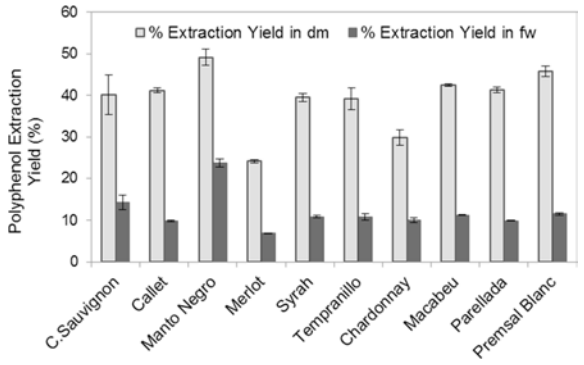

Figure 1. Polyphenol extraction yields. Results are expressed as grams of extract per 100 g of dry matter (dm) and fresh weight (fw) of stem sample.

Total Phenolic Content. The average values and standard deviations of total phenolic content of stem extracts are presented in Table 1. The amount of total phenolic compounds in this winemaking byproduct ranged from 4704 to 11525 mg GA/100 g dm for Merlot and Callet varieties, respectively. Of the 10 varieties considered, Callet stems stood out clearly from the others for their high phenolic content, followed by Syrah ≥ Premsal blanc = Parellada ≥ Manto Negro stem extracts in this ranking order. It is noteworthy to mention that, except for Syrah variety, the stem extracts exhibiting the highest phenolic content derived from the four autochthonous grape varieties investigated in the present study. The stem extracts obtained from Cabernet Sauvignon, Tempranillo, and Macabeu presented intermediate contents, whereas Chardonnay and Merlot stems showed the lowest values ($p < 0.05$). Because all of the stem samples were collected from closed vineyards of the same winery and that the destemming procedure was exactly the same in all cases, the significant differences ($p < 0.05$) observed among their total phenolic contents are mainly due to the intrinsic properties of each grape variety considered in the present study.

In general, the total phenolic content of stem extracts showed the same order of magnitude as that previously reported in the literature.[3,20−22] Nevertheless, the experimental values found in Cabernet Sauvignon, Tempranillo, and Syrah stems were 2−5 times higher than those previously observed for the same grape varieties.[3] In the case of Premsal blanc stems, a similar total phenolic content was reported by Llobera et al.,[21] although the values found by the same authors in Manto Negro stems were approximately 1.5 higher.[20] Total phenolic results similar to those obtained in the present study were established when compared to Roditis and Sharad Seedless stems.[5,23] A wide range of total phenolic contents was proposed for Savatiano white grape stems and for Moschofilero and Agiogitiko red grape stems,[22] which were also in broad agreement with those reported in Table 1. However, Spigno et al.[24] observed total phenolic values considerably lower than those reported in the present research (330 mg GA/100 g dm in Barbera variety). All of these differences may be attributed to the different vintage, geographical origin, and viticultural conditions of the samples and also to the solvent used during the polyphenol extraction process.[25] Moreover, in contrast with Püssa et al.,[26] who reported that stems from red varieties presented higher polyphenol contents than those of white varieties, the present research does not reflect this trend, because the total phenolic results of some white varieties were included in the broad range described for the stems of the red varieties. In fact, there are no significant differences ($p > 0.05$) between some pairs of red and white varieties stems considered in this study, like Tempranillo and Macabeu, Syrah and Parellada, or Merlot and Chardonnay, among others.

Total Proanthocyanidin Content. Results of the total proanthocyanidin content of stem extracts after Bate−Smith reaction are indicated in Table 1. Experimental data ranged between a minimum value of 79.1 ± 5.9 mg/g dm for Chardonnay stems and a maximum of 202.3 ± 6.2 mg/g dm for Callet stems. A trend similar to that observed for the total phenolic content of the stem extracts was obtained, where the Syrah variety, together with the four autochthonous varieties considered in this study, showed the highest values ($p < 0.05$). No significant differences ($p > 0.05$) were observed when stems from red and white varieties were considered separately. Overall, total proanthocyanidin results were consistent with those reported in the literature for stem byproducts, despite using a different analytical method and/or standards. Llobera et al.[20,21] published the total proanthocyanidin content of Manto

Table 1. Total Phenolics, Total Proanthocyanidins, and Flavan-3-ol Profile of the Stem Samples

			flavan-3-ol profile[c]					
	total phenolics[a]	total proanthocyanidins[b]	C	EC	B1	B2	B3	total[d]
Cabernet Sauvignon	7076 ± 327 a	124.9 ± 7.4 ab	49.3 ± 3.4 ab	3.1 ± 0.2 ab	56.4 ± 2.1 ab	2.1 ± 0.2 ab	12.0 ± 0.7 ab	123.0 ± 6.6 a
Callet	11525 ± 886 b	202.3 ± 6.2 c	45.3 ± 7.4 ab	1.6 ± 0.1 ac	45.4 ± 6.4 ac	2.0 ± 0.2 ab	15.6 ± 1.4 b	109.9 ± 15.4 ab
Manto Negro	8470 ± 291 c	165.3 ± 11.4 de	57.5 ± 3.5 ac	2.4 ± 0.1 acd	86.8 ± 3.2 b	2.2 ± 0.2 b	13.2 ± 0.7 ab	162.1 ± 7.7 ac
Merlot	4704 ± 288 d	84.0 ± 2.8 f	12.2 ± 0.5 d	0.6 ± 0.1 c	24.6 ± 1.2 ac	1.1 ± 0.1 a	4.1 ± 0.3 c	42.7 ± 2.1 d
Syrah	9642 ± 804 e	161.4 ± 10.9 dg	114.6 ± 3.1 e	2.4 ± 0.4 ace	132.0 ± 5.5 d	tr	20.8 ± 1.9 d	269.7 ± 10.9 e
Tempranillo	7622 ± 763 af	147.3 ± 4.4 ad	126.9 ± 15.8 ef	11.1 ± 1.9 f	195.8 ± 33.6 e	9.4 ± 1.0 c	23.2 ± 3.2 d	366.3 ± 55.4 f
Chardonnay	4764 ± 398 d	79.1 ± 5.9 f	31.4 ± 3.0 b	1.2 ± 0.1 ac	25.5 ± 4.3 ac	1.5 ± 0.1 ab	5.6 ± 0.3 c	65.2 ± 7.8 bd
Macabeu	7809 ± 718 f	108.8 ± 3.3 bf	9.3 ± 3.3 d	0.5 ± 0.0 c	13.3 ± 2.6 c	1.1 ± 0.0 a	4.5 ± 0.5 c	28.8 ± 6.4 d
Parellada	8924 ± 673 ce	165.2 ± 9.4 dg	133.9 ± 1.8 f	5.8 ± 0.9 g	187.7 ± 9.7 e	4.8 ± 0.1 d	22.2 ± 0.5 d	354.5 ± 13.0 f
Premsal blanc	9002 ± 977 ce	181.4 ± 9.1 ceg	74.0 ± 6.2 c	4.0 ± 0.3 bde	121.8 ± 2.7 d	4.0 ± 0.0 d	10.4 ± 0.1 a	214.2 ± 9.4 ce

[a]Total phenolics expressed as mg GA/100 g dm. [b]Total proanthocyanidins expressed in mg tannins/g dm. [c]Flavan-3-ol concentration expressed in mg/100 g dm. [d]Total individual flavan-3-ol calculated as the sum of C, EC, B1, B2, and B3 individual contents; C, (+)-catechin; EC, (−)-epicatechin; B1−B3, procyanidin dimers; tr, traces; letters following the values in each column show the significant differences among grape varieties ($p < 0.05$).

Table 2. Mean Degree of Polymerization (mDP) and Structural Composition of Stem Polymeric Proanthocyanidins[a]

		general composition		terminal units			extension units	
	mDP	% C	% EC	% C	% EC	% ECG	% C	% EC
Cabernet Sauvignon	5.9 ab	25 ab	74 ab	97 a	tr	3 a	0.11 a	89 a
Callet	4.7 c	29 c	70 c	89 b	7 a	4 b	12 b	88 b
Merlot	6.0 ab	25 a	75 a	97 a	tr	3 a	10 c	90 c
Manto Negro	5.8 b	26 bd	73 bd	97 a	tr	3 a	11 a	89 a
Syrah	6.1 a	22 e	77 e	97 a	tr	3 a	7 d	93 d
Tempranillo	6.9 d	20 f	79 f	95 c	tr	5 b	8 d	92 d
Chardonnay	4.6 c	28 cg	71 cg	89 b	6 a	5 b	11 ac	89 ac
Macabeu	6.2 a	24 h	75 a	83 d	11 b	6 c	13 b	87 b
Parellada	5.0 e	27 dg	72 dg	95 c	2 c	3 a	10 ac	90 ac
Premsal blanc	8.5 f	25 ah	74 a	95 c	tr	4 b	16 e	84 e

[a]C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-*O*-gallate; tr, traces; letters following the values in each column show the significant differences among grape varieties ($p < 0.05$).

Negro and Premsal blanc stems, with 1.6 and 2.3-fold lower values, respectively, for the same grape varieties. As previously observed,[4] a high significant correlation was found between the total phenolic and total proanthocyanidin contents of the stem samples ($r = 0.94$, $p < 0.05$).

HPLC Analysis of Monomeric and Oligomeric Flavan-3-ol. The monomeric and dimeric flavan-3-ol composition in stem samples is shown in Table 1. All of the extracts were analyzed by HPLC to identify and quantify the monomers (+)-catechin and (−)-epicatechin, the dimers B1, B2, B3, and B4, and the trimer C1.

Under the described chromatographic conditions, flavan-3-ols were eluted in the following order: procyanidin B1, procyanidin B3, (+)-catechin, procyanidin B2, and (−)-epicatechin. In contrast with previously reported results for the stem byproducts from Tinta Miúda variety,[9] the procyanidin B4 and the trimer C1 were not detected in any of the stem samples analyzed. The total content of flavan-3-ols in stem samples, calculated by adding up the individual concentration of each above-mentioned compound, ranged from 28.8 to 366.3 mg/100 g dm for Macabeu and Tempranillo varieties, respectively. Such a large interval revealed significant differences among the 10 varieties considered ($p < 0.05$). Specifically, stems from Macabeu, Merlot, and Chardonnay were found to be particularly poor in total flavan-3-ol content, whereas Tempranillo, Parellada, Syrah, and Premsal blanc stems, in this order, presented the highest values. In terms of distribution of the individual compounds, the flavan-3-ol profile presented a higher dimeric than monomeric fraction in all cases, varying from 57 to 70% of the total flavan-3-ols quantified, except for Chardonnay stems, which presented an equal contribution of both fractions. In previously reported data for stems of the Tinta Miúda grape variety,[9] the same general trend of distribution pattern and proportion of both monomeric and dimeric fractions was described.

A general flavan-3-ol trend persisted throughout all of the varieties considered, apart from Chardonnay stems. The most abundant flavan-3-ol was procyanidin B1, accounting for 70−90% of the total dimeric fraction and from 40 to 60% of the total flavan-3-ol content. The monomer (+)-catechin was the second main component, the contribution of which to the total flavan-3-ol content varied between 30 and 42%. As previously observed,[27] when only the monomeric fraction was considered, (+)-catechin was the major flavan-3-ol monomer in grape stems. Nevertheless, in the case of Callet stems, the percentages of procyanidin B1 and (+)-catechin were balanced, whereas for Chardonnay stems, (+)-catechin predominated. With regard to the procyanidin B3, this dimer displayed moderate values from 4.1 to 23.2 mg/100 g dw for Merlot and Tempranillo stems, respectively. Meanwhile, the procyanidin B2 and the monomer (−)-epicatechin were present as minor constituents of stem byproducts, with concentrations lower than 4% of the total flavan-3-ol content. No quantifiable amounts of the procyanidin B2 were found in stems from the Syrah variety.

As observed in Table 1, significant differences ($p < 0.05$) could be noted with regard to the amount of each compound, which has previously been considered to be related to the cultivar.[28] These differences permitted the establishment of a particular flavan-3-ol profile for stems of each grape variety, considered in terms of quantification of the individual compounds. No references to the flavan-3-ol profile of stem byproducts have been found in the literature, apart from that of Sun et al.,[9] whose calculations were based on a fresh matter basis; those of Alonso et al. and Souquet et al.,[3,27] who reported only (+)-catechin and (−)-epicatechin concentrations; and that of Anastasiadi et al.,[10] who also studied the content of procyanidins B2 and B3. In any case, the results obtained in the present research were in broad agreement with these references, according to the order of magnitude of the values and the ranking order of the flavan-3-ol compounds. It is noteworthy to highlight that this is the first time in the literature that a comparative study of the stem flavan-3-ol profile has been performed for several grape varieties in a detailed form.

HPLC Analysis of Mean Degree of Polymerization. Results of mDP and structural composition of stem proanthocyanidins after phloroglucinolysis are presented in Table 2. The experimental mDP values ranged from 4.6 for Chardonnay to 6.9 for Tempranillo, apart from Premsal blanc autochthonous variety, which exhibited a significantly higher value of 8.5. Significant differences ($p < 0.05$) were observed among varieties. However, results showed that mDP could not be used as a factor to differentiate white from red varieties. In general, mDP results obtained for the stem extracts consistently agree with the scarce mDP data reported in the literature for this winemaking byproduct.[27,29,30] Nevertheless, the mDP values measured for both Chardonnay and Merlot stem extracts are slightly lower than that previously observed for the same varieties.[27] These differences could be due to the different vintages, vinegrowing region, and analytical technique used, because the present research carried out the phloroglucinolysis method to calculate the mDP, whereas Souquet et al.[27]

Table 3. Antioxidant Capacity Determined by ABTS, CUPRAC, FRAP, and ORAC Assays for the Stem Samples[a]

	ABTS	CUPRAC	FRAP	ORAC
Cabernet Sauvignon	168.9 ± 5.9 a	226.9 ± 14.8 a	114.8 ± 3.5 a	157.2 ± 20.7 a
Callet	253.2 ± 14.8 b	378.6 ± 16.2 b	170.1 ± 16.8 b	282.1 ± 35.5 b
Manto Negro	198.2 ± 3.7 c	274.2 ± 5.3 c	134.6 ± 9.5 c	192.9 ± 26.3 c
Merlot	109.8 ± 2.0 d	160.6 ± 5.1 d	76.6 ± 2.5 d	101.9 ± 8.5 d
Syrah	203.1 ± 11.8 c	280.5 ± 7.7 e	155.3 ± 7.6 e	243.5 ± 18.1 e
Tempranillo	186.8 ± 11.3 e	254.9 ± 17.2 f	127.4 ± 11.1 c	173.9 ± 18.3 ac
Chardonnay	99.7 ± 4.6 f	145.4 ± 7.2 g	65.4 ± 5.0 f	125.1 ± 11.5 f
Macabeu	131.7 ± 1.9 g	229.2 ± 3.5 a	85.5 ± 7.4 g	146.6 ± 15.3 a
Parellada	223.4 ± 2.3 h	309.9 ± 11.4 h	159.1 ± 4.3 e	224.6 ± 33.4 e
Premsal blanc	218.5 ± 9.0 h	298.5 ± 15.8 h	169.1 ± 13.4 b	143.2 ± 19.6 af

[a]Antioxidant capacities expressed as equivalents of mg Trolox/g dm; letters after the values in each column show the significant differences among grape varieties ($p < 0.05$).

performed the thiolysis method. It is important to point out that, apart from Chardonnay, Merlot, and Cabernet Sauvignon, the stem proanthocyanidin profile of the other seven varieties has not been reported before.

The polymeric proanthocyanidin fraction of all stem samples was mainly constituted by (−)-epicatechin (EC), the proportion of which reached 70−80% of the total polymeric composition, depending on the grape variety. (+)-Catechin (C) was the second main constituent with concentrations around 3-fold lower than those of (−)-epicatechin. In contrast with previous studies,[27,30] (−)-epigallocatechin (EGC) was not detected in any stem extract. However, three terminal subunits were detected in the stem polymeric proanthocyanidins: (+)-catechin, the most abundant one; (−)-epicatechin, the concentration of which could not be quantified for 6 of the 10 grape varieties considered; and (−)-epicatechin-3-*O*-gallate (ECG), which contributed no more than ∼6% in all cases.

The red grape varieties Cabernet Sauvignon, Merlot, Manto Negro, and Syrah presented the same composition for terminal subunits, with the highest (+)-catechin concentrations among the 10 varieties considered. In contrast, stems from the Macabeu variety presented the major terminal (−)-epicatechin participation (∼11%, $p < 0.05$). With regard to the extension subunits, (−)-epicatechin predominated, accounting for between 84 and 93% of the total, according to the grape variety, whereas the percentage of (+)-catechin was lower, representing the rest of the chain subunits. Significant differences ($p < 0.05$) were observed among varieties, noting particularly the low extension (+)-catechin content of Syrah and Tempranillo stems (7.5% in both cases) in comparison with that of Premsal blanc stems, which was 2-fold higher. The stem polymeric proanthocyanidin profile presented in this research can be compared with those previously obtained for the same or different varieties.[27,30]

Antioxidant Capacity. To achieve a greater and more complete vision of the antioxidant capacity of the stem extracts, four different assays were used in the present study: ABTS, CUPRAC, FRAP, and ORAC. The results are shown in Table 3. With regard to the ABTS assay, the greatest antioxidant potential was observed in Callet stems. With an ABTS value of 253.2 ± 14.8 mg Trolox/g dm, Callet stem extracts showed 2.5 times higher antioxidant capacity than that observed for Chardonnay stem extracts, which presented the lowest ABTS value (i.e., 99.7 ± 4.6 mg Trolox/g dm). Significant differences ($p < 0.05$) were detected among the 10 varieties evaluated. However, when the red and white varieties were considered separately, stems did not differ significantly ($p > 0.05$) as observed above for the total phenolic and total proanthocyanidin contents and phenolics composition.

Similarly to the total phenolic and the total proanthocyanidin contents, it is worth noting that four of the five stem extracts exhibiting the highest antioxidant activity measured by ABTS assay were from autochthonous grape varieties. Only Syrah stems intermingled their values as indicated in the following ranking order: Callet > Parellada = Premsal blanc > Syrah = Manto Negro. Meanwhile, Cabernet Sauvignon, Macabeu, Merlot, and Tempranillo stem extracts exhibited intermediate antioxidant abilities according to the ABTS results.

CUPRAC and FRAP values for stem extracts are also depicted in Table 3. As observed, because each assay is based on a different chemical system and/or reaction, antioxidant activity values clearly varied for each stem extract depending on the method used.[15] The antioxidant ability of stem extracts determined as cupric reducing antioxidant capacity (CUPRAC) and as ferric reducing antioxidant power (FRAP) ranged from 145.4 ± 7.2 to 378.6 ± 16.2 mg Trolox/g dm and from 65.4 ± 5.0 to 170.1 ± 16.8 mg Trolox/g dm, respectively. Similar to the ABTS results, Callet stem extracts yielded the highest antioxidant capacity values for both CUPRAC and FRAP methods, whereas Chardonnay exhibited the lowest ones. Even though slight differences were observed in the ranking order for the intermediate values, stems from the four autochthonous varieties still kept their position in the top five of the highest antioxidant activity according to the CUPRAC and FRAP assays. Overall, a similar behavior pattern was observed for the antioxidant capacity of the stem extracts considered, measured by either ABTS, CUPRAC, or FRAP assays.

However, looking at the ORAC values, major differences were detected when compared with the general tendency described by the three previous assays. This discrepancy might be due to the different chemical mechanism of the ORAC assay, which consists of the ability of the sample antioxidants to act as hydrogen donors. Here, Merlot stem extracts, rather than Chardonnay, were found to present the lowest antioxidant potential value (101.9 ± 8.5 mg Trolox/g dm) determined as ORAC; once again, Callet stem extracts clearly stood out from the others for their highest antioxidant capacity (282.1 ± 35.5 mg Trolox/g dm).

To date, investigation of the antioxidant capacity of stem byproducts is very scarce. Furthermore, in addition to the variety of methods that can be used to evaluate it, there is a serious lack of standardization in the assays, making comparisons between the experimental and those values recorded in the literature quite difficult to establish. Alonso et

al.[3] proposed antioxidant capacity ranges (around 90−215, 60−165, and 90−315 mg Trolox/g dm, respectively) measured by ABTS assay, for stems from Cabernet Sauvignon, Tempranillo, and Syrah varieties cultivated under both irrigated and nonirrigated conditions. These are in close agreement with those described in the present study for the same grape varieties and method. Although the DPPH assay was not performed in this research, some authors have reported antioxidant capacity values of stem byproducts by using this analytical method. Because its action mechanism is based on a single-electron transfer as in ABTS, CUPRAC, and FRAP assays, it could be interesting to establish rough comparisons with their values. An antioxidant activity of 495 mg Trolox/g dm has been previously reported in the literature for Manto Negro stem extracts,[20] being 2.5, 1.8, and 3.7 times higher than the values measured by ABTS, CUPRAC, and FRAP assays, respectively, for the same grape variety in the present study. However, the DPPH value described by the same authors for Premsal blanc stems[21] (290 mg Trolox/g dm) was very similar to the CUPRAC value stated in Table 3 for this variety.

To evaluate the uniformity of the expression of the stems' antioxidant capacity based on the four assays applied, Pearson's correlation coefficients were calculated. All combinations among the different methods revealed a high, significant, and positive correlation ($r \geq 0.76$, $p < 0.05$), suggesting that they give comparable and interchangeable values in characterizing the antioxidant capacity of stems. The data obtained using the ABTS and both the CUPRAC and FRAP assays showed the best correlation coefficients ($r = 0.97$ and $r = 0.98$, respectively), although strong correlation was also observed between CUPRAC and FRAP methods ($r = 0.93$). Moreover, the fact that the three assays are based on the same antioxidant action mechanism, mediated by a single-electron transfer, could explain these significant correlations. Further study of the correlations among antioxidant assays revealed that ORAC data correlated less well than the others. This was exhibited by Pearson's correlation coefficients of 0.82, 0.85, and 0.76 when considering the ABTS, CUPRAC and FRAP assays, respectively. These results could be due to the fact that the ORAC assay presents an action mechanism based on a hydrogen transfer, different from that used for the others.

Various authors have reported correlations between the antioxidant ability results found after evaluation with different methods. As generally observed, antioxidant capacity techniques such as ABTS and FRAP are high and positively correlated ($r \geq 0.92$, $p < 0.05$) on a wide range of food products.[15,19,31,32] With regard to the CUPRAC method, comparison of the antioxidant capacity values of grape seed powder based on ABTS and CUPRAC assays[33] denoted a correlation coefficient between them ($r = 0.87$, $p < 0.05$) lower than that reported in the present study. Furthermore, when ABTS was compared to ORAC data in this research, the correlation degree was in agreement with the upper side of a wide range of correlation coefficients that has been reported in the literature depending on the food product tested, varying from <0.10[34] to 0.90.[35] With regard to the FRAP assay, the correlation coefficient with ORAC was slightly higher than those observed by other authors.[19,32] To justify this variance in the bibliography, some authors suggested that the complexity of the sample, containing antioxidants of very different kinds (hydrophilic and lipophilic origin), causes a lower correlation between ORAC and any single-electron transfer assay, because of the different kinetics and reaction mechanisms of the various antioxidants present.[35]

Regardless of the antioxidant capacity assay used, from Tables 1 and 3, it was observed that samples with the highest total phenolic content also presented the highest antioxidant capacity values. Thus, Pearson's correlation coefficients between total phenolics and antioxidant capacity data were found to be high and statistically significant ($r \geq 0.88$, $p < 0.05$). The most important link was observed between total phenolic content and the CUPRAC assay ($r \geq 0.97$, $p < 0.05$). As expected, these results correspond with the claims that the phenolic content of the plant extracts contributes substantially to their antioxidant capacity.[3,5,19,31] A high positive correlation was also observed between the antioxidant capacity and the total proanthocyanidin content of the stem extracts ($r \geq 0.97$, $p < 0.05$), slightly lower when compared to ORAC data ($r = 0.80$, $p < 0.05$).

To the best of the authors' knowledge, stem byproducts have not been previously studied in such a detailed form, either in terms of their phenolic composition (total phenolic and total proanthocyanidin contents or flavan-3-ol and proanthocyanidin profiles) and antioxidant properties or in the number of stem samples considered from different grape varieties, most of them examined in the present study for the first time in the literature.

Stems from the 10 grape varieties considered presented high total phenolic contents ranging from 4704 ± 288 to 11525 ± 886 mg GA/100 g dm for Merlot and Callet varieties, respectively. Total proanthocyanidin content was also important, with values from 79.1 ± 5.9 mg/g dm for Chardonnay stems to 202.3 ± 6.2 mg/g dm for Callet stems. A particular flavan-3-ol profile was observed for stems of each grape variety considered in terms of quantification of the individual compounds, whereas a general trend persisted throughout all of the varieties with the procyanidin B1 standing out clearly as the main flavan-3-ol component. Regardless of the grape variety studied, stem byproducts denoted great antioxidant capacities, the values of which depended on the assay used. No significant differences ($p > 0.05$) were observed when stems from red and white varieties were considered separately.

On the whole, the data discussed above clearly suggest that stem byproducts are a potential and promising source of polyphenols with a high antioxidant capacity. Such data, along with the dietary fiber characterization of stem byproducts previously evaluated for the same 10 grape varieties,[36] result in a complete and useful database for industrial food processing, to upgrade the value of these winemaking residues, improve waste management, and reduce the environmental problems associated with wine production. Stem byproducts have been demonstrated to have high phenolic and tannin contents and high antioxidant capacity, which makes them suitable for a wide range of applications as ingredients of functional or enriched foods. Furthermore, because a large variability was observed in the phenolic profile and/or quantitative amounts of the main components among the 10 grape varieties considered, the present research provides useful information for selecting the most suitable stem byproduct depending on the phenolic compounds or antioxidant properties required.

It is noteworthy that the three autochthonous varieties from the Balearic Islands (Callet, Manto Negro, and Premsal blanc) and that of Catalonia (Parellada) stand out clearly from the others as the most promising polyphenol-rich stem byproducts, especially in the case of the Callet variety. The Syrah variety also exhibited high total phenolic and total proanthocyanidin contents, as well as a great antioxidant capacity, comparable to those of some autochthonous grape varieties.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+34) 971172757. Fax: (+34) 971173426. E-mail: susana.simal@uib.es.

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