Title: Impact of a double post-fermentative maceration with ripe and overripe seeds on the phenolic composition and color stability of Syrah red wines from warm climate

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Abstract: The post-fermentative double addition of Pedro Ximénez cv seeds obtained from natural matured grapes (ripe seeds, RS) and postharvest sun-dried grapes (overripe seeds, OS) were studied as sustainable enological alternatives to conventional vinification (CW) to improve the stability of Syrah wines produced in a warm climate. The phenolic composition was assessed by rapid resolution liquid chromatography, copigmentation/polymerization processes by spectrophotometry, and color quality and stability by Differential Colorimetry. OSW and RSW wines enriched their total phenolic content, being the effect more pronounced with overripe seeds (by 23% versus 10%). OSW differences were found for gallic acid, monomeric flavan-3-ols, and procyanidins compared to CW, and for (+)-catechin, procyanidin B2-3-O-gallate and the tetramer to RSW. Phenolic changes were related to higher color intensity in seed-added wines. OSW having higher percentage of polymeric pigments maintained for longer time the chromatic improvement, being visually darker and more intense than final CW and RSW.

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ABSTRACT

The post-fermentative double addition of Pedro Ximénez cv seeds obtained from natural matured grapes (ripe seeds, RS) and postharvest sun-dried grapes (overripe seeds, OS) were studied as sustainable enological alternatives to conventional vinification (CW) to improve the stability of Syrah wines produced in a warm climate. The phenolic composition was assessed by rapid resolution liquid chromatography, copigmentation/polymerization processes by spectrophotometry, and color quality and stability by Differential Colorimetry. OSW and RSW wines enriched their total phenolic content, being the effect more pronounced with overripe seeds (by 23% versus 10%). OSW differences were found for gallic acid, monomeric flavan-3-ols, and procyanidins compared to CW, and for (+)-catechin, procyanidin B2-3-O-gallate and the tetramer to RSW. Phenolic changes were related to higher color intensity in seed-added wines. OSW having higher percentage of polymeric pigments maintained for longer time the chromatic improvement, being visually darker and more intense than final CW and RSW.

Keywords: sun-dried grapes; overripe seeds; phenolic composition; red wine color; post-fermentative maceration.

1. Introduction

The production of full-bodied red wines with stable and deep color is a major challenge for the wine industry. This sensory and quality attribute is directly related to the phenolic composition of wines. During maceration process, wines acquire the phenolic structure and, hence, the capability to perform a proper aging process since, among other important factors such as formation of low and high molecular weight polymeric pigments, complexation of phenolics (polysaccharides, sugars, etc.) or rearrangements and oxidation of tannins, the copigmentation is crucial in this stage. From the early stages of vinification, copigmentation, a non-covalent phenomenon occurring between anthocyanins and colorless phenolics (copigments), can enhance the color intensity of young wines by 30-50% and confers greater stability to anthocyanins due to the formation of more stable pigments (Boulton, 2001). This chemical conversion causes in most cases the evolution of the wine color, being the first step to its stabilization (De Feitas & Mateus, 2011; Trouillas, Sancho-García, De Freitas, Gierschner, Otyepka & Dangles, 2016). Thus, it is interesting to develop oenological strategies focused on the exogenous addition of phenolics to allow modulating the levels and types of copigments and, therefore, the copigmentation equilibria (Schwarz, Picazo-Bacete, Winterhalter & Hermosín-Gutiérrez, 2005). In this respect, the use of wood chips, enzymes, and enological tannin from seed and skins, are some examples of internationally approved enological practices (OIV, 2012) to improve the sensory profile of wines and to reduce defects.

Grape byproducts, especially those from white grapes, are powerful phenolic sources that can be reused with the aim of promoting higher levels of copigmentation in wines and favor more stable colors (Jara-Palacios, Gordillo, González-Miret, Hernanz, D. Escudero-Gilete & Heredia, 2014a; Pedroza, Carmona, Alonso, Salinas & Zalacain, 2013; Nicolle, Marcotte, Angers & Pedneaulta, 2018). In addition to the potential benefits on color, the use of grape byproducts during vinification has economic and environmental repercussion on winemaking regions.

Seeds obtained from grape pomace have been used in red winemaking by adding during the process to enhance color and tannin extraction due to their richness in monomeric flavanols, catechins, and procyanidins. These phenolic substances have also been shown to have antioxidant properties and potential benefits to human health contributing to the global quality of wines (Jara-Palacios, Hernanz, Cifuentes-Gomez, Escudero-Gilete, Heredia & Spencer, 2015; Jara-Palacios, Hernanz, Escudero-Gilete & Heredia, 2016). However, the content and proportions of the different flavanols in seeds, as well as their polymerization grade, considerably vary with the ripeness grade of the grape (Perez-Magariño & San José, 2006). This factor can influence the capability of seeds to modulate the sensory quality of red wines such as the color enhancement and sensation of astringency. The exogenous addition of seeds from high maturation level (overripe) grapes could be a good strategy for increasing the extraction of adequate antioxidant copigments in wines for color stabilization purposes (Alcalde-Eon, García-Estévez, Ferreras-Charro, Rivas-Gonzalo, Ferrer-Gallego, Escribano-Bailón, 2014; Rivero, Gordillo, Jara-Palacios, González-Miret & Heredia, 2017; Rivero, Jara-Palacios, Gordillo, Heredia & González-Miret, 2019; Rivero et al., 2020). In particular, overripe seeds obtained from high-mature white grapes summited to intensive dehydration by postharvest sun-drying have been proposed as alternative sources of copigments capable to stabilize wine anthocyanins (Rivero et al., 2019). This technological strategy has been shown to be useful in warm climate regions. Jones, White, Cooper, & Storchmann (2005) studied the average temperature during growing season (April-October in the Northern Hemisphere) to define the climate of different regions, suggesting a range of 16.5-19.5 °C for warm climate. In such warm regions, the extreme climate conditions (high night temperatures and severe light exposures intensified by the effect of climate change) cause time discrepancy between the technological ripeness (sugars/acids ratio) and the phenolic ripeness of grapes, leading to unbalanced ripening. Consequently, red grapes usually do not reach sufficient phenolic maturity at harvest (occurring typically earlier in the summer) and the copigmentation and the color stabilization processes in wines do not always occur favorably (Gordillo et al., 2012; Rivero et al., 2017).

88 Even so, the impact on the levels of pigments and copigments in wines, as well as on the

89 copigmentation/polymerization processes, significantly vary depending on the dose applied and the

90 stage of vinification in which such as overripe byproducts are applied, with important consequences on

91 the color quality and stability. Jara-Palacios et al. (2016) confirmed that the addition of overripe seeds

92 during the fermentation improved the phenolic and antioxidant potential of a young red wine.

93 Notwithstanding, these effects were more notable with single seed additions than with double ones (450

94 g and 950 g seeds/150 kg of grapes). Likewise, Rivero et al. (2017) confirmed that the fermentative

95 addition of overripe seeds (3 g/L) led to red wines with significant higher content of anthocyanins and

96 procyanidins than the wines traditionally produced. As consequence, higher color stability and bluish

97 hues was achieved by increasing the pool of copigments and polymerization, although this effect was

98 visually limited. More recently, Rivero et al. (2019) assessed the impact of a post-fermentative

99 maceration with different doses (single and double) and contact time (30/60 days) of such as overripe

100 seeds. Interestingly, a double post-maceration process during 60 days was more effective to increase the

101 content of some copigments in wines (flavanols, benzoic acids and procyanidins) than single maceration

102 during 30 days. In this case, although partial absorption of anthocyanins was observed, wines produced

103 by double seed addition demonstrated visually perceptible color changes compared to control wines

104 (without overripe seed addition).

105 Thus, in the case of using overripe seeds during the first stages of vinification, controlling the conditions

106 of the seed addition is crucial to optimize the potential benefits on structure and color stability compared

107 to traditional maceration.

108 Studies focused on elaborate wines added with overripe seeds in comparison with seeds obtained from

109 natural ripeness process are useful to elucidate the components responsible for these effects. The aim of

110 this work was to evaluate the impact on the phenolic composition and color of red wines from warm

111 climate by the post-fermentative double addition of seed byproducts having different ripeness grade

112   (ripe seeds obtained from grapes submitted to on-vine natural maturation and overripe seeds from grapes

113   submitted to off-vine postharvest sun-drying), in comparison to traditional maceration.

114   **2. Material and methods**

115   **2.1. Grape seed byproducts and winemaking protocols**

116   The grape seeds used as natural sources of copigments in the winemaking experiments were obtained

117   from the pomaces of Pedro Ximénez (PX) white grape summited to different ripening processes (on-

118   vine natural maturation and off-vine postharvest sun-drying) in the Montilla-Moriles Designation of

119   Origin (D.O). The Montilla-Moriles D.O (Córdoba, southwestern Spain) is classified as a semi-

120   continental Mediterranean climate winemaking region with short winters and long, dry, hot summers

121   (the diurnal temperature can reach 40 °C) where traditional sweet wines from Pedro Ximénez (PX)

122   white grapes are elaborated (Rivero et al., 2020).

123   Ripe seeds (RS) proceeded from the pomace of PX mature grapes exposed to natural on-vine ripening

124   until reach 16 ºBé of sugar content, and overripe seeds (OS) from the pomace of high-mature PX dried

125   grapes exposed to 10 days off-vine over-ripening by postharvest sun-drying until reach 23 ºBé of sugar

126   content. Pomaces from PX mature and overripe grapes were provided in enough amounts by a local

127   winemaking Cooperative of the Montilla Moriles D.O., after the elaboration of their respective sweet

128   wines from PX white grapes. The separation procedure consisted of sifting the pomaces (rest of skins,

129   pulp, and seeds) through a mesh (70 cm x 120 cm, approx.) that allowed the RS and OS seeds to be

130   quickly separated from the rest of the pomaces. Once the seeds were separated through the mesh, they

131   were manually cleaned from small rest of solid parts. Around 4 kg of each type of seeds (RS and OS

132   with similar moisture content) were obtained and stored frozen (-20 ˚C) until used for the vinification

133   assays.

134   A young red wine made from *Vitis vinifera* var. Syrah cultivated in Condado de Huelva Designation of

135   Origin (D.O) was used for the post-maceration experiments with ripe and overripe PX seeds. The

136   "Condado de Huelva" D.O. is a restricted wine-producing zone in southwestern Spain with

137 climatological conditions of warm climate (average Tª of growing season 16.9-25.4 °C). It includes

138 approximately 6000 ha of neutral or slightly alkaline soil having a typical Mediterranean climate with a

139 clear Atlantic influence: gentle winters and springs, long and warm summers (average temperature 18

140 °C, minimum over 10 °C in winter and over 40 °C in summer), relative humidity ranging between 60%

141 and 80%, and mean rainfall around 700 mm year^{-1} (Gordillo et al., 2012).

142 Syrah grapes, harvested at optimum technological maturity (density = 13.1 ºBé; total acidity = 5.51 g/L;

143 pH = 3.61) and good sanitary conditions, were destemmed and crushed, and then the crushed mass (must

144 and solid parts) was distributed into tanks for maceration. Alcoholic fermentation was induced by

145 inoculating selected yeast (*Saccharomyces cerevisiae* 25 g/hL, Viniferm BY, Agrovin, Ciudad Real,

146 Spain) and occurred at controlled temperature (20-25 ºC). Fermentation caps were punched down once a

147 day during the on-skin maceration period (6 days). After this, the mash was drawn off to remove the

148 solid parts, and the free run wine was racked to nine 50 L stainless steel tanks to finish the fermentation.

149 To ensure that malolactic fermentation occurs, selected lactic acid bacteria (*Oenococcus oeni*

150 VINIFERM Oe 104, 14 mL/hL, Agrovin, Ciudad Real, Spain) were inoculated at the end of alcoholic

151 fermentation. When fermentative processes finished, sulfur dioxide levels were adjusted (total sulfur

152 dioxide about 100 mg/L and free sulfur dioxide about 60 mg/L in all wines).

153 Based on results from previous studies (Rivero et al., 2019), which proved that to ensure a real double

154 addition of seeds, in contrast to single addition, the best way was adding twice, three types of

155 experimental post-fermentative treatments were performed:

156 - CW (control wine, 3 tanks 50 L): wines made by traditional winemaking (without seed addition).

157 - RSW (Ripe seed wine, 3 tanks 50 L): wines made by double post-fermentative maceration with PX

158 ripe seeds. This procedure consisted in the addition of 600 g of ripe seeds per tank, macerated during

159 30 days, and after removing the seeds, a further second addition of 600 g RS, macerated 30 days more

160 (12 g/L of first seed addition and a second addition of 12 g/L seeds).

161 - OSW (Overripe seed wine, 3 tanks 50 L): wines made by double post-fermentative maceration with

162 PX overripe seeds. This procedure consisted of the addition of 600 g of overripe seeds per tank,

163 macerated during 30 days, and after removing the seeds, a further second addition of 600 g RS,

164 macerated 30 more days (12 g/L of first seed addition and a second addition of 12 g/L seeds).

165 - Wine samples (50 mL) were taken at day 1 (first seed addition, 12 g/L), day 30 (seed removal and

166 second seed addition, 12 g/L), day 60 (end of the post-fermentative seed maceration), and along 5

167 months of stabilization in 50 L stainless steel tanks (90, 120, 140, and 150 days after seed addition).

168 **2.2. Oenological parameters**

169 The conventional oenological parameters of wines (pH, total and volatile acidity, free and total SO_2,

170 malic and lactic acids, and Alcohol degree) were performed according to the Official Methods

171 established by European Union (Table 1).

172 **2.3. Phenolic extraction from ripe and overripe PX seeds**

173 Ripe and overripe PX seeds obtained from the pomaces were extracted with methanol:water (750/250

174 mL/mL) according to the methodology described by Rivero et al. (2017) to assess and compare their

175 phenolic composition and content. The extraction procedure was made in triplicate as follows: 50 g of

176 the homogeneous lyophilized powder of RS and OS seeds were individually homogenized in 250 mL of

177 solvent for 1 h in a shaking apparatus (VWR Incubating minishaker, Barcelona, Spain), and further

178 centrifuged at 4190 g for 15 min. The supernatant was collected and the residue was submitted twice to

179 the same process. Finally, the supernatants were combined and the methanolic extract was concentrated

180 to dryness and freeze dried until the analyses.

181 2.4. **Copigmented and polymerized anthocyanin determination**

182 The contribution of copigmented anthocyanins to the total wine color at pH 3.6 (% copigmented

183 anthocyanins; %CA) and the degree of anthocyanin polymerization (% polymeric pigments; %PP) were

184 determined following the method proposed by Boulton (1996). The pH values of wine samples were

185 first adjusted to 3.6 using 1M NaOH or HCl.

2.5. HPLC-DAD analysis of phenolic compounds

The monomeric anthocyanins and flavonols of samples were determined in triplicate according to the method reported by Rivero et al. (2019). The chromatographic separation and quantification of compounds were performed in an Agilent 1200 chromatographic system, equipped with quaternary pump, UV-VIS diode-array detector, automatic injector, and ChemStation software (Agilent Technologies, Palo Alto, USA). The wine samples were filtered through a 0.45 μm Nylon filter prior to direct injection; then, a volume of 50 μL was injected onto a Zorbax C18 column (250 x 4.6 mm, 5 μm particle size). Acetonitrile, formic acid and water were used as solvents, being 3:10:87 solvent A and 50:10:40 solvent B (mL:mL:mL). The elution profile was 0-10 min with 6% B; 10-15 min with 11% B; 15-20 min with 20% B; 20-25 min with 23% B; 25-30 min with 26% B; 30-35 min with 40% B; 35-38 min with 50% B; 38-46 min with 60% B; and 46-47 min with 6% B. The temperature was set at 40 ℃ and 0.63 mL/min flow rate. All UV-Vis spectra were recorded from 200 to 800 nm with a bandwidth of 2.0 nm, using the external calibration method for the quantification of anthocyanins (520 nm) and flavonols (360 nm) by comparing the areas with the standards malvidin 3-*O*-glucoside and quercetin, respectively. The concentration of compounds in wine samples was expressed as mg/L.

The analyses of flavan-3-ols (monomeric and procyanidins), as well as the hydroxycinnamic and benzoic acids were performed, in triplicate, according to Jara-Palacios, Gordillo, Gonzalez-Miret, Hernanz, Escudero-Gilete & Heredia (2014b) by rapid resolution liquid chromatography (RRLC). After filtration through a 0.45 μm Nylon filter, samples were injected (0.5 μL injection volume) in an Agilent 1290 chromatographic system, equipped with quaternary pump, UV-VIS diode-array detector, automatic injector, and ChemStation software (Agilent Technologies, Palo Alto, USA). A C18 Poroshell 120 column (2.7 μm, 5 cm x 4.6 mm) was used. The solvents were formic acid and water (1:999 mL:mL) as solvent A, and acetonitrile as solvent B at the following gradients: 0-5 min of 5% B linear; 5-20 min of 50% B linear; and 20-25 min of washing, which was followed by re-equilibration of the column. The flow-rate was 1.5 mL/min, and the column temperature was set to 25 °C. Identification of phenolics was

211 performed according to the retention times of the standards (when available), UV-vis spectra and mass

212 spectra, as described by Jara-Palacios et al. (2014b). The quantification was made at 280 nm (flavan-3-

213 ols, procyanidins and benzoic acids) and 320 nm (hydroxycinnamic acids) by external calibration

214 comparing the areas with the gallic acid, *p*-coumaric acid and catechin standards. The concentration of

215 compounds was expressed as mg/L for wine samples and mg/100 g of dry seeds for PX ripe and

216 overripe seeds.

217 In addition, the total anthocyanin, flavonol, phenolic acids, monomeric flavan-3-ol and procyanidin

218 contents were calculated as the sum of individual phenolic compounds identified by HPLC. The Total

219 phenolic content of samples was determined in triplicate by the Folin-Ciocalteau method (Singleton &

220 Rossi, 1965) using an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, USA).

221 **2.6. Colorimetric measurements**

222 The absorption spectra (380- 770 nm) of wines were recorded at constant intervals ($\Delta\lambda$=2 nm) with an

223 Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, USA), using 2 mm path

224 length glass cells and distilled water as reference. The CIELAB parameters were calculated from the

225 absorption spectra by using the original software CromaLab$^{©}$ (Heredia, Álvarez, González-Miret &

226 Ramírez, 2004), following the recommendations of the Commission International de L'Eclairage: the

227 CIE 1964 10° Standard Observer and the Standard Illuminant D65, corresponding to the natural daylight

228 (CIE, 2004). CIELAB parameters were calculated: L* (the correlate of lightness; ranging from 0, black,

229 to 100, white), and two color coordinates, a* (which takes positive values for reddish colors and

230 negative values for greenish ones) and b* (positive for yellowish colors and negative for bluish ones).

231 From these coordinates, correlates of the perceived attributes (that is, with a psychophysical meaning),

232 are calculated: the hue angle (h_{ab}, the correlate of chromatic tonality) and the chroma (C^*_{ab}, the correlate

233 of saturation).

234 By applying Differential Colorimetry (Gordillo et al., 2015), the color differences (ΔE^*_{ab}) among wines

235 during vinification were calculate by the Euclidean distance between two points in the three-dimensional

236 space defined by L*, a*, and b*: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. In addition, the relative

237 contribution of lightness (%ΔL), chroma (%ΔC) and hue (%ΔH), that makes a given color difference

238 (ΔE^*_{ab}) expressed as percentages, were calculated as follows:

239 - Relative contribution of lightness: % $\Delta L = [(\Delta L^*)^2/(\Delta E^*_{ab})^2] \times 100$

240 - Relative contribution of chroma: % $\Delta C = [(\Delta C^*_{ab})^2/(\Delta E^*_{ab})^2] \times 100$

241 - Relative contribution of hue: % $\Delta H = [(\Delta H)^2/(\Delta E^*_{ab})^2] \times 100$

242 being ΔH mathematically deduced from: $\Delta H = [(\Delta E^*_{ab})^2 - ((\Delta L)^2 + (\Delta C)^2)]^{1/2}$

243 **2.7. Statistical analysis**

244 All statistical analyses were performed using Statistica[®] 8.0 software (Stat Soft). Univariate analysis of

245 variance (Tukey test, $p<0.05$) was applied to establish statistical differences for the chemical and

246 colorimetric characteristic of samples.

247 3. **Results and discussion**

248 **3.1. Phenolic composition of wines**

249 The impact of the post-fermentative addition of ripe and overripe PX seeds on the phenolic composition

250 (mg/L) and the percentages of copigmentation and polymerization of Syrah wines (mean±SD, n= 3) are

251 showed in Table 2. Data are reported at seed addition (day 0), at the end of the post-fermentative

252 maceration (60 days) and after 5 months of stabilization (150 days), showing the statistical differences

253 among treatments. At the end of the post-fermentative maceration, wines added with PX seeds (RSW60

254 and OSW60) significantly ($p<0.05$) enriched their total phenolic content in relation to control wines

255 (CW60), whose concentration slightly decreased. However, the magnitude of the positive effect varied

256 depending on the ripeness grade of seeds, which also dissimilarly affected the contents of the different

257 phenolic families. Wines macerated with overripe seeds increased the total phenolic content by 23%

258 (3356.9 versus 2711.09 mg/L, in OSW60 and CW0, respectively). At the same amount added, the

259 increase in Total phenolics reached in wines treated with ripe seeds was comparatively lower (by 10%,

260 2976.2 versus 2711.09 mg/L, in RSW60 and CW0, respectively). This observation can be considered a

11

261 positive effect on the phenolic composition; however, from a global sensory perspective the impact of

262 the seeds addition on the perceived bitterness and astringency of wine could result undesirable, because

263 excessive phenolic extraction could increase these characteristics, and so, it should be assessed.

264 The differences found between OSW60 and RSW60 wines were mainly due to the higher contents of

265 total monomeric flavan-3-ols (404.3 versus 322.12 mg/L) and total procyanidins (10.7 versus 8.6 mg/L)

266 in the formers. The changes in the individual compounds agreed with those observed for the groups of

267 phenolics. Wines macerated with overripe seeds (OSW60) had significant ($p<0.05$) highest contents of

268 most of monomer, dimmer and oligomeric flavan-3-ols than control wines (CW60); and the differences

269 compared to those treated with ripe seeds (RSW60) were significant ($p<0.05$) for (+)-catechin,

270 procyanidin B2-3-O-gallate, and the tetramer. These results disagree with the phenolic composition of

271 PX seeds, in quantitative terms. Ripe seeds had higher contents of total phenolics (3246.4 versus 2984.4

272 mg/100 g dry seed), total phenolic acids (92.36 versus 80.24 mg/100 g dry seed) and total monomeric

273 flavan-3-ols (38.62 versus 34.47 mg/100 g dry seed) than overripe seeds, although the difference was

274 significant ($p<0.05$) only for the phenolic acids. On the other hand, the different proportions of the

275 individual phenolic compounds found between PX seeds (OS and RS) could be related to the effect

276 observed in their respective wines (OSW and RSW). As showed in Figure 1, overripe seeds were

277 proportionally richer ($p<0.05$) in protocatechuic acid derivative, (+)-catechin, procyanidin B2-3-O-

278 gallate and the trimer than ripe seeds. Factors such as seeds protein or polysaccharide can affect the

279 phenolic content of wines. Additionally, the dehydration of grapes and the extreme temperature during

280 the overripening process are factors that irreversible affect the cell structure and textural properties of

281 overripe seeds (Ruiz, Moyano & Zea, 2014; Serratosa, Marquez, Moyano, Zea & Merida, 2014), which

282 could favor a higher extractability and diffusion of some different types of phenolics to wines compared

283 to not overripe seeds.

284 On contrast, the content of total monomeric anthocyanins, which are the compounds directly responsible

285 of color, was found to be lower in seed-treated wines than control ones, being the differences significant

286 ($p<0.05$) for wines macerated with overripe seeds (158.8, 151.8 and 125.6 mg/L in CW60, RSW60 and

287 OSW60, respectively). These results are in agreement with those of Rivero et al. (2019) but disagree

288 those of Canals et al. (2008), Rivero et al. (2017) and Alcalde-Eon, Ferreras-Charro, Ferrer-Gallego,

289 Rivero, Heredia and Escribano-Bailón (2019), which confirm the controversial effects of the pre and

290 post-fermentative addition of grape seeds on the pigment contents of wines. In addition to the grape

291 cultivar and the sun overripe duration, different factors can affect the anthocyanin composition during

292 the seed-added maceration such as the dose, contact time, and vinification stage. According to Gordillo

293 et al., (2014), the partial elimination by adsorption, transformation into new polymeric compounds by

294 polymerization with wine copigments, as well as the degradation by oxidative processes are

295 physicochemical transformations in which anthocyanins can be involved during the addition of grape

296 byproducts to wine. At this respect, wines macerated with PX seeds showed lower percentages of

297 copigmentation (%CA) but higher of polymeric pigments (%PP) than control wines (%CA= 18%,

298 18.3% and 21%; %PP= 43%, 41%, and 39% in OSW60, RSW60 and CW60, respectively). The higher

299 contents of several colorless phenolic copigments (gallic acid, monomer, dimmer, and oligomeric

300 flavan-3-ols) reached in seed-treated wines (especially in OSW) could have favored the interactions with

301 anthocyanins during the post-fermentative maceration period (60 days in this study) and thus, the

302 formation of aggregates among them. This could partially explain the higher values of %PP and lower of

303 monomeric anthocyanins observed at the end of the seed-added maceration.

304 In the case of flavonols, which have been described as effective phenolic copigments, the contributions

305 of overripe and ripe seeds on the total contents of wines were almost negligible due to their scarce

306 presence, in agreement with the findings by Rivero et al. (2019) and Alcalde-Eón et al. (2019).

307 After seed removal, most of the aforementioned pigments and phenolic copigments decreased in all

308 wines (except for gallic acid and EC-gallate), but the stability of the different phenolic families varied

309 among treatments over time. Regarding colorless phenolics, wines added with overripe seeds showed

310 significant ($p<0.05$) higher losses of total monomeric flavan-3-ols and procyanidins during the

311 stabilization period (from seed removal to 150 days of storage) than control wines and those treated with

312 ripe seeds, specially the monomeric forms (by 27%, 12% and 6% in OSW, CW and OSW, respectively).

313 On contrast, the global losses of flavonols were similar between RSW and CW but higher than OSW (by

314 31-29% versus 21%, respectively), although these differences were not significant. Likewise, the

315 behavior of anthocyanin pigments showed that RSW and CW had higher losses of total monomeric

316 forms than OSW (22% versus 19% and 16%, respectively). Especially, RSW stated for suffering the

317 highest reductions of all the anthocyanin groups (by 17% of the non-acylated¸ 21% of the acetated and

318 39% of the coumaroilated derivatives), being the differences significant ($p<0.05$) for the acetated

319 derivatives. Based on the phenolic contents at the seed-removal (day 60), and the subsequent changes

320 previously described, the control wines at the end of the study (CW150) maintained significant ($p<0.05$)

321 higher contents of monomeric anthocyanins (mainly acetylated and p-coumaroilated derivatives) than

322 the wines added with seeds (RSW150 and OSW150). However, they were comparatively poorer in

323 copigments such as gallic acid, (+)-catechin, (-)-epicatechin, procyanidin B2-3-O-gallate, and the trimer.

324 On the other hand, OSW150 maintained higher contents of phenolic acids (mainly gallic acid) and

325 procyanidins than RSW150, which was richer in anthocyanins pigments, (-)-epicatechin and EC-gallate.

326 The gradual formation of polymeric pigments during the storage period was confirmed in all the wines

327 by an increase of the bisulphite-stable color (% PP). Among treatments, OSW had the significant

328 ($p<0.05$) highest values (%PP= 52% versus 46-47% in RSW and CW, respectively), which indicates a

329 higher proportion of more stable pigments in wines with overripe seeds.

330 3.2. **Color evolution during vinification**

331 The evolution of the CIELAB psychophysical color parameters (L* C*$_{ab}$ and h$_{ab}$) in wines during 60

332 days of post-fermentative seed-added maceration and 150 days of stabilization is shown in Figure 2. As

333 observed, the addition of ripe and overripe PX seeds had a significant ($p<0.05$) impact on the

334 quantitative (L* and C*$_{ab}$) and qualitative (h$_{ab}$) color attributes of wines, leading to different color

335 characteristics and stability during vinification. Comparing the color at the end of the post-fermentative

14

336 seed-added maceration (day 60), wines treated with PX seeds had significant ($p<0.05$) lower values of

337 lightness and higher chroma than control wines (L*=72.5, 73.19 and 75.05; C^*_{ab}=25.5, 24.4 and 22.7 in

338 RSW, OSW, and CW, respectively). The magnitude of this effect was more noticeable with ripe seeds.

339 The differences for the L* and C^*_{ab} values between RSW and OSW were no significant (ΔC^*_{ab}

340 increased by 10% and 7%; ΔL^* decreased by 3% and 2.5%, respectively). Although wines treated with

341 seeds had lower contents of monomeric anthocyanins than control wines at the end of the maceration

342 period (Table 2), the effect observed on the chroma and lightness values could be attributed to an

343 increase in the percentage of new polymeric anthocyanins (%PP), which also contribute to the changes

344 in the wine chromatic characteristics (Alcalde-Eón et al., 2019; Jiménez-Martínez, Bautista-Ortín, Gil-

345 Muñoz & Gómez-Plaza, 2019). With regard to the hue, all the wines showed increases from negative

346 towards positive values during the post-fermentative period, which denote a reduction of the blue

347 component of the red color. After 60 days of seed-added maceration, the three wines had very similar h_{ab}

348 values (close to 2°), which confirms that the seed addition had not an immediate effect on the qualitative

349 attribute of color.

350 During the stabilization period (150 days), the quantitative colorimetric parameters evolved in similar

351 way in all the wines, but seed-added wines maintained higher values of chroma and lower of lightness

352 than control wines over time. However, the differences for L* and C^*_{ab} values between OSW and CW

353 tended to increase along the storage while decrease between RSW and CW, indicating different

354 chromatic stability of the treatments. The fact that a higher intensity of color was kept for a longer time

355 in OSW than in RSW wines could be related to the higher enrichment of some phenolic copigments

356 achieved with overripe seeds from the earlier stages of vinification, and the higher proportion of more

357 stable pigments (%PP) along the time. These results agree with those of Rivero et al. (2019), who

358 confirmed the effectiveness of the double post-fermentative maceration of overripe seeds to produce

359 wines chromatically more stable than the wines traditionally produced by increasing the content of some

360 flavanols, benzoic acids and procyanidins.

361 The behavior of hue, however, was quite similar between seed-added wines (OSW and SRW), which

362 tended to decrease at the end of the storage period, making the difference compared to the higher value

363 in control wines. The representation in the (a*b*) color diagram of the three wines from the end of seed

364 maceration (day 60) to the end of stabilization period (day 150) allows to observe a trend in the

365 evolution of color (Figure 3). It can be noticed that practically all CW samples are located in the first

366 quadrant (positive values of a* and b*), which correspond to the redness area of the (a*b*)-plane (h_{ab}=

367 0-10˚), whereas RSW and OSW tended to displace to the fourth quadrant (positive values of a* and

368 negative of b*), which correspond to the red-bluish color region. At the same time, a net rise in the

369 chroma $C*_{ab}$ values was observed in all the wines, being more pronounced in OSW than in CW and

370 RSW. According to this evolution, the color of wines macerated with overripe seeds (OSW) was more

371 intense, darker and red-bluish than control wines (CW) at the end of the storage period, that is,

372 quantitatively and qualitatively different ($p<0.05$). In comparison, the color of wines macerated with ripe

373 seeds was similar in color intensity and lightness than control wines but showed bluer tonality, that is,

374 comparable quantitatively but qualitatively different ($p<0.05$).

375 With the aim of evaluating whether the observed colorimetric changes were visually relevant, the mean

376 color differences ($\Delta E*_{ab}$) among the vinification treatments (CW-RSW; CW-OSW; RSW-OSW) were

377 calculated at each step of the study (30, 60, 90, 120, 140, and 150 days) and showed in Figure 4.

378 Moreover, the relative contributions of lightness (%ΔL), chroma (%ΔC), and hue (%ΔH) to each color

379 difference ($\Delta E*_{ab}$) was calculated to assess the color attribute most influenced by the vinification

380 treatment. At the end of the maceration period (day 60), higher color differences were found between

381 CW-RSW than CW-OSW ($\Delta E*_{ab}$= 3.8 versus 2.5, respectively), which confirmed that ripe seeds

382 produced a higher effect on the wine color during the maceration period than overripe seeds. According

383 to Martinez, Melgosa, Pérez, Hita, and Negueruela (2001), $\Delta E*_{ab}$ around or higher than 3 units indicates

384 color differences perceived by the human eye (average observer, non-trained human eye). Based on this

385 premise, the colorimetric effect produced by ripe seeds can be considered visually appreciable at seed

removal compared to overripe seeds. In both cases, results showed that lightness and chroma were the most overall influential parameter ($\%\Delta L$=45-54%, $\%\Delta C$=44-55%) whereas the weights of the hue modifications were in general comparatively much lower ($\%\Delta H$=1.3-1.5%), which is consistent with the changes observed in the CIELAB color parameters among the three wines. In contrast, the color difference between RSW-OSW was not discernible at this moment (ΔE^*_{ab}=1.3).

The evolution of the color differences between the vinification treatments during the stabilization period support the different colorimetric stability of wines added with ripe and overripe seeds. In general, the ΔE^*_{ab} values between CW-RSW decreased over time while increased in the case of CW-OSW, which means that the color of wines macerated with ripe seeds tended to be similar to control wines along the time of stabilization studied and those macerated with overripe ripe seed tended to differentiate. In particular, the color difference between final CW-OSW could be considered visually discernible (ΔE^*_{ab}= 3.5-4.5) but not between CW-RSW (ΔE^*_{ab}= 1.2-2.5). Similar results were obtained by Rivero et al., (2019), which compared the effectiveness of the post-fermentative maceration of single and double doses of overripe seeds to improve and stabilize the color of wines. At the end of the stabilization period (150 days), it can be observed that the maceration of wines with overripe seeds affected the color in a quantitative and qualitative way, with higher and similar relative variations of lightness and chroma ($\%\Delta L$=44%, $\%\Delta C$=47%) but also an increase of the weight of hue modification ($\%\Delta H$=8%) compared to control wines. Likewise, the higher weight of the hue variation in final wines was confirmed with ripe seeds ($\%\Delta H$=50%), but the overall color differences compared to control ones are virtually negligible in this case.

The comparison of the ΔE^*_{ab} values between RSW-OSW also proved that the color of wines macerated with ripe and overripe seeds tended to be visually different over time (ΔE^*_{ab}>4.5), but the color differences were mainly quantitative ($\%\Delta L$=55%, $\%\Delta C$=43% versus $\%\Delta H$=2% at day 150) in this case.

4. Conclusions

Depending on their ripeness grade, grape seeds from Pedro Ximénez cv. wine byproducts demonstrated different ability to modulate the content and the types of phenolic compounds in Syrah wines from warm climate. The increasing effects on the phenolic composition was achieved by a double post-fermentative maceration of ripe and overripe seeds during 60 days and consisted of the enrichment of total phenolics by increasing the pool of some colorless copigments such as phenolic acids, and most of monomeric flavan-3-ols and procyanidins. Results showed that these effects were more pronounced with overripe seeds obtained from grapes submitted to postharvest sun-drying than with ripe seeds from grapes submitted to on-vine natural maturation. The changes produced in the phenolic composition were reflected in the color of the wines macerated with PX seeds, which were darker and more intense than those elaborated conventionally (without seed addition). Moreover, the addition of ripe and overripe PX seeds provoked a positive effect on the color stability of wines. Differential Colorimetry demonstrated that the effect on the color was higher and more durable (was kept longer over time) in the case of adding overripe seeds, leading to differences visually discernible regarding both conventional and ripe-seeds wines. Nevertheless, further studies could be performed to assess the global impact of the seed addition on others sensory properties, mainly on possible increment of astringency prior to recommend the technique at industrial scale.

Conflict of interest statement

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523

FIGURE CAPTIONS

525 **Figure 1.** Proportions (%) of the phenolic compounds identified in ripe and overripe PX seeds (RS and

526 OS, respectively). Abbreviations: GALL (gallic acid), PROT and PROTdv (protocatechuic acid and

527 derivative), SIR (syringic acid), CAT ((+)-catechin), EC ((-)-epicatechin), B2, B2-GAL, B7

528 (procyanidin dimmers), TRIM (procyanidin trimer). Asterisk indicate significant differences ($p<0.05$,

529 Tukey test).

530 **Figure 2.** Changes in the psychophysical color parameters (means±SD, n=3) for control wines (CW)

531 and wines macerated with ripe and overripe PX seeds (RSW and OSW, respectively) along the

532 vinification process. (a) L^*, lightness; (b) C^*_{ab}, chroma; (c) h_{ab}, hue. Asterisk indicates significant

533 differences ($p<0.05$, Tukey test) compared to control wines.

534 **Figure 3.** CIELAB color space (a^*b^*)-plane for control wines (CW) and wines macerated with ripe and

535 overripe PX seeds (RSW and OSW, respectively) from the end of seed maceration (day 60) to the end of

536 stabilization period (day 150).

537 **Figure 4**. Color differences (ΔE^*_{ab}), with the relative contribution of lightness, chroma, and hue (%ΔL,

538 %ΔC, %ΔH), between control wines (CW) and wines macerated with ripe and overripe PX seeds (RSW

539 and OSW, respectively) at different stages of the vinification (30, 60, 90, 120 and 150 days).

540

Table 1

Table 1. Conventional analytical data (Mean ± SD, n=3) of final red wines (day 150).

Analytical data	CW	RSW	OSW
pH	3.85 ± 0.05	3.86 ± 0.07	3.84 ± 0.01
Total acidity (g/L as tartaric acid)	5.75 ± 0.17	5.04 ± 0.20	5,05 ± 0.07
Volatile acidity (g/L as acetic acid)	0.53 ± 0.02	0.48 ± 0.03	0.52 ± 0.05
Free SO_2 (mg/L)	7.75 ± 0.50	7.80 ± 0.60	9.5 ± 2.53
Total SO_2 (mg/L)	31.00 ± 1.00	32.33 ± 4.51	36.00 ± 7.81
Malic acid (g/L)	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Lactic acid (g/L)	1.22 ± 0.03	1.22 ± 0.07	1.21 ± 0.05
Alcohol by volume (% v/v)	13.50 ± 0.18	13.50 ± 0.18	13.50 ± 0.18

Abbreviations: CW (control wines), RSW (wines added with ripe PX seeds), OSW (wines added with overripe seeds).

Table 2

Table 2. Mean values and standard deviations (n=3) of the phenolic composition (mg/L), and the percentage of copigmentation (%CA) and polymerization (%PP) of control wines (CW) and wines with the post-fermentative addition of Pedro Ximénez seeds (RSW: wines macerated with PX ripe seeds); OSW (wines macerated with PX overripe seeds); at seed addition (0 day), seed removal (60 days) and at the end of stabilization stage (150 days).

	CW0	CW60	RSW60	OSW60	CW150	RSW150	OSW150
Total phenolics (Folin Ciocalteau)	2711.09 ± 109.2	2667.8 ± 20.3 a	2976.2 ± 65.3 b	3356.9 ± 142.1 c	2543.8 ± 35.4 a	2875.5 ± 32.6 ab	2913.0 ± 97.9 b
Total monomeric anthocyanins	179.16 ± 0.97	158.80 ± 9.33 b	151.75 ± 0.99 b	125.56 ± 8.40 a	128.43 ± 0.13 c	118.31 ± 2.87 b	105.35 ± 2.39 a
Total non-acylglc anthoc.	115.54 ± 0.18	96.59 ± 2.84 b	93.88 ± 0.32 b	79.58 ± 3.97 a	84.10 ± 0.46 b	77.71 ± 1.69 b	68.54 ± 1.36 a
Total acetylglc anthoc.	33.18 ± 0.19	30.42 ± 2.27 b	28.45 ± 0.01 b	23.91 ± 1.61 a	24.02 ± 0.09 c	22.64 ± 1.11 b	21.11 ± 0.70 a
Total *p*-coumglc anthoc.	34.44 ± 0.60	31.80 ± 4.27 b	29.41 ± 1.00 b	22.07 ± 2.82 a	30.30 ± 0.42 c	17.97 ± 0.39 b	15.70 ± 0.32 a
Total phenolic acids	132.69 ± 0.44	139.77 ± 4.79 a	152.23 ± 4.33 b	156.64 ± 3.83 b	147.19 ± 1.19 a	151.42 ± 0.75 b	161.89 ± 0.59 c
Total monomeric flavan-*3*-ols	425.1 ± 8.72	320.38 ± 7.84 a	322.12 ± 4.66 a	404.27 ± 10.04 b	281.51 ± 3.72 a	303.94 ± 2.54 b	291.55 ± 7.42 ab
Total procyanidins	8.73 ± 0.22	8.55 ± 0.20 a	8.59 ± 0.17 a	10.66 ± 1.19 b	8.19 ± 0.10 b	7.88 ± 0.02 a	8.01 ± 0.11 b
Total flavonols	28.71 ± 2.22	27.11 ± 080 a	27.07 ± 1.27 a	23.05 ± 2.40 a	18.78 ± 0.39 a	19.14 ± 0.47 a	18.21 ± 0.81 a
% CA	14.83 ± 1.04	21.34 ± 2.22 a	18.30 ± 0.19 a	17.93 ± 2.73 a	17.74 ± 1.35 b	11.67 ± 0.16 a	9.76 ± 0.65 a
% PP	39.98 ± 1.12	39.13 ± 0.53 a	41.06 ± 1.92 a	43.80 ± 3.23 a	46.64 ± 0.88 a	46.31 ± 0.19 a	51.57 ± 0.94 b
Monomeric anthocyanins							
Dp-3-glc	7.95 ± 0.16	7.16 ± 0.03 b	7.11 ± 0.17 b	6.06 ± 0.25 a	6.18 ± 0.05 b	6.28 ± 0.13 b	5.14 ± 0.03 a
Cy-3-glc	1.91 ± 0.27	1.65 ± 0.19 a	1.55 ± 0.03 a	1.41 ± 0.07 a	1.69 ± 0.21 a	1.51 ± 0.29 a	1.27 ± 0.06 a
Pt -3-glc	12.10 ± 0.02	10.12 ± 0.18 b	10.22 ± 0.05 b	8.42 ± 0.43 a	9.64 ± 0.13 c	8.72 ± 0.05 b	7.87 ± 0.13 a
Pn- 3-glc	9.12 ± 0.04	8.06 ± 0.31 b	7.91 ± 0.27 b	6.47 ± 0.21 a	6.85 ± 0.02 c	6.18 ± 0.39 b	5.20 ± 0.12 a
Mv-3-glc	80.45 ± 0.58	69.59 ± 2.68 b	67.07 ± 0.03 b	57.20 ± 3.02 a	59.72 ± 0.08 c	55.02 ± 1.52 b	49.07 ± 1.02 a
Pt-3-acetylglc	2.53 ± 0.16	2.19 ± 0.11 b	2.29 ± 0.07 b	1.88 ± 0.07 a	2.15 ± 0.09 a	2.05 ± 0.58 a	1.90 ± 0.08 a
Pn-3-acetylglc	4.74 ± 0.19	4.30 ± 0.20 c	3.91 ± 0.10 b	3.17 ± 0.10 a	3.56 ± 0.18 a	3.70 ± 0.27 a	3.85 ± 0.09 a
Mv-3-acetylglc	25.91 ± 0.16	23.94 ± 2.18 b	22.25 ± 0.04 b	18.87 ± 1.44 a	18.31 ± 0.36 c	16.88 ± 0.34 b	15.34 ± 0.53 a
Pt -3-*p*-coumglc	3.46 ± 0.03	3.49 ± 0.56 a	3.17 ± 0.03 a	2.60 ± 0.43 a	2.31 ± 0.06 b	2.01 ± 0.20 ab	1.71 ± 0.01 a
Pn- 3-*p*-coumglc	5.74 ± 0.01	5.12 ± 0.80 b	4.92 ± 0.25 ab	3.66 ± 0.43 a	3.36 ± 0.29 b	2.92 ± 0.21 ab	2.74 ± 0.01 a

Mv -3-*p*-coumglc	25.23 ± 0.62	23.19 ± 2.19 b	21.33 ± 0.72 b	15.80 ± 1.96 a	14.63 ± 0.64 c	13.04 ± 0.18 b	11.26 ± 0.29 a
Benzoic acids							
Gallic acid	77.44 ± 0.22	86.08 ± 4.86 a	98.59 ± 4.26 b	103.30 ± 4.07 b	93.66 ± 1.15 a	97.61 ± 0.68 b	108.72 ± 0.53 c
Protocatechuic acid	17.47 ± 0.30	17.35 ± 0.04 a	17.45 ± 0.17 a	17.33 ± 0.07 a	17.28 ± 0.04 b	17.30 ± 0.04 b	17.08 ± 0.04 a
Syringic acid	18.64 ± 0.19	17.45 ± 0.04 a	17.44 ± 0.07 a	17.60 ± 0.19 a	17.52 ± 0.08 a	17.91 ± 0.07 a	17.55 ± 0.20 a
Hydroxycinnamic acids							
Caffeic acid	19.13 ± 0.04	18.89 ± 0.08 b	18.71 ± 0.13 b	18.56 ± 0.17 a	18.76 ± 0.07 a	18.60 ± 0.04 a	18.54 ± 0.15 a
Monomeric flavan-3-ols							
(+)-Catechin	244.32 ± 3.74	221.94 ± 4.46 b	201.47 ± 33.20 a	266.53 ± 1.49 c	188.49 ± 2.23 a	202.12 ± 4.78 b	199.64 ± 3.93 b
(-)-Epicatechin	164.81 ± 3.09	81.46 ± 2.23 a	97.08 ± 8.18 ab	106.53 ± 11.23 b	66.14 ± 1.56 a	74.28 ± 2.27 b	66.35 ± 3.09 a
EC Gallate	15.87 ± 5.45	16.97 ± 3.19 a	23.57 ± 2.06 b	25.72 ± 0.57 b	26.88 ± 0.56 ab	27.54 ± 0.59 b	25.56 ± 0.47 a
Procyanidins							
Procyanidin B2	1.33 ± 0.19	3.49 ± 0.06 a	3.68 ± 0.15 a	4.76 ± 1.19 a	3.27 ± 0.09 c	1.69 ± 0.01 a	2.36 ± 0.08 b
Procyanidin B2-3-*O*-gallate	3.46 ± 0.02	1.23 ± 0.05 a	1.31 ± 0.03 a	2.22 ± 0.05 b	1.20 ± 0.02 a	2.19 ± 0.02 c	1.34 ± 0.03 b
Trimer	1.30 ± 0.05	1.57 ± 0.13 b	1.30 ± 0.02 a	1.25 ± 0.03 a	1.51 ± 0.02 a	1.77 ± 0.02 b	2.09 ± 0.03 c
Tetramer	2.74 ± 0.05	2.26 ± 0.05 a	2.31 ± 0.02 a	2.41 ± 0.08 b	2.27 ± 0.03 a	2.22 ± 0.02 a	2.23 ± 0.03 a
Flavonols							
Myricetin-3-glucuronide	tr.	tr	tr	tr	tr	tr	tr
Myricetin-3-glc	9.97 ± 0.55	9.52 ± 0.29 a	9.53 ± 0.39 a	8.25 ± 0.62 a	7.37 ± 0.07 a	7.43 ± 0.13 a	7.14 ± 0.38 a
Quercetin-3-glucuronide	5.56 ± 0.51	5.26 ± 0.44 a	5.57 ± 0.22 a	4.87 ± 0.82 a	3.50 ± 0.12 a	3.74 ± 0.12 a	3.64 ± 0.27 a
Quercetin-3-glc	7.18 ± 0.36	6.23 ± 0.01 a	6.30 ± 0.11 a	5.62 ± 0.39 a	4.00 ± 0.15 a	4.11 ± 0.08 a	3.97 ± 0.07 a
Laricitrin-3-glc	1.96 ± 0.27	2.02 ± 0.06 b	1.79 ± 0.01 b	1.27 ± 0.15 a	1.26 ± 0.08 a	1.27 ± 0.03 a	1.13 ± 0.06 a
Kaempferol-3-glc	tr	tr	tr	tr	tr	tr	tr
Isorhamnetin-3-glc	1.45 ± 0.21	1.30 ± 0.15 a	1.34 ± 0.26 a	0.98 ± 0.17 a	0.92 ± 0.05 a	0.88 ± 0.05 a	0.74 ± 0.04 a
Syringetin-3-glc	2.59 ± 0.35	2.76 ± 0.19 a	2.55 ± 0.29 a	2.05 ± 0.25 a	1.73 ± 0.05 a	1.73 ± 0.06 a	1.60 ± 0.08 a

Abbreviations: Dp: delphinidin; Cy: cyanidin; Pt: petunidin; Pn: peonidin; Mv: malvidin; glc: glucose; non-acylglc anthoc.: non-acyl glucoside anthocyanins; acetylglc anthoc.: acetylglucoside anthocyanins; *p*-coumglc anthoc.:*p*-coumaroylglucoside anthocyanins; tr: traces.
 Different letter for the same row indicates significant differences according to Tukey test (*p*<0.05) between CW, RSW, and OSW at day 60; and between CW, RSW, and OSW at day 150.
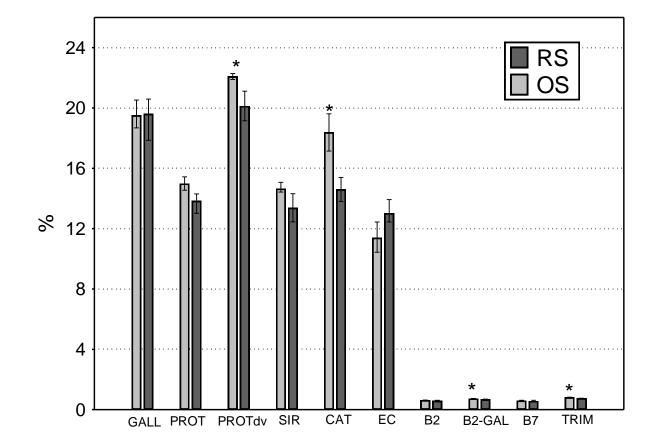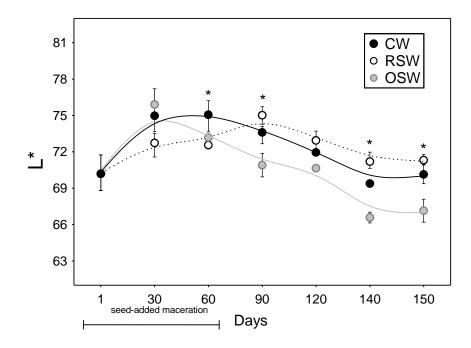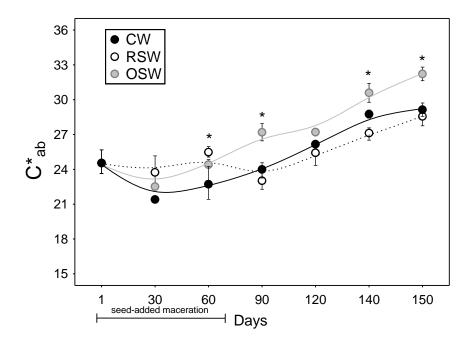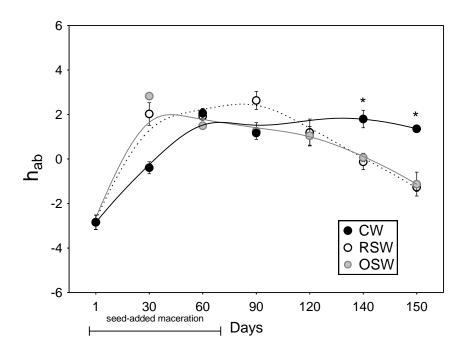
Figure 1.

Figure 2.

A.

B.

C.

Figure 3.

Figure 4.