**Assessment of the color modulation and stability of naturally copigmented anthocyanin-grape colorants with different levels of purification**

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**Abstract**

Grape skins or their by-products from wine production are rich sources of anthocyanins and various colorless phenolics, depending on the grape variety. Phenolics have strong antioxidant and anthocyanin stabilizing properties and help to produce functional anthocyanin colorants with improved stability. This study aimed to assess differences in color expression and stability of anthocyanin colorants from red grape varieties naturally copigmented and with different levels of purity and to compare them to synthetic FD&C Red No. 3. Model juice systems were prepared at pH 3.5 with anthocyanins and phenolic copigments extracted from four *Vitis vinifera* grape varieties ('Tempranillo', 'Syrah', 'C. Sauvignon', and 'Graciano') both crude and purified by C18 solid phase extraction. Attention was focused on differential colorimetry and phenolic composition related to the color. Degradation kinetics of total color were also studied during storage of 17 days in darkness at 25ºC. Grape variety significantly influenced pigment yield, proportion of acylation, and proportion of copigments:pigments ratios in crude extracts; purification modulated the copigment:pigment ratios. This proportion was related to perceptible color variability among colorants and to different stabilities. With the same pigment content, grape varieties richer in skin copigments and higher copigment/pigment ratios ('Syrah' and 'Tempranillo') produced more intensely colored crude extracts whose tonalities ranged from reddish ('Graciano') to red-bluish ('Syrah'), depending on the proportion of acylation. Increasing the purity of the pigments diminished the color variability due to variety, making them less vivid and visually more similar to one another and also to the synthetic colorant. Degradation kinetic studies showed that unpurified grape colorants had higher color stability over time, with the greatest stabilizing effects achieved with varieties richer in skin flavonols ('Tempranillo' and 'Syrah').

**Keywords:** Anthocyanin colorants; copigmentation; grape variety; purity; differential colorimetry.

## 1. Introduction

Color additives are substances from natural or synthetic origin used to impart, restore, or standardize the color and appearance of foodstuffs making them more attractive to consumers (Pasias, 2015). The use of synthetic colorants has unquestionable advantages for the food industry because they are comparatively easier and less expensive to produce than natural colors. From a technological perspective, they typically show higher chemical stability without imparting odor or flavor to products. However, one of the limiting factors of using synthetic colorants is evidence of their potential detrimental effects on human health depending on the dose used (Carocho, Morales & Ferreira, 2015). Studies have shown that synthetic colorants are not themselves toxic, but when used in mixtures, there might be a synergistic effect (Amchova, Kotolova, & Ruda-Kucerova, 2015). Major international food safety authorities have restricted the use of some synthetic colorants to particular foods at the minimum possible dosage (Carocho et al., 2015). Besides regulations, consumers also show a higher preference for food products that use natural ingredients (including colors), which are in general perceived as healthy and safe because many of them have been found to be nutraceuticals (Bearth, Cousin, & Siegrist, 2014; Wrolstad & Culver, 2012).

All these circumstances have strongly influenced the food sector. The search for alternative natural pigments to replace the synthetic colorants is a current market trend, especially within premium foods and in products positioned for children (Carocho et al., 2015; Nielsen & Holst, 2002; Wrolstad & Culver, 2012). Red and yellow colorants account for ~90% of the total amount of colorants added to food (Potera, 2010). Therefore, there is high interest for greater availability of natural red colorants which have increased stability in food matrices due to the continued restrictions of their synthetic counterparts (Giusti & Wrolstad, 2003; Rodríguez-Saona, Giusti, & Wrolstad, 1999).

One class of natural pigments traditionally used by the food industry that provide red colors is anthocyanins, a large group of flavonoids widely spread in nature (Sigurdson, Tang, & Giusti, 2017). Interest in anthocyanins is due to the many attractive colors they can produce and multiple health benefits associated with their consumption (He & Giusti, 2010). As they are water-soluble and innocuous pigments, anthocyanins have a great potential to color food products with added biofunctional value. However, color formulations based on anthocyanins have some limitations. Anthocyanins are sensitive to several different factors such as pH changes, exposure to heat, light, oxygen,

temperature, metals, bleaching agents, etc. (Ioannou, Hafsa, Hamdi, Charbonnel, & Ghoul, 2012). The stabilization of anthocyanins is still a major challenge and constitutes an important topic for the food colorant industry (Cortez, Luna-Vital, Margulis, & Gonzalez de Mejia, 2016).

Grape skins, or their by-products from the wine industry, represent some of the main commercial sources of anthocyanins (classified as E163 number). The major pigments present in *Vitis vinifera* grape skin are delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides and their acylated derivatives with cinnamic acids (Narduzzi, Stanstrup, & Mattivi, 2015). Acylation improves the stability of anthocyanins by protection of the chromophore by intramolecular copigmentation (Giusti & Wrolstad, 2003; Zhao et al., 2017). Grape skins also contain colorless phenolics that can act as cofactors (the so-called copigments) of anthocyanins protecting them through intermolecular copigmentation phenomena; colorless copigments contribute to reduced degradative reactions (Narduzzi et al., 2015; Trouillas et al., 2016). The proportions and amounts of the different pigments and copigments in *Vitis vinifera* grapes are strongly dependent on the grape variety (Narduzzi et al., 2015). These factors have important impacts on the color properties and stability as natural anthocyanin colorants.

Therefore, different methods of extract preparation and purification may also influence the chemical composition of anthocyanin-based colorants. Purification is often necessary to remove other plant components that are simultaneously co-extracted with pigments and could have negative impacts on the sensorial attributes and stability of natural colorants. Conversely, the coexistence of pigments with colorless phenolics in anthocyanin extracts can improve their chemical stability through copigmentation (Jensen, Lopez-de-Dicastillo Bergamo, Payet, Liu, & Konczak, 2011). Moreover, these interactions can also increase the health-promoting properties of natural colorants through additive or synergistic effects, as reported by Seeram, Adams, Hardy, & Heber (2004).

Thus, the main aim of this study was to assess the colorimetric properties of anthocyanin-rich grape colorants according to variety and level of purity and compare them to synthetic FD&C Red N$^{o}$.3. The kinetics of anthocyanin color degradation over time were also investigated from a colorimetric point of view, providing useful information to the food industry about the stabilization of these extracts as natural copigmented colorants.

## 2. Materials and methods

### *2.1. Plant material*

Red grapes (*Vitis vinifera* sp.) used in this study were 'Tempranillo' (TE), 'Syrah' (SY), 'Cabernet Sauvignon' (CS), and 'Graciano' (GR) varieties. TE, SY, and CS varieties were grown in the Condado de Huelva Designation of Origin (Spain), while GR variety was grown in the Rioja Designation of Origin (Spain). Mature grapes of each variety (500 g) were harvested and stored at -20°C until analyzed. Grapes were manually peeled, and the skins were freeze-dried (lyophilizer Cryodos-80, Telstar Varian DS 102, Terrasa, Spain) and pulverized to obtain a homogeneous powder.

### *2.2. Preparation of the crude and purified anthocyanin extracts from grape skin*

Anthocyanins were extracted and purified according to the method of Rodriguez-Saona and Wrolstad (2001). One gram of the skin powder was extracted with 0.01% HCl acidified 70% aqueous acetone (v/v) until the skin powder had no coloration. Extraction was conducted in triplicate for each grape variety. The extracts were filtered through Whatman no. 4 paper (Whatman Inc., Florham, N.J., U.S.A.) and partitioned with 2 volumes of chloroform (Fisher Scientific) in a separatory funnel. The solution was gently mixed and left to stand overnight at 4ºC to ensure adequate separation. The aqueous layer containing anthocyanins was collected, and residual acetone in the samples was evaporated using a rotary evaporator at 30ºC.

The crude anthocyanin extracts (n=12) were brought to 50 mL with acidified water (0.01% HCl), and a fraction of each sample was purified with Sep-Pak C18 cartridge (6 mL, 1 g sorbent; Waters Corp., Milford, MA) to obtain the respective purified anthocyanin extracts (n=12). The cartridge was activated with methanol and washed with acidified water (0.01% HCl) before samples were loaded. Loaded cartridges were washed with acidified water (0.01% HCl) to remove sugars and organic acids and then with ethyl acetate to remove less polar phenolics. Then, anthocyanins were recovered with 0.01% HCl acidulated methanol, which was removed in a rotary evaporator at 35ºC under vacuum.

Model drink solutions were prepared dissolving the concentrated crude ($TE_C$, $SY_C$, $CS_C$, $GR_C$) and purified ($TE_P$, $SY_P$, $CS_P$, $GR_P$) extracts until 25 mL with McIlvaine's buffer (also known as citrate-phosphate buffer, pH 3.5) to a final anthocyanin concentration of 100 mg/L. All the samples (n=24) were filtered through 0.45 μm Millipore membranes, stored in sterilized 20 mL capped vials, and allowed to equilibrate for 2 hours at room

temperature (25ºC±1) in the dark prior to chemical and colorimetric analysis.
Similarly, a solution of FD&C Red No. 3 (Noveon Hilton Davis, Inc., Cincinnati, OH, USA) was also prepared in McIlvaine's buffer (pH 3.5, 100 mg/L) to compare colorimetric characteristics against the natural grape skin colorants.

*2.3. Total Monomeric Anthocyanin and Total Phenolic Contents*

The spectrophotometric determinations of total monomeric anthocyanin (TMA) and total phenolic (TP) contents were performed using a Shimadzu 2450 UV-visible spectrophotometer (Shimadzu, Columbia, MD, USA), using 10 mm path length glass cells and distilled water as reference.
Total monomeric anthocyanin (TMA) content was determined according to the pH differential method (Giusti & Wrosltad, 2001). Samples were diluted with aqueous buffers pH 1.0 and 4.5 (potassium chloride solution, 0.025 M, pH 1; sodium acetate buffer, 0.4 M, pH 4.5) and left standing for 15 min. Then, the absorbance measurements were recorded at 520 and 700 nm. Results of TMA were expressed in milligrams (as malvidin 3-glucoside equivalents) per 100 g of skin (dry and fresh matter: DM and FM, respectively), and in mg/L for model drink solutions, using the following equation:

$$TMA\ (mg/L) = [((A_{520}-A_{700})_{pH1} - (A_{520}-A_{700})_{pH4.5}) \times DF \times 1000 \times MW] / \varepsilon \times P$$

where DF is the dilution factor (15), MW is the molecular weight (493.2 for malvidin 3-glucoside), ε is the molar absorptivity coefficient (20200 $cm^{-1}$ $mg^{-1}$ for malvidin 3-glucoside), and *P* is the cuvette path length.
The total phenolic (TP) content was determined using a modification of the Folin-Ciocalteu method (Singleton & Rossi, 1965). Briefly, 0.25 mL of sample, 1.25 mL of Folin-Ciocalteu reagent, and 3.75 mL of a solution of 20% sodium carbonate were mixed, and distilled water was added to make up a total volume of 25 mL. The solution was homogenized and left to stand for 120 min for the reaction to occur and stabilize. Absorbance of the samples was measured at 765 nm. Gallic acid was employed as a calibration standard, and results were expressed in milligrams of GAE (as gallic acid equivalents) per 100 g of skin (dry and fresh weight: DW and FW, respectively) and in mg/L for model drink solutions.

*2.4. Phenolic determination by HPLC*

HPLC was used to analyze the individual phenolic composition in the different extracts; the system (Shimadzu, Columbia, Maryland, U.S.A.) was equipped with LC-20AD pump, CBM-20A communication module, SIL-20A HT autosampler, CTO-20AC column oven, and SPD-M20A photodiode array detector. LCMS Solution Software (Version 3, Shimadzu, Columbia, Maryland, U.S.A.) was used to analyze results. Separation of phenolic compounds was achieved on a Kinetex reverse-phase EVO C18 column with 5 µm particle size and 100Å pore size in 150 x 4.6 mm column size (Phenomenex®, Torrance, CA, U.S.A).

Prior to injection, samples were filtered through Phenomenex® PhenexTM RC 0.45 µm, 15 mm membrane syringe filters (Torrance, CA, U.S.A.). Flow rate was set to 0.8 mL/min with a run time of 36 min, and the injection volume was 50 µL. Chromatographic solvents (acetonitrile and formic acid) were HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ). Reverse phase HPLC was conducted with a binary gradient using solvents A: 4.5% formic acid and B: acetonitrile. Gradient began at 5% B and was maintained for the first minute, then increased 5-35% from 1-36 min. The column was maintained at 30ºC during analyses.

Spectral data were recorded from 250 to 700 nm over the whole run. The wavelengths of detection were 520 (anthocyanins), 280 nm (flavanols and benzoic acids), 320 nm (cinnamic acids and their tartaric esters), and 360 nm (flavonols). Identification of individual phenolic compounds (low molecular weight) was carried out by comparing their retention times and UV-vis spectra with those of original standards, as described in Gordillo et al., (2014). Peak areas at maxplot (260-700 nm) were integrated and normalized.

*2.5. Colorimetric Analysis*

A ColorQuest XE colorimeter (HunterLab, Hunter Associates Laboratories Inc., Reston, VA, USA) was used to measure the color characteristics of the extracts. The transmittance spectra of samples were recorded at constant intervals (400-700 nm, $\Delta\lambda$=10 nm). The CIELAB parameters were calculated following the recommendations of the Commission Internationale de L'Eclariage: the CIE1964 10° Standard Observer and the Standard Illuminant D65 (CIE, 2004). The CIELAB parameters calculated were: L* (the correlate of lightness; ranging from 0, black, to 100, white) and two color coordinates, a* (which takes positive values for reddish colors and negative values for

greenish ones) and b* (positive for yellowish colors and negative for bluish ones). From these coordinates, other color angular parameters are defined: the hue angle ($h_{ab}$, the correlate of tonality), and the chroma ($C^*_{ab}$, the correlate of color vividness). 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 color.

Differential Colorimetry was applied to assess the color variation among model drink solutions and their color stability during storage. Color-difference formulas ($\Delta E^*_{ab}$, $\Delta L^*$, $\Delta C^*_{ab}$, $\Delta h_{ab}$, $\%\Delta L$, $\%\Delta C$, and $\%\Delta H$) were calculated from the scalar (L*, a*, b*) and cylindrical (L*, $C^*_{ab}$, $h_{ab}$) CIELAB color coordinates of samples, as described in Gordillo et al. (2015). Color differences between pairs of samples were computed by means of the CIE76 color difference formulae: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\ ]^{1/2})$, as well as from the lightness, chroma and hue angle differences ($\Delta L^*$, $\Delta C^*_{ab}$, and $\Delta h_{ab}$, respectively). Specifically, $\Delta h_{ab}$ is the difference between two hues, in sexagesimal degrees.

The relative contribution of the lightness, chroma, and hue, that make up the color difference parameter ($\Delta E^*_{ab}$), was 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 $\Delta H$ mathematically deduced from: $\Delta H = [(\Delta E^*_{ab})^2 - ((\Delta L)^2 + (\Delta C)^2)]^{1/2}$

The parameter total color (E) was calculated as the color difference ($\Delta E^*_{ab}$) between the color of model drink solutions (L*, a*, and b*) and to the color of blank used as white reference (L*=100, a*=0, b*=0).

### *2.6. Degradation kinetics*

Model drink solutions were stored at room temperature (25ºC±1) in the dark, and the changes in color properties were monitored over time (0, 8, 12, and 17 days) as described in Section 2.5. The data obtained for changes of total color (E) during storage were used in modelling of kinetics of color degradation, providing meaningful information on color specifications rather than changes in $\lambda_{max}$.

As previously reported (Buchweitz, Brauch, Carle, & Kammerer, 2013; Loypimai, Moongngarm, & Chottanom, 2016), anthocyanin color degradation was assumed to follow pseudo-first-order reaction kinetics, and linear regression analysis was used to

determine adequacy of the model. The first-order reaction is expressed by the following equation: $ln\ (Et / E_0) = -kt$

where Et and $E_0$ are the total color of samples at time t and t=0 days during storage, respectively, and *k* the kinetic constant. The half-life time $t_{1/2}$ (time needed for 50% degradation of total color) for a first order reaction was calculated using the following equation: $t_{1/2} = \ln (2)/ k$

*2.7. Statistical analysis*

All statistical analyses were performed using Statistica v.8.0 software (StatSoft Inc., 2007). Univariate analysis of variance (Tukey test, $p<0.05$) was applied to establish statistical differences for the phenolic compositions and colorimetric characteristics among samples, according to the grape variety and the extraction conditions.

## 3. Results and discussion

*3.1. Phenolic composition of anthocyanin-based colorants*

*3.1.1. Influence of grape variety*

The detailed phenolic composition of *Vitis vinifera* grape skins according to the variety are shown in Table 1. Results indicate significant ($p<0.05$) quantitative and qualitative differences among them as sources of naturally copigmented anthocyanin-based colorants.

Quantitatively, the average values for total monomeric anthocyanins (TMA) ranged between 360 and 904 mg/100 g FW. The pigment content in the studied grape varieties was found much higher than those described in different cultivars of other sources rich in anthocyanins such as black berry (12-326 mg/100 g FW), blueberries (~120 mg/100 g FW), or red cabbage (145-150 mg/100 g FW) (Ahmadiani, Robbins, Collins, & Giusti, 2014; Noh, Jung, Choe, Hoo Yoon, 2015). The differences in TMA confirms the importance of grape varietal characterization to select the cultivars with higher potential for commercial exploitation. The skin of 'Graciano' (GR) grape variety was the richest source of anthocyanins, having a significantly ($p<0.05$) higher content of TMA, followed by 'Cabernet Sauvignon' (CS) and 'Syrah' (SY). By contrast, the 'Tempranillo' (TE) variety was a comparatively inferior source of anthocyanins (TMA about 2-fold less than the other varieties).

Differences were also observed for the proportions of pigments and phenolic copigments in the crude extracts (Figure 1). SY extracts, having the highest phenolic potential (TP = 4371 mg/100 g DW), was the richest source of copigments (~35%) due to its higher content of flavonols (25%). These proportions were similar in TE and CS (16-17% of copigments), however, GR skin was the poorest source of copigments having the lowest proportions of flavonols (7%) and the highest percentage of anthocyanins (87%).

Regarding the pigment profile, the main differences among crude extracts were due to the different proportions of non-acylated and monoacylated anthocyanins, which can substantially affect their chemical stability (Giusti & Wrolstad, 2003). SY skin provided the highest proportions of more stable anthocyanins (56% of monoacylated), followed by CS, TE, and GR (45%, 30%, and 13% respectively). The proportions of the acylated anthocyanins also differed among grape varieties as a function of the type of acyl group (acetic acid or *p*-coumaric acid). SY and CS contained higher proportions of *p*-coumaroylated derivatives (35% and 28%, respectively), which exhibit much stronger capacity to stabilize anthocyanins than aliphatic ones due to its aromatic nature (Zhao et al., 2017). In the case of the colorless phenolics, flavonols were the compounds that most contributed to varietal differences, which have widely shown strong stabilizing properties by copigmentation (Trouillas et al., 2016).

### *3.1.2. Influence of purification*

Different methods can be used to isolate anthocyanins including solid-phase extraction based on different sorbents (C18, HLB, LH-20) or ion-exchange resins. In this study, solid phase extraction (SPE) with C18 sorbent was selected due to its good balance of efficiency, cost, and simplicity of manipulation. The HPLC chromatograms of CS skin extracts, before and after purification, detection at 520 nm (anthocyanins) and 360 nm (flavonols) are illustrated as a representative example of the effect of purification on the major grape skin phenolics (supplemental information). As evidenced by the chromatographic analysis, the purification process did not affect the anthocyanin profile of the extract but largely removed the main colorless phenolics (flavonols) naturally present. Independent of the grape variety, the same trends were observed for all crude extracts (data not shown). Thus, results confirmed the efficiency of C18 sorbent to purify anthocyanin mixtures from anthocyanin-rich plant materials (He & Giusti, 2011). In general, the contribution of copigments to the global phenolic content was notably

reduced in all samples after purification (to ≤ 11%) while the pigments accounted for 91.4% ± 2.1, as mean value (Figure 1). The differences in anthocyanin profiles among samples remained after purified. In contrast, purification reduced the differences in the proportions of flavonols and consequently the pigment/copigments ratios (Figure 1), which could exert an important influence on the stability of grape colorants as affected by natural copigmentation. The extent of this effect depended on the original phenolic composition of grape skins, being more marked for the varieties originally with higher proportions of copigments (SY, CS or TE). In the case of GR variety, the phenolic composition and pigments/copigments ratio remained quite similar regardless of the level of purity because copigments were minor compounds in the crude extract (Figure 1).

*3.2. Color characteristics of anthocyanin-based colorants*

A colorimetric analysis of model drink solutions colored with the crude and purified skin extracts of each grape variety (100 mg/L of TMA in citrate buffer, pH = 3.5, n=3) was performed in the CIELAB space to assess the effect of grape variety and purification on color. The colorimetric parameters (L*, a*, b*, $C^*_{ab}$, and $h_{ab}$) of samples are summarized in Table 2.

*3.2.1. Influence of grape variety*

There were differences in the color of virtually all the model drink solution samples prepared with the crude extracts as a function of the grape variety (Table 2). Drink solutions colored with the crude extracts of SY, TE, and CS had similar chroma values ($C^*_{ab}$ = 41-43 units) but comparatively higher than those colored with GR extract ($C^*_{ab}$ = 37 units). Thus, with the same pigment concentration, the color of these solutions was distinctively more intense, consistent with the higher proportions of copigments in SY, TE, and CS crude extracts and reflecting the effects of copigmentation on the chroma of anthocyanin pigments (Trouillas et al., 2016).

Despite similar chroma, drink solutions colored with $SY_C$, $TE_C$, and $CS_C$ could be differentiated from one another by hue and lightness. Those from $SY_C$ showed hue values between 0 and -10º, corresponding to purple-red tonalities (positive values of a* and negative values of b*) while those colored with $TE_C$, $CS_C$, and $GR_C$ extracts showed $h_{ab}$ values between 0° and +10°, more pure red colors (positive values of a* and b*). These variations in hue values could be attributed to the dissimilarities in the proportions of monoglucosides and acylated anthocyanins (Table 1), as well as

influences of copigmentation. These findings suggest that higher proportions of monoacylated anthocyanins (56%) and copigments (25%) conferred to SY variety a slightly purple red tonality as colorant. Conversely, less vivid colorants with reddish hue were results of crude extracts with less acylation and copigmentation and richer in red-orange anthocyanidin derivatives (cyanidin and peonidin), such as those of GR variety (Heredia, Francia-Aricha, & Rivas-Gonzalo, 1998). The lowest L* values were obtained in model drink solutions colored with crude extracts of SY and TE (L* values ranking from 66 to 68 units), which appeared the darkest colorants. A similar color-composition relationship was also reported by Ahmadiani et al. (2014), who showed how the color properties of anthocyanin-based colorants from red cabbage differed between different cultivars due to the genetic influence on the anthocyanin profile.

*3.2.2. Influence of purification*

Results showed that the level of purity significantly influenced the colorimetric properties of grape skin colorants both in quantitative (L*, $C^*_{ab}$, and $h_{ab}$) and qualitative terms. In general, model drink solutions colored with the purified extracts through C18 cartridges had higher values of lightness and lower chroma than their respective samples colored with crude extracts (Table 2). These changes showed a clear reduction in the color intensity after removing the copigments from the crude grape skin extracts, consistent with findings of previous studies (Jensen et al., 2011; Sari, Wijaya, Sajuthi, & Supratman, 2012). Hue angle tended to increase after purification indicating a decrease of the purple notes in the case of SY variety and an evolution toward more orange-red hues for TE and CS samples.

From a sensory perspective, these findings mean that for the same variety, the color properties of grape colorants were changed notably in terms of luminosity, tonality, and intensity depending on the level of purity. The color characteristics, however, did not change significantly in grapes low in copigments, as is the case of GR variety.

*3.3. Differential colorimetric evaluation of anthocyanin-based colorants*

In order to assess whether the observed changes in the CIELAB parameters were visually relevant, the CIELAB color difference ($\Delta E^*_{ab}$) was calculated comparing samples by pairs in relation to the grape variety before and after purification. According to Martínez, Melgosa, Pérez, Hita, & Negueruela (2001), $\Delta E^*_{ab}$ values $\geq 3$ units indicate color differences noticeable by the human eye (average observer), which was used as a reference threshold to visually differentiate the color among pairs of samples. The color

differences obtained among our samples are presented in Table 2 and Figure 2.

*3.3.1. Influence of grape variety*

The mean color differences calculated among the model drink solutions colored with the crude extracts indicated that coloring properties among grape skin colorants as a function of the variety were easily distinguished (Figure 2A). The greatest color differences were found between the color provided by GR with respect to the other varieties ($\Delta E^*_{ab}$ values from 6 to 10 units) and the least differences between the pairs SY/CS and SY/TE ($\Delta E^*_{ab}$ values around 3.5 units). The relative contribution of lightness (%ΔL), chroma (%ΔC), and hue (%ΔH) to each $\Delta E^*_{ab}$ value defined the role of each color attribute for a given color variation. The main contribution to the color variation between GR and the other varieties was mainly quantitative (%ΔL + %ΔC ranging from 79% to 92%, as mean values) and to a lesser extent qualitative. However, the weight of the hue modifications were more marked for the pairs GR/SY and GR/CS (%ΔH=21% and 17%, respectively) indicating that the tonality of GR differed more with respect to the SY and CS varieties than to TE (% ΔH = 8%).

*3.3.2. Influence of purification*

The effect of purification by C18 solid phase extraction was assessed by means of the color differences calculated for each variety before and after purification (Table 2). In global terms, purification induced higher color variation in TE ($\Delta E^*_{ab}$ = 7.3 units) than in CS and SY varieties ($\Delta E^*_{ab}$ = 4.7 and 3.5 units, respectively), although in all cases the effect could be considered visually perceptible. According to the trend of the color changes ($\Delta L^*$, $\Delta C^*_{ab}$, and $\Delta h_{ab}$), the losses of chromatic intensity and the variations of tones (higher values of $\Delta C^*_{ab}$, and $\Delta h_{ab}$) were more marked in TE and CS than in SY. These results are interesting because the SY variety originally had higher proportions of copigments than TE and CS, which may indicate the importance of copigmentation. However, the efficiency of purification was significantly higher in TE than in CS and SY (% copigments in purified extracts= 6.8 for TE *versus* 9.8 and 11%, for CS and SY respectively; Figure 1), which could explain the dissimilarities observed for $\Delta E^*_{ab}$ values. At this respect, higher anthocyanin purity could be obtained through C18 cartridges by optimizing the pH of eluting solvents, as previously reported by He and Giusti (2011). On the other hand, the color difference between the crude and purified extracts of GR was almost negligible ($\Delta E^*_{ab}$ = 0.6 units) suggesting that these extracts could be used as natural colorants whether purified or not since they are quantitatively and qualitatively similar.

When the color differences were calculated between the model drink solution colored with the different purified grape extracts as a function of the variety (Figure 2B), a decrease in the $\Delta E^*_{ab}$ values occurred as compared to those obtained with the crude extracts (Figure 2A). Thus, differential colorimetry confirmed that in most cases, increasing the purity of grape extracts tends to diminish the color variability due to variety, being consistent with the effect on the chemical composition. Specifically, lower values of %ΔC and %ΔH among pairs of samples indicate that grape colorants were more similar one another respect to the chroma and hue after purification (Figure 2B). The $\Delta E^*_{ab}$ values were > 3 indicating perceptible color differences probably due to the dissimilarities in the anthocyanin profile among purified colorants.

In addition, differential colorimetry was applied to compare the color of the grape colorants to the synthetic colorant FD&C Red No. 3, as a function of grape variety and purity level. The color characteristics of the FD&C Red No.3 prepared in the same model beverage conditions as the grape colorants (McIlvaine's buffer at pH 3.5, 100 mg/L, n=3) were: L* = 78.9±0.01, $C^*_{ab}$ = 49.5±0.10, and $h_{ab}$ = 6.3º±0.14. The global color differences were calculated between the grape colorants with respect to the synthetic colorant FD&C Red No.3 as follows: $\Delta E^*_{ab} = [(L^*_{GC} - L^*_{FD\&C\ Red\ No.\ 3}-)^2 + (a^*_{GC} - a^*_{FD\&C\ Red\ No.\ 3})^2 + (b^*_{GC} - b^*_{FD\&C\ Red\ No.\ 3})^2]^{1/2}$. The same direction of the difference was considered for the ΔL*, $\Delta^*C_{ab}$ and $\Delta h_{ab}$ parameters (Table 3). The $\Delta E^*_{ab}$ values ranged from 12.6 to 15.0 units; and therefore, grape colorants would be considered visually differentiable to FD&C Red No.3 regardless the grape variety and purification level. Ahmadiani et al., (2014) reported similar color variation values between the colors of anthocyanin colorants from red cabbage with respect to the same synthetic colorant. According to our results, grape colorants provided less vivid color than the synthetic one (negative values of $\Delta C^*_{ab}$). In particular, the differences for the color intensity were more notable after purifying the extracts of all the varieties studied, except for GR. Regarding the hue, increased levels of purity made grape colorants appear more similar to FD&C Red No.3 ($h_{ab}$ = 6.3º, red-orange tonality), being the effect more marked for CS and TE varieties ($h_{ab}$ = 5.8º and 9.8º, respectively). Results are of interest because consumers do not perceive all color additives in the same way and perceptible differences for the color intensity or tonality can decisively influence their acceptance or perception of risk and benefits (Berth et al., 2014). According to Arocas et al. (2013), food products with less vivid colors or paler tonalities have been gaining more acceptability since they are considered to be more natural, healthier, and safer.

*3.4. Kinetics of color degradation during storage and color stability*

In order to predict the color stability of grape colorants as a function on the variety and level of purity, the kinetic parameters for the total color degradation over time (the kinetic constant *k*, half-life time $t_{1/2}$) were calculated (Table 4). The significant ($p$<0.05) high values found for the correlation coefficients in all cases ($R^2 > 0.95$) confirmed that the anthocyanin color degradation followed first order reaction kinetics, under the assayed conditions (Figure 3). Higher *k* values in model solutions colored with purified extracts were obtained for all grape varieties, except for GR. This meant that crude grape colorants from TE, SY and CS varieties provided higher color stability in model drink solutions than their respective purified extracts. Different stability depending on the grape variety can also be observed, being the model drink solutions colored with $SY_C$ and $TE_C$ more stable (lower k values) than $CS_{C.}$ As expected, the *k* values were similar for $GR_C$ and $GR_P$ indicating comparable color stability regardless of purification.

Half-life time ($t_{1/2}$) values were higher in model drink solutions colored with crude extracts of SY, TE and CS by 40%, 26% and 22% in relation to their respective purified extracts (165 *versus* 96 days in SY, 154 *versus* 54 days in TE, and 114 *versus* 85 days in CS, respectively). This finding demonstrates the lower rate of color degradation of grape anthocyanin colorants containing grape skin copigments (crude extracts), being maximized for SY grape variety (highest $t_{1/2}$ value). Similar findings were reported by Chung, Rojanasasithara, Mutilangi, & McClements (2016), who demonstrated that the addition of small quantities of polyphenols (0.2%) to beverage systems colored with purple carrot anthocyanins delayed the rate of color fading. It is worth mentioning that the average half-life for total color obtained with crude grape skin colorants at room temperature (114-165 days, 3-6 months) suggests a great potential to be applied in a wide variety of shelf stable products. Those values were found higher than those reported in other anthocyanin-based colorants stored in similar conditions (110 days of storage at 20ºC in cherry juice concentrates) (Navruz, Türkyılmaz, & Özkan, 2016).

Results of the kinetic parameters were supported by the overall color changes observed in model drink solutions over time, evaluated as the color difference ($\Delta E^*_{ab}$) according to the grape variety and purification during storage (17 days, 25ºC in darkness) (Figure 4). In all cases, the color changes that took place during storage could be considered perceptible ($\Delta E^*_{ab} > 3$). However, $\Delta E^*_{ab}$ values were significantly ($p < 0.05$) lower in

model drink solutions colored with crude extracts from SY and TE varieties indicating higher color stability than their respective purified. On contrast, the protective effect of grape skin phenols on the anthocyanin color stability seemed less notable in the cases of CS and GR varieties. Smaller color differences during storage were achieved with crude extracts, but the differences with respect to purified extracts were not significant.

## 4. Conclusions

Grape skins represent good natural sources for obtaining functional colorants that notably differed in their anthocyanin composition and copigmentation levels depending on the grape variety, and consequently in their colorimetric properties. Purification eliminated phenolic copigments naturally present in the grape skins, which diminished the variability in color intensity, luminosity and tonality of unpurified colorants due to the variety. Differential colorimetry was a useful tool that provided visually relevant information about the color pattern variations and stability of grape colorants according to the grape variety and purification conditions, as well in comparison to synthetic FD&C Red No. 3. The established mathematical model of total color kinetic degradation during storage allowed for prediction of grape colorant stability, showing that the naturally occurring copigments in unpurified extracts aided pigment and color stability. The extract with the highest copigment to anthocyanin ratio ($TE_C$ and SYc) showed the highest half-life, while the extract with the lowest copigment to anthocyanin ratio ($GR_P$) produced the shortest half-life. This research provides valuable information for the food colorant industry since these factors represent natural alternative strategies to modulate the color properties and stability of anthocyanins as colorants and expand their potential applications.

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## FIGURE CAPTIONS

**Fig. 1.** Proportion of the phenolic families in model drink solutions prepared with grape skin extracts according to grape variety (TE, SY, CS, and GR) and purification level (C, crude; P, purified by C18 solid phase extraction).

**Fig. 2**. Color differences ($\Delta E^*_{ab}$) with the relative variation of lightness (%ΔL), chroma (%ΔC), and hue (%ΔH) calculated by pairs of model drink solutions in relation to grape variety (TE, SY, CS, and CR): a) colored with crude extracts, b) colored with purified extracts by C18 solid phase extraction.

**Fig. 3**. First order degradation curves for Total Color (E) of model drink solutions colored with crude (C) and purified (P) extracts from grape varieties (TE, SY, CS, GR), over time (17 days, 25ºC, darkness).

**Fig. 4**. Mean color variation ($\Delta E^*_{ab}$, n= 3±SD) of each model drink solution from the beginning to the end of storage period (15 days, 25ºC in darkness) according to grape variety (TE, SY, CS, GR) and purification level (crude or purified by C18 solid phase extraction). Different letter among pair of bars indicates significant difference ($p < 0.05$) between crude and purified extracts for each grape variety.

**Figure 1.**

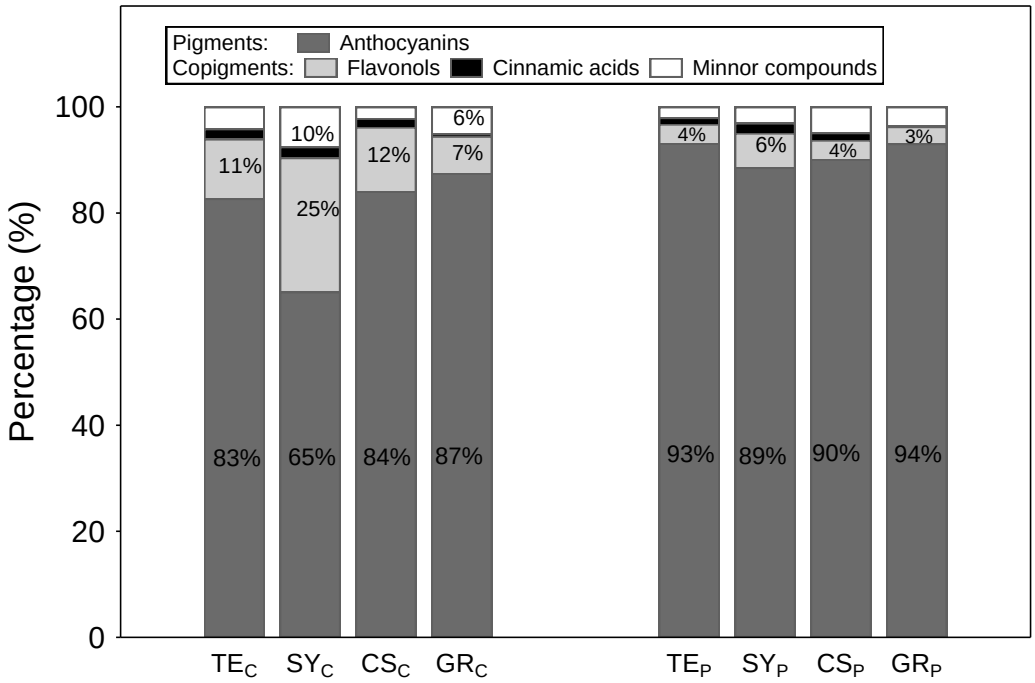
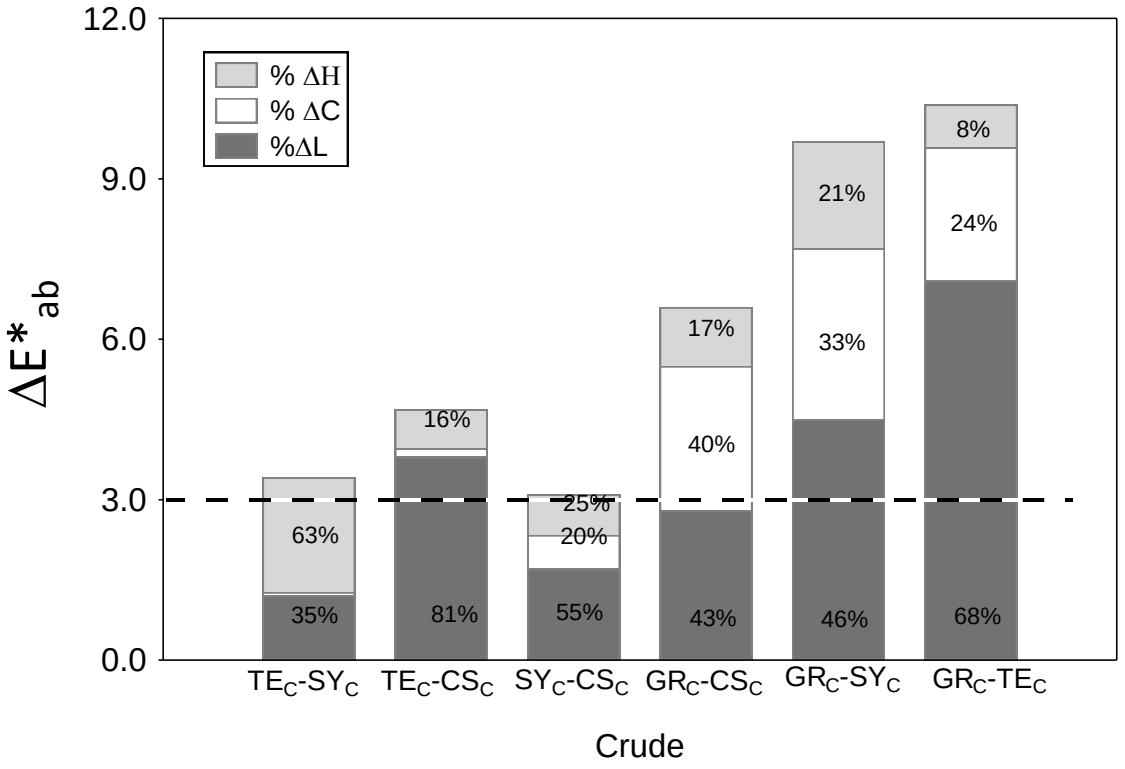

Pigments: Anthocyanins
Copigments: Flavonols Cinnamic acids Minnor compounds
Percentage (%)
100
80
60
40
20
0
11%
83%
10%
25%
65%
12%
84%
6%
7%
87%
4%
93%
6%
89%
4%
90%
3%
94%
TE$_C$ SY$_C$ CS$_C$ GR$_C$
TE$_P$ SY$_P$ CS$_P$ GR$_P$

**Figure 2**

**A**


12.0
9.0
6.0
3.0
0.0
ΔE*ab
% ΔH
% ΔC
%ΔL
63%
35%
16%
81%
25%
20%
55%
17%
40%
43%
21%
33%
46%
8%
24%
68%
TEC-SYC
TEC-CSC
SYC-CSC
GRC-CSC
GRC-SYC
GRC-TEC
Crude

**B**

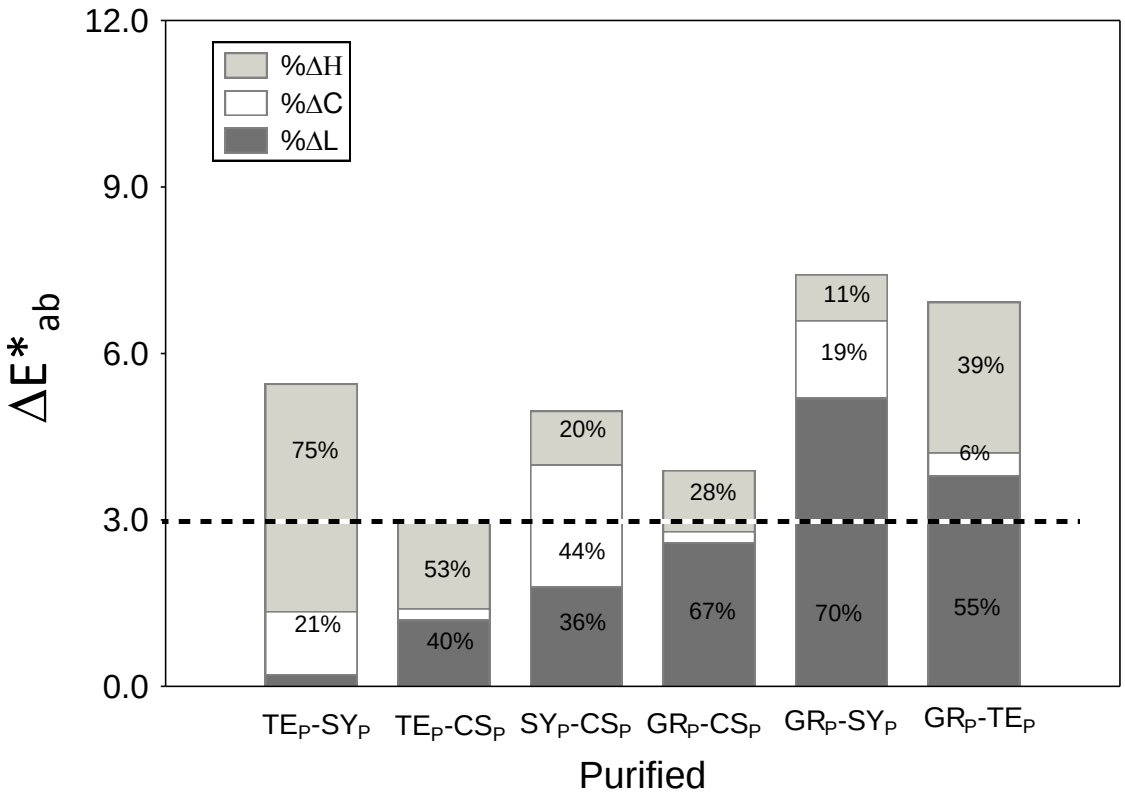
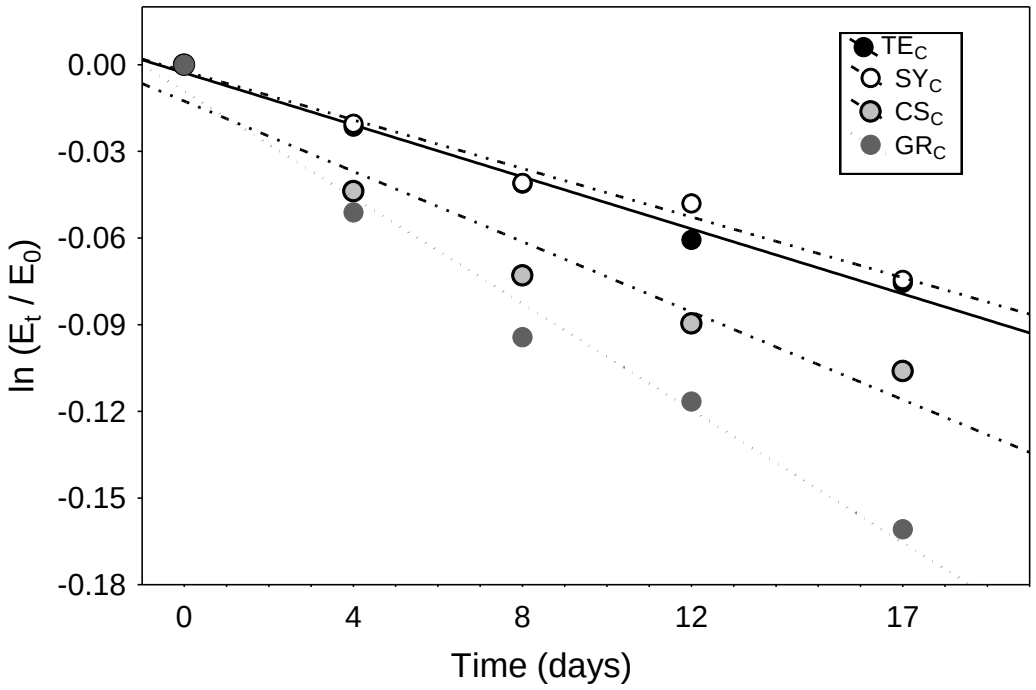

12.0
9.0
6.0
3.0
0.0
ΔE*ab
%ΔH
%ΔC
%ΔL
75%
21%
53%
40%
20%
44%
36%
28%
67%
11%
19%
70%
39%
6%
55%
TEP-SYP
TEP-CSP
SYP-CSP
GRP-CSP
GRP-SYP
GRP-TEP
Purified

**Figure 3.**

A)


TE$_C$
SY$_C$
CS$_C$
GR$_C$
0.00
-0.03
-0.06
-0.09
-0.12
-0.15
-0.18
ln (E$_t$ / E$_0$)
0
4
8
12
17
Time (days)

B)

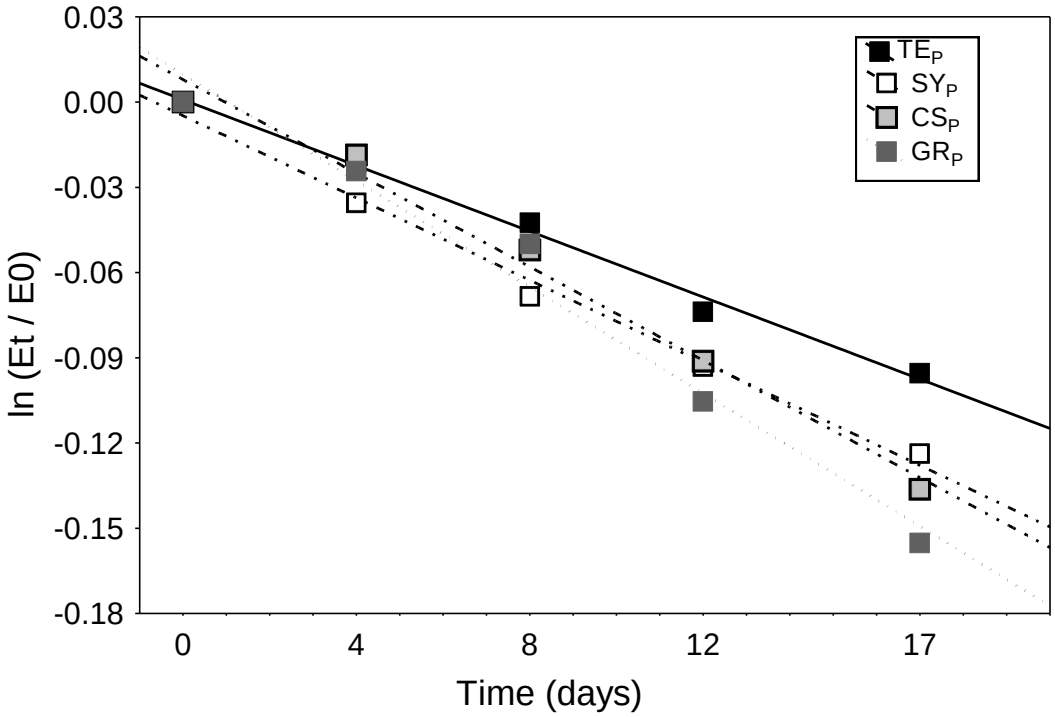

TE$_P$
SY$_P$
CS$_P$
GR$_P$
0.03
0.00
-0.03
-0.06
-0.09
-0.12
-0.15
-0.18
ln (Et / E0)
0
4
8
12
17
Time (days)

**Figure 4**

**Figure 5.**

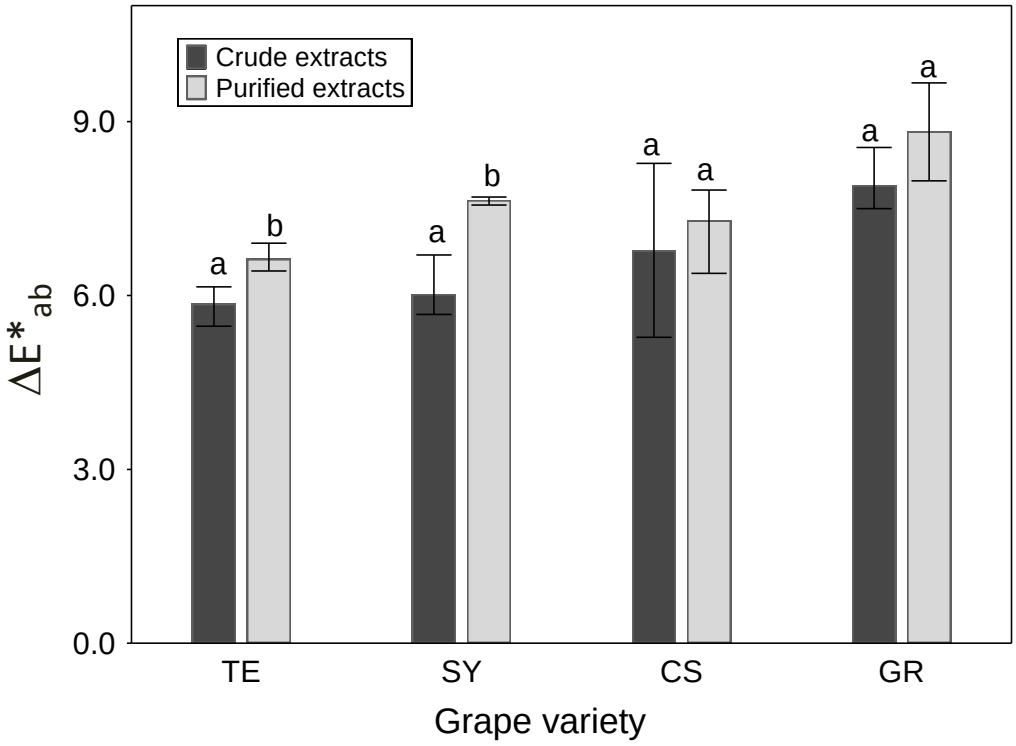


**Table 1**. Total Monomeric Anthocyanin (TMA) and Total Phenolic (TP) contents in grape skin according to grape variety[a], and relative proportion (%) of the individual phenolic compounds (respect to the global peak area of each chemical family at maxplot). Values presented are means and standard deviations (n=3).

| | | Grape variety[a] | | | |
|---|---|---|---|---|---|
| | | **TE** | **SY** | **CS** | **GR** |
| TMA[b] (mg Mv3G/100g of berry skin) | DW[d] | 987.9±28.8a | 1772.1±70.1b | 1857.9±36.8b | 2382.1±34.5c |
| | FW[e] | 360.3±10.5a | 600.2±23.7b | 672.6±13.3c | 903.5±13.1d |
| TP[c] (mg GAE/100g of berry skin) | DW | 3484.1±68.7a | 4371.4±305.3 b | 4163.9±135.7b | 3316.2±176.1a |
| | FW | 1268.1±24.9a | 1480.4±104.9b | 1506.8±49.1b | 1258.2±67.1a |
| **Relative proportions relative to each phenolic family** | | | | | |
| ***Anthocyanins* (%AUC at 520 nm)** | | | | | |
| Delphinidin 3-glucoside | | 6.7±0.5a | 3.0±1.1b | 5.1±0.9ac | 4.7±0.3bc |
| Cyanidin 3-glucoside | | 0.9±0.01a | 0.6±0.04b | 0.7±0.03b | 1.2±0.1c |
| Petunidin 3-glucoside | | 13.7±0.4a | 5.8±0.3b | 8.8±1.2c | 8.3±0.6c |
| Peonidin 3-glucoside | | 3.1±0.3a | 5.8±0.3b | 2.2±0.3a | 11.4±0.9c |
| Malvidin 3- glucoside | | 45.3±1.8a | 28.8±0.7b | 37.8±1.2c | 61.6±1.7d |
| Petunidin 3-acetyl-glucoside | | 1.7±0.02ab | 2.1±0.8a | 3.2±0.2a | 0.4±0.1b |
| Peonidin 3- acetyl-glucoside | | 4.2±0.8a | 0.7±0.5b | 0.4±0.2b | 0.11±0.02b |
| Malvidin 3- acetyl-glucoside | | 4.4±0.6a | 16.8±1.2b | 10.8±1.3c | 3.3±0.5a |
| Petunidin 3-*p*-coumaroyl-glucoside | | 3.6±0.3a | 5.2±0.7b | 4.8±0.3ab | 0.9±0.1c |
| Peonidin 3-*p*-coumaroyl-glucoside | | 1.3±0.2a | 6.2±1.2b | 1.6±0.4a | 1.4±0.2a |
| Malvidin 3-*p*-coumaroyl-glucoside | | 12.9±0.3a | 18.6±2.3b | 16.6±1.4ab | 6.9±0.1c |
| Sum of monoglucosides | | 69.8±1.8a | 44.2±1.8b | 55.1±1.8c | 86.9±1.8d |
| Sum of acylated | | 30.2±1.8a | 55.8±1.8b | 44.9±1.8c | 13.1±1.8d |
| Sum of acetates | | 10.8±1.8a | 20.9±1.8b | 16.9±1.8b | 3.7±1.8c |
| Sum of coumaroylated | | 19.4±1.8a | 34.9±1.8b | 27.9±1.8c | 9.2±1.8d |
| ***Cinnamic acids* (%AUC at 320 nm)** | | | | | |
| t-caftaric acid | | 56.6±0.1a | 56.0 ±0.6a | 51.0±0.7b | 35.2±4.5c |
| coutaric acid | | 43.5±2.1a | 44.0±1.7a | 49.0±0.1b | 64.9±0.9c |
| ***Flavonols* (%AUC at 360 nm)** | | | | | |
| Myricetin 3-glucuronide | | 0.3±0.6a | tr.±0.8 | 1.0±0.4b | 1.6±0.2c |
| Quercetin 3-glucuronide | | 36.5±0.6a | 13.1±0.7b | 26.0±0.1c | 4.4±0.3d |
| Quercetin 3-glucoside | | 4.3±0.4a | 3.6±1.1a | 5.1±0.5a | 15.3±0.3b |
| Laricitrin 3-galactoside | | 4.7±0.1a | 6.0±0.2a | 6.1±0.2a | 6.7±0.1a |
| Laricitrin 3-glucoside | | 41.0±0.6a | 51.0±0.6b | 32.8±0.6c | 42.6±0.6a |
| Laricitrin derivative | | 2.3±0.4ab | 0.7±0.4b | 2.8±0.4ab | 6.5±0.4a |
| Kaempferol 3-glucoside | | 4.4±0.1a | 10.4±0.1b | 9.9±0.1b | 8.5±0.1ab |
| Isorhamnetin 3-glucoside | | 6.5±0.4a | 10.1±0.1b | 4.6±0.5a | 6.0±0.1a |
| Syringetin 3-glucoside | | Tr. | 5.4±0.3a | 11.8±0.1b | 8.4±0.1c |

[a] (TE: 'Tempranillo', SY: 'Syrah', CS: 'Cabernet Sauvignon', GR: 'Graciano'). [b] Total Monomeric Anthocyanins as malvidin-3-glucoside equivalents, determined by the pH differential method, [c] Total Phenolics as as gallic acid equivalents, determined by the Folin-Ciocalteau method. [d] Dry weight, [e] Fresh weight
Different letters in the same row mean significant differences (Tukey test, $p<0.05$); tr.: traces.

**Table 2**. CIELAB colorimetric parameters (L*, a*, b*, $C^*_{ab}$, $h_{ab}$) of model drink solutions (n=3) colored with crude (C) and purified (P) extracts of grape varieties (TE, SY, CS, and GR)[a]. Mean differences of Color, Lightness, Chroma, and Hue ($\Delta E^*_{ab}$, $\Delta L^*$, $\Delta C^*_{ab}$, $\Delta h_{ab}$) calculated between model drink solutions colored with crude and purified extracts for each grape variety.

| | **TE** | | **SY** | | **CS** | | **GR** | |
|---|---|---|---|---|---|---|---|---|
| | **C** | **P** | **C** | **P** | **C** | **P** | **C** | **P** |
| L* | 65.9±0.4a | 69.6±0.01b | 67.9±0.8a | 68.5±0.2a | 70.2±0.2a | 71.5±0.6b | 74.5±0.6a | 74.7±0.6a |
| a* | 42.6±0.4a | 38.0±0.3b | 43.2±0.9a | 40.9±0.8b | 41.4±0.5a | 37.6±0.4b | 36.6±0.7a | 36.8±0.7a |
| b* | 2.3±0.3a | 6.6±0.3b | -0.4±0.5a | 2.1±0.01b | 0.8±0.4a | 4.3±0.06b | 2.6±0.6a | 2.1±0.3a |
| $C^*_{ab}$ | 42.7±0.4a | 38.6±0.3b | 43.2±0.9a | 41.1±0.7a | 41.8±0.5a | 37.8±0.4b | 37.6±0.8a | 36.9±0.8a |
| $h_{ab}$ | +3.1°±0.5a | +9.8°±0.4b | -0.5°±0.6a | +2.6°±0.5b | +1.1°±0.6a | +5.8°±0.05b | +4.2°±0.6a | +3.2°±0.4a |
| | **$TE_C$ - $TE_P$** | | **$SY_C$ - $SY_P$** | | **$CS_C$ - $CS_P$** | | **$GR_C$ - $GR_P$** | |
| $\Delta E^*_{ab}$ | +7.3 | | +3.5 | | +4.7 | | 0.6 | |
| $\Delta L^*$ | -3.7 | | -0.6 | | -1.3 | | -0.2 | |
| $\Delta C^*_{ab}$ | +4.1 | | +2.1 | | +4.0 | | +0.7 | |
| $\Delta h_{ab}$ | -6.7 | | -3.1 | | -4.7 | | +1.0 | |

[a] (TE: 'Tempranillo', SY: 'Syrah', CS: 'Cabernet Sauvignon', GR: 'Graciano'; C: crude, P: purified)
Different letters in the same row for each grape variety (n=3±SD) mean significant differences ($p<0.05$).

**Table 4.** Kinetic parameters for the degradation of Total Colour of model drink solutions colored with crude and purified extracts for each grape variety during 15 days of storage at room temperature (25 ºC) in darkness.

| | $k$ ($d^{-1}$) | $t_{½}$ (d) | $R^2$ |
|---|---|---|---|
| $TE_C$ | 0.0045 | 154.0 | 0.988 |
| $TE_P$ | 0.0058 | 119.5 | 0.993 |
| $SY_C$ | 0.0042 | 165.0 | 0.983 |
| $SY_P$ | 0.0072 | 96.3 | 0.996 |
| $CS_C$ | 0.0061 | 113.6 | 0.950 |
| $CS_P$ | 0.0082 | 84.5 | 0.987 |
| $GR_C$ | 0.0092 | 75.3 | 0.982 |
| $GR_P$ | 0.0094 | 73.2 | 0.984 |

*k* calculated as the first order degradation rate constant, $t_{1/2}$: half-life of the reaction (days)
(TE: 'Tempranillo', SY: 'Syrah', CS: 'Cabernet Sauvignon', GR: 'Graciano'; C: crude, P: purified)

**Table 3.** Mean Color, Lightness, Chroma, and Hue Differences ($\Delta E^*_{ab}$, $\Delta L^*$, $\Delta C^*_{ab}$, $\Delta h_{ab}$) calculated between grape skin colorants (crude and purified for each grape variety[a]) respect to the synthetic colorant FD&C Red No.3.

| | **FD&C Red No. 3** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$TE_C$** | **$TE_P$** | **$SY_C$** | **$SY_P$** | **$CS_C$** | **$CS_P$** | **$GR_C$** | **$GR_P$** |
| $\Delta E^*_{ab}$ | 15.0±0.5a | 14.5±0.3a | 13.8±0.4a | 13.7±0.4a | 12.6±0.3a | 13.7±0.1a | 13.6±0.6a | 13.5±0.6a |
| $\Delta L^*$ | -13.0±0.4a | -9.3±0.1b | -11.0±0.8a | -10.4±0.2a | -8.7±0.2a | -7.4±0.7a | -4.5±0.6a | -4.2±0.6a |
| $\Delta C^*_{ab}$ | -6.8±0.5a | -11.2±0.3b | -6.3±0.9a | -8.3±0.7b | -8.1±0.7a | -11.6±0.3b | -12.8±0.8a | -12.4±0.8a |
| $\Delta h_{ab}$ | -3.1±0.3a | +3.6±0.2b | -6.7±0.1a | -3.2±0.3b | -5.2±0.6a | +0.23±0.1b | -2.0±0.5a | -3.0±0.3a |

[a] (TE: 'Tempranillo', SY: 'Syrah', CS: 'Cabernet Sauvignon', GR: 'Graciano'; C: crude, P: purified)
Different letters in the same row for each grape variety mean significant differences ($p<0.05$)

**Supplementary material for online publication only**

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## Graphical abstract