Copigmentation potential of overripe seeds from sun-dried white grapes on anthocyanins colour and stability by Differential Colorimetry

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***RUNNING TITTLE*: Improving anthocyanin colour by overripe seeds**

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Abstract

Overripe seeds are wine byproducts from grapes submitted to postharvest sun drying, which induce phenolic biosynthesis and polymerisation. Overripe seeds from Pedro Ximénez (PX) and Moscatel (MO) sun-dried grapes were compared as copigments sources to modulate grape anthocyanins (GAs) colour and stability in simulated wine conditions. RRLC/MS analysis proved that overripe seeds contain specific phenolic mixtures capable of inducing positive quantitative and qualitative colour changes in GAs, evidenced by Differential Colorimetry. Copigmentation effects significantly varied depending on the overripe seed variety, related to their qualitative phenolic composition and content. MO extracts richer in gallic acid, catechin and procyanidin B1 led to the stronger colour intensification and perceptible qualitative changes. PX extracts richer in epicatechin, procyanidins B2, B2-GAL and B7 behaved as weak copigments. Overripe seed copigments preserved better GAs colour stability during storage leading to the formation of new anthocyanin-derived pigments, being the stabilising effect stronger with those of MO variety.

Keywords: Postharvest sun drying; overripe seeds; grape byproducts; flavanols; anthocyanins; copigmentation, differential colorimetry

Introduction

In red wines, copigmentation consists of noncovalent interactions between grape anthocyanins (GAs) among themselves or with a wide variety of colourless wine constituents named copigments or cofactors (Boulton, 2001). Copigmentation has mainly two positive effects on anthocyanin colour: 1) an increase of the absorptivity coupled to a bathochromic shift in the visible λ_{max}, and 2) the stabilisation of the red-coloured flavylium cation in the anthocyanin equilibria (Trouillas *et al.*, 2016). In addition, copigmentation is considered the first step in the formation of more stable anthocyanin-derived pigments by covalent links (De freitas & Mateus, 2011). Thus, copigmentation plays an important role on the quality and stability of wine colour. Among wine constituents, phenolic compounds appear to be one of the most efficient copigments due to their planar structure, which can stack more strongly to anthocyanins by intermolecular copigmentation. In this sense, the search for alternative sources of phenolic copigments intended to enhance the chemical and colorimetric stability of anthocyanins in wine is one of the main research areas of oenology (Jara-Palacios *et al.*, 2014a). Previous studies highlighted that wine byproducts (grape pomaces, skins or seeds) obtained from white grapes cultivated in South Europe (the first wine producers worldwide) are one of the most abundant and available sources of phenolics (Carullo *et al.*, 2019). One of the advantages of using those natural bioactive compounds as functional ingredients for food applications is that they can be considered safe in comparison with synthetic additives (Restuccia *et al.*, 2019).

In warm climate regions of Mediterranean countries like southern Spain, white grapes are submitted to postharvest dehydration by direct sun drying in order to increase the sugar content in berries (>20 ºBrix) for elaborating traditional sweet wines (Ruiz *et al.*, 2014*;* Ruiz-Bejarano *et al.*, 2016). The postharvest dehydration is developed during 5 to

10 days, depending on the climatic conditions, which produce intense water loss of berries. The extreme sun conditions also activate the gene expression involved in the phenolic biosynthesis and polymerisation, and consequently, their progressive concentration in grape tissues such as seeds (Serratosa *et al*., 2014; Dumitriu *et al.*, 2015). Thus, overripe seeds obtained after pressing white dried grapes (wine byproducts) represent interesting sources highly concentrated in phenolic compounds (mainly procyanidins and oligomeric flavanols) having potential copigmentation properties. Recently, it has been demonstrated that the addition of overripe seeds during vinification is an environmentally sustainable process to overcome the lack of phenolics in red wines, modulate the copigmentation equilibria, and prevent the colour loss during wine storage (Jara-Palacios *et al*., 2016; Rivero *et al*., 2017; 2019).

However, to date few studies have evaluated the exploitation of overripe seeds byproducts for colour stabilisation purposes. The copigmentation potential of overripe seeds, related to its quantitative and qualitative phenolic composition, depends on the rate and extent of dehydration process, which can affect differently the metabolism of each grape variety and therefore its composition and applications (Serratosa *et al*., 2014; Río-Segade *et al*., 2016). In this sense, further investigations are needed to better understand the contribution of overripe seeds phenolics to the anthocyanin colour and stability.

Currently, an accurate measurement of the colour changes related to perception and acceptability is becoming a mandatory standard for colour applications in the food industry. In particular, spectrophotometric methods have been widely applied as valid and quick tools for assessing the effect of copigmentation on the colour by means of the changes at the $\lambda_{máx}$ of anthocyanins. In comparison, the evaluation of the colour effect in the CIELAB colour space by Differential Colorimetry has demonstrated to provide

an integral colorimetric interpretation of copigmentation by means of the changes produced in the entire visible spectrum (Gordillo *et al*., 2012). Based on the ΔE^*_{ab} parameter, which is visually interpretable, conclusions related to the anthocyanin colour variations and stability can be extracted (Gordillo *et al*., 2015). Likewise, the related colour-differences of lightness, chroma and hue (ΔL^*, ΔC^*_{ab}, Δh_{ab}) have shown to be of practical interest to assess both the quantitative and qualitative colorimetric effects of copigmentation and their incidence on visual perception (Jara-Palacios *et al*., 2014a).

Thus, the aim of this study was to compare the potential of using overripe seeds extracts from two white grape varieties submitted to postharvest sun drying, Pedro Ximénez and Moscatel, as alternative copigments sources to stabilise grape anthocyanins. Our attention was focused on the study of the phenolic composition related to their copigmentation properties and colour stabilisation effects over time at increasing levels, objectively assessed by Differential Colorimetry.

Material and methods

Chemicals and Standards

The chemicals and standards used were (+)-catechin, (-)-epicatechin and gallic acid standards (Sigma Chemical Co., St. Louis, MI, USA), B1 and B2 procyanidin standards (Extrasynthèse, Lyon, France), GAs (grape skin anthocyanin extract, *Vitis vinifera* L., IFC Solutions, New Jersey, NJ, USA), acetonitrile HPLC grade (Fisher Scientific, Waltham, MA, USA), Folin reagent, sodium carbonate and ethanol 96% (Sharlau Chemie S.A., Barcelona, Spain), tartaric acid and sodium chloride (Panreac, Barcelona, Spain), and formic acid HPLC grade (Sigma Chemical Co., St. Louis, MI, USA).

Overripe seeds from sun-dried grapes

Overripe seeds from pomaces of sun-dried white grapes *Vitis vinifera* L. cv. Pedro Ximénez (PX) and Moscatel (MO) were used as natural sources of phenolic

copigments. PX and MO grapes proceeded from two winemaking areas of southern Spain, D.O. Montilla-Moriles (Córdoba, Spain) and D.O. Málaga (Málaga, Spain), respectively, which are classified as semi-continental Mediterranean climate with short winters and long, dry, hot summers (the diurnal temperature can reach 40 ºC).

Ripe grape clusters were harvested, placed in single layers and exposed to sun-drying effect for 10 days. Later, the resulting raisins (24 ºBé of sugar content) were transported to local wineries, destemmed, crushed and pressed to elaborate traditional sweet wines. Immediately after pressing, PX and MO pomaces (mixture of overripe seeds, skins, and rests of pulp) were collected and transferred to the laboratory. Overripe seeds were manually separated from the pomaces, stored frozen (-20 ºC) and further freeze-dried (lyophilizer Cryodos-80, Telstar Varian DS 102, Terrasa, Spain), and ground to powder until extracted.

Copigmentation experiments

All experiments were prepared in wine-like solutions containing 5 g/L tartaric acid in 12% ethanol, buffered with 1 M NaOH to pH 3.6, and ionic strength adjusted to 0.2 M by adding NaCl.

Grape anthocyanins (GAs) were used as pigments and phenolic extracts from PX and MO overripe seeds as copigments. GAs solution was prepared by dissolving purified grape skin extract in wine-like solution. PX and MO phenolic extracts were prepared following the method described by Jara-Palacios *et al.* (2014a). 2g of the homogeneous lyophilized powder of PX and MO overripe seeds were individually macerated in 30 mL of wine-like solution for 12 hours at room temperature (18-20 ºC) with occasional agitation and sonication. The supernatants containing the phenolic compounds were centrifuged (4190 g, 10 min) and filtered through 0.45 μm Nylon filter (5190-5270, Agilent Technologies, Palo Alto, CA, USA).

Pigments/copigments solutions were prepared by adding aliquots of phenolic extracts from PX and MO overripe seeds to the GAs solution at three concentration levels (100, 200, and 400 mg/L of total phenolics). In order to prevent anthocyanin self-association effects in the copigmentation experiments, the final anthocyanin content in all cases was 50 mg/L. Solutions (5 mL) were prepared in triplicate (n=21), stored closed in darkness at 25 ºC. Samples were taken periodically for the chemical and colorimetric analysis at 1, 6, 13, 20, 34, 56, and 90 storage days.

Total phenolic content

The spectrophotometric determination of the Total Phenolic content (TPC) was performed with an Agilent 8453 UV-vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA), using 10 mm path length glass cells and distilled water as reference. The TPC was determined using a modification of the Folin-Ciocalteu method (Singlenton & Rossi, 1965), by measuring the absorbance at 765 nm and using gallic acid as calibration standard. Results were expressed as gallic acid equivalents: mg GAE/100 g of dry seeds (DS), and mg GAE/L in solutions.

RRLC-DAD-MS analysis of phenolic compounds

The phenolic composition of overripe seeds extracts was determined in triplicate according to the method of Jara-Palacios *et al*. (2014b) by rapid resolution liquid chromatography and mass spectrometry (RRLC/MS). The chromatographic system was an Agilent 1290, with a quaternary pump, UV-vis diode-array detector, automatic injector, and ChemStation software (Agilent Technologies, Palo Alto, CA, USA), using a C18 Poroshell 120 column (2.7 μm, 5 cm x 4.6 mm), with an injection volume of 0.4 μL. The solvents were formic acid:water (1:999 mL/mL) as solvent A, and acetonitrile as solvent B, at the following gradients: 0-5 min with 5% B linear; 5-20 min with 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 ºC. Detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer, which was connected to the HPLC equipment via the DAD cell outlet. Phenolic substances were identified by their retention time, UV-vis spectra and mass spectra, as well as by comparing with our data library and standards when available. The quantification was made at 280 nm (flavanols and benzoic acids) by comparing the areas and the retention times with the gallic acid, catechin and procyanidins B1 and B2 standards. The concentration of phenolic compounds was expressed as mg/100 g of dry seeds (DS). The total content of benzoic acid, monomeric flavanol and procyanidins were calculated as the sum of individual phenolics identified.

LC-MS analysis of anthocyanins and derived pigments

The anthocyanin composition and derived pigments in solutions was assessed by LC-MS using an API 3200 Qtrap (AB Sciex, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by Analyst 5.1 software. Analyses were performed in triplicate. An AQUA C18 reversed-phase, 5 μm, 150 mm×4.6 mm column (Phenomenex®, Torrance, CA, USA) thermostatted at 35 ºC was used. Detection was carried out at 520 nm as the preferred wavelength. Spectra were recorded from 220 to 600 nm. Full mass, MS^2 and MS^3 experiments were performed in positive mode (ESI+) accordingly to Alcalde-Eon *et al.* (2014).

Colorimetric analysis

Spectrophotometric measurements of solutions were carried out with a Hewlett-Packard UV-vis HP8453 spectrophotometer (Palo Alto, CA, USA). The absorption spectra (380-770 nm) of samples were recorded at constant intervals ($\Delta\lambda$ = 2 nm) using 10 mm path length glass cells and distilled water as a reference. Each measurement was made in

triplicate. As overripe seed extracts were originally pale yellow, their absorbance spectra were also recorded at each concentration. The absorbance spectra of GAs solution were corrected in the visible range by the absorbance spectra of the overripe seeds extracts to avoid any interference in the measurement of the copigmentation effect. The CIELAB colorimetric parameters of samples (L*, a*, b*, C^*_{ab}, and h_{ab}) were calculated from the absorption spectra by using the original software CromaLab® (Heredia, Alvarez, Gónzález-Miret & Ramírez, 2004) following the recommendations of the Commission International de l'Eclariage (CIE, 2004): 10° Standard Observer and the Standard Illuminant D65, corresponding to natural daylight. CIELAB parameters were calculated: L* (the correlate of lightness; ranging from 0, black, to 100, white) and two colour coordinates, a* (which takes positive values for reddish colours and negative values for greenish ones) and b* (positive for yellowish colours and negative for bluish ones). From L*, a*, b*, other colour parameters are defined: the hue angle (h_{ab}, the correlate of chromaticity or tone), and the chroma C^*_{ab} (the correlate of saturation or intensity of colour). L*, C^*_{ab}, and h_{ab} can be distinguished as quantitative or qualitative parameters as they indicate quantitative (L* and C^*_{ab}) or qualitative (h_{ab}) attributes of colour.

Analysis of Copigmentation by Differential Colorimetry

The effect of copigmentation on the anthocyanin colour and stability was assessed by Differential Colorimetry according to the methodology described in Gordillo *et al*. (2012; 2015), which is based on the application of diverse colour-difference formulas by using the scalar (L*, a*, b*) and cylindrical (L*, C^*_{ab}, h_{ab}) colour parameters, as follows:

The colour difference (ΔE^*_{ab}) between GAs solutions and the same solutions containing increasing content of overripe seed extracts was computed as the Euclidean distance

between two points in the three-dimensional CIELAB: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. The trend of the changes in each colour attribute due to copigmentation was evaluated by pairs of samples by means of the absolute lightness, chroma, and hue differences (ΔL^*, ΔC^*_{ab}, Δh_{ab}). In addition, the relative contribution of lightness (%ΔL), chroma (%ΔC), and hue (%ΔH) that makes a given colour difference (ΔE^*_{ab}), expressed as percentages, were calculated as follows:

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

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

The magnitude of copigmentation was assessed by means of the parameter Total Colour (E). The Total Colour of each sample is calculated as the colour difference (ΔE^*_{ab}) between its colour (L*, a*, and b*) with respect to an achromatic reference (L*=100, a*=0, b*=0): $E = ((L^*-100)^2 + (a^*-0)^2 + (b^*-0)^2)^{1/2}$. Thus, the comparison of the Total Colour of the GAs solutions (E_0) and the Total Colour of the same solutions containing phenolic mixtures from overripe seeds (E_C), by pairs of samples and expressed as the percentage $[(Ec-E_0)/E_0]\times100$, represent a colorimetric index of the magnitude of copigmentation based on Differential Colorimetry.

Statistical Analysis

Analysis of variance (ANOVA, Tukey test, $p<0.05$) were applied in order to evaluate whether significant differences exist for the chemical and colorimetric analysis among the samples, by the Statistica v.8.0 software (Statsoft, 2007).

Results and discussion

Phenolic composition of overripe seeds byproducts

Table S1 shows the phenolic composition (mg/100 g DS) of PX and MO overripe seeds byproducts. Quantitatively, MO overripe seeds had 3-fold higher TPC than those from PX (3806 and 1228 mg/100 g DS, respectively), indicating significant ($p<0.05$) higher phenolic potential. TPC values in MO were also higher than those described in seeds byproducts from freshly harvested grapes, extracted in similar conditions (Özcan *et al.*, 2017; Jara-Palacios *et al.*, 2014a).

Regarding phenolic families, monomeric flavanols were the major compounds found in overripe seeds byproducts (67% w/w), followed by benzoic acids (24-26% w/w) and procyanidins (7-9% w/w). Results only showed trace levels of flavonols (no quantifiable amounts). In particular, MO overripe seeds had higher levels of total flavanols and benzoic acids than those of PX.

Considering individual phenolics, (+)-catechin, gallic acid and (-)-epicatechin were the most abundant compounds. MO overripe seeds presented higher contents in (+)-catechin and gallic acid than PX (321 *vs.* 119 and 118 *vs.* 42 mg/100 g DS, respectively). Conversely, PX overripe seeds was richer in (-)-epicatechin (89 *vs.* 74 mg/100 g DS).

Moreover, the concentration of procyanidins and oligomeric flavanols (trimers and tetramers) differed significantly in the overripe seeds byproducts. Procyanidin B1, procyanidin EC gallate and procyanidin B2 were the major oligomers with concentrations around 2-fold higher in MO extracts. Although PX overripe seeds extracts had higher amount of procyanidin B7, the difference regarding MO extracts was not as pronounced. Great variabilities in the content and type of phenolics have been reported in grape byproducts depending on the variety, winemaking process or type of grape byproduct (Jara-Palacios *et al.*, 2016). In this study, the differences found for the phenolic profile among the overripe seed extracts may be attributed to the

particular interaction of PX and MO varieties with the environmental conditions during postharvest dehydration (Bondada *et al.*, 2017).

Effect of copigmentation in GAs colour characteristics

The magnitude of copigmentation due to overripe seeds extracts was assessed by measuring the increases induced in the Total Colour of the GAs solutions as the ratio $[(Ec-E_0)/E_0]\times100)$, showed in Figure 1. This ratio represents the extent of copigmentation based not only on the changes in a unique wavelength (λ_{max}), but on the modifications in the entire visible spectrum (360-780 nm), providing a more precise evaluation of the colour effect. Results showed that MO overripe seeds extracts induced the highest increases of Total Colour at all concentration tested, being the magnitude of the effect significantly ($p<0.05$) dose-dependent (6% and 12% with 100 and 400 mg/L, respectively). The extent of this effect was higher than the values reported for phenolic extracts of seed byproducts from freshly harvested grapes (white and red varieties), when tested at same concentrations (Jara-Palacios *et al.*, 2014b; González-Manzano *et al.*, 2009). In comparison, PX overripe extracts increased the GAs Total Colour in a lower extent (3-6%), evidencing that they act as weak copigments. The differences found for the copigmentation effects may be due to the different qualitative phenolic composition of the overripe seed varieties. For the same phenolic concentration, MO overripe seed extracts having higher proportions of gallic acid, catechin and procyanidin B1 were more efficient copigments than those of PX, which were comparatively richer in epicatechin, and procydanidins B2, B2-3-*O*-gallate and B7.

The changes on the CIELAB parameters showed the trend of the colour variation due to copigmentation (Table 1). Increasing contents of PX and MO extracts produced progressives increases of chroma (C^*_{ab}) and decreases of lightness (L^*) in GAs solutions, which means an intensification of the colour (more saturated and darker). MO

overripe seeds extracts induced the strongest effects in colour increasing C^*_{ab} values by 6% and decreasing L* by 7% at 400 mg/L. PX overripe seed extracts lead to lower chroma and lightness changes (C^*_{ab} increased by 1.8% and L*decreased by 2% at 400 mg/L), which was consistent with the smaller magnitude of copigmentation. Regarding the hue, both PX and MO extracts increased the h_{ab} values of GAs, indicating changes in the tonality. Similar trend on the hue changes was observed by González-Manzano *et al.*, (2009), which tested seed flavanol mixtures from freshly harvested grapes as copigments of wine anthocyanins. The variation was less marked with the PX extracts (from 7º to 10º at 400 mg/L), indicating that GAs maintained better its original red colour.

Besides, the colour differences (ΔE^*_{ab}) between the GAs solutions and the same solutions containing overripe seeds extracts were calculated by pair of samples in order to find out if the copigmentation effects can be considered visually discernible (Figure 2). According to Martínez *et al.* (2001), ΔE^*_{ab} value > 3 units indicates perceptible colour changes by an average observer. Results confirmed that the colour changes induced by phenolic extracts of MO overripe seeds were visually perceptible at all the concentrations tested (ΔE^*_{ab} = 6.0, 7.6 and 8.8 at 100, 200 and 400 mg/L, respectively). Interestingly, these colour variations were higher than those reported for phenolic extracts of stems, seeds, grape pomace and skins from freshly harvested grapes (ΔE^*_{ab} ~ 1.5, 3.0, 4.5, and 6.0, respectively at 400 mg/L), when applied at the same concentrations in similar conditions (Jara-Palacios *et al.*, 2014b). As expected, phenolic extracts of PX overripe seeds induced lower colour variations in GAs solutions but were visually discernible at concentrations up to 200 mg/L (ΔE^*_{ab}= 2.3, 2.9 and 3.4 at 100, 200, and 400 mg/L, respectively). In particular, the higher values found for the %ΔH (>50%) that make up the ΔE^*_{ab} reflected that the qualitative colour changes induced by

MO overripe seed extracts were more important than the quantitative ones (%ΔC and %ΔL). In the case of overripe seeds from PX, the highest relative contribution to ΔE^*_{ab} was the lightness (%ΔL>60%).

Effect of copigmentation in GAs colour stability

The evolution of the colorimetric parameters (L*, C^*_{ab} and h_{ab}) was studied for 90 days at 25 ºC in darkness (Figure S1). In general, the h_{ab} values increased during storage while the lightness (L*) and chroma (C^*_{ab}) remained more stable in all solutions. However, PX and MO solutions maintained lower values of lightness (L*) and higher of chroma (C^*_{ab}) than GAs solutions (Figure S1A-S1D). Thus, grape anthocyanins preserved darker and more intense colours along storage in the presence of overripe seeds copigments, being the effect more pronounce with those from MO variety. These observations indicate that the initial positive copigmentation effect on the quantitative attributes of colour (intensity and lightness) was maintained over time in a period of 90 days. In comparison, the changes on the hue (h_{ab}) were much more intense than the chroma and lightness modifications (Figure S1E and S1F), and therefore, the colour variation during storage was mainly qualitative. Results showed that GAs maintained higher values of h_{ab} during storage in the presence of overripe seed copigments. Notwithstanding, due to the intense evolution of h_{ab} in GAs solutions without overripe seed copigments, the differences at the end of storage were only significant respect to PX-400 solutions (h_{ab} = 28º and 35º, respectively).

The assessment of the colour differences (ΔE^*_{ab}) in each sample occurring from the end of the storage period respect to the beginning (from day 90 to day 1) allowed comparing the global colour stability between solutions by Differential Colorimetry. Likewise, the evaluation of the lightness, chroma and hue differences (ΔL^*, ΔC^*_{ab}, Δh_{ab}) allowed

assessing if the colour variation during storage were due to quantitative or qualitative changes. Results are shown in Table 2. The lowest ΔE^*_{ab} values (7-9 units) corresponded to GAs solutions containing phenolic extracts of MO overripe seeds, which indicate lower colour variation and therefore, higher colour stability over time. For this overripe seed variety, the stabilizing effect was dose-dependent since the higher phenolic content added to GAs solutions, the lower ΔE^*_{ab} values. In particular, the higher colour stability was mainly due to the lower increases on the hue during storage in MO solutions (Δh_{ab}= +11.8º, +9.3º and +8.9º *vs.* +21º in GAs solutions). In contrast, GAs solutions containing PX overripe seed copigments had the highest values of ΔE^*_{ab} (from 14.5 to 18.5 units) suggesting lower capability to stabilise the anthocyanin colour. In this case, the lower colour stability was mainly due to the higher increases of hue at the highest concentration applied (Δh_{ab}= +25.3º *vs.* +21º in PX-400 and GAs, respectively). The lower values found for ΔL^* and ΔC^*_{ab} confirm that the quantitative changes during storage were similar in all solutions.

Effect of copigmentation in GAs chemical stability

The chemical stability of solutions was assessed by the global changes on the anthocyanin content (mg/L) between day 1 and day 90. The anthocyanin composition of GAs solution was determined: Non-acylated (3-monoglucosides and 3,5-diglucosides: 11% and 46%, respectively) and acylated forms (3-*p*-coumaroylated glucosides and 3-*p*-coumaroylated-5-glucosides, 3% and 17%, respectively) of the five expected grape anthocyanidins (delphinidin, cyanidin, petunidin, peonidin, and malvidin) were identified. A decrease of the different anthocyanin fractions (glucosides and acylated derivatives) and the total anthocyanin content was observed in all of the solutions with storage time (Figure S2), in agreement with previous work (González-Manzano *et al.*, 2009). In general, GAs solutions containing the highest concentration of phenolic

extracts (PX-400 and MO-400) had the highest decreases of total anthocyanins (32 % and 31%, respectively). As reported in literature, the loss of anthocyanins may be due to either reactions of formation of new stable pigments or degradation reactions (Guadalupe & Ayestarán, 2008). Thus, the presence of new anthocyanin-derived pigments was explored in solutions by LC-MS analysis in the final samples (90 days). Two new anthocyanin derived pigments resulting from the acetaldehyde-mediated condensation with flavanols were determined only in MO-400 and PX400 samples. According to their mass spectra and λ_{max} (Table S2), they were identified as malvidin 3,5-diglucoside-ethyl-(epi)-catechin and malvidin 3-*p*-coumaroylglucoside-5-glucoside-ethyl-(epi)-catechin. The content of such as new pigments was 0.8 and 0.57 mg/L in MO-400 and PX-400 solutions (respectively), which partially may explain the higher decreases of monomeric anthocyanins in these samples during storage.

Conclusions

Overripe seeds byproducts from sun-dried white grapes have demonstrated being rich sources to obtain phenolic extracts that can act as effective copigments capable of improve grape anthocyanin colour by perceptible changes, even stronger than other byproducts from freshly harvested grapes. Benefits on anthocyanin colour and chemical stability were also confirmed by Differential Colorimetry and HPLC/MS analysis, related with lower colour variations of GAs solutions during storage and the synthesis of new anthocyanin derived pigments. The extent of the positive effects depended on the particular qualitative phenolic profile of overripe seeds according to the grape variety (Moscatel and Pedro Ximénez) and the dose applied. Thus, results open the possibility to reuse overripe seeds byproducts as wine additives to modulate colour quality and stability, which can provide important economic and environmental benefits to the oenological industry.

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Table 1. Absorbance values (λ_{max} 520 nm), CIELAB colour parameters (L*, a*, b*, C^*_{ab}, h_{ab}) and Total Colour (E) of GAs and the same solutions containing increasing concentration of phenolic extracts from PX and MOS overripe seed extracts (PX: Pedro Ximénez, MO: Moscatel at 100, 200 and 400 mg/L).

	GAs	PX			MO		
		100	200	400	100	200	400
A_{520}	0.77±0.1a	0.81±0.02b	0.82±0.03b	0.83±0.10b	0.86±0.01b	0.88±0.10bc	0.91±0.02c
L*	62.37±0.18a	60.52±0.24b	60.10±0.14bc	59.60±0.14c	58.34±0.36b	57.69±0.07b	56.67±0.46c
a*	38.49±0.02a	38.76±0.32ab	38.98±0.13b	39.03±0.11b	38.70±0.24b	39.06±0.15 bc	39.36±0.31c
b*	4.68±0.01a	6.09±0.16b	6.50±0.10c	6.71±0.08c	9.18±0.28b	10.65±0.44c	11.39±0.41c
C^*_{ab}	38.77±0.03a	39.23±0.24b	39.52±0.13b	39.61±0.12b	39.77±0.30b	40.48±0.05 c	40.98±0.41c
h_{ab}	6.93±0.01a	8.93±0.19b	9.47±0.14c	9.76±0.10c	13.34±0.32b	15.26±0.65c	16.14±0.42c
E^a	54.03±0.09a	55.41±0.5b	55.74±0.02b	56.12±0.15b	57.40±0.06b	57.78±0.52bc	58.81±0.77c

[a] E= Total Colour ($\Delta E^*_{ab} = [(L^*-100)^2 + (a^*-0)^2 + (b^*-0)^2]^{1/2}$

Different letters between columns means significant differences between solutions containing overripe seed extracts (PX and MO) respect to GAs solution. Data are mean values± SD (n=3).

Table 2. Mean colour variations (ΔE^*_{ab}, ΔL^*, ΔC^*_{ab}, Δh_{ab}) during storage from day 1 to day 90 (25 ºC in darkness) of GAs and the same solutions containing increasing concentration (100, 200 and 400 mg/L) of overripe seed extracts from MO and PX (MO: Moscatel, PX: Pedro Ximénez).

	GAs	PX-100	PX-200	PX-400	MO-100	MO-200	MO-400
ΔE^*_{ab}	14.1±0.5a	14.5±1.9a	14.5±1.0a	18.2 ±1.3b	9.1±0.8b	8.0±0.8bc	7.0±0.7c
ΔL^*	+1.4±0.6a	+2.3±0.4b	+1.4±0.6b	+0.5±0.6b	+3.9±0.1bc	+3.2±0.3c	+1.6±0.4c
ΔC^*_{ab}	-0.8±0.5a	+0.2±0.1b	+0.6±0.4b	+2.2±0.3c	-1.2±0.2a	-1.2±0.5a	-0.7±0.4a
Δh_{ab}	+21.1±0.8a	+21.0±2.3a	+20.6±1.7a	+25.23±1.6b	+11.8±1.2b	+9.3±1.9b	+8.9±0.8b

Different letters between columns means significant differences among solutions containing overripe seed extracts (MO and PX at 100,200, 400 mg/L) respect to GAs solution. Data are mean values ±SD (n=3).

Legends to Figures

Figure 1. Magnitude of copigmentation of overripe seed extracts at increasing phenolic levels, assessed by Differential Colorimetry [(E_C-E_0)/E_0]×100; E=Total Colour. Data are mean values ± SD, n=3. (MO: Moscatel, PX: Pedro Ximénez at 100, 200 and 400 mg/L).

Figure 2. Colour differences (ΔE^*_{ab}), with the relative contribution of lightness, chroma, and hue (%ΔL, %ΔC, %ΔH), between GAs solutions and the same solutions containing increasing levels of overripe seed extracts. Data are mean values ± SD, n=3. (MO: Moscatel, PX: Pedro Ximénez at 100, 200 and 400 mg/L).

Figure S1. Evolution of the CIELAB colour parameters of GAs solutions containing increasing concentrations of phenolic extracts from PX and MO overripe seeds, during 90 days storage at 25 ºC in darkness: 3a y 3b) Lightness, L*; 3c y 3d) Chroma, C^*_{ab}; 3e y 3f) Hue, h_{ab}. Data are mean values ± SD, n=3. (MO: Moscatel, PX: Pedro Ximénez at 100, 200 and 400 mg/L).

Figure S2. Global changes (day 1 to day 90) of the total anthocyanin, total glucoside and total acylated contents (mg/L) of GAs solutions containing increasing concentrations of phenolic extracts from PX and MO overripe seeds. Data are mean values ± SD, n=3. (MO: Moscatel, PX: Pedro Ximénez at 100, 200 and 400 mg/L).