J Sci Food Agric 86:915–921 (2006)
DOI: 10.1002/jsfa.2438

Non-galloylated and galloylated proanthocyanidin oligomers in grape seeds from *Vitus vinifera* L. cv. Graciano, Tempranillo and Cabernet Sauvignon

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Abstract: Non-galloylated and galloylated flavan-3-ol composition in seeds from *Vitis vinifera* L. var. Graciano, Tempranillo and Cabernet Sauvignon grapes harvested in 2000, 2001 and 2002 at the same geographical area were determined using normal-phase HPLC coupled with electrospray ionization mass spectrometry (LC/ESI-MS) detection. Non-galloylated and monogalloylated flavan-3-ols up to octamers, and di-, and trigalloylated flavan-3-ols up to heptamers were identified in all grape seeds. Comparisons of the flavan-3-ol composition in three grape varieties harvested in three different years indicate that levels of non-galloylated flavan-3-ols decrease as the degree of polymerization increased, whereas the monogalloylated dimers were present in the highest levels in all varieties and vintages. The levels of other monogalloylated flavan-3-ols varied in different vintages. Tempranillo contained the lowest levels of non-galloylated and monogalloylated flavan-3-ols, whereas Graciano contained the highest levels, with the exception of non-galloylated flavan-3-ols in vintage 2001, and non-galloylated monomers in vintages 2000 and 2002. Grape seeds from vintage 2000 contained the highest levels of both non-galloylated and galloylated structures. Statistical analyses indicate that the distribution of the flavan-3-ols is primarily determined by genetic factors and is also strongly influenced by climate conditions.

Keywords: grape seeds; procyanidin; proanthocyanidin; flavonoids; normal-phase HPLC; mass spectrometry; ESI/MS, LC/MS

INTRODUCTION

Procyanidins, or condensed tannins, are polymeric flavan-3-ol compounds that significantly contribute to critical organoleptic properties such as color, stability, astringency and bitterness of plant-derived foods and beverages.[1–3] Recent studies suggest that procyanidins in food may have health-promoting effects.[4–9] Grape (*Vitis vinifera*) seed are a rich source of procyanidins, and increasingly grape seed extracts are used as active ingredients in a range of nutraceutical products.[10] Grape seed tannins consist of a complex mixture of oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-gallate. Oligomers are linked through the C4–C6 and/or C4–C8 positions (B-type interflavan bonds). The chemical structure of grape seed flavan-3-ols dictates their chemical and physical properties and reactivity. For example, the sensory perception of astringency and bitterness in wine changes in response to the degree of galloylation (DG) and degree of polymerization (DP) of the flavan-3-ols in the wine.[11] Approximately 50% of the flavan-3-ols in red wine are derived from grape seed flavan-3-ols, and these are usually extracted from the grape seeds during the latter stages of wine-making.[12]

The analysis of procyanidins in grape seeds is complex and has relied on reverse-phase HPLC with UV detection at 280 nm.[13–16] This method is successful in separating oligomers of equivalent molecular mass into their isomers up to tetramers. The separation of the higher oligomers (>tetramer) is difficult because the number of isomers concomitantly increases with the degree of polymerization, producing a very broad and unresolved UV-absorbing peak late in the chromatogram. Other methods based on acid-catalyzed degradation in the presence of nucleophilic agents permit quantification of the extension and

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(*Received 22 September 2004; revised version received 9 December 2004; accepted 3 June 2005*)
Published online 1 March 2006

terminal units of grape seed procyanidins, allowing the calculation of the mean DP.[17–20] However, these methods only give average compositional data and provide no information on polymer size distribution.

The most effective HPLC methods for the separation of oligomeric procyanidins (>tetramer) by their DP employs the use of normal-phase chromatography with the UV and fluorescence detection.[21–26] Normal-phase separations on silica allow for the separation of oligomeric procyanidins through decamer based upon their DP. Polymers larger than decamers elute as a single peak at the end of chromatogram. Normal-phase chromatographic methods can separate galloylated forms from non-galloylated forms, since galloylated forms elute later than their non-galloylated counterparts.[27] However, this approach also has limitations as galloylated forms can elute in the region of other oligomers, and because galloylated forms can give a greater UV response than non-galloylated forms.[27,28] Owing to the abundance and complexity of the range of galloylated forms in grape seeds, complete separation and quantitation of procyanidin oligomers based upon their DP has not yet been achieved. Presently, the most effective method for characterizing procyanidins in natural products with a high degree of sensitivity is mass spectrometry (MS) coupled to HPLC.[29–31] The electrospray ionization (ESI) interface is ideally suited for procyanidin analysis using normal-phase LC/MS because it is capable of generating pseudo-molecular ions with no fragmentation from non-aqueous solvents.[32,33] To date, several studies have utilized mass spectral approaches to characterize procyanidins in grape seeds.[22,33–36] For example, Lazarus *et al*.[22] used LC/ESI-MS methods to characterize proanthocyanidins in grape seeds of Pinot Noir, whereas Friedrich *et al*.[33] used this approach to characterize proanthocyanidins extracted from malt. Yang and Chien[34] used HPLC/MS and employed MALDI/TOF MS to characterize proanthocyanidins in grape seeds. Quantitation of higher oligomers, by any method, is still not possible due to a lack of purified standards. In the current study, we employ normal-phase LC/ESI-MS and reconstruction of ion chromatograms to identify and compare relative levels of galloylated and non-galloylated forms of flavan-3-ols in grape seeds from *Vitis vinifera* L. var. Graciano, Tempranillo and Cabernet Sauvignon grapes harvested in 2000, 2001 and 2002 at the same geographical area.

MATERIALS AND METHODS

Chemicals

HPLC-grade acetone, methylene chloride, methanol and acetic acid were obtained from Fisher Scientific (Houston, TX, USA). Reagent-grade, bacteria-free water was generated by a Barnstead E-pure 4-module deionization system (Dubuque, IA).

Grape seeds

Grapes from *Vitis vinifera* L. var. Tempranillo, Graciano and Cabernet Sauvignon were harvested at their technological maturity from vineyards of EVENA (Viticulture and Enology Station of Navarra, Olite, Navarra, Spain) in 2000, 2001 and 2002 vintages. Technological maturity was fixed according to the physical–chemical characteristics of grape varieties for the established vintage schedule in the area. Vines (2.8×1.3 m spacing) were trained to trellis. Row orientation was north–south. Random samples (5 kg) were taken in a 'Z' pattern design to prevent border and center effects. From each row, 50% of the samples were taken from bunches facing east and the other 50% from bunches facing west. For the analysis, 200 berries each were randomly selected from the 5 kg of grapes collected for each grape variety. Grape seeds were manually separated, dried and frozen at −18 °C under nitrogen for subsequent analysis.

Extraction of phenolic compounds

Seeds were ground to a fine powder with a particle size of <50 μm. Lipids from the ground grape seeds (0.35 g) were removed by extracting twice with 15 mL of hexane for 15 min for each time. The lipid-free solid was air-dried and the procyanidins were extracted using 15 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v). The mixture was vortexed and sonicated for 30 min and left for 30 min at room temperature. The extract was then centrifuged at 3000 rpm for 15 min at 20 °C. The supernatant was filtered (Whatman no. 1) and concentrated to 5 mL using a rotary evaporator under partial vacuum at 40 °C. Samples were then freeze-dried. Extraction was performed in triplicate on the starting material. The seed powder (from 0.35 g grape seeds) was reconstituted with 80 mL of water, sonicated (30 min) and filtered (Whatman no. 1 filter). Strata C18-E 6 mL solid-phase-extraction columns (SPE; Phenomenex, Torrance, CA, USA) were used to isolate procyanidins in the reconstituted powder. SPE columns were preconditioned with 15 mL of methanol, followed by 15 mL of water/acetic acid (99.5:0.5 v/v). Samples were loaded onto the preconditioned SPE column and washed with nanopure water (~40 mL). After drying columns under vacuum for 1–2 min, flavan-3-ols were eluted from the SPE column with 6 mL of acetone/water/acetic acid (70:29.5:0.5 v/v/v). The recovery for the SPE column was calculated using a composite standard of procyanidin oligomers extracted from cocoa (unpublished results). The recovery was over 92% for all the flavan-3-ols up to decamers.

Normal-phase LC/ESI-MS analysis of flavan-3-ols

Normal-phase HPLC analysis of the grape seeds and cocoa standard was carried out under conditions similar to those described by Hammerstone *et al*.[29] Procyanidins were separated using

Shimadzu Scientific (Columbia, MD, USA) HPLC equipment and a Phenomenex (Torrance, CA, USA) Luna 5 μm silica column (250 mm × 2.0 mm). The binary mobile phase consisted of solvent A composed of methylene chloride/methanol/water/acetic acid (82:14:2:2 v/v/v/v), and solvent B composed of methanol/water/acetic acid (96:2:2 v/v/v). Separation was performed using a linear gradient of B into A at a flow rate of 0.2 mL min^{-1} as follows: time 0–30 min, 0–18% B; time 30–45 min, 18–31% B; and 45–50 min, 31–88% B. The column was re-equilibrated between injections with the equivalent of 10 mL of the initial mobile phase. The HPLC system was interfaced through an electrospray system (ESI) to a ZSPRAY Micromass Quattro LC (Beverely, MA, USA). The LC/MS conditions were optimized at negative mode as follows: capillary voltage, 3.2 kV; cone voltage, 30V; source temperature, 150 °C; desolvation gas temperature, 300 °C. Ammonium acetate (10 mmol L^{-1}) was employed as an ionization reagent via a tee in the eluant stream at a flow rate of 0.03 mL min^{-1}. MS data were collected from 100 to 3500 *m/z* and processed using MassLynx software (version 3.5).

Determination of flavan-3-ols

Flavan-3-ols were identified by their mass-to-charge ratio (*m/z*) obtained from their mass spectra. Chromatographic peak area corresponding to non-galloylated and galloylated oligomers was integrated by extracting the masses of each oligomeric form from the total ion chromatogram (TIC) and creating reconstructed ion chromatograms (RIC) and summing the area for each DP of the non-galloylated and galloylated oligomers. Multiply charged forms of the each oligomer were also extracted from TIC and corresponding peak areas were added to the total summed area. Compositional differences between varieties and years are based upon this area and are therefore only comparative.

Statistical analysis

ANOVA analysis was performed using the program SPSS for Windows (version 10.0, SPSS, Inc. Chicago, IL, USA) to compare the levels of flavan-3-ols ($n = 3$) among grape varieties and harvest years.

RESULTS AND DISCUSSION

Identification of flavan-3-ols in grape seeds

Due to the weak acidic nature of procyanidins, operating the ESI system in negative ion mode produces proton dissociation much more easily than protonation operating in positive mode and gives simpler mass spectra due to the absence of intense adducts ion species.[29,35,36] ESI generates multiple-charge ions $(M\text{-}zH)^{z-}/z$ with no fragmentation and permits the identification of non-galloylated and galloylated procyanidins with increasing degrees of polymerization.

Non-galloylated and monogalloylated flavan-3-ols, monomers through octamers, and di-, and trigalloylated flavan-3-ols through heptamers were identified in all grape seed extracts of Graciano, Tempranillo and Cabernet Sauvignon grapes (Table 1). Figure 1 demonstrates RICs corresponding to the oligomeric series of non-galloylated flavan-3-ols, monomer through octamer, found in these three grape varieties. RICs were constructed by extracting 289 Da, *m/z* for the flavan-3-ol monomers (i.e. catechin and epicatechin) and 577, 729 Da for dimers, etc. (see Table 1). Mass spectra demonstrate singly charged ions corresponding to the non-galloylated procyanidin oligomers through hexamers. Additionally, spectra demonstrated the presence of procyanidin oligomers through pentamers for mono-, and digalloylated flavan-3-ols, and tetramers for trigalloylated

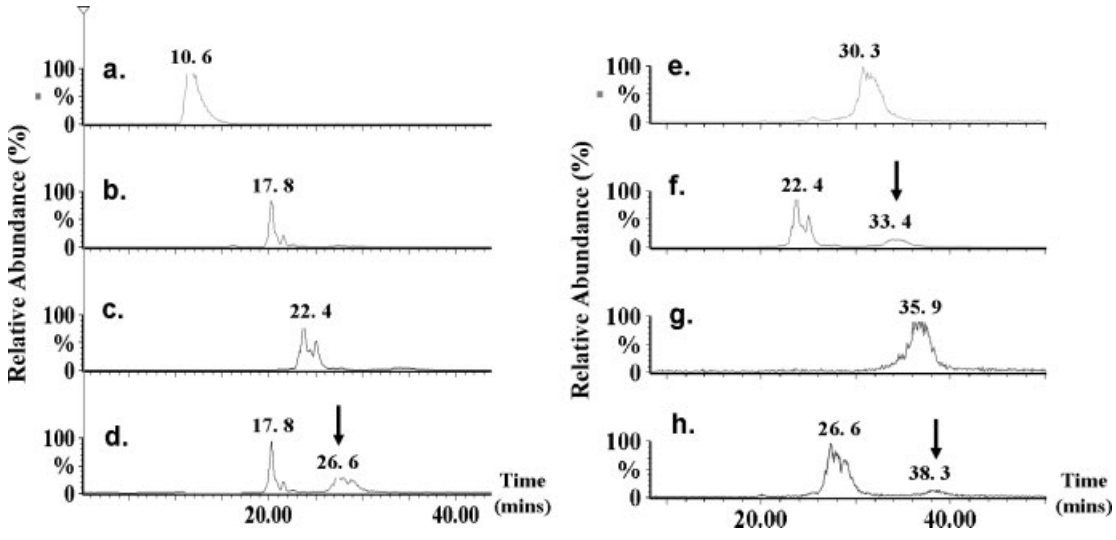

Figure 1. Reconstructed ion chromatograms (RICs) demonstrating t_R corresponding to non-galloylated procyanidin oligomers, indicated by arrows where necessary: (a) monomers $[M-H]^-$ 289 *m/z*; (b) dimers $[M-H]^-$ 577 *m/z*; (c) trimers $[M-H]^-$ 865 *m/z*; (d) tetramers $[M-H]^-$ 1153 *m/z*; (e) pentamers $[M-H]^-$ 1441 *m/z*; (f) hexamers $[M-H]^-$ 1729 *m/z*, (g) heptamers $[M-2H]^-$ 1008 *m/z*; and (h) octamers $[M-2H]^-$ 1152 *m/z*.

Table 1. Distribution of procyanidins in Graciano, Tempranillo and Cabernet Sauvignon grape seeds

	Compound[a]		*m/z* of ions		
R_t (min)	DP	DG	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$
10.6	1	0	289		
16.7	1	1	441		
17.8	2	0	577		
20.7	2	1	729		
22.4	3	0	865		
23.0	2	2	881		
25.4	3	1	1017		
26.6	4	0	1153	576	
27.8	3	2	1169	584	
28.8	4	1	1305	652	
30.3	5	0	1441	720	
30.5	3	3	1321	660	
31.9	4	2	1457	728	
32.6	5	1	1593	796	
33.4	6	0	1729	864	
33.5	4	3	1609	804	
35.5	5	2	1745	872	
35.6	6	1		940	
35.9	7	0		1008	
36.5	5	3		948	
36.5	6	2		1016	
37.2	7	1		1084	
38.3	8	0		1152	768
39.6	6	3		1092	
39.6	7	2		1160	773
41.0	8	1		1228	819
41.2	7	3		1236	824

[a] DP, degree of polymerization; DG, degree of galloylation.

procyanidins. Doubly charged ions (separated by 144 Da) (Table 1) were observed in tetramers through octamers for non-galloylated and monogalloylated procyanidins and in trimers through heptamer for di-, and trigalloylated procyanidins (Table 1). Triply charge ions were seen for non-galloylated and monogalloylated octamers and di- and trigalloylated heptamers (Table 1). Lazarus *et al.*[22] reported the same singly and multiply charged species in grape seed supplement pills analyzed by HPLC/ESI/MS, but Yang and Chien[34] only detected up to trimer signals in a grape seed extract.

In the current study, we distinguished between singly charged and multiply charged species having the same or similar molecular masses (e.g. the doubly charged digalloylated hexamer at *m/z* 1016 and the singly charged monogalloylated trimer at *m/z* 1017), based on differences in the HPLC retention times of these compounds.

Flavan-3-ol composition

The ESI/MS response of a procyanidin oligomer may be influenced by their size and structure (i.e. galloylation and linkages). Complete quantification of procyanidin oligomers has not yet been achieved as reference standards of non-galloylated and galloylated oligomers are not available.[29,30,32–36] To date, quantification of oligomeric procyanidins by normal-phase HPLC/ESI-MS has been limited to non-galloylated procyanidin oligomers since these can be standardized against non-galloylated procyanidin isolated from cocoa.[23–26,31,37] In this study, the peak area obtained from RICs is used to make relative comparisons of levels of oligomers (non-galloylated flavan-3-ols up to hexamers and mono-galloylated up to pentamers) present in seeds from different grape varieties (Graciano, Tempranillo and Cabernet Sauvignon) of vintages 2000, 2001 and 2002 (Fig. 2). The average variation coefficient from these three varieties was ~13%.

Non-galloylated flavan-3-ols

In all three grape varieties and vintages studied, as the DP increased the levels of non-galloylated flavan-3-ols decreased (Fig. 2). Regardless of the vintage, Tempranillo had the lowest levels of flavan-3-ol monomers and of procyanidin oligomers. For vintages 2000 and 2002 Graciano grapes had the highest levels of the different flavan-3-ols, excluding monomers. For the vintage 2001, Cabernet Sauvignon contained the highest levels of flavan-3-ol monomers and procyanidin oligomers (Fig. 2). With respect to the vintages, the levels of non-galloylated flavan-3-ols oligomers followed the same trend for these three varieties. Vintage 2000 had the highest levels of procyanidin oligomers as compared to vintages 2001 and 2002. These results indicate that flavan-3-ol biosynthesis is dependent on both variety and vintage.

Two-way ANOVA analysis was applied to the data to assess the variety and climate influence on the flavan-3-ol composition of grape seeds. Significant differences in variety ($F > 3.5$, $P < 0.05$), vintage ($F > 3.5$, $P < 0.05$) and their interaction (variety *year) ($F > 2.9$, $P < 0.05$) were found for all the flavan-3-ols studied (Table 2). As the DP increased, the *F*-value increased for the three factors studied, indicating that the higher polymerized procyanidins are influenced more by variety and climatic conditions. In general, the *F*-values also indicate that climatic conditions had a greater effect on procyanidin levels than the varietal character, except for hexamers.

Monogalloylated flavan-3-ols

The levels of monogalloylated flavan-3-ols appeared to be approximately 10-fold lower than those of non-galloylated flavan-3-ols (Fig. 2). Although we found that the ionization efficiency of galloylated flavan-3-ols was consistent across samples, an apparent lower response could also result from a decreased ionization efficiency of the galloylated forms as compared to the non-galloylated forms. Without authentic standards this cannot be tested. Nonetheless these comparisons indicate that monogalloylated dimers are present in the highest levels in all varieties and vintages. Because ionization efficiency decreases with the increase of molecular size, it is highly unlikely that

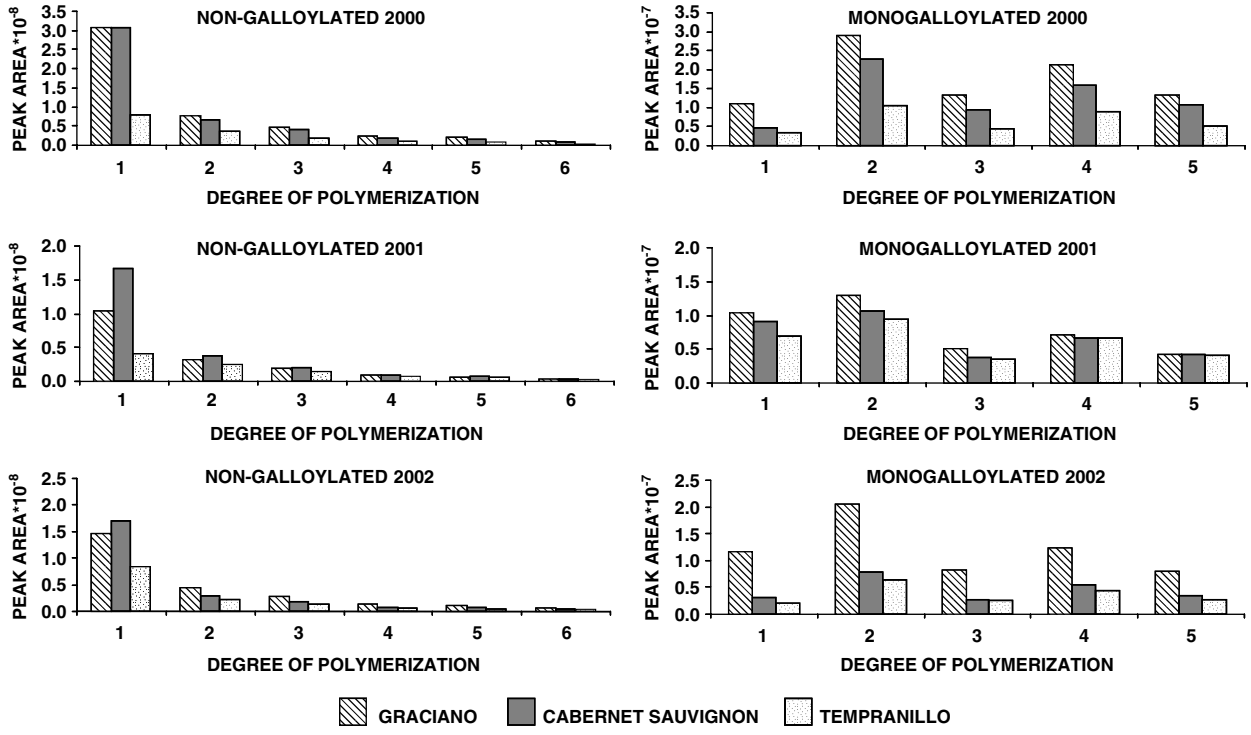

Figure 2. A relative comparison of the peak area corresponding to non-galloylated and monogalloylated flavan-3-ol oligomers isolated in seed extracts from *Vitis vinifera* L. grapes harvested in 2000, 2001 and 2002 vintages.

Table 2. *F*-values obtained from two-way ANOVA analysis of flavan-3-ols in peak Graciano, Tempranillo and Cabernet Sauvignon grapes harvested in 2000, 2001 and 2002

	DP[a]	*F* (variety)	*F* (year)	*F* (variety * year)
Non-galloylated procyanidin	1	72	52	10
	2	75	124	13
	3	88	139	21
	4	90	178	28
	5	99	159	24
	6	212	189	46
Mono-galloylated procyanidin	1	131	28	28
	2	133	108	26
	3	276	278	53
	4	87	160	24
	5	131	28	13

[a] DP, degree of polymerization.
F-values indicate significant differences at the 0.05 level in variety ($F > 3.5$), vintage ($F > 3.5$) and their interaction (variety * year) ($F > 2.9$).

this increase results from a higher ionization efficiency of dimers as compared to monomers. The levels of other monogalloylated flavan-3-ols varied in different vintages (Fig. 2). Regardless of the vintage, Graciano contained the highest levels of monogalloylated flavan-3-ols, followed by Cabernet Sauvignon and Tempranillo. As seen for non-galloylated flavan-3-ols, levels of the different classes of monogalloylated flavan-3-ols were higher for vintage 2000 and lower for vintages 2001 and 2002.

Two-way ANOVA showed significant differences in variety ($F > 3.5$, $P < 0.05$), vintage ($F > 3.5$, $P < 0.05$) and their interaction (variety * year) ($F > 2.9$, $P < 0.05$) for all flavan-3-ols studied (Table 2). As seen for the *F*-values, the varietal character and the climatic conditions have variable effects on the biosynthesis of monogalloylated flavan-3-ols depending on the DP (Table 2).

Distribution of flavan-3-ols by variety and year

The distribution of flavan-3-ols was determined in the grape seeds of these three varieties for each vintage year and are given in Table 3. Distribution percentages were calculated as the sum of the area corresponding to individual class of oligomer (based upon its DP) divided by the total sum of the area identified as flavan-3-ols. The flavan-3-ol distribution among vintages was similar for Graciano and Cabernet Sauvignon, but varied for Tempranillo (Table 3). Cabernet Sauvignon demonstrated the highest levels of monomers, followed by Graciano and Tempranillo. Santos-Buelga *et al.*[14] and Monagas *et al.*[38] reported that a key characteristic of the Tempranillo variety is a high percentage of dimers in comparison to other varieties. In the current study we observed the same trend, but only in the vintages 2000 and 2001. The proportion of dimers in Graciano was higher than in Cabernet Sauvignon. Tempranillo also presented the highest proportion of trimers, tetramers and pentamers. The percentage of monogalloylated procyanidins in grape seeds from these three varieties was also determined after dividing the sum of total monogalloylated flavan-3-ols by the sum of total flavan-3-ols. Levels varied slightly with vintage year for Graciano (15%, 19% and 20% for 2000,

Table 3. Distribution (%) of procyanidins according to their degree of polymerization in grape seeds extracts

	Graciano			Tempranillo			Cabernet Sauvignon		
	2000	2001	2002	2000	2001	2002	2000	2001	2002
Monomer	55.8 ± 0.5[a]	54.0 ± 0.7	51.8 ± 0.5	44.4 ± 2.4	37.8 ± 1.2	58.8 ± 1.6	60.6 ± 1.3	63.0 ± 0.6	66.0 ± 7.5
Dimer	18.6 ± 0.5	21.4 ± 0.6	20.8 ± 0.5	24.7 ± 0.6	26.6 ± 1.1	18.0 ± 1.1	17.5 ± 1.8	17.5 ± 0.4	14.7 ± 1.2
Trimer	10.4 ± 0.4	10.9 ± 0.1	11.3 ± 0.3	12.6 ± 0.9	14.5 ± 0.9	10.6 ± 0.6	9.6 ± 0.3	8.6 ± 0.1	8.8 ± 2.9
Tetramer	7.7 ± 0.3	7.2 ± 0.4	8.1 ± 0.1	9.8 ± 0.7	11.2 ± 0.6	6.9 ± 0.5	6.7 ± 0.4	5.6 ± 0.4	5.3 ± 1.8
Pentamer	5.8 ± 0.3	5.0 ± 0.4	6.1 ± 0.2	7.0 ± 0.5	7.8 ± 0.5	4.6 ± 0.3	4.6 ± 0.6	4.1 ± 0.2	4.1 ± 1.3
Hexamer	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	2.0 ± 0.2	1.2 ± 0.2	1.0 ± 0.4	1.2 ± 0.1	1.1 ± 0.4

[a] Mean ± SD ($n = 3$).

2001 and 2002 vintages, respectively), Tempranillo (17%, 24% and 13% for 2000, 2001 and 2002 vintages, respectively) and Cabernet Sauvignon (12%, 12% and 9% for 2000, 2001 and 2002 vintages, respectively).

CONCLUSIONS

Non-galloylated and monogalloylated flavan-3-ols up to octamers, and di- and trigalloylated flavan-3-ols up to heptamers, were identified in seed extracts from grapes of Graciano, Tempranillo and Cabernet Sauvignon. Based on relative comparisons of the levels of flavan-3-ols (MS peak areas), it was found that the distribution of flavan-3-ols was largely determined by genetic factors, and was also influenced by climate conditions. Levels of the non-galloylated flavan-3-ols appear to decrease as the DP increased, whereas levels of monogalloylated flavan-3-ols appear to decrease in an order variable with the vintage. For the geographic area studied, Tempranillo contained the lowest levels of non-galloylated and monogalloylated flavan-3-ols, whereas Graciano contained the highest levels, with the exception of non-galloylated procyanidins in vintage 2001, and non-galloylated monomers in vintage 2002. Grape seeds from vintage 2000 contained the highest levels of both non-galloylated and galloylated forms. The relative influence of factors such as grape variety, cultivar, age of the vineyard, and climatic conditions on grape seed procyanidin composition (DP, DG) can be studied using the HPLC/ESI-MS methodology reported in this paper. Utilities can be also found in the analysis of food ingredients and dietary supplements made of grape seeds.

ACKNOWLEDGEMENTS

The authors are grateful to Mr Juián Suberviola (EVENA, Navarra, Spain) for providing the grape samples. The authors also thank the Ministerio de Ciencia y Tecnología for an FPI predoctoral scholarship to VN, and the Spanish Comisión Interministerial de Ciencia y Tecnología (CICYT) (Project AGL2003-07394-C02-02).

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