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Monomeric, Oligomeric, and Polymeric Flavan-3-ol Composition of Wines and Grapes from *Vitis vinifera* L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon

MARÍA MONAGAS,† CARMEN GÓMEZ-CORDOVÉS,*,† BEGOÑA BARTOLOMÉ,† OLGA LAUREANO,‡ AND JORGE M. RICARDO DA SILVA‡

Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain, and Laboratorio Ferreira Lapa (Sector Enología), Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisbon, Portugal

The monomeric, oligomeric, and polymeric flavan-3-ol composition of wines, grape seeds, and skins from *Vitis vinifera* L. cv. Graciano, Tempranillo, and Cabernet Sauvignon has been studied using (1) fractionation by polyamide column chromatography followed by HPLC/ESI-MS analysis, (2) fractionation on C_{18} Sep-Pak cartridges followed by reaction with vanillin and acid-catalyzed degradation in the presence of toluene-α-thiol (thiolysis). The content of monomers ((+)-catechin and (−)-epicatechin), procyanidin dimers (B3, B1, B4, and B2), trimers (T2 and C1), and dimer gallates (B2-3-*O*-gallate, B2-3′-*O*-gallate, and B1-3-*O*-gallate) ranged from 76.93 to 133.18 mg/L in wines, from 2.30 to 8.21 mg/g in grape seeds, and from 0.14 to 0.38 mg/g in grape skins. In wines, the polymeric fraction represented 77−84% of total flavan-3-ols and showed a mean degree of polymerization (mDP) value of 6.3−13.0. In grapes, the polymeric fraction represented 75−81% of total flavan-3-ols in seeds and 94−98% in skins and showed mDP values of 6.4−7.3 in seeds and 33.8−85.7 in skins. All the monomeric flavan-3-ols and oligomeric procyanidins found in wines were also present in seeds, although differences in their relative abundances were seen. The skin polymeric proanthocyanidins participated in the equilibration of the wine polymeric proanthocyanidin fraction, especially contributing to the polymer subunit composition and mDP.

KEYWORDS: Proanthocyanidins; flavan-3-ol; monomers; oligomers; polymers; thiolysis; mDP; wine; grapes; extraction; solid-phase extraction

INTRODUCTION

Proanthocyanidins, also called condensed tannins, are transferred from the solid parts of the grape (skins, seeds, and stems) into the must during winemaking operations (crushing, maceration, and fermentation). They are partly responsible for the wine organoleptic properties, including color, astringency, and bitterness, as well as for the physiological effects associated with its consumption (*1*−*6*). Proanthocyanidins participate in oxidative and enzymatic browning reactions, in haze formation and interactions with proteins, and in condensation reactions during the wine aging process (*3*, *7*, *8*).

The quantity, structure, and degree of polymerization of grape proanthocyanidins differ, depending on their localization in the grape tissues (*9*−*16*). While seed tannins are oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate linked by C4−C8 and/or C4−C6 bonds (B type) (*12*), skin tannins also contain (−)-epigallocatechin and trace amounts of (+)-gallocatechin and (−)-epigallocatechin gallate (*14*, *15*). Therefore, wine contains both procyanidins and prodelphinidins (*17*, *18*). The seeds contain higher concentrations of monomeric, oligomeric, and polymeric flavan-3-ols than the skins (*9*, *11*, *14*, *19*−*21*). However, the skin tannins have a much higher degree of polymerization than that from the seeds (*15*, *22*, *23*) and are more easily transferred into wine (*19*). Whereas it is known that the proanthocyanidin concentration of wines is mainly determined by the grape proanthocyanidin content and by other factors such as the extraction or winemaking techniques and the aging conditions (*9*, *11*, *24*−*26*), the structural features (composition, degree of polymerization, galloylation, cis/trans ratio, etc.) of wine proanthocyanidins have been less studied than those of the solid parts of the grapes.

The aim of this paper was to determine the monomeric, oligomeric, and polymeric flavan-3-ol composition of wines from *Vitis vinifera* L. cv. Graciano and Tempranillo, Spanish original varieties (also known as Tinta Miúda and Tinta Roriz or Aragonêz in Portugal, respectively), and Cabernet Sauvignon, all equally elaborated from grapes from the same geographical

* To whom correspondence should be addressed. Tel: (34) 91 562 29 00. Fax: (34) 91 564 48 53. E-mail: cgcordoves@ifi.csic.es.
† Instituto de Fermentaciones Industriales, CSIC.
‡ Instituto Superior de Agronomia, Universidade Técnica de Lisboa.

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area and vintage. For this, a combination of techniques has been used: (1) fractionation by polyamide column chromatography followed by HPLC/ESI-MS analysis and (2) fractionation on C_{18} Sep-Pak cartridges followed by reaction with vanillin and acid-catalyzed degradation in the presence of toluene-α-thiol. The similarities and differences between the flavan-3-ol compositions of wines and of the corresponding grape seeds and skins are discussed.

MATERIALS AND METHODS

Materials. (+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and ethyl gallate were purchased from Extrasynthèse (Genay, France). Procyanidin dimers B1, B2, B3, and B4, B1-3-*O*-gallate, B2-3-*O*-gallate, B2-3′-*O*-gallate, trimer C1 ((−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin), and trimer T2 ((−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(+)-catechin) were previously isolated from a grape seed extract and their structures elucidated as previously described (*10*). Vanillin was obtained from Merck (Darmstadt, Germany) and toluene-α-thiol (benzyl mercaptan) from Fluka (Buchs, Switzerland). Solvents of HPLC grade were purchased from Lab-Scan (Dublin, Ireland) and Sharlau (Barcelona, Spain).

Grape Samples. *Vitis vinifera* L. cv. Tempranillo, Graciano, and Cabernet Sauvignon (vintage 2000) were harvested at their technological maturity from the vineyards of EVENA (Viticulture and Enology Station of Navarra) located in Olite (Navarra, Spain). Two groups of 100 berries, randomly selected from the collected samples of each variety, were used for the study. Grape skins and seeds were manually separated, lyophilized, and frozen at −18 °C under nitrogen for subsequent analysis.

Winemaking. Wines were elaborated at EVENA (Viticulture and Enology Station of Navarra). A lot of 220 kg of grapes of each variety was destemmed, crushed, and collected into 200 L stainless steel wine vats. Semi-industrial scaled fermentations were performed with a yeast inoculum of 25 g/hL (80% EVENA *Saccharomyces cerevisiae* Na33 yeast strain; 20% Lallemand *Saccharomyces bayanus* EC118 yeast strain) at a temperature up to 27 °C. The cap was punched down twice a day until it remained submerged during a 14 day maceration period. At the end of the alcoholic fermentation, the wines were racked and stabilized for a period of 1 month at −2 °C. The wines were then filtered through SEITZ K250 plate filters (2.5−3.0 μm) (Sert Schenk Filter System GmB, Bad Krevznach, Germany) and finally bottled after correcting the free SO_2 level to 30 mg/L. Wines were analyzed after 1.5 years of bottling and storage at 13 °C and 80−85% relative humidity.

Extraction of Phenolic Compounds from Solid Parts of the Grape. Seeds and skins were ground using a coffee-bean miller. Phenolic compounds were extracted following the method described by Bourzeix et al. (*9*). Ground seeds (2.5 g) and skins (7.0 g) were separately immersed in 20 mL of methanol, containing 1 g/L of ascorbic acid to avoid oxidation, and stored at −24 °C for 24 h. The liquid was decanted, and the residue was then extracted with 20 mL of methanol/water (80/20, v/v) for 4 h. After this period, the liquid was again decanted and the residue treated with 20 mL of methanol/water (50/50, v/v) for another 4 h. The process was repeated with 20 mL of distilled water at −24 °C for 15 h. After this period, the residue was finally extracted with 20 mL of acetone/water (75/25, v/v). All the extractions were performed under a nitrogen atmosphere and with no agitation. Extracts were finally combined.

Fractionation of Phenolic Compounds by Polyamide Column Chromatography. Five milliliters (5 mL) of wine and seed or skin extracts (representing 0.14 g of seeds and 0.42 g of skins) were fractionated on a polyamide column (Macherey-Nagel, Duren, Germany) as described by Ricardo da Silva et al. (*27*). Phenolic acids were first eliminated by elution with 80 mL of phosphate buffer, pH 7.0 (67 mM). Monomeric flavan-3-ols were eluted with 50 mL of ethyl acetate/water (30/70, v/v) and oligomeric procyanidins with 50 mL acetone/water (75/25, v/v). The fractions were taken to dryness, dissolved in 1.2 mL of methanol/water (50/50, v/v), filtered through a 0.45 μm membrane, and finally injected into the HPLC column. Polymeric flavan-3-ols remained adsorbed in the polyamide support. A new column was used for each preparation.

HPLC Analysis of Monomeric and Oligomeric Flavan-3-ols. The equipment used for the HPLC analysis consisted of a Konik Instruments UV−vis detector (Uvis 200), a Waters 717Plus autosampler, and a Merck Hitachi L-6200A pump, coupled to a Konikron data treatment system. Separation was performed on a reversed-phase Merck (Darmstadt, Germany) C_{18} Lichrosphere 100 column (250 mm × 4.6 mm, 5 μm) at room temperature. For mononeric flavan-3-ols, a gradient consisting of solvent A (water/acetic acid, 97.5/2.5, v/v) and solvent B (acetonitrile/solvent A, 80/20, v/v) was applied at a flow rate of 0.9 mL/min as follows: 7−25% B linear from 0 to 31 min followed by washing (methanol/water, 50/50, v/v) from 32 to 50 min and reequilibration of the column from 51 to 65 min under initial gradient conditions. For oligomeric procyanidins, a gradient consisting of solvent A (distilled water) and solvent B (water/acetic acid, 90/10, v/v) was applied at a flow rate of 1.0 mL/min as follows: 10−70% B linear from 0 to 45 min, 70−90% B linear from 45 to 70 min, 90% B isocratic from 70 to 82 min, 90−100% B linear from 82 to 85 min, 100% B isocratic from 85 to 90 min, followed by washing (methanol/water, 50/50, v/v) from 91 to 100 min and reequilibration of the column from 101 to 120 min under initial gradient conditions. Detection was performed at 280 nm. Identification and quantification of monomeric flavan-3-ols and oligomeric procyanidins was carried out as previously described (*10*, *27*, *28*).

High-Performance Liquid Chromatography-Electrospray Mass Spectrometry (HPLC/ESI-MS). A Hewlett-Packard series 1100 (Palo Alto, CA) chromatography system equipped with a diode array detector (DAD) and a quadrupole mass spectrometer (Hewlett-Packard series 1100 MSD) with an electrospray interface was used. Separation was performed on a reversed-phase Waters Nova-Pak C_{18} (300 mm × 3.9 mm, 4 μm) column at room temperature. The solvent gradient described above for oligomeric procyanidins was applied at a flow rate of 0.7 mL/min. DAD detection was performed from 220 to 380 nm. The ESI parameters were as follows: drying gas (N_2) flow and temperature, 10 L/min and 340 °C; nebulizer pressure, 40 psi; capillary voltage, 4000 V. The ESI was operated in negative mode, scanning from *m*/*z* 100 to 3000 using the following fragmentation program: from *m*/*z* 0 to 200 (100 V) and from *m*/*z* 200 to 3000 (200 V).

Fractionation of Phenolic Compounds by C_{18} Sep-Pak Cartridges. Wines and seed or skin extracts (prepared as described above under Extraction of Phenolic Compounds from solid Parts of the Grape) were fractionated by Waters C_{18} Sep-Pak cartridges (Waters, Milford, MA) following the method described by Sun et al. (*29*). Five mililiters (5 mL) of wine and seed or skin extracts were concentrated to dryness using a rotary evaporator at <30 °C. The residue was dissolved in 20 mL of phosphate buffer pH 7.0 (67 mM). When necessary, the pH was adjusted to pH 7.0 with NaOH or HCl solutions. Two C_{18} Sep-Pak cartridges connected in series (top, Waters Sep-Pak Plus tC_{18} environmental cartridge; bottom, Waters Sep-Pak Plus tC_{18} cartridge) were conditioned with methanol (10 mL), distilled water (2 × 10 mL), and phosphate buffer pH 7.0 (10 mL). The samples were then passed through the cartridges at a flow rate not higher than 2 mL/min. Phenolic acids were first eliminated by elution with 10 mL of phosphate buffer at pH 7.0. After the cartridges were dried with N_2, elution of monomeric and oligomeric flavan-3-ols (fractions FI + FII) was carried out with 25 mL of ethyl acetate, followed by the elution of polymeric proanthocyanidins (fraction FIII) with 15 mL of methanol. The ethyl acetate fraction was taken to dryness under vacuum, redissolved in 3 mL of phosphate buffer, pH 7.0, and finally redeposited onto the same series of cartridges preconditioned as described above. The cartridges were dried with N_2, and monomers (FI) were separated from oligomers (FII) by sequential elution with 25 mL of diethyl ether and 15 mL of methanol. The three fractions were evaporated to dryness under vacuum and redissolved in 3−5 mL of methanol. Sample fractionation was performed in duplicate. The total flavan-3-ol content of each fraction was determined by the vanillin assay. Polymeric flavan-3-ols (FIII) were also characterized by acid-catalyzed degradation in the presence of toluene-α-thiol.

Determination of the Total Flavan-3-ol Content by the Vanillin Assay. The vanillin assay was performed according to the method described by Sun et al. (*30*). A 2.5 mL portion of H_2SO_4/methanol (25/75, v/v) solution and 2.5 mL of 1% (w/v) vanillin in methanol

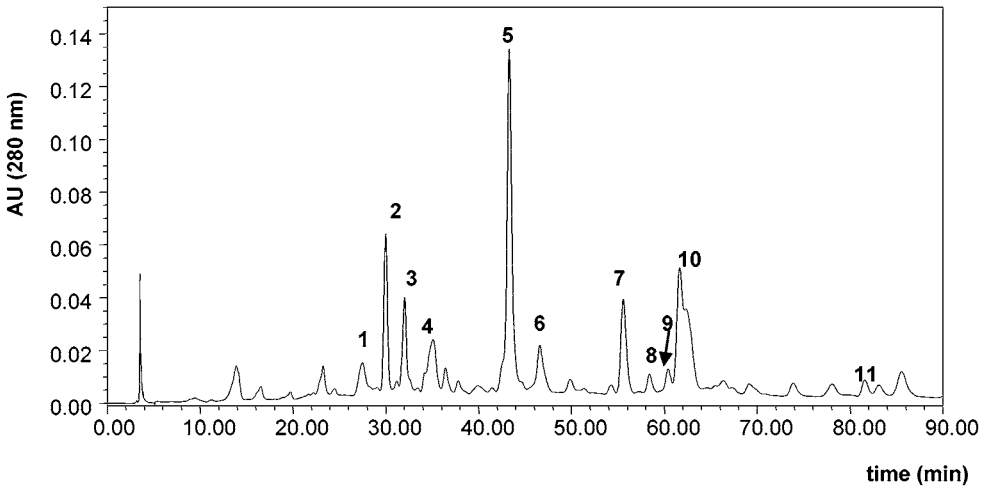

Figure 1. HPLC chromatogram of the oligomeric procyanidin fraction of Graciano grape seeds obtained by polyamide column chromatography at 280 nm. Peak numbers are referenced to **Table 1**.

were added to 1 mL of the sample. A blank was prepared in the same way, but adding methanol instead of vanillin. For FI fractions, the absorbance at 500 nm was read after a reaction time of 15 min at 30 °C using a Unicam UV−vis UV4 spectrophotometer (Unicam, Cambridge, U.K.). For FII and FIII fractions, the reaction was performed at room temperature and left until the maximum absorbance value at 500 nm was reached. Quantification was performed by means of standard curves prepared from monomers (for FI), oligomers (for FII), and polymers of flavan-3-ol (for FIII) isolated from grape seeds, as previously described (*30*). Samples were analyzed in duplicate.

Acid-Catalyzed Degradation of Polymeric Proanthocyanidins (FIII) in the Presence of Toluene-α-thiol. Acid-catalyzed degradation in the presence of toluene-α-thiol was perfomed under the conditions described by Kennedy et al. (*31*), but with some modifications. A 100 μL portion of sample was placed in a 1.0 mL screw-cap vial and mixed with 100 μL of toluene-α-thiol (5% for seed and wine fractions; 12% for skin fractions) in methanol containing HCl (0.2 M). The mixture was placed in a 55 °C water bath for 7 min. The thiolyzed sample was cooled under running water and immediately analyzed by reversed-phase HPLC. The instrument was a Waters (Milford, MA) liquid chromatography system equipped with a 600-MS controller, a 717Plus autosampler, and a 996 photodiode-array detector (DAD). Separation was performed on a reversed-phase Waters Nova-Pak C_{18} column (300 mm × 3.9 mm, 4 μm) at room temperature. A binary gradient consisting of solvent A (water/formic acid, 98/2, v/v) and solvent B (acetonitrile/water/formic acid, 80/18/2, v/v/v) was applied at a flow rate of 1.0 mL/min as follows: 5−30% B linear from 0 to 40 min, 30−50% B linear from 40 to 60 min, 50−80% B from 60 to 70 min, followed by washing (solvent B) and reequilibration of the column from 75 to 97 min. DAD detection was performed from 220 to 600 nm. Peak identification was also performed by HPLC/ESI-MS under the same conditions described above, but applying a flow rate of 0.7 mL/min. The ESI parameters and operation mode were the same previously described under this section. Both flavan-3-ol monomers (terminal units) and toluene-α-thiol adducts (extension units) released from the depolymerization reaction were quantified at 280 nm by calibration curves made from flavan-3-ol standards ((+)-catechin, (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin gallate), as proposed by Kennedy et al. (*31*). Acid-catalyzed degradation was performed in duplicate.

RESULTS AND DISCUSSION

Identification of Monomeric Flavan-3-ols and Oligomeric Procyanidins by HPLC/ESI-MS. Monomeric flavan-3-ols and oligomeric procyanidins fractionated by polyamide column chromatography were identified by means of HPLC/ESI-MS data and by comparison with the retention time and spectral features of flavan-3-ol reference compounds which have been previously isolated from a grape seed extract and their structures elucidated by enzymatic hydrolysis with tannase, by complete acid hydrolysis, by partial acid-catalyzed degradation with phloroglucinol and phenylmethaniol, by FAB-MS, and by ^{1}H NMR (*10*, *28*). The monomeric fraction only contained (+)-catechin and (−)-epicatechin in both seeds and skins. In wines, another compound identified as ethyl gallate (*m*/*z* 197, 169; λ_{max} = 272 nm) was also found. The ion fragment at *m*/*z* 169 corresponded to the gallic acid moiety after the cleavage of the ester bond. The occurrence of ethyl gallate in wine might be associated with the esterification of gallic acid and the ethanol produced during yeast fermentation. Although it has been recently identified in white wines (*32*), to our knowledge this is the first time it has been reported in red wines.

Table 1. Compounds Identified in the Oligomeric Procyanidin Fraction, Obtained by Polyamide Column Chromatography, of Wines and Seeds from Tempranillo, Graciano, and Cabernet Sauvignon Grape Varieties

peak no.	t_R (min)	$[M-H]^-$ (*m*/*z*)	fragments (*m*/*z*)	compd
1	27.5	577	425, 289	procyanidin dimer B3
2	30.0	577	425, 289	procyanidin dimer B1
3	32.0	865	713, 577, 289	procyanidin trimer T2
4	35.1	577	425, 289	procyanidin dimer B4
5	43.3	577	425, 289	procyanidin dimer B2
6	46.6	729	577, 441	procyanidin dimer B2-3-*O*-gallate
7	55.6	729	577, 441	procyanidin dimer B2-3′-*O*-gallate
8	58.4	289		(–)-epicatechin
9	61.6	729	577, 441	procyanidin dimer B1-3-*O*-gallate
10	62.6	865	713, 577, 289	procyanidin trimer C1
11	81.6	441	289, 169	(–)-epicatechin-3-*O*-gallate

HPLC/ESI-MS analysis confirmed the presence of dimeric ($[M-H]^-$ *m*/*z* 577), dimer gallate ($[M-H]^-$ *m*/*z* 729) and trimeric ($[M-H]^-$ *m*/*z* 865) procyanidins (**Figure 1**; **Table 1**). A compound with $[M-H]^-$ at *m*/*z* 441 also confirmed the presence of the monomer (−)-epicatechin-3-*O*-gallate in the oligomeric fraction. Retro-Diels−Alder fission of the heterocyclic ring for dimeric and trimeric procyanidins resulted in the ions *m*/*z* 425 and *m*/*z* 713, respectively (*33*), while the ion corresponding to its subsequent water elimination was only detected for dimeric procyanidins (*m*/*z* 407) (**Figure 2a**,**b**). Under our MS conditions, trimeric procyanidins only underwent one-stage RDA fission, since the characteristic ions (*m*/*z* 425 and 407) that resulted for the dimeric procyanidins

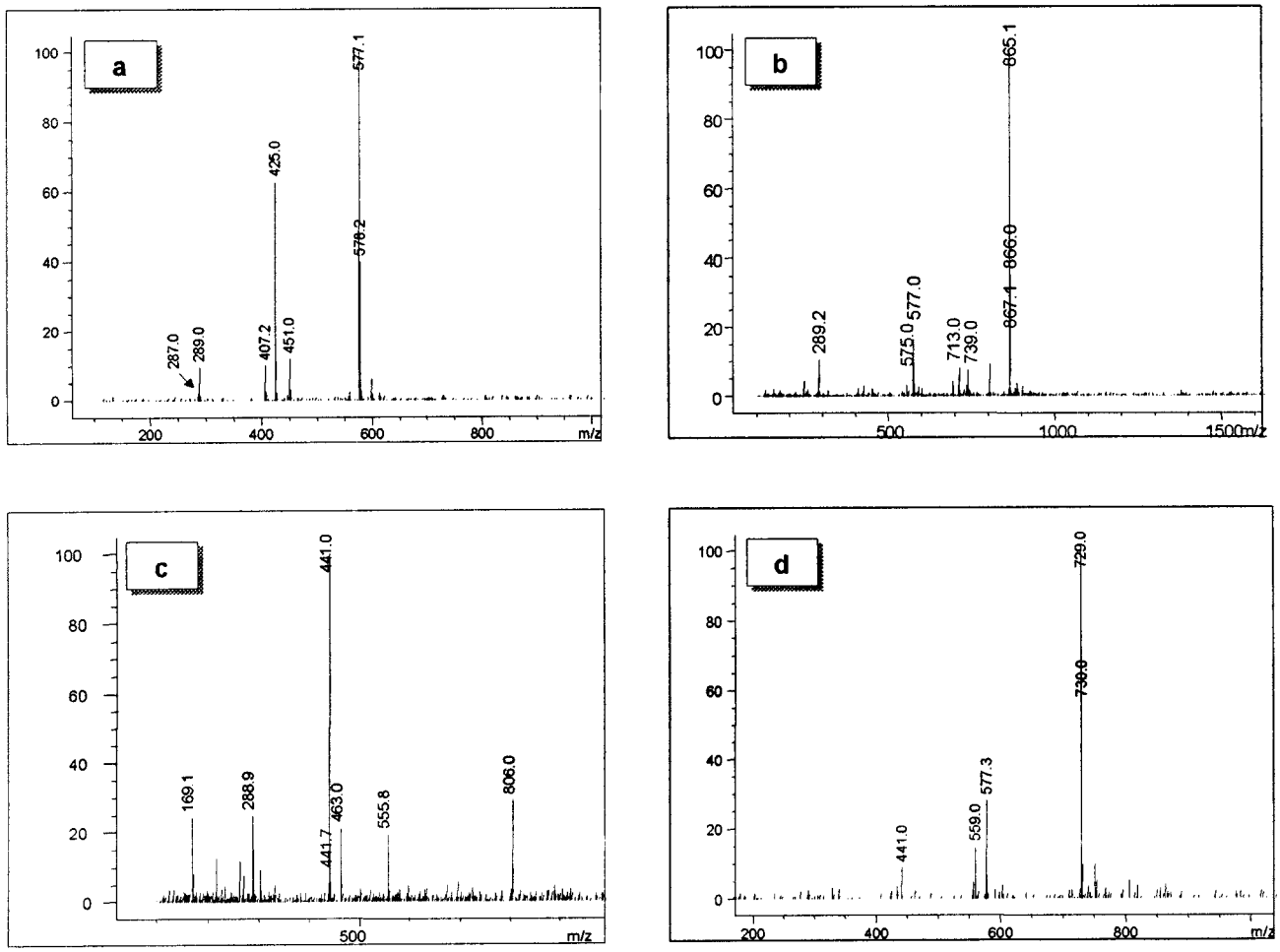

Figure 2. Mass fragmentation pattern of procyanidins under ESI-MS in negative mode: (**a**) dimer; (**b**) trimer; (**c**) (–)-epicatechin-3-*O*-gallate; (**d**) dimer gallate.

were not observed. Ions detected at *m*/*z* 451 (for dimers) and at *m*/*z* 739 (for trimers) resulted from the loss of a fragment equivalent to a phloroglucinol unit ($C_6H_6O_3$) and have been interpreted as an inversion of the reaction involved in the biosynthesis of proanthocyanidins (*34*). Both types of fragmentation are considered to occur in the top unit of the procyanidin molecule (*34*). Interflavanic bond cleavage of dimeric procyanidins through the quinone−methine mechanism resulted in ion fragments detected at *m*/*z* 287 ($[M_{top} - 3H]^-$, methylenic quinone) and *m*/*z* 289 ($[M_{base} - H]^-$, flavan-3-ol monomer) (**Figure 2a**), the latter ion being more abundant than the former one, which also agrees with the observation of other authors (*34*). For trimeric procyanidins, interflavanic fragmentation resulted in the ion fragments detected at *m*/*z* 575 ($[M_{top-middle} - 3H]^-$) and *m*/z 289 (**Figure 2b**). The ion fragment at *m*/*z* 577 corresponded to $[M_{middle-base} - H]^-$, although the other ion generated by this cleavage (*m*/*z* 287) was not detected.

In the case of epicatechin-3-*O*-gallate, fragment ions resulting from the cleavage of the ester bond were detected at *m*/*z* 289 for the (−)-epicatechin unit and at *m*/*z* 169 for the gallic acid moiety (**Figure 2c**). Dimer gallates presented ions corresponding to the loss of a gallic acid moiety (*m*/*z* 577) and to the product of a subsequent water elimination (*m*/*z* 559) (**Figure 2d**). In addition, the product resulting from the loss of a flavan-3-ol unit was also observed at *m*/*z* 441. However, further fragmentation of *m*/*z* 441 into *m*/*z* 289 and 169 was not observed under our conditions.

The confirmation of major monomeric and oligomeric flavan-3-ols by HPLC/ESI-MS in fractions obtained by polyamide column chromatography further enhances the reliability of a method intensively used for the fractionation and quantification of these compounds in grapes and wines (*9*, *11*, *16*, *21*, *26*, *27*).

Monomeric and Oligomeric Flavan-3-ol Composition. In terms of monomeric flavan-3-ols, (+)-catechin was present in higher concentration than (−)-epicatechin for the three wines studied (**Table 2**). Among the oligomeric procyanidins, B1 ((−)-epicatechin-(4β→8)-(+)-catechin) was the most abundant followed by procyanidin B2 ((−)-epicatechin-(4β→8)-(−)-epicatechin), which is in accordance with previous studies (*11*). Concerning the gallates, B2-3-*O*-gallate showed the highest concentration in the different wines. Graciano wines presented the highest levels of total flavan-3-ols (monomers and oligomers), followed by Cabernet Sauvignon and Tempranillo wines (**Table 2**). However, in terms of distribution, Cabernet Sauvignon presented the highest proportion of monomeric flavan-3-ols (47%), followed by Tempranillo (38%) and Graciano (31%) wines. The percentage of procyanidin dimers (B3, B1, B4, and B2) was higher in Graciano and Tempranillo wines (63 and 55%, respectively) than in Cabernet Sauvignon (45%). Cabernet Sauvignon wines presented the highest proportion of dimer gallates, while the trimer content was similar among the different varieties.

In contrast to wines, (−)-epicatechin concentration in seeds was higher or similar to that of (+)-catechin (**Table 2**). Procyanidin B2 was the most abundant dimer in the seeds, as

Table 2. Levels of Monomeric and Oligomeric Flavan-3-ols in Wine, Seeds, and Skins from Tempranillo, Graciano, and Cabernet Sauvignon Grape Varieties[a]

	Tempranillo			Graciano			Cabernet Sauvignon		
	wine	seed	skin	wine	seed	skin	wine	seed	skin
				Monomers and Others[a,b]					
(+)-catechin	17.25 ± 1.17	0.46 ± 0.01	0.061 ± 0.001	28.29 ± 1.91	1.32 ± 0.05	0.104 ± 0.004	29.53 ± 1.59	3.14 ± 0.11	0.206 ± 0.007
ethyl gallate	3.47 ± 0.04	nd	nd	9.57 ± 0.13	nd	nd	3.82 ± 0.23	nd	nd
(−)-epicatechin	12.04 ± 0.76	0.62 ± 0.01	0.079 ± 0.001	13.21 ± 0.38	3.12 ± 0.03	0.235 ± 0.009	12.52 ± 0.56	2.71 ± 0.08	0.171 ± 0.005
(−)-epicatechin-3-*O*-gallate	tr	0.022 ± 0.001	nd	tr	0.027 ± 0.000	nd	tr	0.014 ± 0.001	nd
				Oligomers[a,b]					
procyanidin dimer B3	5.75 ± 0.08	0.15 ± 0.01	nd	0.88 ± 0.04	0.21 ± 0.02	nd	1.93 ± 0.05	0.20 ± 0.01	nd
procyanidin dimer B1	20.24 ± 1.15	0.15 ± 0.01	nd	35.01 ± 0.73	0.44 ± 0.02	nd	24.14 ± 0.33	0.42 ± 0.01	nd
procyanidin trimer T2	1.09 ± 0.18	0.06 ± 0.00	nd	0.67 ± 0.07	0.18 ± 0.01	nd	1.11 ± 0.12	0.18 ± 0.01	nd
procyanidin dimer B4	4.03 ± 0.61	0.20 ± 0.02	nd	16.94 ± 0.76	0.28 ± 0.01	nd	1.94 ± 0.37	0.18 ± 0.01	nd
procyanidin dimer B2	11.95 ± 0.48	0.43 ± 0.02	nd	31.70 ± 0.59	1.29 ± 0.05	nd	12.55 ± 0.65	0.80 ± 0.01	nd
procyanidin dimer B2-3-*O*-gallate	2.95 ± 0.10	0.06 ± 0.01	nd	3.48 ± 0.20	0.15 ± 0.01	nd	4.83 ± 0.68	0.13 ± 0.00	nd
procyanidin dimer B2-3′-*O*-gallate	0.13 ± 0.01	0.05 ± 0.00	nd	0.23 ± 0.01	0.24 ± 0.01	nd	0.27 ± 0.02	0.11 ± 0.01	nd
procyanidin dimer B1-3-*O*-gallate	0.22 ± 0.02	0.003 ± 0.000	nd	0.39 ± 0.01	0.04 ± 0.01	nd	0.21 ± 0.02	0.02 ± 0.01	nd
procyanidin trimer C1	1.27 ± 0.04	0.10 ± 0.01	nd	2.37 ± 0.08	0.53 ± 0.01	nd	0.55 ± 0.01	0.30 ± 0.02	nd
				Totals[b]					
total flavan-3-ols	76.93	2.30	0.14	133.18	7.82	0.34	89.56	8.21	0.38
% monomers	38.1	47.9		31.2	57.1		47.0	71.4	
% dimers	54.6	40.2		63.5	28.4		45.3	19.4	
% monomer gallates		0.9			0.1			0.06	
% dimer gallates	4.3	4.9		3.1	5.5		5.9	3.3	
% trimers	3.1	6.9		2.3	9.1		1.9	5.9	

[a] Abbreviations: nd, not detected; tr, traces. [b] In units of mg/L for wine and mg/g dw for seeds and skins.

reported by other authors (*9*, *11*, *13*, *20*, *21*, *25*, *26*). Among procyanidin dimer gallates, B1-3-*O*-gallate showed lower concentration than any of the other B2-3-*O*-gallates. In terms of distribution, seeds showed a higher percentage of monomers and trimers than wine but a lower percentage of dimers. Among varieties, Graciano and Cabernet Sauvignon presented similar and higher concentration of flavan-3-ols, respectively, when compared to Tempranillo (**Table 2**). Only monomeric flavan-3-ols were detected in the grape skins of the three varieties studied (**Table 2**). Although reported by some authors (*9*, *11*, *14*, *19*, *20*, *35*), skin oligomeric proanthocyanidins have always been difficult to study, due to their low concentration, the high complexity of their crude extracts (which also contain highly polymerized flavan-3-ols, flavonols, and anthocyanins), and their possible bonding with other matrix substances (pectin) (*14*, *36*). The results presented strengthen the idea that the monomeric and oligomeric flavan-3-ols of the seeds could contribute to the flavan-3-ol profile found in the respective wines, if appropriate maceration time and fermentation temperature are applied (*19*). However, it is important to note that these compounds are easily extracted from the skin during must fermentation, also contributing to the wine flavan-3-ol composition (*19*).

Separation of Proanthocyanidins According to Their Degree of Polymerization. In contrast to simple oligomers, polymeric proanthocyanidins (DP > 5) are very difficult to resolve by HPLC techniques since the number of possible isomers increases with the degree of polymerization. Therefore, only fractions containing mixtures of polymers can be isolated, using purification techniques such as normal-phase HPLC, adsorption chromatography on Fractogel TSK HW-40, and C_{18} Sep-Pak cartridges (*12*, *15*, *23*, *29*). **Figure 3** depicts the flavan-3-ol content, determined by the vanillin reaction, of wine, seed, and skin fractions (monomeric, oligomeric, and polymeric) obtained by C_{18} Sep-Pak cartridges. As seen above, Graciano wine presented higher levels of monomeric plus oligomeric flavan-3-ols when compared to Cabernet Sauvignon and Tempranillo wines (**Figure 3a**). In relation to the grape seeds, the vanillin results also revealed that the monomeric and oligomeric flavan-3-ol content of Tempranillo seeds was the lowest one when compared to Graciano and Cabernet Sauvignon (**Figure 3b**). The monomeric and oligomeric flavan-3-ol concentration in the skins was similar for the three varieties studied, with the exception of the oligomeric fraction in Tempranillo (**Figure 3c**).

The polymeric flavan-3-ol fraction represented the highest proportion of the total flavan-3-ols content in the different wines (77−84% of total flavan-3-ols), seeds (75−81%), and skins (94−98%) (**Figure 3**), which agrees with previous studies (*19*). Wine polymeric flavan-3-ol concentration was similar for the three varieties, while in seeds, Graciano content was higher, followed by Cabernet Sauvignon and Tempranillo. The skins showed greater differences among varieties, with Tempranillo standing out when compared to Graciano and Cabernet Sauvignon. It is interesting to note that the lower concentration of monomeric, oligomeric, and polymeric flavan-3-ols presented in Tempranillo seeds was compensated by a respective higher content in the skins when compared to Graciano and Cabernet Sauvignon. Although present in very little concentration, the skin polymers are easily extracted during fermentation, therefore being an important source of proanthocyanidin to the resulting wines (*19*). In contrast, according to Sun et al. (*19*), the seed polymeric fraction does not seem to experience significant variation during fermentation.

Acid-Catalyzed Degradation of Polymeric Proanthocyanidins (Fraction III) in the Presence of Toluene-α-thiol. Acid-catalyzed cleavage in the presence of nucleophilic reagents has revealed important information about polymeric proanthocyanidin subunit composition, mean degree of polymerization, and average molecular mass (*12*, *15*, *22*, *23*). The data concerning the structural composition and characteristics of wine, seed, and skin polymeric proanthocyanidins after toluene-α-thiolysis are

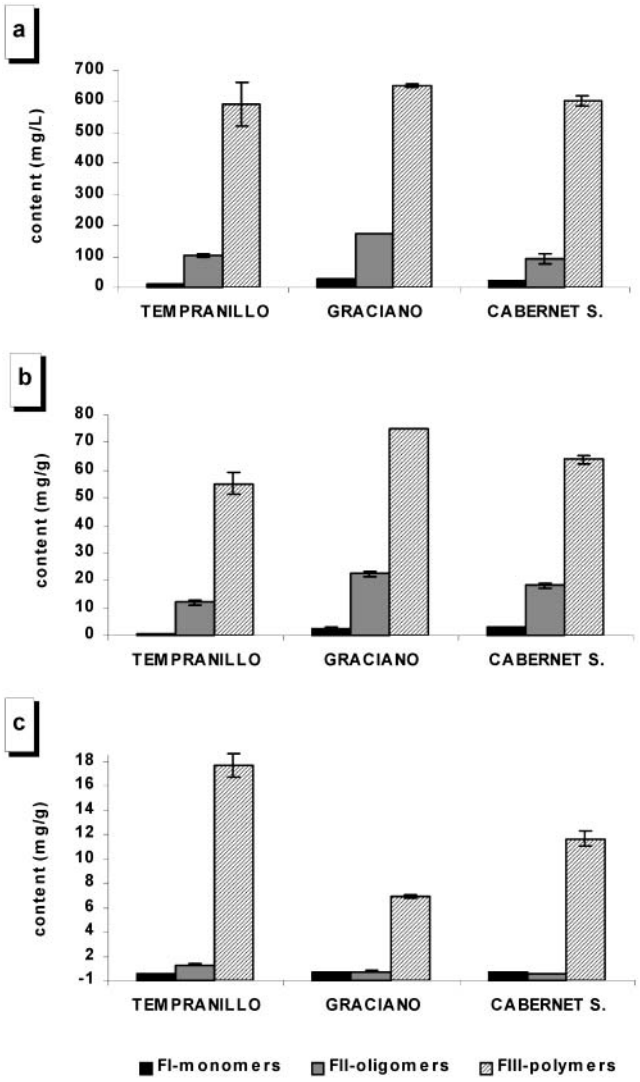

Figure 3. Flavan-3-ol content of the monomeric, oligomeric, and polymeric fractions of (**a**) wine, (**b**) seeds, and (**c**) skins from Tempranillo, Graciano, and Cabernet Sauvignon grape varieties. Bars represent the standard deviation.

presented in **Table 3**. (+)-Catechin (*m*/*z* 289), (−)-epicatechin (*m*/*z* 289), and (−)-epicatechin-3-*O*-gallate (*m*/*z* 441, 289, 169) were found as terminal units, whereas the corresponding benzyl thioethers of (+)-catechin (*m*/*z* 411, 287), (−)-epicatechin (*m*/*z* 411, 287), (−)-epigallocatechin (*m*/*z* 427, 303), and (−)-epicatechin gallate (*m*/*z* 563, 439, 287) were found as extension units.

(−)-Epicatechin was indeed the most abundant subunit in wines, seeds, and skins (**Table 3**). However, in terms of structural composition, wine polymeric proanthocyanidins resembled more the profile found in the skins than that of seeds. This was particularly obvious in the presence of (−)-epigallocatechin as extension unit and in the low proportion of (−)-epicatechin gallate, absent as a terminal unit, in comparison to the seeds. Wine mDP values were similar to or higher than (6.9−13.0) those of seeds (6.4−7.1) but much lower than those of skins (33.8−85.7) (**Table 3**). These results are consistent with data concerning mDP values of polymeric proanthocyanidins reported in the scarce literature found for wines (6.4−15.6) (*17*, *37*) and in the numerous publications found for grape seeds (2.7-18.6) (*11*, *22*, *23*, *31*) from other *V. vinifera* L. varieties. However, literature data concerning mDP values of skin polymeric proanthocyanidins largely vary, ranging from approximately 11 to 83, depending on the fractionating technique employed and on the grape variety and vintage (*15*, *22*, *23*). In addition, the existence of pectin-bond proanthoyanidin polymers in the skins, as well as their possible association with anthocyanins, has been described (*36*) and could influence this measurement.

Table 3. Structural Composition and Characteristics of the Wine, Seed, and Skin Polymeric Proanthocyanidin Fraction (C_{18} Sep-Pak Cartridge, Fraction III) from Tempranillo, Graciano, and Cabernet Sauvignon Grape Varieties[a]

	terminal units			extension units							
	Cat	Ec	EcG	Cat	Ec	EcG	EGC	mDP	aMM	% G	% P
						Wine					
TEM	3.9	3.8	0.0	3.5	74.7	2.8	11.3	13.0	3832.1	2.8	11.3
GRA	6.0	8.6	0.0	2.7	71.8	2.8	8.2	6.9	2017.7	2.8	8.2
CS	6.5	4.9	0.0	3.4	71.2	3.4	10.6	9.0	2647.3	3.4	10.6
						Seed					
TEM	6.1	5.5	2.5	9.6	64.5	11.8	0.0	7.1	2191.8	14.3	0.0
GRA	5.8	5.6	2.3	6.5	71.1	8.7	0.0	7.3	2233.2	10.9	0.0
CS	7.2	6.0	2.4	7.9	66.0	10.5	0.0	6.4	1970.1	12.9	0.0
						Skin					
TEM	1.2	0.2	0.0	3.0	79.4	2.9	13.3	72.3	21302.7	2.9	13.3
GRA	2.5	0.5	0.0	1.2	78.7	6.5	10.7	33.8	10129.9	6.5	10.7
CS	1.1	0.2	0.0	2.1	61.6	3.8	31.2	85.7	25609.0	3.8	31.2

[a] Abbreviations: TEM, Tempranillo; GRA, Graciano; CS, Cabernet Sauvignon; Cat, (+)-catechin; Ec, (−)-epicatechin; EcG, (−)-epicatechin-3-*O*-gallate; EGC, (−)-epigallocatechin; mDP, mean degree of polymerization; aMM, average molecular mass; % G, percentage of galloylation; % P, percentage of prodelphinidins. Terminal and extension units are given in units of proportional composition (mol %).

Differences among the three wines were similar to those presented for skins, in that the polymeric proanthocyanidins from Graciano wine were considerably less polymerized and lower in prodelphidin content when compared to Tempranillo and Cabernet Sauvignon wines. However, in contrast to skins, Tempranillo wine polymers exhibited a higher mDP than Cabernet Sauvignon, which could be associated with the higher concentration of skin polymeric proanthocyanidins of the former when compared to the latter (**Figure 3c**).

In summary, this paper reports valuable data concerning HPLC/ESI-MS fragmentation patterns of oligomeric procyanidins in grape and wine fractions obtained by polyamide column chromatography. Data concerning the flavan-3-ol content and distribution of wines elaborated under the same conditions, and that of respective grape seeds and skins, from three varieties (Tempranillo, Graciano, and Cabernet Sauvignon) cultivated in the same geographical area is given. All the monomeric flavan-3-ols and oligomeric procyanidins found in wines were also present in seeds, although differences in their relative abundance were seen. The skin polymeric proanthocyanidins participated in the equilibration of the wine polymeric proanthocyanidin fraction, especially contributing to the polymer subunit composition and mDP. The distribution of monomers and procyanidin dimers, but not of procyanidin dimer gallates and trimers, was different among the wines studied. In relation to the polymeric fraction, the largest differences among varieties were found in the solid parts of the grape, especially in the skins. This study could find application in winemaking practices, providing guidelines with respect to the regulation of the tannin content of wines according to the grape variety used.

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