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RESEARCH ARTICLE

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Vine vigor and cluster uniformity on *Vitis vinifera* L. seed procyanidin composition in a warm Mediterranean climate

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Abstract

Seed procyanidin composition of *Vitis vinifera* L. var. 'Carignan' and 'Grenache' was analyzed to assess the impact of vintage climatology, plant vigor and bunch variability on the quality of grapes. This study was carried out over 2007 and 2008 vintages in Terra Alta denomination of origin (DO). This region is located in northeastern Spain and characterized by a Mediterranean climate with a continental tendency. Procyanidin composition of seeds from four vineyards was analyzed by rapid resolution liquid chromatography (RRLC-DAD-TOF/MS). Vintage, vigor and ripeness uniformity had an influence on the procyanidin concentration in seeds. Flavan-3-ol polymerization increased during the warm year, together with a notable dependence on the variety and vine vigor. In warmer years and low vigor, 'Grenache' seed composition is likely to be more vulnerable than 'Carignan'. High levels of flavan-3-ol monomers and low polymerization characterized the seeds of the temperate year.

Additional key words: grapevine; Grenache; Carignan; ripeness heterogeneity; flavan-3-ol; polymerization; RRLC-DAD-TOF/MS.

Introduction

Flavan-3-ols [monomeric catechins and oligo-polymeric proanthocyanidins (PAs)] are a large family of phenolic compounds that can be found in the skins and seeds of grapevine (*Vitis vinifera* L.) berries (Prieur *et al.,* 1994; Thorngate & Singleton, 1994). These compounds are mainly responsible for the gustatory impact and color stability of wine. The concentration of PAs in grapes depends on the cultivar and the vintage, and is influenced by viticultural and environmental factors such as shading or canopy temperature (Cohen & Kennedy, 2010; Chira *et al.,* 2011). The highest concentration of most flavan-3-ols occurs during the first phase of berry growth (Kennedy *et al.,* 2001; Koyama & Goto-Yamamoto, 2008), with a decline from veraison to harvest (Romeyer *et al.,* 1986; De Freitas & Glories, 1999). Accumulation of flavan-3-ols in seeds, start very early in berry development, during blooming, and continues until 1-2 weeks after veraison (Kennedy *et al.,* 2002). This period of flavan-3-ols biosynthesis coincides with the formation of the monomeric catechins: catechin and epicatechin, which are widely considered to combine during ripening to give proanthocyanidins (Bogs *et al.,* 2005). There are differences between seed PAs and skin PAs. Seed PAs consist of (+)-catechin (C), (–)-epicatechin (EC), and (–)-epicatechin-3-*O*-gallate subunits (ECG) (Fig. 1). Grape skin PAs also contain (–)-epigallocatechin (EGC) and small amounts of gallocatechin (Souquet *et al.,* 1996). In addition, skin PAs have a higher mean degree of polymerization (mDP) and a lower proportion of galloylated subunits than those from seeds (Di Stefano, 1995; Moutonet *et al.,* 1996). It is well known that the greater mDP and the greater percentage of galloylation will cause a greater sensa-

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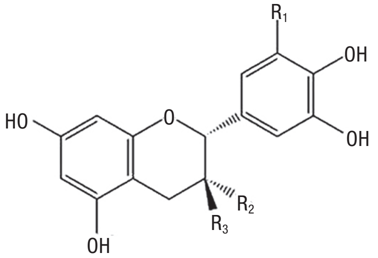
Abbreviations used: Car (Carignan); DO (denomination of origin); ET_0 (evapotranspiration); GDD (growing degree days); Gre (Grenache); H (high); L (low); mDP (mean degree of polimerization); PAs (proanthocyanidins); ThAmplitude (thermal amplitude); daysT > 40°C (number of days with a temperature higher than 40°C).



Flavan-3-ols	R_1	R_2	R_3
(+)-catechin	H	H	OH
(–)-epicatechin	H	OH	H
(–)-epicatechin-3-*O*-gallate	H	*O*-Gallate	H
(–)-epigallocatechin	OH	OH	H

Figure 1. Flavan-3-ol monomeric units in grapevines (skin and seed).

tion of astringency (Vivas & Glories, 1996; Vidal *et al.,* 2003).

During the later stages of ripening, the extractable levels of PAs start to decline (Downey *et al.,* 2003). Physiologically, the decreasing extractability of PAs, particularly from grape skins, represents a decrease in the bitterness and astringency of PAs in the berry and is likely a part of the seed dispersal strategy that includes sugar accumulation and anthocyanin biosynthesis in the berry (Downey *et al.,* 2006). The decrease in extractable PAs during ripening is the result of polymerization of the PAs (Coombe & McCarthy, 2000; Fournand *et al.,* 2006). However, the actual mechanism of what causes this decrease in extractable PAs has yet to be elucidated (Dixon *et al.,* 2005; Lepiniec *et al.,* 2006). It is well known that the grape growing region, location of vines in the vineyard, bunch position into the vine canopy, and berry position into the bunch generate some differences in the ripening rate (Smart *et al.,* 1985; Haselgrove *et al.,* 2000; Le Moigne *et al.,* 2008) and affecting the wine quality. Several studies have focused on the different environmental changes, viticultural practices and berry composition at different stages of maturity, on the PAs content of grapes and on the wine phenolic composition (Harbertson *et al.,* 2002; Cortell *et al.,* 2005; Ristic *et al.,* 2007; Cohen *et al.,* 2008). However, there is a lack of research about procyanidin content from distal parts (top and bottom) of the bunch. Consequently, the aim of this study was to determine the level of the uniformity in procyanidin content of two different varieties cultivated under the effects of vigor and in two vintages in a warm and dry Mediterranean grape growing region (Terra Alta DO, northeastern Spain).

Material and methods

Site details

Terra Alta Denomination of Origin (DO) is located in the precoastal mountains, in the province of Tarragona (Spain). This region has a Mediterranean climate with a continental tendency characterized by temperatures varying sharply from day to night and from summer to winter and receiving very low annual rainfall. According to the heat summation method, based on temperature and developed by Amerine & Winkler (1944), Terra Alta can be classified as Climate Region IV. The typical soils of the region, known as *panal* (mixture of silt and limestone), belong to the Entisols order according to the American Soil Taxonomy (USDA, 1998).

Plant material and experimental design

The study was carried out in 2007 and 2008 vintages. It was conducted in 'Carignan' (Car) and red 'Grenache' (Gre) varieties, in which two different levels of vigor were considered, low (L) and high (H). In total, four combinations (vigor/variety) were established: L-Car (alt. 370 m), H-Car (alt. 305 m), L-Gre (alt. 236 m), and H-Gre (alt. 422 m). In order to classify vineyards into vigor levels, growth and yield variables were measured. Three plot replications of each vigor/variety combination were randomly distributed in the vineyards, with each elementary plot consisting of 30 vines, where each replication was used for sampling as described in the "Fruit sampling and analysis" section. Two vines of each replication were used for vigor measurements (growth, berry weight, yield, pruning weight, length of shoots and total leaf area) with a total of six vines per treatment. Total leaf area (measuring the length of the leaf main nerve) was calculated as described by Sánchez-de-Miguel *et al*., 2011). This previous characterization allowed us to classify the vineyards into two vigor levels (L and H).

All vineyards had a minimum slope, which ranged between 4% and 7%. Vine spacing in L-Car and H-Car was 1.4 m (between vines) × 2.8 m (between rows); and 1.2 m × 2.8 m in L-Gre and H-Gre. Ten-year-old vines were not irrigated. Plants were bush-trained and pruned to 5-7 buds in L-vigor plants and 9-11 buds in H-vigor plants. L-Gre treatment was located in Xeric petrocalcid soil and grafted on *110R* rootstock (resistant to limestone at 17%). L-Car/*110R* vines were growing on Xerofluvent soil. Soils of low vigor vineyards were shallow, had moderate stoniness and good drainage capacity. H-Gre vines were growing in Xerorthent soil and grafted on *41B* rootstock (resistant to limestone at 40%). H-Car/*41B* combination was placed in deeper soil (Xerofluvent) than L-Car. Soils of H-vigor vineyards were deeper, with higher clay content and without stones, allowing for greater water retention.

Climatic data

Meteorological data were provided by the Meteocat weather station located in Batea village (lat. 41.09°, long. 0.32°, alt. 382 m) (http://www.ruralcat.net/agrometeo/html/agrometeobc90.htm). Values of average growing degree days (GDD), annual mean temperature (Tm), total annual rainfall and evapotranspiration (ET_0) were collected over 10 years (Table 1).

Additionally, a HOBO weather station (www.onsetcomp.com) was installed in each treatment. The following meteorological data: minimum temperature (Tmin), maximum temperature (Tmax), mean temperature (Tm) and relative humidity (%RH), were recorded every 15 min to characterize the specific conditions of each vineyard. The weather stations were placed between two vines in the same row, with temperature and humidity data loggers close to the canopy. Meteorological data collected in each treatment allowed us to define the climatic conditions occurring along the growing season and thus to determine the conditions in every phenological grape stage: fruit set, veraison and harvest. Based on the data collected, we calculated the number of days with an average temperature higher than 35°C (daysTmax > 35°C) and higher than 40°C (daysTmax > 40°C), and number of days with thermal amplitude higher than 20°C (daysThAamplitude > 20°C).

Fruit sampling and analysis

In order to analyze the pulp composition and phenolic maturity of the distal parts of the grapes during ripening, samples of four bunches from the three replications per vineyard were randomly collected from different positions in the canopy, with a total of 12 bunches per vineyard. Bunches were stored in plastic bags and kept refrigerated (3-4°C). Samplings were carried out approximately every week from veraison to harvest in order to have measurements at the same physiological stage, to compensate for the ripening delay between vineyards, getting comparable results among treatments.

Samples of each replication were divided in two parts (top and bottom half of the bunch) as previously described in Edo-Roca *et al.* (2013). For each part, a sample of 100 berries was used to determine the sugar level (degree Brix), acidity (g L^{-1} tartaric acid) and pH according to OIVV (1990); another sample of 300 berries was used to analyze the phenolic maturity according to the method described by Nadal (2010).

Determination and identification of procyanidins by RRLC-DAD-TOF/MS

Chemicals

All solvents were of HPLC grade. Water, methanol and formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Standard gallic acid was purchased from Sigma Aldrich, (+)-catechin from Fluka and dimer monogallate, procyanidin C1 (trimer), epicatechin gallate and procyanidin B2 (dimer) from Polyphenols Biotech (Villenave d'Ornon, France).

Table 1. Annual and long-term (10 years) meteorological data of the experimental site

	Mean temperature (°C)			GDD (°C)			Rainfall (mm)			ET_0 (mm)		
	Annual	**Spring**	**Summer**	**Annual**	**Spring**	**Summer**	**Annual**	**Spring**	**Summer**	**Annual**	**Spring**	**Summer**
2007	14.6	15.7	22.5	2,040	564	1,150	384	190	16	1,058	330	448
2008	14.2	14.6	22.8	1,921	459	1,179	588	301	61	1,021	326	446
2000-2009	14.7			2,136			470			1,036		

Spring: From March 20th to June 20th. *Summer:* from June 21st to September 20th.

Sample seed extraction

Approximately 100 berries from each replicate were hand-pressed to separate the pulp, the skin and the seeds. Seeds were washed three times with Milli-Q water, dried on filter paper, lyophilized and crushed to finally obtain a fine powder. Methanol solution (50 mL) was added to 1 g of seed powder. Samples were stirred and ultrasonicated for 15 minutes to completed extraction, and then centrifuged at 8,000 rpm at 5°C for 7 min. The supernatants were combined and dried under a nitrogen stream for their subsequent analysis by HPLC. The resulting fraction was dissolved into 20% of methanol, 0.1% of formic acid (98%) and Milli-Q water and it was filtered through 0.22 mm PVDF filter. Finally, filtered samples were injected in RRLC-DAD-TOF/MS (rapid resolution liquid chromatography coupled with diode array detection and electrospray ionization time-of-flight mass spectrometry).

Instrumentation

Seed procyanidins were analyzed on a Rapid Resolution Liquid Chromatograph RRLC 1200 (Agilent Technologies, USA). The RRLC was coupled to a TOF mass spectrometer G6220A (Agilent Technologies) equipped with an electrospray interface. Detection was done by a DAD (diode array detector).

Chromatographic conditions

According to Valls *et al*. (2009) methodology, a volume of 3 µL of each sample was injected onto a Zorbax Eclipse Plus C18 column (Agilent Technologies). The phenolic compounds were identified according to their order of elution, the retention times of pure compounds (gallic acid, catechin, procyanidin dimer B2, dimer monogallate, procyanidin trimer C1 and epicatechin gallate) and their molecular masses. The analyses were performed between 280 and 306 nm wavelength.

Mass spectrometry

The ionization of the compounds was carried out by electrospray in negative mode. Nitrogen was used as a drying gas and also as a nebulizing gas at an inlet pressure of 60 psi and a temperature of 350°C. Analyses were carried out in scan mode from 100 to 1,600 m/z.

Mean degree of polymerization (mDP)

The flavanol fraction was estimated according to $\text{mDP} = [\Sigma(Ni \cdot Ui)]/Nt$, where Ni is the amount of flavanols of each group (*i.e.,* monomers, dimers B, dimers gallate and trimers), Ui is the number of elementary units in each group of flavanols (*i.e.,* 1, 2, or 3 for monomers, dimers, and trimers, respectively), and Nt is the amount of total flavanols in the sample $(= Ni)$. The method used to calculate mDP has been previously described by González-Manzano *et al.* (2006).

Statistical analysis

Analysis of the variance (ANOVA) was conducted using SPSS 19.0. Significant differences were identified by Tukey's test. Factorial multivariate analysis results show the effects of vigor and uniformity separately as well as interaction among them ($p \leq 0.1$; $p \leq 0.05$; $p \leq 0.001$).

Results

Climatic characterization and effect on vine development

Annual and 10-year meteorological data of the experimental site is shown in Table 1; rainfall was considerably different between both vintages (384 mm in 2007 and 588 mm in 2008). Annual Tm for *spring* period was 1°C higher in 2007, showing 15.7°C in 2007 *vs*. 14.6°C in 2008. Consequently this characterization defines 2007 as warmer and drier than 2008.

To better understand how vintage climatology affected the maturation of vineyards, three periods were defined: *I*, *II* and *III*; where *I* refers to the period between fruit set and veraison; *II* from veraison to advanced ripeness (one week prior to harvest); and *III* is the last stage of ripening (the last week before harvest) (Table 2). Vineyards of L-Car and L-Gre had lower %RH than H-Car and H-Gre during the grape growing season for both years. In general, temperatures were

Table 2. Climatic characterization of all treatments in both vintages

	2007						2008					
	Low vigor			High vigor			Low vigor			High vigor		
	I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
Carignan												
Tmin (°C)	16.7	16.0	12.6	15.0	13.3	12.9	16.8	16.0	10.4	15.7	12.4	5.3
Tmax (°C)	29.9	28.9	28.6	30.0	29.8	24.8	29.6	28.2	20.3	28.8	25.4	20.3
Tm (°C)	23.0	22.0	20.6	22.8	21.4	19.0	23.3	21.7	14.8	22.4	18.9	12.8
ΔGDD (°C)	786	357	64	885	341	58	790	478	30	753	362	19
daysT > 35°C	3	3	0	1	4	0	3	1	0	1	0	0
daysT > 40°C	0	0	0	0	0	0	0	0	0	0	0	0
ThAmplitude (°C)	13.2	12.9	15.9	14.2	16.4	12.0	13.0	12.2	9.9	13.4	13.0	15.0
days ThAmplitude > 20°C	1	1	0	6	10	0	1	0	0	2	2	0
RH (%)	51.8	52.1	55.6	53.1	56.3	68.5	59.1	61.1	55.6	62.0	69.5	78.5
Grenache												
Tmin (°C)	15.5	16.5	15.0	17.1	16.3	13.7	15.3	17.1	16.1	17.4	17.5	15.8
Tmax (°C)	29.7	32.7	33.5	29.9	29.4	28.7	29.1	32.4	33.2	29.2	29.1	17.3
Tm (°C)	22.5	24.3	23.5	23.0	22.3	20.3	22.0	24.6	24.1	22.9	22.5	21.3
ΔGDD (°C)	716	442	92	769	353	14	691	380	100	766	287	65
daysT > 35°C	4	12	3	4	3	0	4	6	0	0	0	0
daysT > 40°C	0	0	2	0	0	0	0	0	0	0	0	0
ThAmplitude (°C)	14.2	16.2	18.5	12.9	13.1	15.0	13.8	15.3	17.2	11.8	11.7	11.4
days ThAmplitude > 20°C	5	8	4	0	0	0	2	3	0	0	0	0
RH (%)	49.4	48.5	49.7	53.7	51.9	53.8	59.8	58.0	61.2	63.5	65.6	68.8

T: temperature. GDD: growing degree days; daysT > 35°C: number of days with temperature higher than 35°C. ThAmplitude: thermal amplitude. Days ThAmplitude: m daysT > 40°C: number of days with thermal amplitude higher than 40°C. RH: relative humidity.

higher in L-vigor treatments than in H-vigor treatments. Tm was higher in L-Car than in H-Car treatment during the summer season (periods *I*, *II* and *III*); but in L-Gre, Tm was higher in periods *II* and *III* (from veraison to harvest date). L-Car registered lower thermal amplitude (ThAmplitude) than H-Car, contrary to the treatments of 'Grenache'.

Comparing varieties, 'Grenache' phenology stages happened earlier than the 'Carignan' phenology (Table 3). Actually, the length of the growing period (from bud break to leaf drop) was longer in 'Carignan' than in 'Grenache'. Dates of phenological phenomena occurred later in 2008 than in 2007 for both cultivars. Veraison showed a delay in 2008 in both varieties, although the duration of the period was the same. 'Carignan' showed a clear delay on harvest date in 2008. Phenology stages were found to start sooner in low vigor vineyards than in high vigor vineyards for both vintages and in both varieties.

The results of growth and yield variables (berry weight, yield, pruning weight, the length of shoots and the total leaf area) verify clearly the higher vigor of the vineyards previously selected in our research trial (Table 4). The ANOVA ($p \leq 0.05$) of vine vigor variables showed that the most vigorous grapevines grew and produced consistently more than the weakest.

Table 3. Dates of fruit set, veraison and harvest for each treatment on both vintages

Treatment	2007			2008		
	Fruit set	Veraison	Harvest	Fruit set	Veraison	Harvest
L-Car	6-Jun	6-Aug	12-Sep	10-Jun	11-Aug	29-Sep
H-Car	9-Jun	18-Aug	25-Sep	16-Jun	20-Aug	8-Oct
L-Gre	29-May	26-Jul	3-Sep	1-Jun	29-Jul	1-Sep
H-Gre	7-Jun	6-Aug	12-Sep	13-Jun	12-Aug	12-Sep

Table 4. Vine vigor characterization (mean values)

	Lenght of shoots (cm)	Total leaf (m^2 $vine^{-1}$)	Berry weight (× 10 g)	Yield (kg $vine^{-1}$)	Prunning weight (g)
2007					
L-Car	73.7^b	3.0^b	15.7^b	2.9^b	282^b
H-Car	115.3^a	4.0^a	22.9^a	5.1^a	710^a
2008					
L-Car	102.9^b	3.9^b	20.0^b	2.3^b	420^b
H-Car	131.1^a	5.7^a	21.9^a	4.9^a	734^a
2007					
L-Gre	102.3^b	2.6^b	15.6^b	2.6^b	264^b
H-Gre	133.7^a	5.9^a	20.4^a	4.4^a	659^a
2008					
L-Gre	68.4^b	3.4^b	14.5^b	2.9^b	318^b
H-Gre	114.3^a	5.3^a	19.4^a	6.1^a	744^a

Values with different letters in a single group are significantly different ($p \leq 0.05$).

Evolution of seed procyanidins from veraison to harvest

The performance of the HPLC RRLC-DAD-TOF/MS analysis allowed determining and identifying a list of 15 compounds (Table 5). The phenolic compounds were identified according to their retention times and molecular masses.1

Figs. 2a and 2b show the kinetics of monomers, dimers and trimers of procyanidins from veraison to harvest for ‘Carignan’ and ‘Grenache’, respectively. Note that the decrease of monomers occurred in two phases. The slopes in the first phase (between $m = -0.11$ and $m = -0.28$) were more pronounced than the slopes of the second phase (between $m = -0.25 \cdot 10^{-2}$ and $m = -0.05$). The low vigor vines of ‘Carignan’ (L-Car/2007 and L-Car/2008) with yields ranging between 2.3 and 2.6 kg $vine^{-1}$ (Table 4), showed a decrease of seed monomers in the first phase ($m = -0.18$ and $m = -0.25$) more pronounced than the high vigor vines (H-Car/2007 and H-Car/2008, yield ranging between 4.9 and 5.1 kg $vine^{-1}$) ($m = -0.11$ and $m = -0.13$). In regard to ‘Grenache’, the low vigor vines (L-Gre/2007 and L-Gre/2008), with the yield ranging between

Table 5. Compounds identified in the procyanidin fraction of seeds from ‘Carignan’ and ‘Grenache’ obtained by chromatography.

Peak	t_R (min)	$[M-H]^-$ (m z^{-1})	Compound
1	0.6	865	Procyanidin trimer T1
2	0.8	169	Gallic acid
3	1.9	577	Procyanidin dimer B3
4	2.1	577	Procyanidin dimer B1
5	2.4	865	Procyanidin trimer T2
6	2.8	289	Catechin
7	3.4	577	Procyanidin dimer B4
8	3.7	577	Procyanidin dimer B2
9	4.7	729	Procyanidin dimer monogallate
10	5.0	289	Epicatechin
11	5.0	865	Procyanidin trimer C1
12	5.1	577	Procyanidin dimer B
13	5.7	881	Procyanidin dimer digallate
14	6.2	441	Epicatechin gallate
15	6.7	577	Procyanidin dimer B

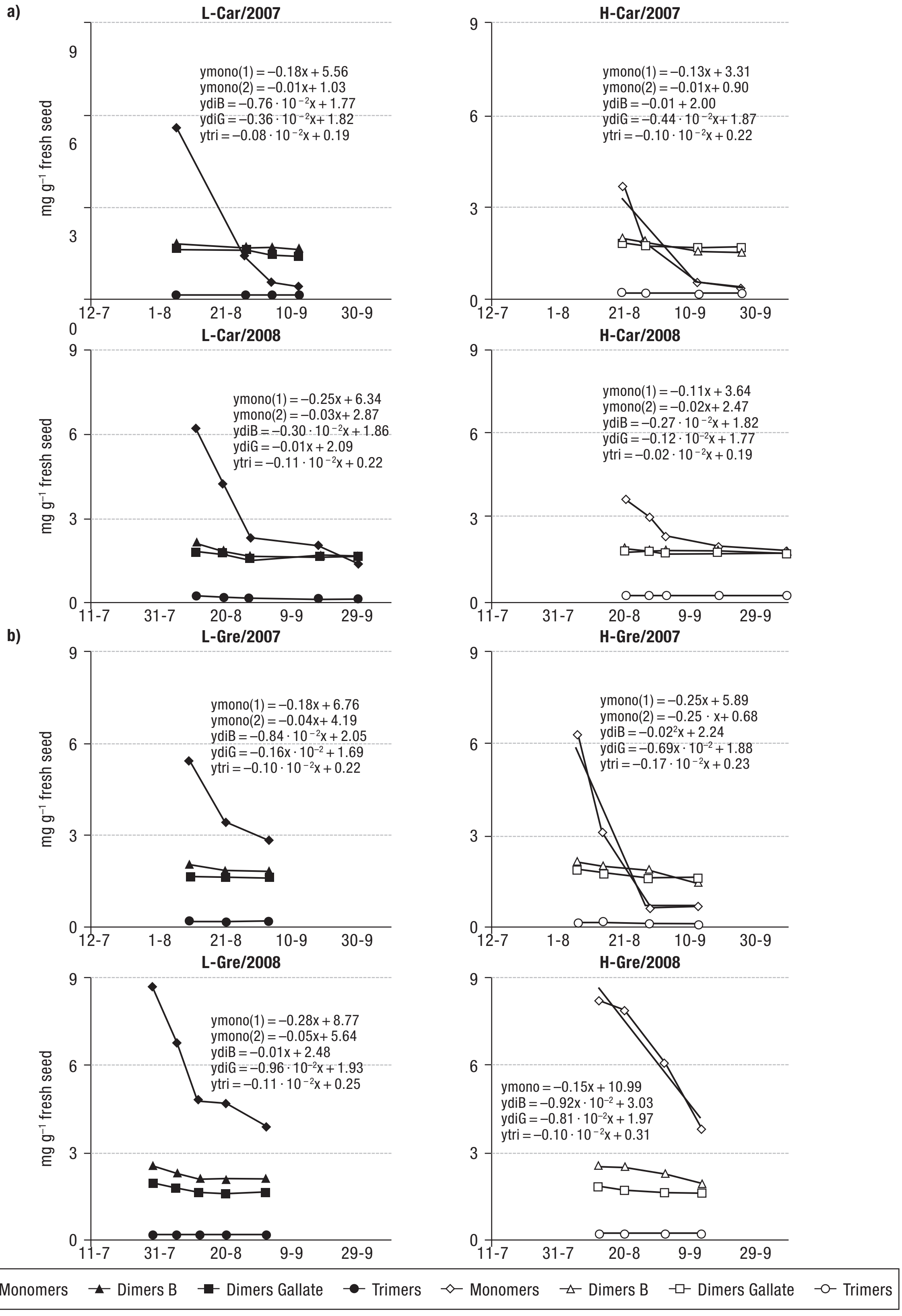


Figure 2. Procyanidins of 'Carignan' (a) and of 'Grenache' (b). Years 2007 and 2008. Concentration of monomers (mono), dimers B (diB), dimers gallate (diG) and trimers (tri) from fresh seed during fruit ripening.

2.6 and 2.9 kg vine^{-1} (Table 1), showed a fast decrease in seed monomers in the first phase (m = –0.18 and m = –0.28), and H-Gre/2007 treatment (with 4.4 kg vine^{-1}) also presented a rapid decrease in seed monomers (m = –0.25), showing a similar trend to those of low vigor. However, it should be noticed that the same treatment but different vintage (H-Gre/2008) showed a kinetic pattern showing a single slope (m = –0.15).

Total PAs (sum of monomers, dimers and trimers) showed a diminution of concentration during ripening in both varieties (Figs. 3a and 3b). This trend is mainly due to the decrease in flavan-3-ol monomers (Figs. 2a and 2b).

Concerning 'Carignan', L-Car plants showed higher PAs concentration at the beginning of ripening (period *II*) than the H-Car plants for both vintages, even if the total procyanidins did not vary at the end of maturation. PAs for L-Car and H-Car followed clearly a two-slope pattern decrease. Both in 2007 and in 2008, during the first two weeks after veraison the slope was higher than in the following weeks (Figs. 3a and 3b). Until the end of ripeness the slope showed low and even values. In 2007, 'Grenache' followed the same pattern than L-Car and H-Car and in 2008 patterns of L-Gre followed showed two markedly different slopes (Figs. 3a and 3b). The main difference can be shown in the H-Gre/2008 treatment, where the diminution of total PAs along the maturation followed a unique pattern ($m_{\text{H-Gre}}$ = –0.19).

Seed procyanidin composition at harvest

'Grenache' accumulated more flavan-3-ol monomers and oligomers than 'Carignan'. The level of mo-

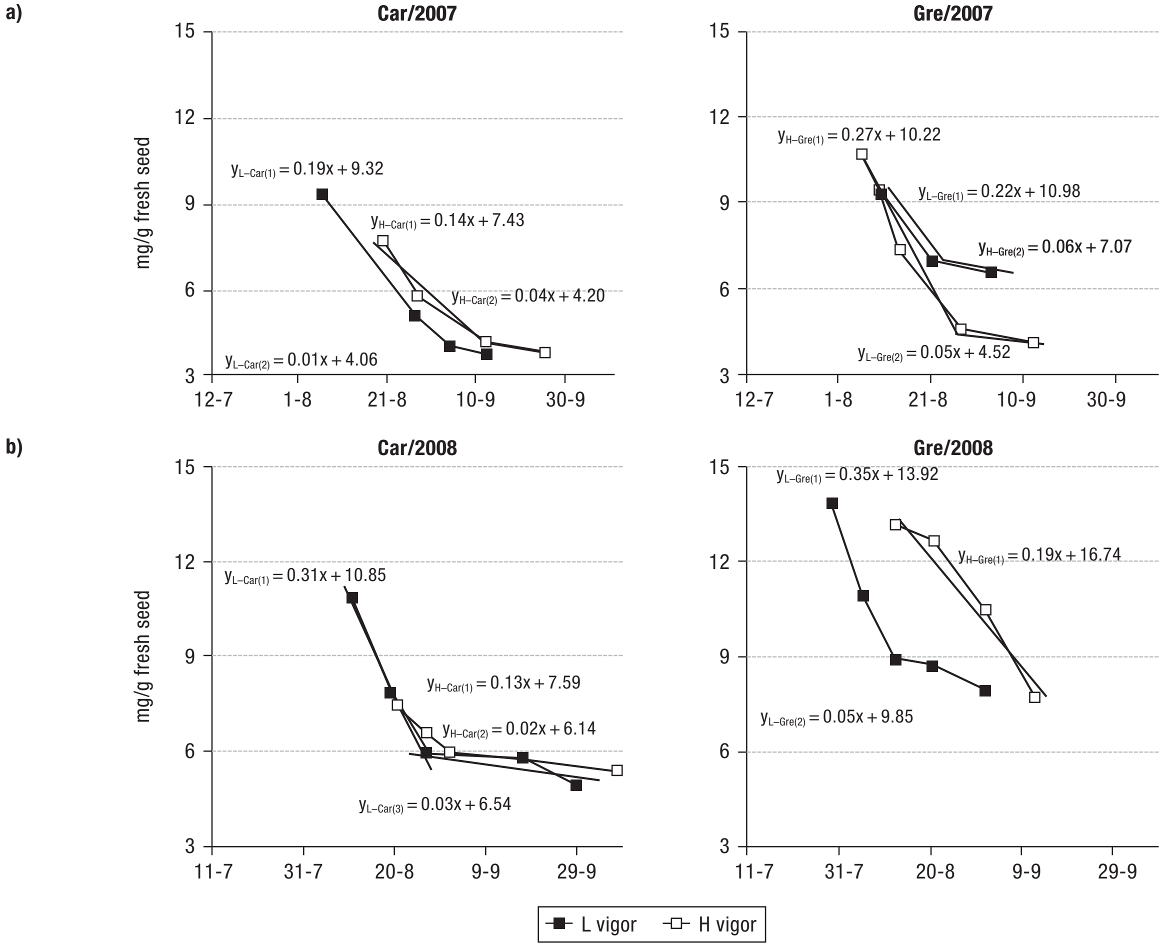


Figure 3. Concentration of total procyanidins (mg g^{-1}) from fresh seed during fruit ripening for 'Carignan' and 'Grenache' in 2007 (a) and 2008 (b).

Table 6. Pulp composition (°Brix and TA), procyanidin content (monomers, dimers B, dimers gallate and trimers) and total PAs (proanthocyanidins as the sum of monomers, dimers and trimers) and mDP (mean degree of polimerization) in seeds at harvest according to vintage and vigor effect for 'Carignan' and 'Grenache'

	°Brix	TA (g L^{-1} tartaric)	Monomers (%)	Dimers B (%)	Dimers gallate (%)	Trimers (%)	Total PAs (mg g^{-1})	mDP
2007								
L-Car	21.8^{a}	7.2^{a}	13.1^{b}	38.1	44.6	4.0	3.79	1.90
H-Car	21.3^{b}	5.8^{b}	14.9^{a}	37.7	43.0	4.0	3.91	1.89
2008								
L-Car	23.8^{a}	7.7^{a}	28.1^{b}	32.8	34.9^{a}	3.5	4.92^{b}	1.74
H-Car	22.5^{b}	6.8^{b}	32.1^{a}	31.8	31.7^{b}	3.4	5.44^{a}	1.69
2007								
L-Gre	26.0^{a}	4.9^{b}	44.0^{a}	27.7^{b}	24.6^{b}	2.9^{b}	6.66^{a}	1.58^{b}
H-Gre	25.7^{b}	5.7^{a}	19.0^{b}	36.3^{a}	39.9^{a}	3.8^{a}	4.08^{b}	1.84^{a}
2008								
L-Gre	23.2^{a}	4.3^{b}	48.9	26.5	20.7	2.8	8.06	1.52
H-Gre	22.0^{b}	5.7^{a}	49.1	25.6	21.3	2.7	7.82	1.51

Values with different letters in a single group are significantly different ($p \leq 0.05$).

nomeric flavan-3-ols in temperate/2008 vintage was higher than in warm/2007 (Table 6). Consequently, the mDP was higher in the warm than in the temperate vintage for both varieties.

Results of seed composition in 'Carignan' showed that the percentage of monomers in 2008 was twice the relative amount of 2007. In both years, the percentage of monomers was higher in H-Car than in L-Car. On the other hand, dimers gallate were higher in L-Car/2008 than in H-Car/2008. The concentration of total PAs was significantly smaller in L-Car/2008 than in H-Car/2008. In warm vintage/2007, total concentration of PAs did not statistically vary between the vigor treatments. Within vigor, the mDP index showed no statistically different values between L-Car and H-Car in both vintages in spite of the tendency of the less vigorous treatment (L-Car) to achieve higher polymerization than the more vigorous. The mDP value was close to 2, indicating the predominance of dimers over trimeric and monomeric forms, also indicated by the highest percentage of dimers (Table 6).

In contrast to 'Carignan', the proportion of monomers in high vigor 'Grenache' (H-Gre/2007) was reduced significantly to about half of the amount of that of low vigor (L-Gre/2007; Table 6). The percentage of dimers B and dimers gallate doubled the percentage of monomers in the seeds of H-Gre/2007, and were significantly higher than those measured in L-Gre. The total procyanidins attained in H-Gre/2007 were significantly lower and, as expected, achieving an mDP higher than in L-Gre/2007. In 2008, mDP in 'Grenache' was about 1.50 and there were no statistical differences between the low and high vigor. Thus the amount of monomers and dimers did not vary in the 'Grenache' seeds in 2008.

Seed procyanidin composition from distal parts of bunch at harvest

Regarding the uniformity in seed ripeness of each of the berries within a bunch (Tables 7 and 8), it was found that the relative amounts in seed flavan-3-ol monomers and procyanidin oligomers of the distal parts were significantly variable in the warm and dry (2007) vintage but not in the temperate (2008).

The seed composition of 'Carignan' was not completely uniform in 2007 (Table 7). In L-Car/2007, statistical differences were evident in the percentage of monomers, which was lower in top side than in bottom seeds. Opposite, in H-Car/2007, the top seeds reached twice the percentage of monomers and higher total PAs than the bottom seeds. On the other hand, the percentage of dimers gallate was lower in top seeds than in bottom in H-Car/2007. Nevertheless, the mDP was statistically equal for both sides in all treatments. In temperate vintage, both top and bottom seeds ripened evenly.

Table 7. Pulp composition (°Brix and TA), procyanidin content (monomers, dimers B, dimers gallate and trimmers) and total PAs (proanthocyanidins as the sum of monomers, dimers and trimmers) and mDP (mean degree of polimerization) in seeds at harvest according to top and bottom sides of 'Carignan' bunches

	°Brix	**TA (g L^{-1} tartaric)**	**Monomers (%)**	**Dimers B (%)**	**Dimers gallate (%)**	**Trimers (%)**	**Total PAs (mg g^{-1})**	**mDP**
2007								
L-Car								
Top	21.9^{a}	7.3	12.5^{b}	38.5	44.9	4.0	3.76	1.91
Bottom	21.5^{b}	7.2	14.0^{a}	37.6	44.1	4.0	3.83	1.89
H-Car								
Top	20.3^{b}	5.6^{b}	17.7^{a}	36.9	41.0^{b}	3.9	4.14^{a}	1.85
Bottom	21.5^{a}	5.8^{a}	9.6^{b}	39.2	46.9^{a}	4.1	3.54^{b}	1.94
2008								
L-Car								
Top	23.4	7.7	28.3	32.7	35.0	3.5	4.89	1.74
Bottom	24.4	7.7	28.0	32.9	34.8	3.6	4.95	1.74
H-Car								
Top	22.3	7.0	31.6	31.9	32.1	3.4	5.34	1.70
Bottom	22.8	6.7	32.9	31.6	31.1	3.4	5.60	1.68

Values with different letters in a single group are significantly different ($p \leq <0.05$).

Seed ripeness of 'Grenache' showed similar heterogeneity between distal parts in 2007 (Table 8), as well as 'Carignan' (Table 7). H-Gre/2007 top side showed a notably higher relative percentage of monomers than the bottom side, oppositely to L-Gre/2007. The same pattern was also observed in the total PAs concentration. The percentage of dimers gallate was higher in top seeds than those in bottom in L-Gre/2007 opposi-

Table 8. Pulp composition (°Brix and TA), procyanidin content (monomers, dimers B, dimers gallate and trimmers) and total PAs (proanthocyanidins as the sum of monomers, dimers and trimmers) and mDP (mean degree of polimerization) in seeds at harvest according to top and bottom sides of 'Grenache' bunches

	°Brix	**TA (g L^{-1} tartaric)**	**Monomers (%)**	**Dimers B (%)**	**Dimers gallate (%)**	**Trimers (%)**	**Total PAs (mg g^{-1})**	**mDP**
2007								
L-Gre								
Top	26.2^{a}	4.7^{b}	41.7^{b}	28.2	26.5^{a}	3.0	6.21^{b}	1.60
Bottom	25.4^{b}	5.3^{a}	47.7^{a}	26.8	21.7^{b}	2.8	7.60^{a}	1.53
H-Gre								
Top	25.9^{a}	5.7	20.5^{a}	36.0	38.7^{b}	3.7	4.26^{a}	1.81
Bottom	25.5^{b}	5.8	15.7^{b}	37.1	42.6^{a}	3.8	3.85^{b}	1.87
2008								
L-Gre								
Top	23.6^{a}	4.1	49.5	26.3	20.3	2.8	8.22	1.51
Bottom	22.1^{b}	4.7	47.5	27.1	21.5	2.8	7.72	1.53
H-Gre								
Top	22.6	5.6	48.8	25.9	21.1	2.8	7.89	1.51
Bottom	21.3	6.0	49.4	25.3	21.5	2.7	7.71	1.51

Values with different letters in a single group are significantly different ($p \leq <0.05$).

tely to H-Gre/2007. The mDP did not vary among the seeds from the same bunch.

Effect of vintage, vigor and uniformity on seed procyanidin composition

Factorial multivariate analysis demonstrated that the vintage was the most influential factor on the seed composition (data not shown). Furthermore, there were two factors (vigor and uniformity) that were influential to the composition of the seed. Therefore, the factorial multivariate analysis was also made with vigor (low and high), uniformity (top and bottom) and their interaction (Vigor * Uniformity) in each vintage for both varieties (Table 9).

Carignan

In the warm vintage 2007, vigor had major effects ($p \leq 0.001$) on concentrations of monomers and mDP (Table 9). In addition, we found in 2007 a slight ($p \leq 0.05$) impact by vigor on dimers gallate and total PAs content. The uniformity had major effects on monomers, dimers, total PAs and mDP, and a slight effect on trimers ($p \leq 0.1$). However, the interaction of Vigor * Uniformity significantly affected all variables. In 2008, the importance of vigor was more pronounced than in 2007. Conversely, uniformity had no significance on dimers B and trimers. Moreover, the interaction of both factors in this vintage (2008) was smaller on dimers B ($p \leq 0.05$) and trimers ($p \leq 0.1$).

Grenache

The most important factor after the vintage effect was vigor, affecting all variables in both vintages. In the warm vintage, uniformity did not demonstrate influence neither dimers B nor trimers. The same result was found for the trimers in 2008. However, the interaction (Vigor * Uniformity) statistically affected all variables in both vintages.

Discussion

Climatic characterization and the effect on vine development

Climatic data of temperature and humidity allowed differentiation of vineyards: low vigor treatments we-

Table 9. Significance of differences within vigor and uniformity, and their interaction

	Monomers (%)	Dimers B (%)	Dimers gallate (%)	Trimers (%)	Total PAs (mg g^{-1})	mDP
Carignan						
2007						
Vigor	***	ns	**	ns	**	***
Uniformity	***	***	***	*	***	***
Vigor * Uniformity	***	***	***	**	***	***
2008						
Vigor	***	***	***	**	***	***
Uniformity	***	ns	***	ns	***	***
Vigor * Uniformity	***	**	***	*	***	***
Grenache						
2007						
Vigor	***	***	***	***	***	***
Uniformity	***	ns	***	ns	***	**
Vigor * Uniformity	***	***	***	**	***	***
2008						
Vigor	***	***	***	***	***	***
Uniformity	***	*	***	ns	***	***
Vigor * Uniformity	***	***	***	***	***	***

PAs: proanthocyanidin. mDP: mean degree of polimerization. *, **, *** indicate significance at $p \leq 0.1$, $p \leq 0.05$, $p \leq 0.001$, respectively. ns: not significant.

re warmer and drier than high vigor treatments (Table 4). The temperature and humidity environmental conditions in periods *I*, *II* and *III* around the grapevine had an influence on the berry development; for instance, a high-accumulated temperature and low humidity at the end of a ripening period caused a speeding up of the harvest date. The effect was more pronounced in 'Grenache' grapes. Particularly in 'Grenache', dry climatic conditions during ripening caused an accelerated accumulation of sugars in the berry (ranging 1-1.5 ºBrix) and the maturation of the seed was promoted.

Evolution of seed procyanidins from veraison to harvest

The evolution of seed procyanidins from veraison to harvest ($Y_{L\text{-}Car}$, $Y_{L\text{-}Gre}$, $Y_{H\text{-}Car}$ and $Y_{H\text{-}Gre}$) indicate that the kinetics of both monomers and total seed PAs decreased faster in 'Grenache' than 'Carignan' and showed two different patterns of decrease (Figs. 2a, 2b, 3a and 3b).

Most of the earlier research on total seed PAs has been conducted to define a kinetic model with two different phases. Kennedy *et al.* (2000a,b) and Harbertson *et al.* (2002) showed that 'Cabernet sauvignon' and 'Shiraz' seeds exhibit a two-slope pattern of diminution. De Freitas & Glories (1999) also found a two-slope pattern for 'Ugni blanc' and 'Sémillon', with a steeper slope during the first two weeks after veraison. On one hand, this model is consistent and reinforces our model found in 'Carignan' (L-Car and H-Car) and in low vigor 'Grenache'. On the other hand, kinetics followed by H-Gre/2008 suggests susceptibility to climatology linked to 'Grenache' for obtaining ripened seeds.

As long as the maturation of seed advances, polymerization processes result in the extension of procyanidins reaching their highest amount at harvest (Kennedy *et al.,* 2000a; Pastor-del-Río & Kennedy, 2006). According to Saint-Criq-Gaulejac *et al.* (1997) the more subunits procyanidins have, the more difficult the extraction is. Therefore, during ripening, the amount of procyanidins actually decreases, probably because of a diminution of their extractability.

Furthermore, the evolution of each variety concerning seed procyanidins from veraison to harvest (Figs. 3a and 3b) depended on vigor and, consequently, on the environment around the grape. In vineyards of 'Grenache', decreasing temperatures during ripening (period *II* and *III*) in high vigor treatments and, especially in 2008, would have an effect on the kinetics of the seed, given the high concentrations of monomers found at harvest. In this case, a lower seed maturity occurred.

Seed procyanidin composition at harvest

Although it is known that 'Grenache' accumulates more flavan-3-ol monomers and oligomers than 'Carignan' (Romeyer *et al.,* 1986), both varieties showed similar behavior in the accumulation of total seed procyanidins content as concluded from the results in Table 6. In temperate vintage, seed procyanidins content was higher because the concentration of monomers remained elevated. The polymerization of seed procyanidins was not favored in temperate conditions, contrary to warmer and drier vintages.

'Carignan' showed a delay in the seed maturity *vs.* the pulp in temperate vintage. In warm vintage, pulp and seed maturation occurred in parallel. Given the effect of vintage, 'Carignan' reached values of higher °Brix in 2008 due to the longer lasting ripeness compared to the °Brix values in 2007 (Table 6). Consequently, maturity of seeds in 2008 was not completed at the time of harvest. Moreover, under high vigor conditions maturation occurred later than in low vigor, in both the seed and the pulp. This is shown by the lower °Brix and higher amount of monomers in H-Car than in L-Car regardless of the vintage (discussion of pulp maturity process has been evaluated in Edo-Roca *et al.*, 2013). For instance, warm vintage would cause the pulp and the seeds to mature at the same time for 'Carignan' regardless of the vigor, but in temperate vintage the ripeness of pulp and seeds would not be favored by high vigor conditions (and high yields). In fact, grape ripeness of H-Car/2008 would not have achieved the grape juice optimal standards of quality in these conditions of vigor and vintage because 'Carignan' has a long growing period and it requires high heat summation to complete the maturity. Actually, the increase of GDD in L-Car/2008 treatment was higher than in H-Car/2008 in all the three periods established (*I*, *II*, *III*). Furthermore, it should be noted that in periods *II* and *III* of 2007, Tm of H-Car was 21.4°C and 19.0°C, respectively (Table 4); whilst in 2008, Tm was much lower (18.9°C and 12.8°C, respectively).

The elevated percentage of monomers in the seeds of 'Grenache' in 2008 showed a delay in seed ripeness compared with the pulp, which achieved acceptable values of °Brix, regardless of vigor (Table 6). In 2007, the high vigor conditions favored the reduction of monomers, causing an increase in mDP. Therefore, in warm vintage, the seeds ripened differently depending on the vigor effect, with the high vigor and warm vintage (H-Gre/2007) presenting the best conditions to mature. 'Grenache' (L-Gre and H-Gre) reached the same level of PAs at harvest of 2008 (L-Gre, 8.06 mg g^{-1} and H-Gre, 7.82 mg g^{-1}) but not in 2007 (L-Gre, 6.66 mg g^{-1} and H-Gre, 4.08 mg g^{-1}). In the L-Gre treatment, Tmax in period *II* of 2007 exceeded 35°C for 6 days; whilst in the H-Gre treatment the temperatures remained milder during ripening (periods *II* and *III*) (Table 4). Consequently, the accumulation of a relatively low concentration of monomers in 2007 suggests that the warm vintage conditions and high vigor, in the case of 'Grenache', allowed the optimum ripening of the seed. In contrast, the warm vintage in the low vigor vines revealed unripe seeds, indicating sensitivity in this variety during extreme climate conditions, when the high maximum temperatures and low relative humidity were registered in this vineyard during ripening. The different pattern observed in 'Grenache' and 'Carignan' let us to consider a viticultural management specifically for each variety in order to improve the seed ripeness.

Seed procyanidin composition from distal parts of bunch at harvest

In the current study, it was found that in the warm and dry vintage, top seeds ripen better in the low vigor conditions (L-Car/2007 and L-Gre/2007), yielding the lowest levels of monomers measured in this study. In contrast, in high vigor (H-Car/2007 and H-Gre/2007), the seeds of the bottom side were more mature at harvest (Tables 7 and 8). Moreover, the differences between the °Brix of distal parts of the bunch did not always correspond to a greater difference in levels of monomers and oligomers of the seeds in the top and bottom half of a bunch.

Effect of vintage, vigor and uniformity on seed procyanidin composition

According to the results from the multivariate factorial analysis (Table 9), regardless of the vintage, vigor is the most influential factor on the maturation of 'Grenache' seeds. Instead, seed ripeness for 'Carignan' does not depend on vigor when the vintage is warm. In warm vintage, the seeds of 'Carignan' were more influenced by the effect of the interaction Vigor * Uniformity than each factor individually.

Conclusions

Based on the current study it is clear that the evolution of total seed procyanidins from veraison to harvest depends primarily on the diminution of flavan-3-ol monomers. The kinetics of this evolution depends on the variety, vintage and vigor. During seed maturation, monomers diminish faster in low vigor vines. At harvest, procyanidin content in Grenache remains always higher than in Carignan. Under temperate vintage conditions, both 'Carignan' and 'Grenache' maintain a high level of flavan-3-ol monomers and a low polymerization, suggesting that the seeds have not fully matured. Under warm conditions (very high temperatures and drought) and in high vigor vines, seed procyanidin polymerization was favored only in 'Grenache'. Seed ripeness depends first on the vintage and to a lesser extent on the vigor, for both 'Carignan' and 'Grenache'. Procyanidin seed composition from the distal parts of the bunch also depends on vine vigor and vintage. In temperate vintage (2008), seeds ripped homogeneously in both parts of the bunch. In warm vintage (2007), the top seeds ripened better in the low vigor, whereas bottom seeds ripened better in the high vigor.

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