Effect of early leaf removal on *Vitis Vinifera* L. *cv.* Tempranillo seeds during ripening based on chemical and image analysis

M. Inmaculada Talaverano Arroba[a*], Francisco J. Rodríguez-Pulido[b], M. Esperanza Valdés Sánchez[a], Esther Gamero Samino[a], Daniel Moreno Cardona[a], M. José Jara-Palacios[b], Francisco J. Heredia Mira[b]

[a]Technological Institute of Food and Agriculture, Government of Extremadura (Spain), Hortofruenol Research Group, Avda. Adolfo Suárez, S/N, 06071 Badajoz, Spain.

[b]Food Colour and Quality Laboratory, Dept. Nutrition and Food Science, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain.

*Corresponding author: Phone: +34 954556495; Fax.: +34 954556110 E-mail: heredia@us.es

Abstract

Phenolic composition, color and morphology variables were monitored during ripening ~~on~~ grape seed of *Vitis vinifera* L: *cv.* Tempranillo. The aim of study was determinate the effect of limitation induced by early leaf removal (ELR) *vs.* non-defoliated (ND) control vines. The ultimate goal of this research was to assess ~~a prediction of phenolic composition~~ based on variables obtained by image analysis. ELR ~~brought about an advancement of maturation~~ and ~~also had lower concentration of phenols~~ and smaller and darker seeds. (+)-Catechin and total cinnamic acid contents as well as L* and aspect ratio were the most significant parameters for distinguishing treatments. Furthermore, ~~A~~rea, length, width, *L**, *a**, *b** and heterogeneity predict the chemical composition in seed grapes. Although it is not yet a substitute for chemical analysis, it could become a quick way to estimate the chemical characteristics of grape seed during maturation. The methodologies proposed in this work can be powerful tools for winemakers.

Keywords

Early Leaf Removal, Grape Seeds, Image Analysis, Phenols

INTRODUCTION

The phenolic compounds located in grape skin and seeds give different properties to red wine depending on the stage of maturation ~~of these solid parts~~ (Robichaud and Noble, 1990). Qualitative and quantitative phenolic composition of grapes depends on multiple factors, including climate, variety, soil, water availability, and degree of ripeness (Bautista-Ortín et al., 2012). Several studies have shown that phenols in seeds accumulate before the onset of ripening or veraison, reaching a maximum around veraison and decreasing towards harvest (Ferrer-Gallego et al., 2010; Kennedy et al., 2000b). Moreover, it has also been reported that poorly ripened berries have lower phenol extractability from skin and higher extractability from seeds (Peryot des Gachons and Kennedy, 2003). It has been found that seed phenols decline during grape ripening and that the amount of extracted phenols declines at the same time as maturity. (Kennedy et al., 2000a; Kennedy et al., 2000b).

Changes in seed coat colour and morphology have also been related to developmental changes in berry anthocyanins and total skin phenolics, suggesting that external appearance and colour of seeds may be used as an additional indicator of overall berry ripeness (Ferrer-Gallego et al., 2010; Ristic and Iland, 2005). Usually, colour is determined by tristimulus colorimetry and expressed in terms of *L*, a* and b** variables, corresponding to the uniform colour space CIELAB (CIE, 2004). The instruments used for measuring colour, require ~~homogenize the sample~~, to achieve uniform colour, ~~so it becomes a tedious and complicated task to measure the colour of heterogeneous materials, or of~~ small objects such as grape seeds. For this purpose, the use of digital imaging is advantageous. Moreover, digital image analysis ~~has useful complements since not only colour but~~ other characteristics such as shape, texture and homogeneity ~~can be determined~~ (Zheng and Sun, 2008).

Vineyard ~~practice~~ is another aspect that affects phenolic compounds, colour and morphology of grape during seed and grape development (Roby and Matthews, 2004). Early defoliation is a viticultural practice that proved effective for regulating yield and improving grape quality. It is usually carried out pre-bloom, unlike traditional leaf removal, which is typically done between fruit set and veraison on high density canopies to improve fruit exposure and air circulation (Tardaguila et al., 2008). Crop regulation is achieved in early defoliated vines through reduced fruit set, producing smaller and looser clusters that are less susceptible to Botrytis rot (Poni et al., 2006). In these two studies, grape quality also improved in defoliated vines as soluble solids and anthocyanin concentrations increased.

Unfortunately, very little is known about the relationships between such practices and the quantity and composition of seed phenols, colour and morphology. In order to understand these relationships, it is important to understand first the pattern of accumulation and modification of these phenols during ripening. This study was designed to establish how ~~the~~ source limitation induced by early leaf removal affects phenolic composition, colour and morphology of grape seed in *Vitis vinifera* L.; *cv*. Tempranillo *vs*. non-defoliated (ND) control vines from veraison to postharvest in 2010 vintage. The main aim of this work was monitor changes in phenols, colour and morphology of berry seed during ripening. Once it has been shown that these variables have a clear and measurable evolution during ripening, the ultimate goal was to determine the correlation between phenolic composition and appearance (colour and morphology) of berry seeds from different agronomic techniques. Because the wine industry requires the use of simple, rapid and reliable analytical procedures involving minimal or no sample preparation to aid in making harvesting decisions, the methodologies proposed in this work can be powerful tools for winemakers.

MATERIAL AND METHODS

Plant material and experimental design

The experiment was conducted on *Vitis vinifera* L. *cv*. Tempranillo red grape berries grown in Extremadura (Spain) in 2010. The experimental vineyard was in Guadajira (38ºN, 6ºW, n 198 m a.s.l) and vines were trained to a vertical trellis on a bilateral cordon system oriented in an ~~East-West~~ direction (104º SE-76º NW). The vineyard was planted in 2001 on Richter-110 rootstock at a spacing of 2.5 m by 1.2 m (3000 vines ha^{-1}). Irrigation treatment was done by replacing crop evapotranspiration (ET_c) at a rate of 100% ET_c. Drip irrigation was applied with pressure-compensated emitters of 4 L h^{-1} located in a single row 120 cm apart.

The experimental design was a split-plot with four replicates. The plots had six rows with eighteen vines per row. The main plot consisted of two treatments: early leaf removal (ELR) and control or non-defoliated (ND) treatment. Early leaf removal consisted of ~~hand-removing~~ the first seven basal leaves from main shoot (seven basal nodes) before flowering.

Sampling

Vitis vinifera L. Tempranillo grape samples were collected in 2010 in each experimental plot at six different developmental stages: from veraison to over-ripeness. The first sampling was performed when the ELR reached approximately 20 ºBrix. Each experimental plot had four replicates (n = ~~4~~), except some stage from ND treatment which samples were not considered optimal for performing the analysis (II stage n = 3, V and VI stage n = 2). Sampling was carried out as follows: 100 berries were collected from both sides of the vines in a row within the vineyard. Edge rows and the first two vines in each row were avoided. The samples (whole grapes) were immediately frozen and stored at -80°C until analyses were performed.

Must analysis

Total soluble solids (TSS) (ºBrix) by refractometry, total acidity (TA) by ~~titrimetry~~ (expressed as tartaric acid) and pH by a pH-meter, were analysed at different maturation stages in the must samples. Total phenolic compounds (TPC, expressed as gallic acid, mg g^{-1} of berry fresh weight) and anthocyanins (expressed as malvidin glucoside, mg g^{-1} of berry fresh weight) were extracted and determined using methods proposed by the Australian Wine Research Institute (Iland et al., 2005). Berry tannin concentration (expressed as catechin, mg g^{-1} of berry fresh weight) was determined according to Sarneckis et al. (2006). All analyses were made in triplicate.

Phenolic extraction and analysis

Phenolic extraction was carried out as described in Nawaz et al. (2006) with some modifications. Grape seeds were manually separated, freeze-dried and ground to obtain a homogeneous powder. Sample (2 g) was homogenized in 10 mL of 75% ethanol, ~~kept under shaking~~ for 1 h in a shaking apparatus (VWR Incubating minishaker), and further centrifuged at 4190*g* for 5 min. The supernatant was collected and the residue submitted to the same process using 7.5 mL of 95% ethanol as solvent. The extracts (2 mL) were combined and concentrated (Eppendorf® Concentrator plus/Vacufuge® plus) to dryness and further re-dissolved in 1 mL of water-methanol-acetic acid (88:10:2, v/v/v). The extracts were injected directly into the chromatographic system after filtration through a 0.45 µm filter. All analyses were performed in triplicate.

Analysis of the individual phenolic compounds was performed according to the methodology described by Hernanz et al., (2007) with some minor modifications. High Performance Liquid Chromatography analyses were carried out in an Agilent 1100 series HPLC system (Palo Alto, CA) equipped with a diode-array detector, which was

set to scan from 200 to 770 nm, and a C18 Zorbax ODS column (5 µm, 4.6x 250 mm), using an injection volume of 10 µL.

The solvents were water-methanol-acetic acid (88:10:2, v/v/v, solvent A) and methanol-water-acetic acid (70:28:2, v/v/v, solvent B) at the following gradient: 0-60 min, 100 % B linear; 60-70 min, 50 % A and 50 % B linear; 70-75 min, 100 % A linear; 75-80 min, 100 % B linear; 80-90 min, washing and re-equilibration of the column. The flow was 1.0 mL min^{-1}, and the temperature of the column was set at 20 °C.

Identification of phenolic compounds was achieved by comparing their retention times and spectra with those of appropriate standards. Quantification was carried out by external calibration from the areas of the chromatographic peaks obtained by UV detection at the following wavelengths: 280 nm for benzoic acids and flavanols, 320 nm for cinnamic acid derivatives and 370 nm for flavonols. The corresponding calibration curves were made up for the following phenolic compounds: (-)-epicatechin (r^2 < 0.9998), gallic acid (r^2 < 0.9999) and caffeic acid (r^2 < 0.9999). The range of the linear calibration curves was 10 to 500 mg L^{-1}, with limit to detection (LOD) of 1.703 mg L^{-1} and limit to quantification (LOQ) of 5.677 mg L^{-1} for (-)-epicatechin. The range of the linear calibration curves was 1 to 25 mg L^{-1}, with LOD of 0.069 mg L^{-1} and LOQ of 0.231 mg L^{-1} for gallic acid. The range of the linear calibration curves was 0.5 to 45 mg L^{-1}, with LOD of 0.144 mg L^{-1} and LOQ of 0.481 mg L^{-1} for caffeic acid. The different phenolic compounds analysed were tentatively identified according to their order of elution, retention times of pure compounds. Quantification of other compounds was made using the calibration curves belonging to the most similar compound. (-)-Epicatechin, (+)-catechin, and procyanidins B1 and B2 were quantified with the calibration curve of epicatechin. Gallic acid, ethyl gallate, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid and syringic acid were quantified as gallic acid; and

caffeic acid, *p*-coumaric acid, and *m*-coumaric acid as caffeic acid. Standards, epicatechin, gallic acid and caffeic acid, were acquired from *Sigma* Aldrich®, Analyticals Carlo Elba® and Fluka®, respectively.

Image acquisition

The DigiEye® imaging system based upon the calibrated digital camera was used (Luo et al., 2001). This device consists of a closed illumination box, specially designed by Veri Vide Ltd. (Leicester, UK), and a digital camera (10.2 megapixel Nikon® D80 with Nikkor® 35 mm f/2D objective) connected to a computer (Pentium IV 3.00 GHz processor) via USB. The cabinet was equipped with two CIE D65 standard illuminant emulators, which allow the samples to be consistently illuminated under stable lighting conditions. To obtain morphological and appearance parameters, and the CIELAB coordinates from the RGB colour space data, software DigiFood® (Heredia et al., 2006) was used. Image processing was performed according to the methodology described by Rodríguez-Pulido et al. (2012b). Images of seeds corresponding to 100 berries per sample were acquired using the DigiEye system (n = 318-713, depending on the number of seeds per sampling).

Statistical treatment

Significant differences between ELR and ND treatments were determined by Student's T-test at the same stage. The differences between ripening stages were assessed by one-way analysis of variance (ANOVA) with Tukey's test. General discriminant analyses (GDA) and multiple linear regressions (MLR) were performed with Statistica Version 8.0 software (Stat-Soft, 2007). Student's T-test and ANOVA were performed using the SPSS Program Version 17.0 for Windows software package (SPSS Inc., Chicago, III, U.S.A).

RESULTS AND DISCUSSION

Must and berry analysis

Table 1 shows the values corresponding to the mean of must TSS, pH, TA, ºBrix/TA ratio, TPC, anthocyanins, tannins, mean weight of 100 berries, mean weight of seeds per 100 berries and mean number of seeds per 100 berries in each treatment.

The content of TSS the two treatments underwent a significant increase during study. ELR vines accounted for the increase in berry ºBrix values compared with control canopies, in agreeing with the results found by other authors in Graciano and Carignan vines (Tardáguila et al., 2010). This trend was maintained in all stages, although, the TSS were only significantly higher in stages II and VI (Table 1). ELR ~~treatment brought about an advancement of harvesting~~ by thirteen days, thus optimum ripeness (~~criteria established in area at~~ 23-24 ºBrix) was reached at stage II, as shown by ~~several~~ studies (Tardáguila et al., 2008; Poni et al., 2006). One commonly used index in assessing grape maturity is the ratio of °Brix to titratable acidity ~~showed significantly higher values in~~ ELR than ND treatment ($p < 0.05$ ~~in all stages, except in VI stage of the study~~).

A gradual increase in the pH of grapes was observed between the first and the

A gradual increase in the pH of grapes was observed between the first and the

last sampling dates for all treatments. Also, the titratable acidity was found to decrease to a minimum value at the last sampling dates. ELR grapes showed the lowest titratable acidity values ($p < 0.05$ in I, III, IV and V stage) and the greatest pH values ($p < 0.05$ in II, III, IV and V stage). These results ~~accord~~ with reports that ~~highly improved~~ cluster exposure in ELR vines is explained by temperature-driven enhanced degradation of organic acids (Kliewer, 1971).

The content of TPC in both treatments showed differences significantly ($p < 0.01$) between stages, with slight fluctuations. The anthocyanins concentrations presented significant ($p < 0.01$) variation during maturation in ELR treatment, which

211 recorded the highest values at harvest time. By contrast, the content of tannins displayed
212 significant differences ($p < 0.001$) in ND treatment with the lowest values in the fourth
213 stage. The

214 **Table 1.** Evolution of berry characteristics recorded in Tempranillo grapes subjected to non-defoliation (DN) or early leaf removal (ELR)

215 (Mean±Std. Dev.).

Parameters	Treatments	Stage I DOY 216	Stage II DOY 230 [1]	Stage III DOY 252	Stage IV DOY 256 [2]	Stage V DOY 264	Stage VI DOY 271	*Significance between stages*[5]
Must Soluble solids (ºBrix)	ND	18.92 ± 0.85 ^{c}	21.42 ± 0.41 ^{b}	23.03 ± 1.12 ^{ab}	22.97 ± 1.12 ^{ab}	22.93 ± 1.82 ^{ab}	24.32 ± 0.35 ^{a}	***
	ELR	20.49 ± 1.11 ^{c}	23.50 ± 0.40 ^{b}	24.15 ± 0.72 ^{ab}	24.35 ± 0.40 ^{ab}	23.98 ± 0.83 ^{b}	25.62 ± 0.43 ^{a}	***
	Significance[4]	*ns*	***	*ns*	*ns*	*ns*	***	
pH	ND	3.49 ± 0.10 ^{bc}	3.63 ± 0.02 ^{bc}	3.78 ± 0.06 ^{ab}	3.81 ± 0.15 ^{a}	3.76 ± 0.04 ^{ab}	3.75 ± 0.00 ^{ab}	**
	ELR	3.65 ± 0.08 ^{b}	3.75 ± 0.05 ^{b}	3.90 ± 0.05 ^{ab}	4.05 ± 0.03 ^{ab}	4.05 ± 0.06 ^{ab}	4.29 ± 0.44 ^{a}	***
	Significance	*ns*	**	*	*	**	*ns*	
Titratable Acidity (mg L^{-1} Tartaric acid)	ND	7.40 ± 0.46 ^{a}	5.19 ± 0.29 ^{b}	5.11 ± 0.28 ^{b}	4.78 ± 0.23 ^{b}	4.76 ± 0.12 ^{b}	5.23 ± 0.58 ^{b}	***
	ELR	6.38 ± 0.58 ^{a}	4.76 ± 0.46 ^{b}	4.34 ± 0.19 ^{b}	4.10 ± 0.20 ^{bc}	3.87 ± 0.39 ^{b}	4.70 ± 0.79 ^{b}	***
	Significance	*	*ns*	**	**	*	ns	
Ratio (ºBrix/Titratable Acidity)	ND	2.57 ± 0.26 ^{b}	4.13 ± 0.17 ^{a}	4.52 ± 0.27 ^{a}	4.82 ± 0.38 ^{a}	4.82 ± 0.27 ^{a}	4.68 ± 0.49 ^{a}	***
	ELR	3.24 ± 0.46 ^{c}	4.97 ± 0.48 ^{b}	5.57 ± 0.39 ^{ab}	5.95 ± 0.34 ^{ab}	6.23 ± 0.49 ^{a}	5.53 ± 0.76 ^{ab}	***
	Significance	*	*	**	**	**	*ns*	
Total phenolics compounds (mg g^{-1} Gallic acid)	ND	1.86 ± 0.25 ^{a}	1.17 ± 0.22 ^{b}	*nd*[3]	1.82 ± 0.39 ^{a}	1.67 ± 0.23 ^{ab}	1.39 ± 0.12 ^{ab}	**
	ELR	1.91 ± 0.35 ^{ab}	2.12 ± 0.37 ^{a}	*nd*	1.58 ± 0.07 ^{b}	1.91 ± 0.42 ^{ab}	1.92 ± 0.21 ^{ab}	*ns*
	Significance	*ns*	**		*ns*	*ns*	*	
Anthocyanins (mg g^{-1} Malvidin glucoside)	ND	0.58 ± 0.21	0.48 ± 0.19	*nd*	0.77 ± 0.22	0.65 ± 0.12	0.54 ± 0.07	*ns*
	ELR	0.50 ± 0.10 ^{b}	0.85 ± 0.11 ^{a}	*nd*	0.58 ± 0.05 ^{b}	0.68 ± 0.06 ^{ab}	0.66 ± 0.14 ^{ab}	**
	Significance	*ns*	*		*ns*	*ns*	*ns*	
Tannins (mg g^{-1} catechin)	ND	4.30 ± 0.69 ^{a}	4.54 ± 0.98 ^{a}	*nd*	2.20 ± 0.28 ^{b}	3.88 ± 0.36 ^{a}	2.35 ± 0.12 ^{b}	***
	ELR	5.83 ± 3.02	3.85 ± 1.08	*nd*	4.43 ± 1.29	4.13 ± 1.00	3.17 ± 0.54	*ns*
	Significance	*ns*	*ns*		*	*ns*	*ns*	
Weight 100 berries (g)	ND	197.30 ± 5.89	223.37 ± 23.36	196.07 ± 2.97	216.40 ± 30.20	213.24 ± 4.58	231.52 ± 35.11	*ns*
	ELR	185.07 ± 31.39	166.25 ± 8.34	193.80 ± 22.85	188.00 ± 14.18	189.88 ± 18.68	193.29 ± 16.47	*ns*
	Significance	*ns*	*	*ns*	*ns*	*ns*	*ns*	
Weight seeds/100 berries (g)	ND	6.52 ± 0.66	6.13 ± 0.20	6.68 ± 0.89	6.50 ± 0.51	6.45 ± 0.27	6.89 ± 0.50	*ns*
	ELR	6.45 ± 0.42	6.84 ± 0.30	5.86 ± 0.59	5.57 ± 0.48	6.59 ± 1.52	6.10 ± 1.03	*ns*
	Significance	*ns*	**	*ns*	*	*ns*	*ns*	
No. of seeds / 100 berries	ND	143.25 ± 12.72	156.00 ± 14.61	156.00 ± 12.83	178.25 ± 23.04	159.00 ± 17.32	172.50 ± 13.28	*ns*
	ELR	147.00 ± 13.93	154.50 ± 11.09	154.25 ± 5.19	169.75 ± 34.27	165.25 ± 15.69	170.75 ± 13.23	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	

216 [1]Data of harvest of ELR treatment.

217 [2]Data of harvest of ND treatment.

[3]Data non determined.

[4]Means separated within rows by t-test: *, **,***, *ns*: significant at $p < 0.05$, 0.01, 0.001, not significant, respectively, between treatments.

[5]One-way ANOVA: *, **,***, *ns*: significant at $p < 0.05$, 0.01, 0.001, not significant, respectively, between samples at different ripening stages for the same treatment.

Different superscriptletters [a,b,c,d], on the same line indicate significant differences($p < 0.05$) in the content of the compounds between samples at different ripening stages according to the Tukey's test.

content of TPC, anthocyanins and tannins showed higher values in ELR treatment at several maturity stages. Then the trend matched previous findings for the other cultivars (Poni et al., 2006). These higher concentrations could be related to smaller berry size trend induced by early defoliation in all stages (Table 1), which would presumably ~~allow~~ a higher skin:plum ratio (Clingeleffer et al., 2000).

Finally, the weight berries and seeds, as well as the number of seeds per 100 berries did not present any significant variation during maturation. Moreover, there was a positive relationship between seed and berry masses per 100 berries for both treatments for stages I, III, V, and VI, which is in agreement with previous findings by Roby and Matthews (2004). There ~~were no difference significantly to~~ the number of seed per 100 berries, although the trend in ELR treatment was minor number of seed than in ND for stages II, III, IV, and VI.

Phenolic compounds analysis in grape seed

~~Table 2 shows the changes in the composition of thirteen different phenolic compounds in grape seed for the ELR and ND treatments throughout ripening.~~ Seeds noted for their high flavanol content (from 3.90 to 2.29 mg g^{-1}) according to Rodríguez-Montealegre et al. 2006. Within flavanols, ~~for both treatments,~~ the highest concentration was found in procyanidin B2 compounds, followed by (+)-catechin. These results ~~coincide~~ with those reported by other authors in other red grape varieties such as Touriga Francesa (Mateus et al., 2001). At the end of berry maturation, procyanidin B2 was always found to be the major compound, which is in agreement with previous works for several varieties (De Freitas and Glories, 1999). Benzoic and cinnamic acids were present in seeds in low amounts (from 1.05 to 0.76 mg g^{-1} and from 0.04 to 0.05 mg g^{-1}, respectively) and their concentrations did not suffer noticeable changes during ripening.

250 **Table 2.** Evolution during ripening of phenol contents in seeds in Tempranillo grapes

251 subjected to non-defoliation (DN) or early leaf removal (ELR) Mean±Std. Dev (mg g^{-1}).

Compund	Treatments	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI	*Significance between stages*[4]
Flavanols								
(+)-Catechin	ND	0.740 ± 0.036	0.707 ± 0.097	0.673 ± 0.091	0.665 ± 0.128	0.631 ± 0.041	0.701 ± 0.106	*ns*
	ELR	0.649 ± 0.144	0.645 ± 0.120	0.538 ± 0.079	0.580 ± 0.117	0.527 ± 0.132	0.588 ± 0.144	*ns*
	Significance[3]	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
(-)-Epicatechin	ND	0.457 ± 0.093^{b}	0.273 ± 0.058^{ab}	0.234 ± 0.033^{a}	0.286 ± 0.094^{ab}	0.231 ± 0.084^{a}	0.273 ± 0.031^{ab}	*
	ELR	0.286 ± 0.072	0.169 ± 0.026	0.171 ± 0.053	0.159 ± 0.041	0.216 ± 0.138	0.192 ± 0.109	*ns*
	Significance	*	*	*ns*	*	*ns*	*ns*	
Procyanidin B1	ND	0.730 ± 0.048^{a}	0.595 ± 0.035^{ab}	0.555 ± 0.036^{b}	0.603 ± 0.120^{ab}	0.557 ± 0.007^{ab}	0.610 ± 0.075^{ab}	*
	ELR	0.575 ± 0.071	0.520 ± 0.067	0.427 ± 0.045	0.446 ± 0.056	0.418 ± 0.115	0.437 ± 0.128	*ns*
	Significance	*	*ns*	**	*ns*	*ns*	*ns*	
Procyanidin B2	ND	1.981 ± 0.476	1.451 ± 0.219	1.358 ± 0.041	1.424 ± 0.505	1.239 ± 0.222	1.410 ± 0.522	*ns*
	ELR	1.659 ± 0.293	1.396 ± 0.461	1.155 ± 0.076	1.272 ± 0.066	1.396 ± 0.684	1.314 ± 0.609	*ns*
	Significance	*ns*	*ns*	**	*ns*	*ns*	*ns*	
Total Flavanols	**ND**	3.908 ± 0.571	3.026 ± 0.361	2.819 ± 0.111	2.979 ± 0.836	2.658 ± 0.273	2.995 ± 0.673	*ns*
	ELR	3.170 ± 0.555	2.731 ± 0.593	2.290 ± 0.207	2.456 ± 0.228	2.557 ± 1.030	2.532 ± 0.978	*ns*
	Significance	*ns*	*ns*	**	*ns*	*ns*	*ns*	
Benzoic Acids								
Gallic Acid	ND	0.146 ± 0.010	0.154 ± 0.017	0.144 ± 0.017	0.155 ± 0.032	0.124 ± 0.005	0.148 ± 0.054	*ns*
	ELR	0.111 ± 0.039	0.136 ± 0.032	0.118 ± 0.022	0.116 ± 0.019	0.130 ± 0.032	0.138 ± 0.039	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
Protocatechuic Acid	ND	0.316 ± 0.069	0.312 ± 0.022	0.290 ± 0.026	0.297 ± 0.050	0.295 ± 0.001	0.301 ± 0.070	*ns*
	ELR	0.310 ± 0.022	0.330 ± 0.052	0.287 ± 0.007	0.304 ± 0.028	0.276 ± 0.047	0.289 ± 0.054	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
4-Hydroxybenzoic Acid	ND	0.313 ± 0.035	0.280 ± 0.027	0.250 ± 0.019	0.270 ± 0.037	0.248 ± 0.000	0.283 ± 0.028	*ns*
	ELR	0.235 ± 0.086	0.225 ± 0.032	0.197 ± 0.018	0.215 ± 0.023	0.199 ± 0.043	0.222 ± 0.057	*ns*
	Significance	*ns*	*ns*	**	*	*ns*	*ns*	
Vanillic Acid	ND	0.088 ± 0.013	0.095 ± 0.006	0.089 ± 0.017	0.092 ± 0.016	0.089 ± 0.007	0.104 ± 0.010	*ns*
	ELR	0.066 ± 0.006	0.080 ± 0.019	0.069 ± 0.012	0.069 ± 0.016	0.073 ± 0.017	0.083 ± 0.020	*ns*
	Significance	*	*ns*	*ns*	*ns*	*ns*	*ns*	
Syringic Acid	ND	0.191 ± 0.033	0.142 ± 0.018	0.127 ± 0.007	0.132 ± 0.049	0.109 ± 0.014	0.125 ± 0.032	*ns*
	ELR	0.120 ± 0.026	0.121 ± 0.039	0.092 ± 0.017	0.101 ± 0.017	0.110 ± 0.056	0.103 ± 0.052	*ns*
	Significance	*	*ns*	*	*ns*	*ns*	*ns*	
Total Benzoic Acid	ND	1.052 ± 0.124	0.983 ± 0.043	0.901 ± 0.065	0.947 ± 0.177	0.866 ± 0.016	0.962 ± 0.195	*ns*
	ELR	0.842 ± 0.159	0.893 ± 0.154	0.763 ± 0.060	0.806 ± 0.084	0.789 ± 0.179	0.836 ± 0.210	*ns*
	Significance	*ns*	*ns*	*	*ns*	*ns*	*ns*	
Derivative Acid								
Ethyl Gallate	ND	0.005 ± 0.001^{a}	0.003 ± 0.001^{ab}	0.004 ± 0.001^{ab}	0.003 ± 0.001^{ab}	0.002 ± 0.000^{b}	0.004 ± 0.000^{ab}	*
	ELR	0.004 ± 0.001^{a}	0.003 ± 0.001^{ab}	0.003 ± 0.001^{ab}	0.003 ± 0.002^{ab}	0.002 ± 0.000^{b}	0.003 ± 0.000^{ab}	*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
Cinammic Acids								
Caffeic Acid	ND	0.038 ± 0.003	0.032 ± 0.001	0.029 ± 0.003	0.037 ± 0.007	0.033 ± 0.003	0.028 ± 0.005	*ns*
	ELR	0.025 ± 0.005	0.030 ± 0.001	0.028 ± 0.003	0.029 ± 0.008	0.025 ± 0.004	0.027 ± 0.005	*ns*
	Significance	**	*	*ns*	*ns*	*ns*	*ns*	
p-Coumaric Acid	ND	0.010 ± 0.001	0.010 ± 0.001	0.011 ± 0.002	0.009 ± 0.001	0.008 ± 0.001	0.010 ± 0.001	*ns*
	ELR	0.012 ± 0.002	0.009 ± 0.002	0.008 ± 0.001	0.009 ± 0.000	0.009 ± 0.002	0.008 ± 0.001	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
m-Coumaric Acid	ND	0.001 ± 0.001	0.005 ± 0.002	0.004 ± 0.003	0.002 ± 0.001	0.002 ± 0.000	0.002 ± 0.000	*ns*
	ELR	0.002 ± 0.002	0.003 ± 0.002	0.002 ± 0.001	0.001 ± 0.001	0.002 ± 0.000	0.002 ± 0.000	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
Total Cinamic Acids	ND	0.049 ± 0.003	0.047 ± 0.004	0.045 ± 0.006	0.048 ± 0.006	0.044 ± 0.004	0.042 ± 0.006	*ns*
	ELR	0.039 ± 0.004	0.042 ± 0.003	0.039 ± 0.002	0.040 ± 0.001	0.036 ± 0.005	0.038 ± 0.005	*ns*
	Significance	**	*ns*	*ns*	*ns*	*ns*	*ns*	
Total Phenolic								
	ND	5.015 ± 0.678	4.060 ± 0.392	3.770 ± 0.177	3.977 ± 1.019	3.570 ± 0.261	4.002 ± 0.862	*ns*
	ELR	4.055 ± 0.702	3.669 ± 0.743	3.096 ± 0.267	3.306 ± 0.315	3.384 ± 1.208	3.409 ± 1.192	*ns*
	Significance	*ns*	*ns*	**	*ns*	*ns*	*ns*	

2522 Meaning of letters and symbols as in Table

5

2

2532

5

3

Slight significant differences between stages were found (last column of Table 2), (-)-epicatechin and procyanidin B1 in ND treatment and ethyl gallate compound for both treatments. However, there was a tendency to find higher values in early stages than final stages in agreement with other studies (Ferre-Gallego et al., 2010; Rodríguez-Pulido et al., 2012a).

~~The treatment of defoliation applied affected decreasing~~ total flavanols, also procyanidin B1, procyanidin B2, and (-)-epicatechin at various maturity stages. Likewise, 4-hydroxybenzoic acid, vanillic acid, syringic acid as well as total benzoic acid showed significant differences between treatments at different stages, presenting lower values in ELR than ND treatment. Finally, ELR treatment affected significantly to caffeic acid and total cinnamic acids at the beginning of the study. Those changes may be attributable to the advancement of maturity in defoliation treatments (Table 1). ~~Thus, the lignification of seed could be higher,~~ and it was difficult for the solvent to access the inner integument (Cadot et al., 2006), which might explain the lower amount of phenols extracted in the ELR samples. As ripening advanced, differences between ELR and ND treatments became less pronounced, so the solidification of the outer cells, which are rich in phenols, could have affected the extraction of these compounds.

Image analysis

The colorimetric data and morphological variables of digital images are shown in Table 3. The phenomenon of browning appears as a decrease in the quantitative colorimetric variable chroma (C^*_{ab}) as well as the qualitative colorimetric variable hue (h_{ab}) during maturation (Table 3). Furthermore, changes in hue are usually related to qualitative changes in chemical composition (Heredia et al., 1998), which would be in agreement with the chemical changes described previously. Similar results have been reported in

red cultivar by several authors (Ferrer-Gallego, et al. 2010; Rodríguez-Pulido et al., 2012a,b).

Table 3. Evolution during ripening of CIELAB values and morphological parameters measured grape seeds in Tempranillo grapes subjected to

Parameters	Treatments	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI	*Significance between stages*[4]
	ND	46.46 ± 1.49	47.16 ± 0.97	43.91 ± 1.89	46.00 ± 1.30	45.01 ± 0.19	43.25 ± 3.71	*ns*
L*	ELR	45.21 ± 0.54^{a}	42.45 ± 2.75^{ab}	43.67 ± 0.75^{ab}	40.62 ± 2.48^{b}	43.49 ± 1.46^{ab}	43.53 ± 1.15^{ab}	*
	Significance[3]	*ns*	*	*ns*	*	*ns*	*ns*	
	ND	9.69 ± 0.49	8.73 ± 0.19	9.08 ± 0.45	9.30 ± 0.21	9.28 ± 0.07	9.55 ± 0.46	*ns*
a*	ELR	9.07 ± 0.34	9.06 ± 0.33	9.17 ± 0.46	9.29 ± 0.31	9.59 ± 0.55	9.33 ± 0.27	*ns*
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
	ND	20.62 ± 0.74^{a}	20.17 ± 0.75^{ab}	19.27 ± 1.18^{ab}	19.06 ± 0.42^{ab}	18.32 ± 0.61^{b}	18.48 ± 0.05^{ab}	*
b*	ELR	20.53 ± 0.16^{a}	19.64 ± 0.20^{ab}	18.27 ± 0.36^{c}	17.26 ± 0.69^{c}	18.39 ± 0.96^{bc}	17.79 ± 0.56^{c}	***
	Significance	*ns*	*ns*	*ns*	***	*ns*	*ns*	
	ND	22.95 ± 0.86	22.10 ± 0.75	21.45 ± 1.27	21.37 ± 0.40	20.68 ± 0.56	20.96 ± 0.23	*ns*
C^*_{ab}	ELR	22.59 ± 0.26^{a}	21.76 ± 0.29^{ab}	20.61 ± 0.44^{c}	19.77 ± 0.65^{c}	20.92 ± 1.08^{bc}	20.29 ± 0.55^{c}	***
	Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	
	ND	64.48 ± 0.52^{ab}	66.33 ± 0.55^{a}	64.11 ± 0.65^{b}	63.46 ± 0.73^{bc}	62.58 ± 0.69^{bc}	61.96 ± 1.37^{c}	***
h_{ab}	ELR	65.87 ± 0.79^{a}	64.84 ± 0.76^{a}	62.50 ± 1.27^{b}	60.90 ± 1.31^{b}	61.55 ± 1.04^{b}	61.49 ± 0.54^{b}	***
	Significance	*	*	*ns*	*	*ns*	*ns*	
	ND	6.82 ± 0.47^{c}	6.85 ± 0.50^{bc}	6.91 ± 0.44^{ab}	6.82 ± 0.45^{c}	6.78 ± 0.41^{c}	6.99 ± 0.49^{a}	***
Length (mm)	ELR	6.46 ± 0.45^{c}	6.73 ± 0.44^{a}	6.57 ± 0.48^{b}	6.51 ± 0.49^{bc}	6.51 ± 0.49^{bc}	6.51 ± 0.44^{bc}	***
	Significance[3]	***	***	***	***	***	***	
	ND	3.98 ± 0.40^{ab}	4.03 ± 0.41^{a}	3.95 ± 0.40^{b}	4.00 ± 0.43^{ab}	3.87 ± 0.37^{d}	4.02 ± 0.43^{ab}	***
Width (mm)	ELR	4.05 ± 0.43^{ab}	4.08 ± 0.39^{a}	4.01 ± 0.38^{bc}	4.00 ± 0.39^{bc}	3.96 ± 0.40^{cd}	3.93 ± 0.41^{d}	***
	Significance	**	*ns*	**	*ns*	**	**	
	ND	20.09 ± 2.53^{ab}	20.22 ± 2.62^{ab}	19.78 ± 2.40^{b}	20.08 ± 2.71^{ab}	19.04 ± 2.23^{d}	20.54 ± 2.92^{a}	***
Area (mm^2)	ELR	19.68 ± 2.52^{b}	20.22 ± 2.27^{a}	19.35 ± 2.29^{bc}	19.33 ± 2.45^{bc}	19.01 ± 2.44^{cd}	18.76 ± 2.36^{d}	***
	Significance	**	*ns*	**	***	*ns*	***	
	ND	1.78 ± 0.21^{ab}	1.76 ± 0.21^{b}	1.81 ± 0.22^{a}	1.78 ± 0.22^{ab}	1.81 ± 0.19^{a}	1.82 ± 0.22^{a}	***
Aspect ratio	ELR	1.66 ± 0.21^{b}	1.70 ± 0.21^{a}	1.68 ± 0.20^{ab}	1.68 ± 0.20^{ab}	1.70 ± 0.21^{a}	1.71 ± 0.21^{a}	***
	Significance	***	***	***	***	***	***	
	ND	1.37 ± 0.17^{bc}	1.36 ± 0.10^{c}	1.39 ± 0.10^{a}	1.36 ± 0.09^{c}	1.39 ± 0.08^{ab}	1.39 ± 0.21^{a}	***
Roundness	ELR	1.30 ± 0.10^{d}	1.34 ± 0.10^{ab}	1.34 ± 0.09^{ab}	1.32 ± 0.08^{c}	1.33 ± 0.09^{bc}	1.35 ± 0.12^{a}	***
	Significance	***	***	***	***	***	***	
	ND	10.27 ± 3.46^{b}	10.94 ± 3.18^{a}	10.07 ± 3.03^{b}	10.63 ± 3.64^{ab}	10.66 ± 3.37^{ab}	10.17 ± 3.67^{b}	***
Heterogeneity (%)	ELR	9.06 ± 2.51^{d}	9.81 ± 2.89^{bc}	10.58 ± 2.79^{a}	9.57 ± 2.44^{c}	10.15 ± 2.95^{ab}	10.07 ± 3.28^{b}	***
	Significance	***	***	**	***	*	*ns*	

non-defoliation (DN) or early leaf removal (ELR) at each stage of maturation. (Mean±Std. Dev., n=318-713) (CIELAB Units.).

281 Meaning of letters and symbols as in Table 1.

Seeds belonging to ELR treatment had lower values of L*, b* and hab, though only significant differences were found at stages I, II, and IV stage. In general, seeds belonging to ELR treatment showed the darkest colour. This could be related with advancement of maturity in this treatment, since seed coat colour can be used as an indicator of not only maturation but also of overall berry ripeness.

The morphology of seeds remained fairly stable throughout the research, without major changes, as reported by other authors (Ristic and Iland, 2005). The present study began at veraison, which is associated with cessation of seed growth and continued with seed drying and maturation until post-harvest. The values of morphological parameters measured in seeds are similar to results for Syrah and Tempranillo in South-Western Spain (Rodriguez-Pulido, et al. 2012b).

In general, seeds ELR were smaller than ND ~~ones.~~ This fact might be related to reduction of berry size due to defoliation, as earlier findings report for other red varieties such as Trebbiano (Poni et al., 2006), Graciano (Tardáguila et al., 2010) and Sangiovese (Palotti et al., 2011).

Statistical treatment

Chemical analysis results were subjected to forward stepwise General Discriminant Analysis (GDA) to determine ability to distinguish ELR and ND treatments from these data (n = 43). The model included total phenols, procyanidins B1, gallic acid, caffeic acid, procyanidin B2, total cinammics acids and (+)-catechin, these last two being significant at $p < 0.05$ (Table 4). Similarly, image analysis data were also subjected to forward stepwise GDA to determine ability to distinguish ELR and ND treatments (n = 43). The stepwise model included the variables Heterogeneity, Length, *a**, *L**, and

Aspect Ratio, these last two being significant at $p < 0.05$ (Table 4). The high level of significance of Aspect Ratio is worthy of note, and for this reason, analysis was repeated once only. By using the five variables from image analysis included in the model, it was possible to classify 95.83 % of the ELR samples and 94.74 % of the ND samples. In fact, it was possible to classify the samples using only the Aspect Ratio variable with almost the same level of accuracy as when using all the variables (91.66 % of the ELR samples and 89.47 % of the ND samples). This fact means that the treatment significantly altered the shape of the seed. Since the sampling was made at veraison, it would be of interest to study how the treatment affects seeds in earlier stages, when actual seed development takes place.

Table 4. *P*-values for General Discriminant Analysis obtained from chemical and image analysis variables included in the model (in order of significance). Values in bold were significant at $p<0.05$, (n=43).

Chemical analysis	***p-values***	**Image analysis**	***p-values***
Catechin	**0.002354**	Aspect ratio	**0.000013**
Total cinnamic acids	**0.011318**	L*	**0.038286**
Procyanidin B2	0.070049	a*	0.220789
Caffeic acid	0.091910	Length	0.250108
Galic acid	0.118918	Heterogeneity	0.302673
Procyanidin B1	0.132982		
Total phenols	0.237930		

Moreover, in order to combine the two sets of data, a forward stepwise Multiple Linear Regression (MLR) was applied with the aim of predicting the chemical

composition from the variables obtained by image analysis regardless of the agronomic conditions (n = 43). ~~Table 5 shows the most noteworthy results of this analysis.~~ Although values for R^2 were not higher than 0.75, they were significant ($p < 0.05$) in all cases. Of the variables obtained by image analysis, Area, Length, Width, *L**, *a**, *b**, and Heterogeneity were present in almost all cases.

Table 5. Results of Multiple Linear Regression of the most significant compounds showing R^2 and the independent variables included in each case, (n = 43).

		Independent variables	
Dependent variable	**R^2**	**Included and significant at *p<0.05***	**Included but not significant**
Catechin	0.645	Area, L*	Length
Procyanidin B1	0.714	Area, L*, Width	C^*_{ab}
Procyanidin B2	0.759	a*, heterogeneity, L*	Area, h_{ab}, Aspect Ratio, Length, Width
Galic acid	0.699	Length, L*, b*	Roundness, Aspect Ratio, Width, a*, Heterogeneity
Total Flavanols	0.747	Area, a*, L*, Heterogeneity	
Total benzoic acids	0.746	L*	Area, a*, Length, Aspect Ratio, Heterogeneity, C^*_{ab}, Width, b*

CONCLUSION

~~To sum up, the results~~ suggest that ELR treatment ~~showed~~ lower level ~~phenolic in seed~~ and darker colour attributable to the advancement of maturity compared to ND treatment. Also smaller seed was found in ELR treatment. The results obtained are evidence that the

336 treatment significantly altered the phenolic composition and shape of the seed.
337 Consequently, (+)-catechin and total cinnamic acids could be variables includes in the
338 model for differentiation of treatment. In addition, the variables analyzed by image
339 analysis permitted a good differentiation of treatments, in particular, Aspect Ratio
340 variable. Furthermore, Area, Length, Width, L*, a*, b*, and Heterogeneity obtained by
341 image analysis could be used for predicting the chemical composition regardless of the
342 agronomic conditions. It has not yet replaced conventional chemical analysis but it is an
343 attractive alternative due to its simplicity, versatility and low-cost. By controlling the
344 affected variable, it could become a way to assess the chemical characteristics of grape
345 seeds during maturation, thus saving time and chemical reagents and allowing the winery
346 to make quick decisions, for example in determining the moment for harvesting. The
347 methodologies proposed in this work can be powerful tools for winemakers.

348 ACKNOWLEDGEMENTS

349 This work was supported by Government of Extremadura, European Regional
350 Development Fund , "Instituto Nacional de Investigación y Tecnología Agraria y
351 Alimentaria" (INIA) (Project RTA 2008-00037-C04) and Hortofruenol
352 Reseach Group (Project GR10006). M.I.T are grateful to the INIA for his research
353 contract (DOC-INIA) and for her doctoral scholarship.

354 REFERENCES

355 Bautista-Ortín, A.B., Rodríguez-Rodríguez, P., Gil-Muñoz, R., Jiménez-Pascual, E.,
356 Busse-Valverde, N., Martínez-Cutillas, A., López-Roca, J.M., Gómez-Plaza, E.,
357 2012. Influence of berry ripeness on concentration, qualitative composition and
358 extractability of grape seed tannins. Aust. J. Grape Wine Res. 18, 123-130.

359 Bogs, J., Downey, M.O., Harvey, J.S., Ashton, A.R., Tanner, G.J., Robinson, S.P., 2005.
360 Proanthocyanidins synthesis and expression of genes encoding
361 leucoanthocyanidinreductase and anthoyanidinreductase in developing grape
362 berries and grapevine leaves. Plant Physiol. 139, 652-663.
363 Cadot, Y., Minana-Castello, M.T., Chevalier, M., 2006. Anatomical, histological, and
364 histochemical changes in grape seeds from *Vitis vinifera* L. *cv* Cabernet Franc
365 during fruit development. J. Agric. Food Chem. 54, 9206-9215.
366 Clingeleffer, P.R., Kristic, M., Sommer, K., 2000. Production efficiency and relationships
367 among crop load, fruit composition and wine quality. In: Proceedings of the
368 ASEV 50th Anniversary Annual Meeting, J. ed. Rantz, by Am. Society for
369 Enology and Viticulture, Pp. 318-322, Davis.
370 De Freitas, V.A.P., Glories, Y., 1999. Concentration and compositional changes of
371 procyanidins in grape seeds and skin of white *Vitis vinifera* varieties. J. Sci. Food
372 Agric. 79, 1601-1606.
373 Ferrer-Gallego, R., García-Marino, M., Hernández-Hierro, J.M., Rivas-Gonzalo, J.C.,
374 Escribano-Bailón, M.T., 2010. Statistical correlation between flavanolic
375 composition, colour and sensorial parameters in grape seed during ripening. Anal.
376 Chim. Acta. 660, 22-28.
377 Heredia, F.J., Francia-Aricha, E.M., Rivas-Gonzalo, J.C., Vicario, I.M., Santos-Buelga,
378 C., 1998. Chromatic characterization of anthocyanins from red grapes. I pH effect.
379 Food Chem. 63, 491-498.
380 Heredia, F.J., González-Miret, M.L., Álvarez, C., Ramírez, A., 2006. DigiFood®, No. SE-
381 01298.

382 Hernanz, D., Recamales, A., Meléndez-Martínez, J.A., González-Miret, M.L., Heredia,
383 F.J., 2007. Assessment of the Differences in the Phenolic Composition of Five
384 Strawberry Cultivars (Fragaria x ananassa Duch.) Grown in Two Different
385 Soilless Systems. J. Agric. Food Chem. 55, 1846-1852.
386 Iland, P., Bruer, N., Wilkes, E., Edward, G., 2005. Chemical Analysis of grapes and
387 wine: Techniques and Concepts, 1st Ed. Winetitles: Broadview, Australia.
388 Kennedy, J.A., Matthews, M.M., Waterhouse, A.L., 2000a. Changes in grape seed
389 polyphenols during fruit ripening. Phytochemistry. 55, 77-85.
390 Kennedy, J.A., Troup, G.J., Pilbrow, J.R., Hutton, D.R., Hewitt, D., Hunter, C.R., Ristic,
391 R., Iland, P.G., Jones, G.P., 2000b. Development of seed polyphenols in berries
392 from *Vitis vinifera* L. *cv*. Shiraz. Aust. J. Grape Wine Res. 6, 244-254.
393 Kliewer, W.M., 1971. Effect of day temperature and light intensity on concentration of
394 malic and tartaric acids in Vitis vinifera L. grapes. J. Hortic. Sci**.** 96, 372-377.
395 Luo, M.R., Cui, F.H., Li, C., 2001. British Patent Entitled Apparatus and Method for
396 Measuring Colour (DigiEye System) Derby University Enterprises Limited
397 (0124683.4).
398 Mateus, N., Marques, S., Gonçalves, A.C., Machado, J.M., De Freitas, V., 2001.
399 Proanthocyanidin composition of red *Vitis vinifera* varieties from the Douro
400 Valley during ripening: influence of cultivation altitude. Am. J. Enol. Vitic. 52,
401 115-121.
402 Nawaz, H., Sh,i J., Mittal, G., Kakuda, Y., 2006. Extraction of polyphenols from grape
403 seeds and concentration by ultrafiltration. Sep. Purif. Technol. 48, 176-181.

404 Peryot des Gachons, C., Kennedy, J.A., 2003. Direct method determining seed and skin
405 proanthocyanidin extraction into red wine. J. Agric. Food Chem. 51, 5877-5881.
406 Poni, S., Casalini, L., Bernizzoni, F., Civardi, S., Intrieri, C., 2006. Effects of early
407 defoliation on shoot photosynthesis, yield components, and grape composition.
408 Am. J. Enol. Vitic. 57, 397-407.
409 Ristic, R., Iland, P., 2005. Relationships between seed and berry development of *Vitis*
410 *Vinifera* L. *cv* Shiraz: Developmental changes in seed morphology and phenolic
411 composition. Aust. J. Grape Wine Res. 11, 43-58
412 Robichaud, J.L., Noble, A.C., 1990. Astringency and bitterness of selected phenolics in
413 wine. J. Agric. Food Chem. 53, 343-353.
414 Roby, G., Matthews, M.A., 2004. Relative proportions of seed, skin and flesh, in ripe
415 berries from Cabernet Sauvignon grapevines grown in a vineyard either well
416 irrigated or under water deficit. Aust. J. Grape Wine Res. 10, 74-82.
417 Rodríguez-Montealegre, R., Romero-Peces, R., Chacón-Vozmediano, J.L., Martínez-
418 Gascueña, J., García-Romero, E. 2006. Phenolic compounds in skins and seeds of
419 ten grape *Vitis vinifera* varieties grown in a warm climate. J. Food Compos. Anal.
420 19, 687-693.
421 Rodríguez-Pulido, F.J., Ferrer-Gallego, R., González-Miret, M.L., Rivas-Gonzalo, J.C.,
422 Escribano-Bailón, M.T., Heredia, F.J., 2012a. Preliminary study to determine the
423 phenolic maturity stage of grape seeds by computer vision. Anal. Chim. Acta.
424 732, 78-82.

425 Rodríguez-Pulido, F.J., Gómez-Robledo, L., Melgosa, M., Gordillo, B., González-Miret
426 M.L., Heredia, F.J., 2012b. Rippeness estimation of grape berries and seeds by
427 image analysis. Comput. Electron. Agr. 82, 128-133.
428 Sarneckis, C.J., Dambergs, R.G., Jones, P., Mercurio, M., Herderich, M.J., Smith, P.A.,
429 2006. Quantification of condensed tannins by precipitation with methyl cellulose:
430 development and validation of an optimised tool for grape and wine analysis.
431 Aust. J. Grape Wine Res. 12, 39-49.
432 Tardáguila, J., Diago, M.P., Martinez de Toda, F., Poni. S, Vilanova, M., 2008. Effects of
433 timing of leaf removal on yield, berry maturity, wine composition, and sensory
434 properties of Grenache wines grown in dry farmed conditions. J. Int. Sci. Vigne
435 Vin 42, 221-229.
436 Tardáguila, J., Martinez de Toda, F., Poni, S., Diago, M.P., 2010. Impact of early leaf
437 removal on yield and fruit and wine composition of *Vitis vinifera* L. Graciano and
438 Carignan. Am. J. Enol .Vitic. 61, 372-381.
439 Zheng, C., Sun, D.W. (2008). Object measurement methods, In: Da-Wen, Ed. S,
440 Computer Vision Technology for Food Quality Evaluation. Pp. 57-80. Academic
441 Press, Amsterdam.