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Pre-fermentative addition of an enzymatic hydrolyzate of grape seeds in warm climate winemaking. Effect on the differential colorimetry, copigmentation and polyphenolic profile

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Running title: Seeds hydrolyzate in winemaking

ABSTRACT

The effect of adding an enzymatic hydrolyzate of grape seeds (EH-GS) during fermentation on Syrah wine elaborated in warm climate has been evaluated. Our attention was focused on the polyphenolic composition and differential and tristimulus colorimetry applied to colour data. This is the first attempt to employ this oenological alternative to avoid common colour losses of red wines elaborated in warm climate. The addition of 250 g (simple dose, SW) of EH-GS to 120 Kg of fermentation mash promoted a significant (p<0.05) increase in the total polyphenolic content of stored wines, especially in benzoic acids, hydroxycinnamic acid derivatives, flavonols and anthocyanins. That fact could favour the higher copigmentation percentage and maximum colour stabilization (C^*_{ab}), without significantly change the tonality of wines. Unexpectedly, the use of a double quantity (DW) of EH-GS resulted in chroma even significantly lower than control wines (CW), showing visually perceptible colour changes (ΔE^*_{ab}>3 CIELAB units).

Keywords: enzymatic grape seed hydrolyzate; polyphenolic compounds; CIELAB; differential tristimulus colorimetry; warm climate.

INTRODUCTION

In warm climate, the high temperatures make difficult to obtain high quality red wines due to the usual colour instability over time. This fact is produced because both phenolic and technologic maturities do not coincide at the moment of harvesting as occurred in colder viniculture zones (López, Sánchez, Díaz, Ramírez, & Morales, 2007). Thus, seeds remain unripen and, as a consequence, copigmentation phenomena (which contribute to colour stabilization) is hampered by the shortage of pigments and copigments (Boulton, 2001). Therefore, fall of colour normally occurred after some months of storage in either bottle or barrels.

Numerous studies about implementation of wines with tannins from natural sources have been developed to counteract its natural shortfall and avoid colour losses (Vivas & Glories, 2003). In that way, grape seeds and pomace, notwithstanding of being a by-product, are rich on tannins and other polyphenolic compounds (González-Centeno, Rosselló, Simal, Garau, López, & Femenia, 2010; José Jara-Palacios, Hernanz, González-Manzano, Santos-Buelga, Escudero-Gilete, & Heredia, 2014) that could participate on colour stabilization of red wines. So, several studies have been developed about addition of seeds or pomace from white grape varieties to red wine with that purpose (Canals, Del Carmen Llaudy, Canals, & Zamora, 2008; Cliff, Stanich, Edwards, & Saucier, 2012; Gao, Yang, Li, Zhang, & Liu, 2013; Revilla, Ryan, Kovac, & Nemanic, 1998). That fact could be viable because, in warm climate, the harvest of red and white grape varieties coincides in time, being available white grape pomace and seeds to be added to red winemaking. Concretely, our research group is focused on the improvement of colour stability of red wines elaborated in warm climate to counteract the tannin deficit. That is the case of the pre-fermentative addition of American oak chips on Tempranillo fermentation mash (Gordillo, Cejudo-Bastante, Rodríguez-Pulido,

Lourdes González-Miret, & Heredia, 2013), and the addition of Pedro Ximenez white grape pomace to Syrah red grapes (Gordillo, Cejudo-Bastante, Rodríguez-Pulido, Jara-Palacios, Ramírez-Pérez, González-Miret, et al., 2014).

Furthermore, another practice of implementation is the addition of commercial ready-to-use oenological tannins preparations extracted from natural sources. In that sense, Chamorro, Viveros, Alvarez, Vega, & Brenes (2012) characterized the grape skin and seed extracts after the addition of different enzymes, such as carbohydrases and tannases, and pectinase, cellulase and tannase (Fernández, Vega, & Aspé, 2015). Besides, the addition of enzymes in winemaking were also carried out, demonstrating an improvement of colour extraction and stability of Sangiovese red wines (Canuti, Puccioni, Giovani, Salmi, Rosi, & Bertuccioli, 2012), and the occurrence of higher quantities of flavonols and caftaric acid in Monastrell wines (Bautista-Ortín, Martínez-Cutillas, Ros-García, López-Roca, & Gómez-Plaza, 2005). Also, an enhancement of the amount of procyanidins was observed when polygalacturonase and cellulase were added to Tannat, Monastrell and Cabernet Sauvignon wines (Favre, Peña-Neira, Baldi, Hernández, Traverso, Gil, et al., 2014).

However, the disadvantage of using commercial tannins is that, in many occasions, they are extracted with organic solvents that involve environmental and health risks. Although several authors have studied how minimize the use of organic solvents (Guerrero, Marín, Mejías, & Barroso, 2006; Xia, Deng, Guo, & Li, 2010), nowadays there are eco-friendly and solvent-free alternative procedures to reach optimal extraction of compounds. In that sense, Rodriguez-Rodriguez, Justo, Claro, Vila, Parrado, Herrera, et al. (2012) developed and patented an enzymatic method of extraction of phenolic compounds from grape pomace using an endoproteases mixture (trypsin- and chymotrypsin-like) (Parrado Rubio, Romero Ramírez, & Bautista Palomas, 2006).

These authors proved its higher stability, antioxidant properties and bioactivity, and phenols release in comparison with those traditionally extracted. This technique could resolve, on the one hand, the problems of the low extractability of polyphenolic compounds from seeds to wine (because the hydrolyzate is completely soluble in water), and, on the other hand, could avoid the use of organic solvents for extracting polyphenolic compounds. Moreover, to the best of our knowledge, the effect of adding this enzymatic hydrolyzate in winemaking has not been already studied.

With the objective of stabilizing the colour of red wines, the main goal of this work was to study the effect of the addition of a soluble enzymatic hydrolyzate of grape seeds during the fermentation of Syrah grapes cultivated in "Condado de Huelva" Designation of Origin (Spain). Grape seeds are chosen as natural source to reinforce wines with tannins, compounds that normally are present at low quantities because the immaturity of seeds at the harvest moment in warm climate winemaking. Our attention was focused on the study of chromatic characteristics by applying differential colorimetry and the polyphenolic composition related to the colour. It is highlighted that this is the first attempt to use this kind of product in winemaking and scrutinize their efficiency on the colour stabilization of wines elaborated in warm climate.

MATERIAL AND METHODS

Chemical and solvents

Methanol of HPLC grade was purchased from J. T. Baker (Baker Mallinckrodt, Mexico), and formic acid and Folin-Ciocalteau reagent were supplied by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade was obtained by a Milli-Q plus water purification system (Millipore Corp., Bedford, MA, USA). With regard of standards, malvidin-3-glucoside, (+)-catechin, (-)-epicatechin, gallic acid, caffeic acid, and quercetin were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Enzymatic hydrolysis of grape seeds

The product was prepared according to an enzymatic process patented for grape pomace (Parrado Rubio, Romero Ramírez, & Bautista Palomas, 2006; Rodriguez-Rodriguez, et al., 2012). 26.25 Kg of grape seeds (supplied by Viñaoliva Sociedad Cooperativa, Almendralejo, Badajoz, Spain) were submitted to enzymatic hydrolysis by using an endoproteases mixture (trypsin- and chymotrypsin-like) as hydrolytic agent in a bioreactor with controlled temperature and pH (60 ºC, pH 8) during 2 h, using the pH-stat method. After several procedures (separation of solids by centrifugation, filtration, and concentration), the final product was concentrated to dryness using a rotatory evaporator. As a result, a completely soluble in water syrup was obtained, which was lyophilized to obtain a fine brown powder. 26.25 Kg of dry and free-pulp seeds yielded approximately 2.4 Kg of lyophilizate.

Winemaking

This study was carried out with grapes from *Vitis vinifera* grape cv. Syrah cultivated in "Condado de Huelva" Designation of Origin, in south-western Spain. Around 1250 Kg were manually harvested in a good maturity (12.4 ºBaumé) and in good sanitary conditions. The grapes were destemmed and crushed, and the resulting must were distributed in nine stainless steel tanks of 220 L for skin maceration. Three types of vinifications were carried out: (a) three tanks were submitted to the addition of 250 g of the enzymatic hydrolyzate of grape seeds (EH-GS) (simple dose, SW), and (b) 500 g of EH-GS to other three tanks (double dose, DW). Taking into account that around 120 Kg of fermentation mash were used, that the grapes have around 5 % of grape seeds, and that the yield of the process was 2.4 Kg hydrolyzate / 26.25 Kg grape seeds, the doses of hydrolyzate added corresponded to the half and the same quantity of seeds that the fermentation mash already had, respectively (i.e., the supplemental addition of 2.5 and 5

% but in the form of enzymatic hydrolyzate). Other three tanks contained untreated fermentation mash, with 100 % of Syrah, were considered as control (CW) (c).

During the skin maceration, a manual punch down of the content of each tank was carried out once a day during 7 days. Once alcoholic fermentation was spontaneously developed for all wines in this stage, the mash was drawn off and the solid parts were separated from the wine. Malolactic fermentation begun after 4 days of skin removal, which lasted nine days, being confirmed by enzymatic measurements of malic and lactic acid contents. Then, the wines were then racked to stainsteel tanks of 50 L. The wine characteristics were monitored in different moments of the process: the initial point of grape crushing, during the skin-maceration stage and over the stabilisation stage. All the sample replicates were analysed in triplicate.

The official methods established by European Union were used to analysed the conventional oenological parameters such as pH, total and volatile acidity and free and total SO_2 (UE, 2003).

HPLC-DAD analysis of polyphenolic compounds

An Agilent 1200 chromatographic system, equipped with a quaternary pump, and UV-vis diode-array detector, an automatic injector, and ChemStation software (Palo Alto, CA), was used to the HPLC separation, identification and quantification of anthocyanins, flavonols, monomeric flavan-3-ols and hydroxycinnamic acid derivatives. Prior direct injection, the samples were filtered through a 0.45 μm Nylon filter (E0034, Análisis Vínicos, Spain). All analyses were made in triplicate.

The anthocyanin identification was carried out following the method proposed by Heredia, Escudero-Gilete, Hernanz, Gordillo, Meléndez-Martínez, Vicario, et al. (2010), based on the retention times and malvidin-3-glucoside standard. Acetonitrile-formic acid-water (3:10:87) as solvent A and acetonitrile-formic acid-water (50:10:40) as

171 solvent B were used. The elution profile was as follows: 0-10 min 94% A - 6% B; 10-15
172 min 70% A - 30% B; 15-25 min 60% A – 40% B; 25-35 min 55% A - 45% B; 35-40
173 min 50% A - 50% B; 40-42 min 40% A - 60% B; 42-43 min 94% A - 6% B. The
174 samples were injected (50 μl), in triplicate, onto a reversed-phase column Zorbax C18
175 (250 x 4.6 mm, 5 μm particle size), thermostatted at 38 ºC. UV-Vis spectra were
176 recorded from 200 to 800 nm with a bandwidth of 2.0 nm. The quantification was made
177 at 525 nm by comparing the areas and the retention times with the malvidin 3-glucoside
178 standard.
179 The method developed by Gordillo, Cejudo-Bastante, Rodríguez-Pulido, Lourdes
180 González-Miret, & Heredia (2013) was used for the identification of the polyphenolic
181 compounds (hydroxycinnamic acid derivatives, monomeric flavan-3-ols and flavonols).
182 This method is a modification of that described by (Castillo-Muñoz, Gómez-Alonso,
183 García-Romero, & Hermosín-Gutiérrez, 2007), with an identification based on retention
184 times and HPLC-DAD-ESI-MS^n. A volume of 50 μL of wine was injected in triplicate
185 onto a Zorbax C18 column (250 x 4.6mm, 5 μm particle size), maintained at 40 ºC, with
186 a flow rate of 0.63 mL/min. Acetonitrile-formic acid-water (3:10:87) as solvent A and
187 acetonitrile-formic acid-water (50:10:40) as solvent B were used. The elution profile
188 was as follows: 0 min 94% A - 6% B; 5 min 89% A - 11% B; 10 min 89% A - 11% B;
189 15 min 80% A - 20% B; 20 min 77% A - 23% B; 25 min 74% A - 26% B; 30 min 60%
190 A - 40% B; 35 min 50% A - 50% B; 38 min 40% A - 60% B; 46 min 94% A - 6% B.
191 UV-Vis spectra were recorded from 200 to 800 nm with a bandwidth of 2.0 nm. The
192 quantification was made at 280, 320 and 360 nm by comparing the areas and the
193 retention times with the gallic acid, caffeic acid, and quercetin standards, respectively.
194 The analysis of procyanidins and benzoic acids were carried out by RRLC after
195 filtration through a hydrophilic PVDF Millex-HV 0.45 μm syringe filter (Millipore,

196 Bedford, MA, USA). An Agilent 1260 chromatograph (Agilent Technologies, Palo
197 Alto, CA, USA) equipped with a diode array detector, which was set to scan from 200
198 to 770 nm, was used for the analysis of procyanidins and benzoic acids of wines and
199 enzymatic hydrolyzate of grape seeds. A C18 Poroshell 120 column (2.7 μm, 5 cm x 4.6
200 mm), using an injection volume of 15 μL, was employed for the separation of
201 compounds. The solvents were 0.1% formic acid in water (solvent A) and acetonitrile
202 (solvent B) at the following gradient: 0–5 min, 5% B linear; 5–20 min 50% B linear;
203 20–25 min, washing and re-equilibration of the column. The flow-rate was 1.5 mL/min
204 and the temperature of the column was set at 25 ºC, according to the method proposed
205 by José Jara-Palacios, Hernanz, González-Manzano, Santos-Buelga, Escudero-Gilete, &
206 Heredia (2014). The identification was made according to the retention times of
207 standards (when available), UV-vis spectra and mass spectra, as described Jara-
208 Palacios, González-Manzano, Escudero-Gilete, Hernanz, Dueñas, González-Paramás, et
209 al. (2013). The quantification of the polyphenolic compounds was carried out by
210 external calibration with polyphenolic standards at 280 nm.
211 Total anthocyanins, flavonols, benzoic acids, hydroxycinnamic acid derivatives,
212 monomeric flavan-3-ols and procyanidins were calculated as sum of individual
213 polyphenolic compounds identified by HPLC. Folin-Ciocalteau reagent was used for the
214 analysis of total phenolics (Singleton & Rossi, 1965). Total tannin assay was carried out
215 according to the method described by Abdel-Hammed (2009).
216 **Spectrophotometric colour measurement**
217 A Hewlett-Packard UV-vis HP8452 spectrophotometer (Palo Alto, CA) was used to
218 determine the whole visible spectrum (380-770 nm) at constant intervals ($\Delta\lambda$=2 nm),
219 using 2 mm path length glass cells and distilled water as reference. The original
220 software CromaLab© (Heredia, Álvarez, González-Miret, & Ramírez, 2004) was

221 employed to obtain the CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, and h_{ab}), following the
222 Commission Internationale de L'Eclariage's, CIE, recommendations (CIE, 1986): the
223 CIE 1964 10° Standard Observer and the CIE Standard Illuminant D65. Euclidean
224 distance between two points in the three-dimensional space define by L*, a*, and b*
225 were used for calculating colour differences (ΔE^*_{ab}): $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 +$
226 $(\Delta b^*)^2]^{1/2}$.

227 **Copigmented and polymerized anthocyanins**

228 The contribution of copigmentation to the total wine color at pH 3.6 (% copigmented
229 anthocyanins, %CA) and the degree of anthocyanin polymerization (% polymerized
230 anthocyanins, %PA) were determined following the method proposed by (R. B.
231 Boulton, 1996). The pH values of the wine sample were previously adjusted to pH 3.6
232 using 1 M NaOH or HCl. Total wine color at a pH value of 3.6 is assumed to be A^{acet},
233 the measure of absorbance at 520 nm (using water as a blank) after addition of 20 µL of
234 10% acetaldehyde to 2 mL of wine sample, and keeping for 45 min. The wine colour
235 without the copigmented anthocyanins effect is A^{20}, the absorbance measured at 520 nm
236 of the wine sample diluted 1:20 with a buffer solution (24 ml pure ethanol is added to
237 176 ml distilled water, dissolve 0.5 g of potassium bitartrate into the solution. The
238 solution pH is adjusted to 3.6 with HCl or NaOH as needed). The reading is corrected
239 for the dilution by multiplying by 20. That dilution leads to the dissociation of the
240 copigment complex while the contributions of the free anthocyanins and the polymeric
241 pigments remain. All absorbance readings are converted to 10 mm pathlength. The
242 following data were calculated:

243 $$\% \text{ Copigmentation} = [(A^{acet} - A^{20})/A^{acet}] \times 100$$

244 $$\% \text{ Polymerization} = (A^{SO2}/A^{acet}) \times 100$$

245 **Statistical Analysis**

All statistical analyses were performed using Statistica v.8.0 software (Statistica, 2007). Univariate analysis of variance (ANOVA) was applied using the general linear model program to establish whether mean values of the sample data differed significantly each other. The means values of each set of samples (n = 3) were compared by the Tukey test at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

The effect of the pre-fermentative addition of an enzymatic hydrolyzate of grape seed to red wines has been scrutinized. A follow-up along different vinification stages (day 0, initial point; and skin-maceration of 2, 4 and 7 days) and stabilization time (15, 22, 30, 37, 45, 60, 75, 90, 105, 120 and 150 days) has been conducted. An in-deep study of polyphenolic compounds, copigmentation and polymerization, CIELAB parameters and differential tristimulus colorimetry has been carried out.

Enological parameters

Both alcoholic and malolactic fermentations were correctly developed for all wines, in the light of the values of density and malic acid (around 998 g/L and < 0.1 g/L, respectively). Low values of the volatile acidity were reported, always situated below the limit (1.2 g/L) established by EU. In addition, optimal values of free and total sulfur dioxide content were reported for all wines (around 20 and 80 mg/L, respectively).

Polyphenolic profile of the enzymatic hydrolyzate of grape seed (EH-GS)

The polyphenolic characterization and total phenolics and tannins (mg/100 g dry extract) of the EH-GS are showed in Table 1. A total of eleven polyphenolic compounds have been identified and quantified in the enzymatic hydrolyzate, belonging to several families: benzoic acids (gallic acid and protocatechuic acid), hydroxycinnamic acid derivatives (*p*-coumaric acid), monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin) and a large extent of procyanidins forms (procyanidins B1, B2, B4 and B7,

procyanidin B2 3-*O*-gallate and procyanidin trimer 2). Any flavonol and anthocyanin has been identified in EH-GS.

As it can be seen, the polyphenolic composition of EH-GS represent only the 0.03 % of the dry matter, being gallic acid and procyanidins B1, B4 and B2 3-*O*-gallate the most predominant polyphenols, followed by protocatechuic acid. The rest of polyphenolic compounds contributed with a lower percentage, being practically negligible the presence of *p*-coumaric acid (Table 1).

Taking into account that the content of polyphenolic compounds of seeds (characterized and quantified by Jara-Palacios, Hernanz, González-Manzano, Santos-Buelga, Escudero-Gilete, & Heredia (2014)) and the consideration of an extractability average around 5% from seeds to wine (Rodríguez-Pulido, Hernández-Hierro, Nogales-Bueno, Gordillo, González-Miret, & Heredia, 2014), it could be affirmed that the main differences among seeds and enzymatic hydrolyzate of seeds were found in the content of benzoic acids and some flavan-3-ols. Thus, gallic and protocatechuic acids showed a higher concentration in EH-GS, contrarily to that observed in (+)-catechin. Besides, procyanidins B4 and trimer 2 showed a superior amount in the EH-GS, observing lower quantity of procyanidin B2-*O*-gallate, possibly owing to the use of enzymes in the obtaining of the hydrolyzate. The rest of procyanidins remain in the same order of magnitude. With regard to total phenolics, although seeds and EH-GS showed similar values (around 60 mg/g dry matter) (Jara-Palacios et al., 2014), the complete solubility of EH-GS in wine make that the availability of phenolic compounds is much higher than that provided by seeds.

Polyphenolic profile of wines

The polyphenolic profile of control wines and wines submitted to the addition of EH-GS did not differ in qualitative terms. Several types of polyphenolic compounds have

been identified in wines, belonged to benzoic acids, hydroxycinnamic acid derivatives, monomeric flavan-3-ols, procyanidins, flavonols and anthocyanins. Benzoic acids (gallic and protocatechuic acid), hydroxycinnamic acid derivatives (GRP, *trans*-caftaric, *trans*-coutaric and *p*-coumaric acids), and monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin) were the expected, well-known, compounds normally occurred in wines (Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2007). Besides, the procyanidins identified in the EH-GS have been also found in wines. Among flavonols, myricetin and quercetin were identified as their 3-glucuronide and glucoside forms and only the last one form for the rest of flavonols (kaempherol, isorhamnetin and syringetin) (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2007). No aglycons of flavonols were identified in Syrah wines. Native grape anthocyanins were detected, including non-acylated, acetylated and *p*-coumaroylated derivatives of the five expected anthocyanidins (delphinidin, cyaniding, petunidin, peonidin and malvidin) (Cejudo-Bastante, Pérez-Coello, & Hermosín-Gutiérrez, 2011; Gordillo, López-Infante, Ramírez-Pérez, González-Miret, & Heredia, 2010).

Polyphenolic Evolution

Table 2 summarizes the mean concentration (mg/L) of the colorless polyphenolic compounds (benzoic acids, hydroxycinnamic acid derivatives, monomeric flavan-3-ols, procyanidins and flavonols) and the total phenolic content (as mg GAE/L) of control Syrah wines and those with the supplement addition of a simple and double dose of EH-GS (SW and DW, respectively). Data are reported at the beginning of the treatment, after skin removal (SR) and at the end of the treatment (5 months of stabilization time). As well, Table 3 exposes the amount of anthocyanin compounds (mg/L) and the percentage of copigmentation and polymerization of the wines. Statistical analysis among samples is also included in the tables in order to scrutinize the possible

321 significant differences among wines. Furthermore, the evolution over time of the main
322 families of polyphenolic compounds (benzoic acids, hydroxycinnamic acid derivatives,
323 monomeric flavan-3-ols, procyanidins, flavonols, and anthocyanins) as sum of
324 individual compounds by HPLC at different vinification stages and stabilization time is
325 exposed in Fig. 1.
326 At the beginning of the treatment, the addition of the enzymatic hydrolyzate product
327 provoked a significant ($p < 0.05$) higher content of total phenolic content (as Folin-
328 Ciocalteau measurement) (CW, 1185.78 ± 25.12; SW, 1836.94 ± 89.82; DW, 1951.91 ±
329 193.64) (Table 2). Among the phenolic compounds, the hydroxycinnamic acid
330 derivatives could be contributed to this fact (Fig. 1), mainly due to GRP and *trans*-
331 caftaric. That fact could be owing to the enzymatic activity of the hydrolyzate; GRP is
332 formed by the reaction between *trans*-caftaric (or coutaric) acid and glutathione
333 (tripeptide contained in GRP) in the presence of PPO (polyphenol oxidase) (Cejudo-
334 Bastante, Pérez-Coello, & Hermosín-Gutiérrez, 2010). The enzymatic hydrolyzate
335 contained enzymatic activity such as proteases (which could release glutathione), and
336 hydrolases, that could favor the hydrolysis of GRP and release *trans*-caftaric acid,
337 increasing its content in the resulting wines.
338 Moreover, the in-deep study about the changes of the levels of benzoic acids,
339 hydroxycinnamic acid derivatives, monomeric flavan-3-ols, procyanidins, monomeric
340 flavonols, procyanidins and anthocyanins over time permitted to establish the
341 vinification stages more affected to polyphenolic profile by the addition of the
342 enzymatic grape seed hydrolyzate (Fig. 1).
343 The fermentative phase (0-7 days) did not exert a remarkable impact among the three
344 kind of wines on polyphenolic compounds and physicochemical transformations in
345 which they are involved (copigmentation and polymerization), without significant ($p <$

346 0.05) differences in any time-point (Fig. 1). At the moment of the skin removal (SR, day
347 7), only punctual significant ($p < 0.05$) differences were observed as a consequence of
348 the enzymatic hydrolyzate addition; concretely in *trans*-caftaric and gallic acid (Table
349 2) and in the non-acylated delphinidin-3-glucoside and the *p*-coumaroylated derivative
350 of peonidin (Table 3), fact that was maintained during the first stages of the stabilization
351 time (15-60 days). The quantity of enzymatic hydrolyzate only exerted a significant (p
352 < 0.05) effect on protocatechuic acid and *p*-coumaric acid at the skin removal
353 (DW>SW>CW). However, procyanidin B1, B4 and trimer 2 achieved the significantly
354 ($p < 0.05$) highest content in SW. This fact could be due to the enzymatic activity
355 (hydrolases or proteases and pectinases), releasing gallic acid or coumaric acid from
356 their esters or slightly increasing the extraction from grape. The lower content of total
357 phenolics in SW and DW after skin removal (Table 2) could be due to possible
358 saturation of the medium, pigment sedimentation or partial adsorptions of some
359 phenolic compounds (such as higher molecular weight proanthocyanidins) by cell wall
360 material (Le Bourvellec, Guyot, & Renard, 2004; Bindon, Smith, Holt, & Kennedy,
361 2010; Bindon, Smith, & Kennedy, 2010).
362 However, it was after 75 days of storage when the effect of the addition of EH-GS to the
363 fermentation mash was noticeable, affecting to the main chemical families of
364 polyphenolic compounds (Fig. 1). Although a gradual decrease of the content of
365 hydroxycinnamic acid derivatives, procyanidins, flavonols and anthocyanins over time
366 were observed in the three types of wines, the loss was significantly lower in the
367 presence of enzymatic hydrolyzate. Thus, the higher amount of anthocyanins and
368 copigments (phenolic compounds) in treated aged wines were in concordance with their
369 higher percentage of copigmentation (Table 1), influencing on a greater chemical
370 stabilization (Gómez-Míguez, González-Manzano, Teresa Escribano-Bailón, Heredia, &

371 Santos-Buelga, 2006). Besides, the content of benzoic acids increased over time, with
372 significantly ($p < 0.05$) higher concentrations when the quantity of hydrolyzate
373 increased (DW) (Fig. 1), being the protocatechuic acid the main responsible.
374 However, not always higher quantities of EH-GS reported advantages, because negative
375 effects in the content of monomeric flavan-3-ols, procyanidins and flavonols were
376 observed, likely for a possible saturation of the medium and subsequent precipitations.
377 Despite of the lower content of copigments (phenolic compounds) in DW, a
378 significantly ($p < 0.05$) higher percentages of copigmentation and polymerization were
379 observed. This fact could be due to the presence in the medium of other compounds
380 with planar polarizable nuclei derived from the enzymatic hydrolysis, which, together
381 with the higher content of anthocyanins, could form intermolecular copigmentation
382 reactions (Darias-Martín, Carrillo, Díaz, & Boulton, 2001). As affirmed Escribano-
383 Bailón & Santos-Buelga (2012), a wide variety of substances can act as copigments, e.g.
384 organic acids, amino acids, nucleotides, metals; phenolic compounds especially
385 flavonoids, including anthocyanins themselves. Likewise, the significantly ($p <0.05$)
386 higher percentage of polymerization in these wines led us to think that adding a double
387 dose of enzymatic hydrolyzate (DW) reached a higher proportion of more stable
388 pigments than CW and SW (Gordillo, et al., 2014).
389 As a summary, an increase on the content of polyphenolic compounds was produced by
390 adding the enzymatic grape seed hydrolyzate, much more when 250 g (SW) was
391 considered, obtaining wines rich on benzoic acids (gallic and protocatechuic acids),
392 hydroxycinnamic acid derivatives (such as *trans*-caftaric acid), flavonols (quercetin-3-
393 glucuronide and 3-glucoside derivatives of myricetin, isorhamnetin and syringetin) and
394 anthocyanins (malvidin-3-*p*-coumaroyl-glucoside). Those compounds, well described as

copigments by several authors (Gutiérrez, Lorenzo, & Espinosa, 2005), could be related to the significantly higher percentage of copigmentation found in SW wines (Table 2). Further, the losses of anthocyanins by possible adsorptions when seeds were added to the must in order to improve wine colour (Gordillo, et al., 2014) were not manifested with the use of EH-GS, resulting the addition of the enzymatic hydrolyzate of grape seeds a promising winemaking technique for the amelioration of wine quality.

Colour evolution

The evolution of CIELAB colour parameters (L^*, C^*_{ab} and h_{ab}) during alcoholic fermentation and stabilization time for control wines (CW) and wines submitted to the addition of enzymatic hydrolyzate (SW and DW) have been represented in Fig. 2.

All wines showed a similar evolution over time, i.e., a diminution of lightness (L^*) and an increase of chroma (C^*_{ab}) and hue (h_{ab}) during skin-maceration process (7 days). Afterwards, it is highlighted the remarkable increase of hue in the course of the stabilization stage.

The fact of adding EH-GS to the fermentation mash provoked significant ($p < 0.05$) differences in lightness at the beginning of the treatment (0 days), and in chroma and hue when the quantity of EH-GS was higher (DW) (Table 4). During the course of alcoholic fermentation (0-7 days) and the first days of stabilization, the quantity of EH-GS excessively influenced on CIELAB parameters. Whereas the addition of a single dose of enzymatic hydrolyzate (SW) did not produce significant differences on hue and chroma (when compared with wines traditionally elaborated, CW), higher quantities of EH-GS (DW) produced a negative effect in both lightness and chroma, not to mention the brownish tonality of the resulting wines (increase of around 8 º) (Fig. 2) (Table 4).

The panorama significantly changed after a period of stabilization (150 days) (Table 4). By adding a simple dose of hydrolyzate (SW), not only wines did not vary the hue after

420 the stabilization period (5 months), but also they reached a significantly higher values of
421 C^*_{ab} and lower lightness, favoring the colour stabilization. This positive trend could be
422 specially related to the significantly ($p < 0.05$) higher quantity of some copigments
423 (flavonols, hydroxycinnamic acid derivatives and benzoic acids, and total polyphenols)
424 (Table 2), to the formation of tannin-anthocyanin adducts and other polymeric pigments
425 (taking into account the higher values of polymerization observed in these wines) (Table
426 3). As a result, a better copigments/pigment ratio was achieved, and, hence, color
427 stability (Malien-Aubert, Dangles, & Amiot, 2002). Other authors also observed a
428 beneficial effect on the colour when seeds were directly added to red wines (Kovac et
429 al., 1995, 2005) or by the addition of oenotannin from grape seeds (Canuti, Puccioni,
430 Giovani, Salmi, Rosi, & Bertuccioli, 2012).
431 However, this positive behavior was not observed when higher quantities of hydrolyzate
432 was considered (DW), having even lower values of chroma and higher of lightness
433 compared to CW, evidencing the chromatic instability of those wines after five months
434 of storage. This loss of colour could be due to the formation of brown pigments or
435 possible co-precipitation of proteins and phenolic compounds (Charlon et al., 2002). In
436 fact, significantly ($p < 0.05$) lower content of monomeric flavan-3-ols and procyanidins
437 were reported in the last stages of stabilization period, phenomena also reported by
438 Gordillo et al. (2014) when grape pomace was added to Syrah wines.
439 The assessment of the colour differences (ΔE^*_{ab}) that took place from the skin removal
440 to the end of stabilization period (5 months) permitted to establish the possible visually
441 differentiation among wines. In terms of total colour, the lowest values of colour
442 difference (ΔE^*_{ab}) was attributed between CW and SW (data not shown), indicating
443 lower color variation and, thus, higher color stability. Although colour differences were
444 appreciable by human eye (ΔE^*_{ab}>3 CIELAB units) (Martínez, Melgosa, Pérez, Hita, &

445 Negueruela, 2001) over fermentation maceration, they remarkably dropped over the
446 stabilization period, reaching values below the visual appreciation threshold after five
447 months of storage (ΔE^*_{ab} = 2.65 u). That fact evidenced that the addition of single dose
448 of hydrolyzate (SW) could reach color stabilization (significantly ($p < 0.05$) higher
449 values of chroma with lower variations over time) without visually appreciable color
450 variations. The winemaking treatment that made the difference in terms of colour was
451 DW, owing to they maintained the visual appreciable differences with the rest of wines
452 (CW and SW), not only during the alcoholic fermentation but after the stabilization
453 period (5 months) (CW/DW, ΔE^*_{ab} = 8.00 u; SW/DW, ΔE^*_{ab} = 10.31 u). This fact
454 could be related with the remarkable decrease of phenolic compounds (copigments) in
455 DW (such as procyanidins, flavonols and flavan-3-ols), variating the copigmentation
456 complexes and, hence, wine color stabilization. Besides, the colour differences could be
457 also due to other component of the enzymatic hydrolyzate: procyanidins could yield
458 brown pigments and various types of anthocyanin-tannin adducts some of which may
459 precipitate; and the proteins or other macromolecules containing the hydrolyzate may
460 co-precipitate with some phenolic compounds (Charlon et al., 2002), producing
461 remarkable changes on the final colour of wines. The role of each colour attribute
462 respect $\Delta^2 E^*_{ab}$ was calculated at this moment (as percentage of the quadratic increases
463 of lightness, chroma and hue). The addition of a high quantity of enzymatic hydrolyzate
464 (DW) mainly affected to colour in a quantitative way after 5 months, with similar
465 quadratic variations of lightness and chroma, and practically negligible of hue ($\%\Delta^2 L$ =
466 67.4 and 57.3, $\%\Delta^2 C$ = 25.7 and 37.4, $\%\Delta^2 H$ = 7.5 and 5.5 for CW/DW and SW/DW,
467 respectively).

468 **CONCLUSIONS**

This study demonstrated that the addition of a grape seed enzymatic hydrolyzate might constitute a promising technique on the colour stability of red wines, which wines from warm climate normally lack. Treated wines experimented higher values of chroma and lower of lightness without significant variation on the tonality, probably due to the major content of colourless polyphenols that could act as copigments. However, higher quantities of enzymatic hydrolyzate did not suppose the purported colour stability in term of final colour, and a detrimental final colour quality of the wines was achieved. This novel research could be another step forward to improve the production of high-quality red wines from warm climate.

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Table 1

Table 1. Content (mg/100 g dry matter) and standard deviations of benzoic acids, hydroxycinnamic acid derivatives (HACD), monomeric flavan-3-ols and procyanidins, and total polyphenolics and tannins (n = 3) of the enzymatic hydrolyzate of grape seeds (EH-GS).

	EH-GS
Benzoic acids	
Gallic acid	5.03 ± 0.36
Protocatechuic acid	2.77 ± 0.19
HACD	
GRP	nd
Trans-caftaric acid	nd
Trans-coutaric acid	nd
p-coumaric acid	0.08 ± 0.02
Monomeric flavan-3-ols	
(+)-catechin	1.23 ± 0.12
(-)-epicatechin	1.90 ± 0.57
Procyanidins	
Procyanidin B1	4.37 ± 0.60
Procyanidin B2	1.43 ± 0.01
Procyanidin B4	3.08 ± 0.06
Procyanidin B7	1.71 ± 0.31
Procyanidin B2 3-*O*-gallate	4.47 ± 0.48
Procyanidin trimer 2	1.59 ± 0.35
Total phenolics (Folin-Ciocalteau)	6604.83 ± 909.59
Total tannins	2142.80 ± 93.45

HACD, hydroxycinnamic acid derivatives; GRP, grape reaction product (2-*S*-glutathionyl-caftaric acid).

Table 2

Table 2. Mean concentration (mg/L) and standard deviations of benzoic acids, hydroxycinnamic acid derivatives (HACD), flavan-3-ols and flavonols, and total phenolics ($n = 3$) in wines, at the beginning (0 days), at skin removal (SR) and after 5 months of stabilization (150 days).

	stage	CW		SW		DW	
Benzoic acids							
Gallic acid	0 days	12.97 ± 1.63		12.02 ± 0.23		12.63 ± 0.47	
	SR	21.65 ± 0.29	a	23.47 ± 0.18	b	24.71 ± 0.90	b
	150 days	28.07 ± 1.00	a	31.46 ± 1.06	b	32.06 ± 0.52	b
Protocatechuic acid	0 days	4.65 ± 0.39	a	5.11 ± 0.09	b	6.54 ± 0.18	c
	SR	4.84 ± 0.04	a	5.57 ± 0.04	b	6.21 ± 0.07	c
	150 days	5.28 ± 0.19	a	6.27 ± 0.20	b	7.03 ± 0.11	c
HACD							
GRP	0 days	22.79 ± 0.29	a	29.73 ± 0.27	b	29.40 ± 0.39	b
	SR	26.38 ± 2.26		25.26 ± 0.97		25.91 ± 1.40	
	150 days	4.79 ± 2.51		8.17 ± 1.89		8.23 ± 0.34	
Trans-caftaric acid	0 days	15.75 ± 1.07	a	19.31 ± 0.24	b	19.23 ± 0.50	b
	SR	19.93 ± 0.60	a	22.84 ± 0.03	b	22.10 ± 0.33	a
	150 days	5.38 ± 2.93		8.19 ± 1.44		8.11 ± 0.25	
Trans-coutaric acid	0 days	3.33 ± 0.53		3.48 ± 0.03		3.37 ± 0.04	
	SR	5.30 ± 0.75		5.77 ± 0.76		5.92 ± 0.40	
	150 days	34.36 ± 2.29		32.47 ± 1.79		29.41 ± 0.86	
p-coumaric acid	0 days	6.65 ± 0.47	a	7.62 ± 0.15	b	8.16 ± 0.01	c
	SR	5.67 ± 0.02	a	5.77 ± 0.03	b	5.97 ± 0.03	c
	150 days	15.00 ± 1.12		16.04 ± 2.35		16.50 ± 2.50	
Monomeric flavan-3-ols							
(+)-catechin	0 days	1.59 ± 0.47		1.34 ± 0.13		1.22 ± 0.03	
	SR	7.48 ± 1.14		7.58 ± 0.28		6.90 ± 0.10	
	150 days	10.31 ± 0.98	b	12.00 ± 0.67	b	2.73 ± 0.06	a
(-)-epicatechin	0 days	4.00 ± 1.41		3.12 ± 0.30		2.84 ± 0.48	
	SR	12.53 ± 2.02		13.67 ± 2.23		11.51 ± 0.42	
	150 days	14.75 ± 1.25	b	12.73 ± 2.61	b	10.83 ± 0.64	a
Procyanidins							
Procyanidin B1	0 days	21.55 ± 5.15		24.53 ± 1.58		23.34 ± 0.00	

	SR	28.23	±	0.93	a	30.60	±	0.60	b	28.91	±	1.04	a
	150 days	30.50	±	5.59		32.68	±	4.68		27.43	±	0.77	
Procyanidin B2	0 days	11.88	±	0.95		12.78	±	0.11		12.55	±	0.00	
	SR	13.40	±	0.62		13.86	±	0.30		13.00	±	0.15	
	150 days	11.91	±	0.99		12.60	±	0.90		11.12	±	0.48	
Procyanidin B4	0 days	11.59	±	0.53		11.74	±	0.11		11.88	±	0.11	
	SR	12.80	±	1.12	a	13.45	±	0.15	b	11.81	±	0.00	a
	150 days	13.69	±	0.23	b	13.69	±	0.09	b	13.30	±	0.00	a
Procyanidin B7	0 days	tr				tr				tr			
	SR	9.79	±	0.12		9.98	±	0.09		9.83	±	0.31	
	150 days	11.28	±	0.30		11.25	±	0.56		11.53	±	0.24	
Procyanidin B2 3-O-gallate	0 days	10.37	±	0.04		10.74	±	0.84		10.70	±	0.20	
	SR	12.17	±	0.90		12.68	±	1.05		12.08	±	0.93	
	150 days	13.60	±	0.38		14.15	±	0.88		14.11	±	0.21	
Procyanidin trimer 2	0 days	14.12	±	1.37		15.01	±	0.32		15.23	±	0.63	
	SR	14.59	±	0.68	a	15.92	±	0.48	b	15.03	±	0.23	a
	150 days	14.59	±	1.41	a	18.35	±	1.36	b	15.38	±	2.78	a
Flavonols													
Myricetin-3-glucuronide	0 days	tr				tr				tr			
	SR	tr				tr				tr			
	150 days	tr				tr				tr			
Myricetin-3-glucoside	0 days	1.61	±	0.50	b	1.11	±	0.02	a	0.88	±	0.29	a
	SR	5.36	±	0.92		5.71	±	0.76		4.89	±	0.73	
	150 days	6.57	±	1.58	a	9.15	±	1.23	b	4.55	±	0.51	a
Quercetin-3-glucuronide	0 days	2.59	±	0.86		1.96	±	0.05		1.18	±	1.01	
	SR	3.97	±	0.86		4.61	±	0.33		4.46	±	0.47	
	150 days	6.05	±	2.43	a	10.27	±	0.79	b	3.30	±	0.28	a
Quercetin-3-glucoside	0 days	4.47	±	1.30		3.47	±	0.02		2.20	±	0.88	
	SR	10.43	±	1.08		11.46	±	0.39		10.21	±	0.46	
	150 days	7.89	±	2.58		9.96	±	2.34		4.37	±	0.32	
Kaempherol-3-glucoside	0 days	tr				tr				tr			
	SR	tr				tr				tr			
	150 days	tr				tr				tr			

Isorhamnetin-3-glucoside	0 days	0.39	±	0.42		0.17	±	0.09		tr			
	SR	2.61	±	0.48		3.05	±	0.30		2.51	±	0.18	
	150 days	2.69	±	1.16	a	3.10	±	0.73	b	1.08	±	0.08	a
Syringetin-3-glucoside	0 days	tr				tr				tr			
	SR	1.73	±	0.40		2.07	±	0.14		1.71	±	0.12	
	150 days	2.31	±	0.96	a	3.41	±	1.07	b	1.36	±	0.15	a
Total phenolics (Folin-Ciocalteau)	0 days	1185.78	±	25.12	a	1836.94	±	89.82	b	1951.91	±	193.64	b
	SR	2734.47	±	4.90	b	2266.42	±	20.38	a	2073.52	±	24.90	a
	150 days	2009.06	±	215.89	a	2735.27	±	132.25	b	2066.28	±	159.27	a

CW, control wines; SW and DW, wines fermented with a single and double dose of hydrolyzed grape seeds; tr, traces; GRP, grape reaction product (2-*S*-glutathionyl-caftaric acid); HACD, hydroxycinnamic acid derivatives. Different letters in the same row denote significant differences ($p < 0.05$).

Table 3

Table 3. Mean concentration (mg/L) and standard deviations of anthocyanin compounds, and percentages of copigmentation and polymerization of wines (n = 3), at the beginning (0 days), at skin removal (SR) and after 5 months of stabilization (150 days).

	stage	CW		SW		DW	
Delphinidin-3-glucoside	0 days	13.86 ± 2.41		17.62 ± 0.26		16.08 ± 0.32	
	SR	14.54 ± 0.55	a	17.95 ± 1.71	b	16.45 ± 1.35	b
	150 days	10.43 ± 1.78		10.99 ± 1.85		10.67 ± 2.32	
Cyanidin-3-glucoside	0 days	9.11 ± 0.45		9.16 ± 0.32		9.26 ± 0.13	
	SR	8.36 ± 0.11		8.59 ± 0.26		8.26 ± 0.06	
	150 days	7.76 ± 0.00		7.76 ± 0.00		7.77 ± 0.01	
Petunidin-3-glucoside	0 days	18.22 ± 3.08		22.63 ± 3.85		18.69 ± 0.39	
	SR	25.16 ± 3.44		30.34 ± 3.07		25.51 ± 1.74	
	150 days	17.50 ± 3.54		22.00 ± 4.24		24.12 ± 0.03	
Peonidin-3-glucoside	0 days	24.77 ± 3.67		34.33 ± 0.57		28.76 ± 2.02	
	SR	23.77 ± 1.57		26.44 ± 2.77		28.03 ± 2.85	
	150 days	15.00 ± 1.41		18.57 ± 3.47		19.63 ± 0.04	
Malvidin-3-glucoside	0 days	120.57 ± 24.88		131.47 ± 4.89		118.66 ± 6.03	
	SR	215.23 ± 22.25		249.61 ± 17.12		233.06 ± 11.00	
	150 days	115.00 ± 35.36		175.00 ± 21.21		219.16 ± 0.01	
Petunidin-3-acetyl-glucoside	0 days	9.66 ± 0.55		9.68 ± 0.47		9.11 ± 0.04	
	SR	12.44 ± 1.49		11.96 ± 0.80		12.26 ± 0.49	
	150 days	14.25 ± 0.63		16.97 ± 0.18		14.04 ± 0.05	
Peonidin-3-acetyl-glucoside	0 days	15.84 ± 1.92		13.70 ± 0.61		11.94 ± 0.04	
	SR	19.62 ± 2.24		19.43 ± 1.96		21.59 ± 2.22	
	150 days	16.18 ± 4.08		19.62 ± 0.56		18.53 ± 0.04	
Malvidin-3-acetyl-glucoside	0 days	58.33 ± 10.22		48.31 ± 2.40		44.16 ± 2.69	
	SR	105.08 ± 12.34		122.54 ± 10.78		116.25 ± 6.99	
	150 days	67.50 ± 10.61		100.00 ± 14.14		109.18 ± 0.00	
Petunidin-3-*p*-coumaroyl-glucoside	0 days	8.26 ± 0.03	c	8.03 ± 0.01	b	7.76 ± 0.00	a
	SR	10.00 ± 1.74		10.07 ± 0.91		9.79 ± 0.94	
	150 days	13.00 ± 3.47		16.50 ± 2.12		10.75 ± 0.00	
Peonidin-3-*p*-coumaroyl-glucoside	0 days	10.88 ± 0.76	b	8.83 ± 0.31	a	8.29 ± 0.22	a
	SR	12.38 ± 0.45	a	15.82 ± 1.57	b	13.27 ± 1.22	b

	150 days	11.50	±	2.12		14.00	±	1.41		15.51	±	0.02	
Malvidin-3-*p*-coumaroyl-glucoside	0 days	24.02	±	4.94		16.01	±	0.81		11.89	±	0.74	
	SR	39.77	±	7.83		51.10	±	6.78		54.36	±	5.54	
	150 days	29.00	±	3.66	a	45.00	±	3.07	b	52.91	±	0.13	c
Sum of glucoside derivatives	0 days	186.53	±	34.48		216.21	±	9.90		191.44	±	8.88	
	SR	287.06	±	27.48		332.93	±	21.46		311.31	±	15.59	
	150 days	166.24	±	56.57		237.64	±	41.19		282.02	±	2.90	
Sum of acetyl derivatives	0 days	83.83	±	12.69		71.69	±	3.47		65.21	±	2.77	
	SR	137.14	±	13.54		153.93	±	12.15		150.09	±	9.55	
	150 days	97.93	±	22.38		136.59	±	14.88		141.74	±	0.09	
Sum of *p*-coumaroyl derivatives	0 days	43.15	±	5.73	b	32.87	±	0.49	a	27.93	±	0.96	a
	SR	62.15	±	9.29		77.00	±	8.42		77.42	±	6.98	
	150 days	53.50	±	4.31	a	75.50	±	6.36	b	79.17	±	0.12	b
% copigmented anthocyanins (%CA)	0 days	8.63	±	2.01		9.21	±	0.88		8.05	±	1.32	
	SR	9.67	±	2.57		11.04	±	0.66		8.52	±	2.04	
	150 days	32.85	±	1.45	a	57.78	±	7.64	b	87.01	±	4.59	c
% polymerized anthocyanins (%PA)	0 days	42.64	±	5.89		41.26	±	13.17		33.53	±	1.02	
	SR	59.80	±	7.20		68.20	±	13.16		67.54	±	9.47	
	150 days	63.46	±	5.71	a	67.23	±	6.11	a	76.75	±	6.89	b

CW, control wines; SW and DW, wines fermented with a single and double dose of hydrolyzed grape seeds. Different letters in the same row denote significant differences ($p < 0.05$).

Table 4

Table 4. Mean values and standard deviations of lightness (L^*), chroma (C^*_{ab}), and hue (h_{ab}) (n = 3), at the beginning (0 days), at skin removal (SR) and after 5 months of stabilization (150 days).

	stage	CW			SW			DW		
L^*	0 days	81.08	± 0.06	b	79.26	± 0.14	a	80.51	± 0.10	a
	SR	73.00	± 1.72		71.54	± 1.32		74.22	± 0.56	
	150 days	72.67	± 1.53	b	70.11	± 1.72	a	79.24	± 1.37	b
C^*_{ab}	0 days	26.21	± 0.09	b	27.13	± 0.37	b	22.36	± 0.04	a
	SR	30.69	± 2.27	b	30.59	± 1.50	b	26.05	± 0.36	a
	150 days	22.83	± 0.76	b	26.15	± 2.52	c	18.78	± 1.24	a
h_{ab}	0 days	-9.19	± 0.03	b	-9.06	± 0.27	b	-4.63	± 0.05	a
	SR	-8.15	± 0.14	b	-7.36	± 0.13	b	-5.69	± 0.55	a
	150 days	0.57	± 0.67		-0.06	± 1.59		2.76	± 1.80	

CW, control wines; SW and DW, wines fermented with a single and double dose of hydrolyzed grape seeds. Different letters in the same row denote significant differences ($p < 0.05$).

Figure 1

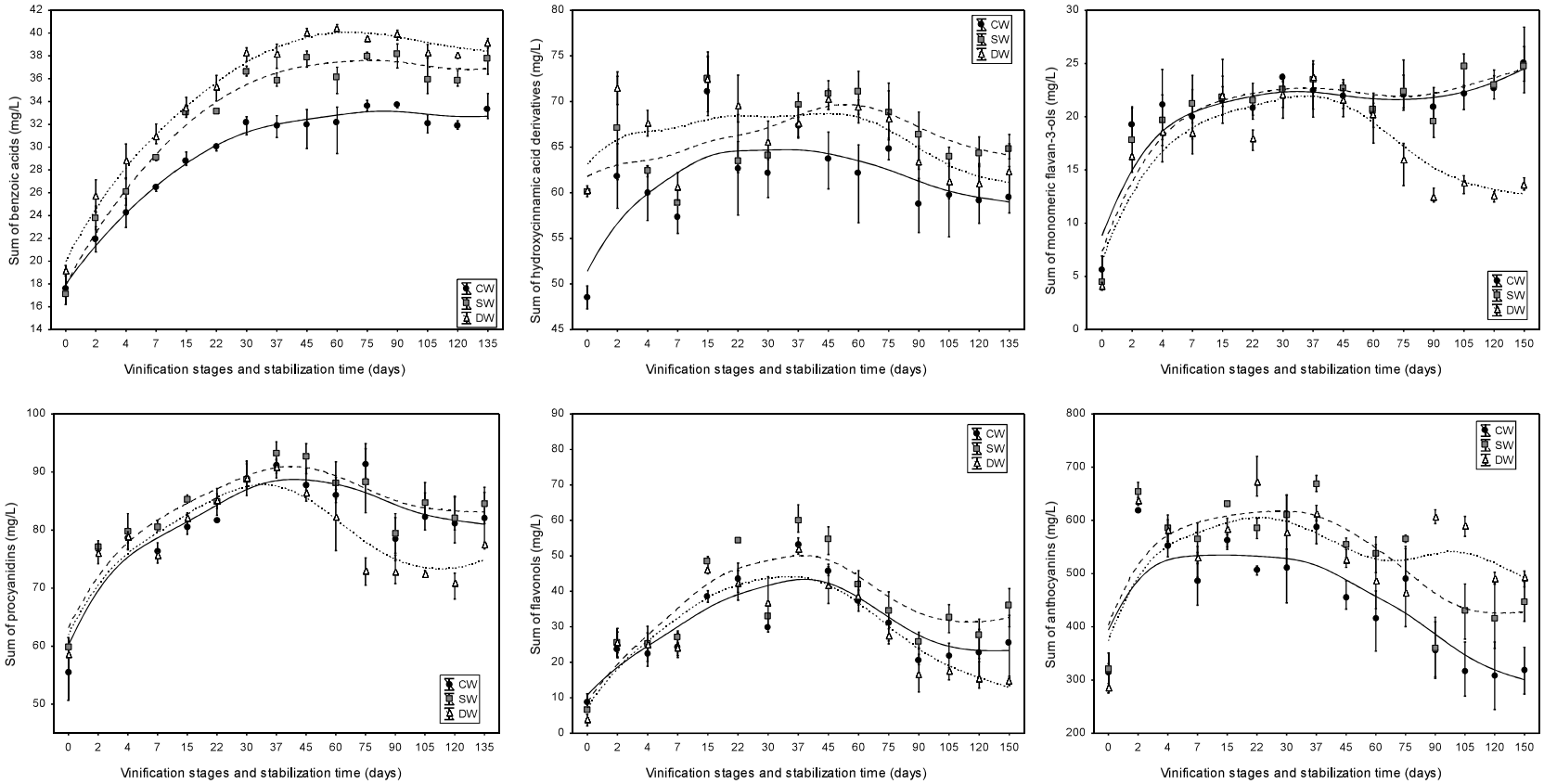
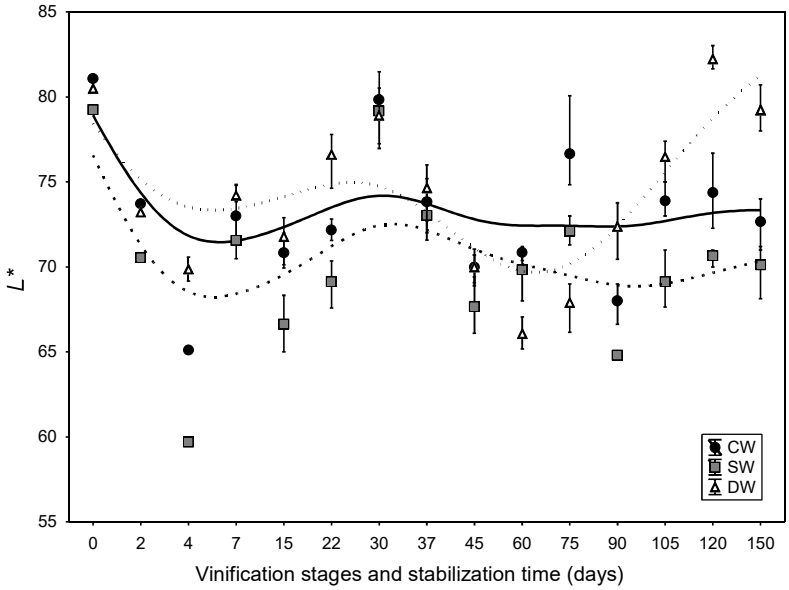
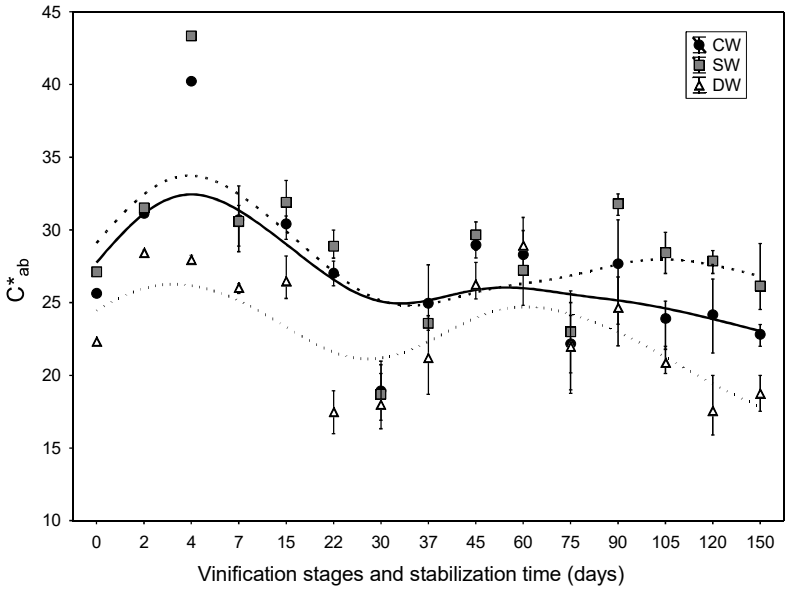


Figure 1. Evolution over time of the main polyphenolic families (mg/L ± SD, *n* = 3) in control wines (CW) and wines with the pre-fermentative addition of an enzymatically hydrolyzate of grape seeds in a simple (SW) and double dose (DW) (means ± SD, *n* = 3).

Figure 2


L*
85
80
75
70
65
60
55
0 2 4 7 15 22 30 37 45 60 75 90 105 120 150
Vinification stages and stabilization time (days)
CW
SW
DW


C*ab
45
40
35
30
25
20
15
10
0 2 4 7 15 22 30 37 45 60 75 90 105 120 150
Vinification stages and stabilization time (days)
CW
SW
DW

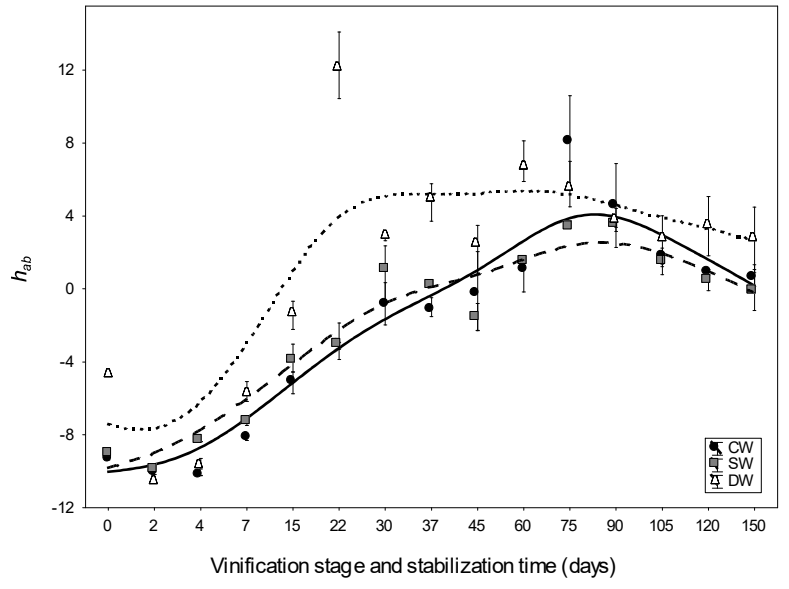

hab
12
8
4
0
-4
-8
-12
0 2 4 7 15 22 30 37 45 60 75 90 105 120 150
Vinification stage and stabilization time (days)
CW
SW
DW

Figure 2. Evolution of CIELAB parameters (means ± SD, *n* = 3) during vinification in control wines (CW) and after the pre-fermentative addition of hydrolyzed grape seed extract (single and double doses, SW and DW, respectively) (means ± SD, *n* = 3).