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Anthocyanin copigmentation and color of wine: The effect of naturally obtained hydroxycinnamic acids as cofactors

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ABSTRACT

Copigmentation of anthocyanins accounts for over 30% of fresh red wine color, while during storage, the color of polymeric pigments formed between anthocyanins and proanthocyanidins predominates. Rosmarinic acid and natural extracts rich in hydroxycinnamic acids, obtained from aromatic plants (*Origanum vulgare* and *Satureja thymbra*), were examined as cofactors to fresh Merlot wine and the effect on anthocyanin copigmentation and wine color was studied during storage for 6 months. An increase of the copigmented anthocyanins that enhanced color intensity by 15–50% was observed, confirming the ability of complex hydroxycinnamates to form copigments. The samples with added cofactors retained higher percentages of copigmented anthocyanins and higher color intensity, compared to the control wine, up to 3 months. However, the change in the equilibrium between monomeric and copigmented anthocyanins that was induced by added cofactors, did not affect the rate of polymerization reactions during storage.

1. Introduction

Color is one of the most important organoleptic characteristics of red wine, affecting the quality evaluation of the product. Anthocyanins in the flavylium cation form, produce the red color of wine (Hermosín Gutiérrez, Sánchez-Palomo Lorenzo, & Vicario Espinosa, 2005). Nonetheless, the color of an anthocyanin solution is defined by the proportions of the different anthocyanin forms, namely the red flavylium cation, the violet quinonoidal bases, the colorless water or sulfite adducts, and the yellow chalcones. At the pH value of wine, malvidin-3-glucoside occurs mostly as the colorless hemiketal (75%), while the three other forms (flavylium, chalcone and quinonoidal base) are minor. Thus, the intense red wine color and its preservation over time require some pigment stabilizing mechanisms to take place (Cheynier, 2006).

The phenomenon of copigmentation is due to molecular associations between pigments and other (usually noncolored) organic molecules present in the solution that are often reported as cofactors (Boulton, 2001). It causes stabilization of the colored structural forms of the anthocyanins and enhances their color (Figueiredo-González, Cancho-Grande, & Simal-Gándara, 2013a; Gómez-Míguez, González-Manzano, Escribano-Bailón, Heredia, & Santos-Buelga, 2006). Copigmentation can account for between

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30% and 50% of the color in young red wines (Boulton, 2001). During storage or aging a decrease in copigmented anthocyanins and an increase in polymerized ones is observed for all red wine varieties (Dobrei, Poiana, Sala, Ghita, & Gergen, 2010; Hermosín Gutiérrez et al., 2005) with consequent changes in color characteristics of the product. Cofactors include a variety of compounds, such as phenolic acids, flavonoids (in particular derivatives of flavonols and flavones), amino acids, alkaloids, and anthocyanins themselves (self-association) (Rustioni, Bedgood, Failla, Prenzler, & Robards, 2012).

Gómez-Míguez et al. (2006) studied the effectiveness of seven phenolic compounds (catechin, epicatechin, procyanidin B2, caffeic acid, *p*-coumaric acid, myricetin and quercetin) as cofactors of malvidin-3-*O*-glucoside, using a cofactor/pigment molar ratio of 1:1, in model solutions simulating wine. Despite their relatively low concentration, all of them were able to induce a hyperchromic shift characteristic of a copigmentation process. Moreover, Álvarez, Aleixandre, García, Lizama, and Aleixandre-Tudó (2009) reported that the prefermentative addition of cofactors (caffeic acid, rutin, (+)-catechin and flavanols extracted from white grape skin or seed) increased anthocyanin copigmentation reactions and produced wines with more intense color, higher anthocyanin concentration, superior contribution of anthocyanins to the color of the wine, and less astringency. Flavanols from grape skin or seed could be promising cofactors for industrial applications, as they can be obtained from natural, winery by-products. However, Darias-Martin, Carrillo, Diaz and Boulton (2001), indicated that the prefermentative addition of catechin enhanced wine color only by 10%, while caffeic acid lead to an increase by 60%. Hydroxycinnamic acids, such as *p*-coumaric and caffeic acid, have also been reported by Bloomfield, Heatherbell, and Nikfardjam (2003) as cofactors that enhance the color of Cabernet Sauvignon and Pinot Noir wines. Also, rosmarinic acid has been studied as a cofactor in cranberry, strawberry, raspberry and lingonberry juices by Rein and Heinonen (2004) and its enhanced effect compared to other cinnamic acids, such as ferulic and sinapic acid, was noted. However the effect of rosmarinic acid as cofactor has never been studied in wine.

Therefore the present study was undertaken, in order to further examine the effect of hydroxycinnamic acids on anthocyanin copigmentation and color enhancement of red wine. Rosmarinic acid as well as natural extracts rich in hydroxycinnamates were examined. Two characteristic Greek aromatic herbs belonging to the Lamiaceae family, namely *Origanum vulgare* (Greek oregano) and *Satureja thymbra* (pink savory) were used as sources of hydroxycinnamates. Additionally to the magnitude of copigmentation, the effect of the added cofactors on the changes in copigmented versus polymerized anthocyanins during storage of fresh wine was studied. The subsequent change in the color of red wine was also examined. Wine is usually consumed several months after production, and the decline of copigmented anthocyanins and wine color over storage time, as affected by cofactors has not been examined, to the best of our knowledge.

2. Materials and methods

2.1. Reagents and standards

Standard malvidin-3-*O*-glucoside was purchased from Extrasynthèse (Genay, France) and quercetin dihydrate as well as rosmarinic acid from Sigma–Aldrich (Steimheim, Germany). The rest standards and reagents were the following: gallic acid (98% (w/w), Acros Organics, Fair Lawn, New Jersey), Folin–Ciocalteu reagent (Merck, Darmstadt, Germany), hydrochloric acid (37% (v/v), Sigma–Aldrich, Seelzem, Germany), formic acid (98–100%, Merck, Darmstadt, Germany), sodium carbonate (Mallinckrodt, St. Louis, Missuri). Water, acetonitrile and methanol used for chromatography analyses were of HPLC and MS grade (Fisher Chemical, Leicestershire, UK).

2.2. Wine

Fresh wine from monovarietal grapes of the cultivar Merlot (*Vitis vinifera*) that were harvested on 2014 from Peloponnese (southern Greece) was used. The vinification process was conducted on Demou winery (Madinia, Arkadia, Peloponnese, Greece). The wine was obtained one month after the end of alcoholic fermentation and had alcoholic strength 12.8% vol., total acidity 5.3 g/L (tartaric acid eq.) and pH 3.6.

2.3. Aromatic plants ethanol extracts preparation

Dried leaves of *O. vulgare* ssp. *hirtum* (carvacrol chemotype) and *S. thymbra* were obtained from the Agricultural Research Centre of Northern Greece (member of the Helenic Agricultural Organization-DEMETER) and subjected to water-steam distillation in a pilot scale (17 L) distiller to remove the essential oil. The distillation allowed the complete deodorization of the herbs. The oil-free herbs were dried in a ventilated oven at 38 °C and ground in a laboratory mill (Retch ZM 1; Haan, Germany), equipped with a 0.5 mm sieve. The ground material (70 g) was extracted in a Soxhlet apparatus, for 6 h, with ethyl acetate (350 mL) to remove most of the flavonoid aglycones (Kouri, Tsimogiannis, Bardouki, & Oreopoulou, 2007) and subsequently with ethanol (350 mL) to recover the phenolic acids and flavonoid glycosides.

Ethanol extracts were transferred into a 500 mL volumetric flask after filtration and diluted with ethanol to the volume. The green colored pigments of the extracts that are mainly chlorophylls were removed by means of solid phase extraction, using mini columns of graphitized carbon (Bond Elut Carbon, 250 mg, 5 mL, Agilent Technologies, Santa Clara, California, USA). The obtained extracts had a slight yellow color, and were dried in a rotary evaporator under vacuum (Büchi RE; Büchi Laboratoriums Technik AG, Flawil, Switzerland). The dried extracts were kept in sealed glass vials, in the refrigerator until further processed for analysis or addition to wine.

2.4. Experimental procedure

An appropriate amount of each extract was dissolved in 500 mL of fresh wine to obtain added total phenol concentration of 650 mg/L expressed as gallic acid equivalents (GAE). Rosmarinic acid was also added at a concentration of 650 mg/L in fresh wine. Two replicates of each sample were prepared.

After the addition of cofactors, samples (20 mL each) of all four series (in duplicate replications), namely control wine (CW), wine with 650 mg/L rosmarinic acid (RosW), wine with 650 mg/L savory extract (EsW) and wine with 650 mg/L oregano extract (EocW) were packaged in laminated bags (OPP 20 μm/ink/adhesive/PET MET 12 μm/adhesive//PE 75 μm STC) under modified atmosphere (50% N_2–50% CO_2) by using a Boss NT42N MAP unit (Bad Homburg, Germany). Duplicate samples were removed at definite time intervals and proceeded for analysis.

2.5. Analytical procedures

2.5.1. Total phenol content (TP)

The Folin–Ciocalteu method (Waterhouse, 2005) was used for the quantification of TP after dilution of samples (1:10) in ethanolic solution 10% (v/v). The results were expressed as GAE, through construction of a reference curve. All samples were analyzed in duplicate and the presented results are mean values.

2.5.2. Quantification of flavan-3-ols

The total flavanol content was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Li, Tanner, & Larkin, 1996). Wine samples properly diluted, were added to DMACA solution (0.1% in 1 N HCl in MeOH). The mixture was vortexed and allowed to react at room temperature for 10 min. Following, the absorbance at 640 nm was measured against a blank prepared similarly without DMACA. The measurements were interpolated in a catechin calibration curve and the total flavanol content was expressed as mg/L catechin.

2.5.3. Determination of phenolic composition by HPLC–DAD–ESI-MS/MS

The identification of wine phenolic compounds was performed on a Varian 212-LC chromatography system, coupled to an ion trap mass spectrometer equipped with an electrospray interface and a diode array detector. System control and data acquisition was performed using the Varian Workstation software (Varian Inc., Palo Alto, California) and coupled to Varian Workstation data processing software. Samples were injected after filtration (0.2 μm, PVDF syringe filters, Teknokroma, Barcelona, Spain) on a reversed phase Hypersil C18 column (ODS 5 μm, 250 × 4.6 mm, MZ Analysentechnik, Mainz, Germany). The chromatographic separation of compounds was based on the method proposed by Bimpilas, Tsimogiannis, Balta-Brouma, Lymperopoulou, and Oreopoulou (2015) for wine samples, whereas a method proposed by Tsimogiannis, Samiotaki, Panayotou, and Oreopoulou (2007) was used for aromatic plant extracts. The injection volume was 20 μL and the DAD detection of anthocyanins was accomplished at 520 nm, flavonols/flavones at 360 nm and hydroxycinnamic acids at 320 nm. The quantification of individual anthocyanins, flavonols, tartaric esters of phenolic acids (caffeic and *p*-coumaric acids), as well as complex hydroxycinnamic acids (rosmarinic, salvianolic acids) was based on the respective reference curves constructed with malvidin-3-*O*-glucoside, quercetin, caffeic and rosmarinic acid at the abovementioned wavelengths.

2.5.4. Determination of monomeric, copigmented and polymeric anthocyanins, according to their contribution to wine color

The contribution of the monomeric (non-copigmented), copigmented and polymeric anthocyanins to the total wine color was determined following the method proposed by Boulton (2001). The wine samples were adjusted to pH 3.6 and membrane filtered (0.45 mm pore size). 20 μL of 20% (w/v) acetaldehyde was added to 2 mL of wine and the sample was allowed to stand for approximately 45 min. To another 2 mL sample of wine, 160 μL of 5% (w/v) SO_2 was added. Finally 100 μL of wine sample was placed into 1900 μL of bitartrate buffer. The absorbance of each sample was measured at 520 nm in a 10 mm glass cuvette using a Unicam Helios spectrophotometer (Spectronic Unicam EMEA, Cambridge, UK), and the respective measures were A^{acet}, A^{SO_2} and A^{wine}. A^{wine} reading was corrected for the dilution by multiplying by 20. The following equations were used to calculate the percentage of each fraction to color contribution:

$$\%\,\text{copigmented anthocyanins} = \frac{A^{acet} - A^{wine}}{A^{acet}} \cdot 100\%$$

$$\%\,\text{monomeric anthocyanins} = \frac{A^{wine} - A^{SO_2}}{A^{acet}} \cdot 100\%$$

$$\%\,\text{polymeric anthocyanins} = \frac{A^{SO_2}}{A^{acet}} \cdot 100\%$$

2.5.5. Color evaluation

Color evaluation of the samples was performed by using a spectrophotometer (Spectronic Unicam EMEA, Unicam Helios alpha, Leeds, UK). Optical density of undiluted wine samples was measured at 420, 520, and 620 nm, using a 1 mm optical path glass cell. Colorimetric calculations were performed according to the formulas proposed by Glories (1984):

$$\text{Color Intensity}: CI = A_{420} + A_{520} + A_{620}$$

$$\text{Hue}: T = A_{420}/A_{520}$$

where A_{420}, A_{520} and A_{620} are the absorbance values at 420, 520 and 620 nm respectively.

Differences between control wine (CW) and samples with added cofactors (CofW) were evaluated according to the following formulas:

$$\%\Delta A_{520} = \frac{A_{520}^{CofW} - A_{520}^{CW}}{A_{520}^{CW}}\%$$

$$\%\Delta CI = \frac{CI^{CofW} - CI^{CW}}{CI^{CW}}\%$$

$$\%\Delta T = \frac{T^{CofW} - T^{CW}}{T^{CW}}\%$$

2.6. Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range test were applied to detect differences among the four series of samples during storage, in terms of % color contribution of different groups of anthocyanins (monomeric, copigmented and polymeric), color parameters (A_{520}, CI and hue), and total flavan-3-ols. Analyses were performed with the STATISTICA software (version 10, StatSoft®Inc., United States). Differences were considered to be significant at $p < 0.05$. Moreover linear regression analysis was used to determine the ratio of the formation of polymeric anthocyanins, (SigmaPlot software, version 11.0, Systat®Inc., Germany).

3. Results and discussion

3.1. Effect of the addition of aromatic plant cofactors in fresh wine

3.1.1. The phenolic profile of fresh wine

Total phenols in fresh wine, determined with the Folin–Ciocalteu assay, amounted to 1600 ± 35 mg/L GAE, whereas total flavan-3-ols amounted to 290 ± 16 mg/L catechin equivalents (Table 1). The DMACA method was used for the determination of total flavanols because it has a great advantage over the widely used vanillin assay, since there is no interference by anthocyanins. Further, it provides higher sensitivity and specificity (Li et al., 1996).

Merlot wine has a complex anthocyanin profile that was analyzed by HPLC–DAD–ESI-MS/MS and the quantification of individual compounds expressed as malvidin-3-*O*-glucoside equivalents is shown in Table 1. In total 14 anthocyanins were identified and quantified. The composition of anthocyanins was studied thoroughly since differences have been reported concerning the ability of individual anthocyanins to form copigments (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009; González-Manzano, Santos-Buelga, Dueñas, Rivas-Gonzalo, & Escribano-Bailón, 2008; Vaadia, 1997). He et al. (2012), reported that the greater the degree of methoxylation in the B ring of the anthocyanin molecule, the greater was the extent of self-association and that the self-association effect of malvidin-3-*O*-glucoside was thermodynamically favored over intermolecu-

Table 1
Analysis of fresh merlot wine before the addition of cofactors, and quantification of anthocyanins (expressed as malvidin-3-glucoside eq.), flavonols (expressed as quercetin eq.), and hydroxycinnamic acids (expressed as caffeic acid eq.).

Parameter	Content in wine [mg/L]
Total phenols	1600 ± 35
Total flavan-3-ols	290 ± 16
Delphinidin-3-glucoside	19.0 ± 1.0
Cyanidin-3-glucoside	Trace
Petunidin-3-glucoside	28.7 ± 0.9
Peonidin-3-glucoside	22.2 ± 1.5
Malvidin-3-glucoside	203.2 ± 2.8
Delphinidin-3-acetyl glucoside	8.4 ± 0.7
Cyanidin-3-acetyl glucoside	Trace
Petunidin-3-acetyl glucoside	11.0 ± 0.9
Peonidin-3-acetyl glucoside	16.6 ± 1.1
Malvidin-3-acetyl glucoside	61.5 ± 1.9
Petunidin-3-coumaroyl glucoside	9.2 ± 0.4
Peonidin-3-coumaroyl glucoside	11.8 ± 0.8
Malvidin-3-coumaroyl glucoside	27.3 ± 1.4
Malvidin-3-caffeoyl glucoside	Trace
Total monomeric anthocyanins	418.9 ± 4.6
Caftaric acid	11.1 ± 0.7
Coutaric acid	3.6 ± 0.6
Caffeic acid	Trace
p-Coumaric acid	Trace
Total hydroxycinnamic acids	14.7 ± 0.9
Myricetin-3-glucuronide	Trace
Myricetin-3-glucoside	2.6 ± 0.1
Quercetin-3-glucuronide	1.6 ± 0.1
Quercetin-3-glucoside	3.1 ± 0.3
Laricitrin-3-glucoside	1.8 ± 0.3
Myricetin	2.8 ± 0.5
Syringetin-3-glucoside	1.7 ± 0.1
Quercetin	1.9 ± 0.6
Total flavonols	16.3 ± 0.9

lar interaction with cofactors, while the *p*-coumaroyl group of malvidin-3-*O*-(6″-*p*-coumaroyl)-glucoside prevents self-association.

Malvidin was detected in the 3-*O*-glucoside form and moreover acylated by acetic, *p*-coumaric and caffeic acid and amounted to almost 70% of the total anthocyanin content. The 3-*O*-glucosides, 3-*O*-(6″-acetyl) glucosides and 3-*O*-(6″-*p*-coumaroyl) glucosides of petunidin and peonidin were detected as well, whereas delphinidin and cyanidin were found only in the first two forms. The total monomeric anthocyanin content of the wine, calculated as the sum of individual compounds by HPLC, amounted to 418.9 ± 4.6 mg/L. It should be noticed that monomeric anthocyanins quantified by HPLC account also for a fraction of copigmented anthocyanins quantified as monomeric. Copigments are stacked molecular aggregations, primarily accomplished by hydrophobic interactions. The dilution of wine samples during the chromatographic analysis can cause partial dissociation of copigments and the respective anthocyanins are measured as monomeric molecules. Boulton (2001) reported the limited success of comparing the anthocyanin content of wines determined by HPLC, with those of various spectrophotometric methods due to copigments.

The main flavonols detected in Merlot were quercetin and myricetin in glucosylated, glucuronated as well as in their aglycone forms. Moreover, glucosides of laricitrin and syringetin were detected. Caftaric and coutaric acid were the main hydroxycinnamates quantified in fresh Merlot wine, while their non-esterified forms, caffeic and *p*-coumaric acids were detected in traces.

3.1.2. The phenolic profile of aromatic plant extracts and enriched wines

Aromatic plants of the Lamiaceae family, such as *O. vulgare* and *S. thymbra*, present a high polyphenolic content with major constituents belonging to the subgroup of hydroxycinnamates, like rosmarinic acid (Janicsák, Máthé, Miklóssy-Vári, & Blunden, 1999; Petersen & Simmonds, 2003). The total phenolic content of the extracts expressed in gallic acid equivalents was found to be 274 ± 5 g/kg (d.b.) in oregano and 289 ± 6 g/kg (d.b.) in savory. The main phenolic compound in both extracts was rosmarinic acid, whereas both extracts were rich in other complex cinnamic acids like salvianolic acid A and lithospermic acid, which are expected to act as cofactors as well, considering the fact that they are both caffeic acid derivatives. Apart from hydroxycinnamates, both extracts presented a low flavone content. Apigenin 6,8-di-*C*-glucoside was the major constituent, while apigenin 7-*O*-diglucuronide, luteolin and quercetin glucosides were also detected, as well as the respective aglycones that were found in traces. Therefore, the effect of the addition of *S. thymbra* and *O. vulgare* on the anthocyanins of wine cannot only be attributed to hydroxycinnamic acids but partially to their flavone and flavonol content. Since the concentration of the latter groups of compounds was significantly lower than that of phenolic acids, their contribution to the formation of copigments is expected to be low. However their effect on the color of wine cannot be neglected since their maximum absorbance is close to the area of yellow color (420 nm).

All wine samples were analyzed by HPLC, just after the addition of the cofactors, and the quantification of the main constituents is presented in Table 2. As can be seen, hydroxycinnamic acids are the predominant compounds in both samples with added extracts, while the EocW sample presented slightly higher amounts of all the compounds compared to the EsW.

3.1.3. Effect of the added cofactors on anthocyanins copigmentation of fresh wine

The % monomeric, copigmented and polymeric anthocyanins in fresh wine and the respective changes by the addition of cofactors is presented in Fig. 1A. The spectrophotometric analysis of fresh wine, with no additives, showed that 45% of the anthocyanins are found as monomeric, 28% have formed copigments, and 29% were already polymerized, indicative to the fact that polymerization reactions between anthocyanins and flavan-3-ols initiate really early, even during the alcoholic fermentation.

The self-association of anthocyanins and especially malvidin-3-*O*-glucoside (which presents the highest content in wine, 203.2 mg/L) could be responsible for a part of the copigmented fraction in CW. Nonetheless, it has been indicated that for the self-aggregation of the anthocyanins to take place, they should be found in the solutions at concentrations greater than 1 mmol/L (González-Manzano et al., 2008; He et al., 2012). Thus, the effect of self-association on the color of wine is arguable. On the other hand, flavonols, flavan-3-ols, hydroxycinnamic acids (caffeic, *p*-

Table 2
Quantification of cofactors in wine samples.

Parameter	RosW (mg/L)	EocW (mg/L)	EsW (mg/L)
Rosmarinic acid	650	423.4 ± 5.9	341.0 ± 2.9
Lithospermic acid[a]	–	36.9 ± 1.8	56.1 ± 2.6
Salvianolic acid A[a]	–	33.4 ± 2.7	85.1 ± 7.6
Unidentified hydroxycinnamic acids[a]	–	28.2 ± 1.7	10.7 ± 2.2
Total added hydroxycinnamic acids[a]	–	522.0 ± 7.3	492.9 ± 9.1
Apigenin 6,8-di-C-glucoside[b]	–	17.5 ± 1.0	13.9 ± 0.9
Luteolin 7,4′-di-*O*-glucuronide[b]	–	2.2 ± 0.4	1.9 ± 0.1
Apigenin 7-*O*-diglucuronide[b]	–	2.6 ± 0.3	–
Luteolin[b]	–	Trace	2.8 ± 0.6
Apigenin[b]	–	Trace	2.4 ± 0.2
Quercetin	–	Trace	2.1 ± 0.5
Rutin[b]	–	Trace	Trace
Total added flavones/flavonols[b]	–	25.0 ± 2.2	24.1 ± 1.4

[a] Expressed as rosmarinic acid equivalents.
[b] Expressed as quercetin equivalents.

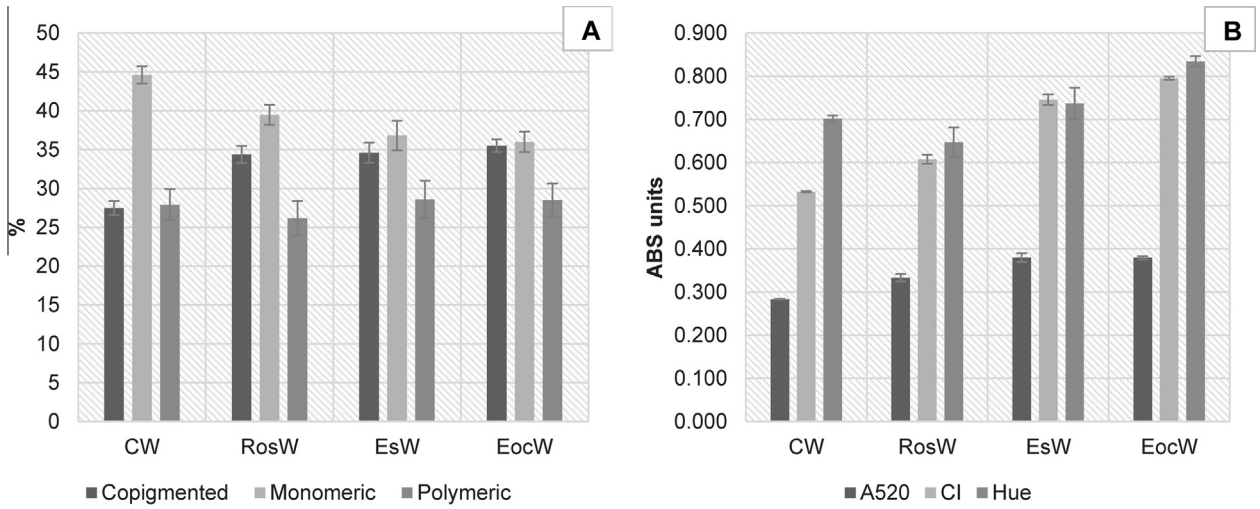

Fig. 1. The effect of the addition of cofactors on the anthocyanins (A) and the color parameters (B) of fresh wine.

coumaric) and hydroxycinnamoyl derivatives are considered to be principal cofactors in young red wines (Favre et al., 2014; He et al., 2012), and partially account for copigments found in CW. Flavanols are relatively poor cofactors, compared to other flavonoids like flavonols or flavones and even more compared to hydroxycinnamic acids. The comparatively small ability of flavanols to act as cofactors is attributed to their non-planar structure that impedes a close approach to the anthocyanin and reduces the potential surface area available for hydrophobic stacking (González-Manzano et al., 2009; Liao, Cai, & Haslam, 1992). On the other hand, flavonols and hydroxycinnamic acids are reported to be the best cofactors in wine (Darias-Martin et al., 2001; Gómez-Míguez et al., 2006). The concentration of total flavonols and total hydroxycinnamates in CW amounted to 16.3 ± 0.9 mg/L quercetin equivalents and 14.7 ± 0.9 mg/L caffeic acid equivalents, respectively, whereas flavan-3-ols to 290 ± 16 mg/L. Therefore, since the former are present in much lower levels than flavan-3-ols, flavan-3-ols are still the major cofactors in wine.

After the addition of cofactors, polymeric anthocyanins content remained practically constant in all samples, equal to the one of CW, while a reduction on % monomeric, followed by an increase of % copigmented was observed (Fig. 1A). In particular, monomeric anthocyanins were 39% in RosW, 37% in EsW and 36% in EocW. The respective values for % copigmented were 34% for RosW, 35% for EsW and 36% for EocW. These changes were statistically significant compared to CW, while there were no significant differences between RosW, EsW and EocW. Given that the added amount of cofactors in all cases was similar, and that the main constituents in all samples were hydroxycinnamic acids, these results can be explained. Moreover the claim that complex phenolic acids like lithospermic and salvianolic were expected to act as cofactors, bearing a similar behavior to rosmarinic acid was confirmed.

3.1.4. Effect on color of fresh wine

Copigmentation results in color modification in young red wines, promoting an increase of 5–20 nm in the maximum absorption wavelength (bathochromic effect), and causing a shift towards higher intensities (hyperchromic effect). The stacking of anthocyanin molecules in the copigmentation complexes produces a sandwich configuration, physically limiting water access to the chromophore of the anthocyanins, thereby limiting the formation of colorless hydrated forms (chalcone or carbinol pseudobase). Thus, copigmentation can result in a greater color intensity of anthocyanin solutions than theoretically could be expected from the anthocyanin concentration and media pH effects (González-Manzano et al., 2009; He et al., 2012; Jackson, 2008).

Given the fact that all added cofactors in RosW, EsW and EocW, namely hydroxycinnamic acids, flavones, or flavonols, do not absorb at 520 nm, had it not been for copigmentation effects, no spectrophotometric changes in this particular wavelength should be expected compared to CW. However the colorimetric measures presented a significant change of A_{520}, as indicated in Fig. 1B. A_{520} of CW was measured equal to 0.284 absorbance units, whereas the respective values for samples with added cofactors were 0.333 for RosW, and 0.380 for both EsW and EocW. A similar trend was observed for the CI values. CI of CW was 0.533 absorbance units, while for RosW was 0.608, for EsW 0.746 and for EocW 0.796. The statistical evaluation of these results with Duncan's multiple range test ($p < 0.05$) indicated significant differences not only of all samples against CW, but also of EocW and EsW versus RosW. The fact that the hyperchromic shift, especially at 520, appears to be greater for samples with added aromatic plant extracts, should be attributed to the nature of the different cofactors among samples. Gómez-Míguez et al. (2006) reports that the magnitude of the copigmentation effect has been found to depend on the nature of the cofactor, indicating that flavonols are the best copigments in red wines due to their planar polyphenolic nucleus, which can tightly stack onto anthocyanins, increasing the absorptivity between 45% and 55% in equimolar mixtures with malvidin-3-*O*-glucoside. Thus, however low is the concentration of flavones and flavonols in EsW and EocW, their contribution in color change might be significant.

The determination of hue in samples with added cofactors and the respective difference from CW (%ΔT) was indicative of the differentiation between RosW and the samples with added extracts from aromatic plants. The %ΔT for EsW and EocW were 5.0% and 18.8%, respectively, while the calculated value of RosW was negative (−7.8%). The negative value of RosW is due to the increase of A_{520} while A_{420} remained constant, and is indicative of the copigmentation effect. On the contrary in both EocW and EsW, the spectral absorption of flavones and flavonols, with maxima around 360 nm, has significant contribution to A_{420} and that is the reason of the hue increase of these compounds. In other words this positive difference of hue for EocW and EsW is attributed to the yellow color of flavonoids, and is irrelevant to the formation of copigments. It should be also mentioned that the higher increase in CI values when either extract is added to wine is also partially attributed to the same reason.

3.2. Effect of cofactors during storage of fresh wine

3.2.1. Variations in anthocyanin content during storage of wine

The change of anthocyanin content and color of wine samples was studied during storage for 6 months. The storage time was defined by the total monomeric anthocyanin content, which decline with time due to polymerization reactions with proanthocyanidins (Figueiredo-González, Cancho-Grande, & Simal-Gándara, 2013b). The quantification of total monomeric anthocyanins by HPLC was indicative of this fact, since in CW they initially amounted to 418.9 ± 4.6 mg/L, whereas after 6 months their concentration was 21.2 ± 0.3 mg/L. The total phenolic content remained practically constant during storage, since the reactions between the phenolic substances of wine are mainly polymerization reactions (anthocyanins–proanthocyanidins) rather than degradation or oxidation reactions so they have minor effect on the total number of hydroxyl groups that are measured by the Folin–Ciocalteu assay.

The response of DMACA assay was indicative of the polymerization reactions that are taking place during storage of wine. This method measures the color of the product formed by the reaction between the aldehyde reagent and the C8 of flavanol structures, namely monomers, dimers, trimers (proanthocyanidins) or polymers (tannins). The most common covalent linkage between flavanols to form linear oligomers and polymers, occurs between the C4 of the pyran ring of one flavanol with C8 of the A ring of another. However, bonding of flavanols between C4 and C6 sites is possible and permits branching of the polymer (Jackson, 2008; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). The DMACA assay theoretically responds only to the flavanols that are not substituted at the C8 of the A ring, and thus, concerning oligomers or polymers, only one unit per chain may react with the aldehyde reagent, regardless of its length unless there is some branching (Ivanova, Vojnoski, & Stefova, 2012). After 6 months of storage, the DMACA index of all series of samples presented a decrease, indicating that less C8 positions are available due to polymerization. The obtained values were 213 ± 4 catechin equivalents for CW; 210 ± 5 for RosW; 213 ± 3 for EsW; 213 ± 7 for EocW, while the initial respective measurements after the addition of cofactors were: 290 ± 16, 286 ± 2, 278 ± 4, 282 ± 2 mg/L.

The change of anthocyanins during storage for all series of samples is presented in Fig. 2. A decrease of % copigmented and % monomeric anthocyanins with the simultaneous increase of % polymeric was observed in all cases. The differences in copigmented and monomeric anthocyanins between CW and wines with added cofactors were statistically significant during the first 3 months of storage. After 6 months of storage, copigmented anthocyanins were 8 ± 2% in CW, 11 ± 1% in RosW, 8 ± 2% in EsW and 7 ± 5% in EocW. The reduction of % copigmented anthocyanins is in accordance with the results of Hermosín Gutiérrez et al. (2005), who reported that, the percentage of copigmented anthocyanins in Cabernet Sauvignon reduced by 25–43% during 3 months of storage of fresh wine, while in Syrah the respective reduction was 34–44%. On the other hand Dobrei et al. (2010) reported a reduction of only 3% of copigmented anthocyanins during storage of a Merlot variety for 4 months (i.e. from 35% to 32%). These contradictory results indicate that the evolution of anthocyanins is influenced by parameters like storage/aging conditions and abundance of procyanidins that may increase or decrease the rates of polymerization reactions between anthocyanins and procyanidins.

Polymerized anthocyanins amounted to more than 80% in all samples after 6 months of storage. Linear regression analysis was used to determine the rates of increase of % polymeric anthocyanins ($R^2 > 0.98$). The rate for CW was 9.7 ± 0.3% increase per month, while the respective values for samples with added cofactors were: 9.9 ± 0.6%, RosW; 9.6 ± 0.5%, EsW; 8.8 ± 0.7%, EocW. No significant differences were determined among the different series of samples ($p > 0.05$), in other words the initially different amount of % copigmented anthocyanins of CW compared to RosW, EsW, and EocW, did not influence the evolution of polymeric anthocyanins quantitatively.

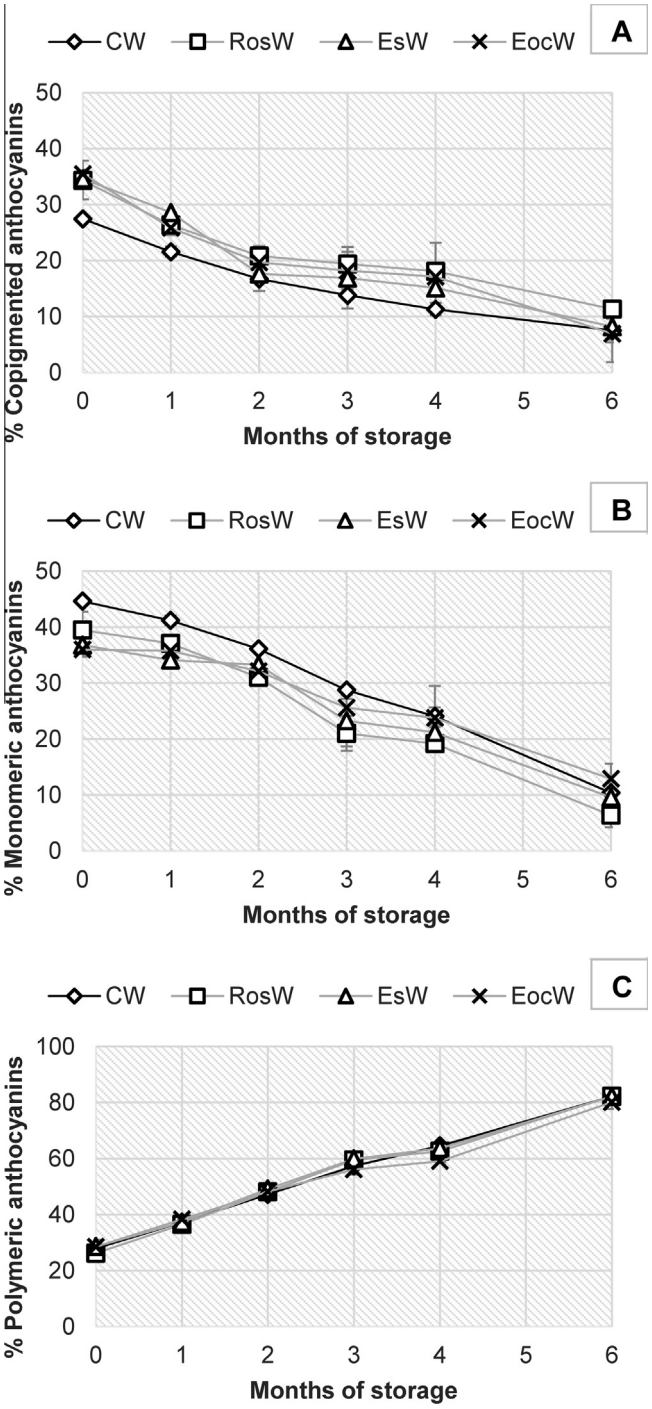

Fig. 2. The evolution of copigmented (A), monomeric (B) and polymeric (C) anthocyanins of wine during storage.

During wine storage, there is a chemical equilibrium between monomeric and copigmented anthocyanins (Eq. (1), Rustioni et al., 2012). Moreover, monomeric anthocyanins react so as to form polymeric pigments (Eq. (2)):

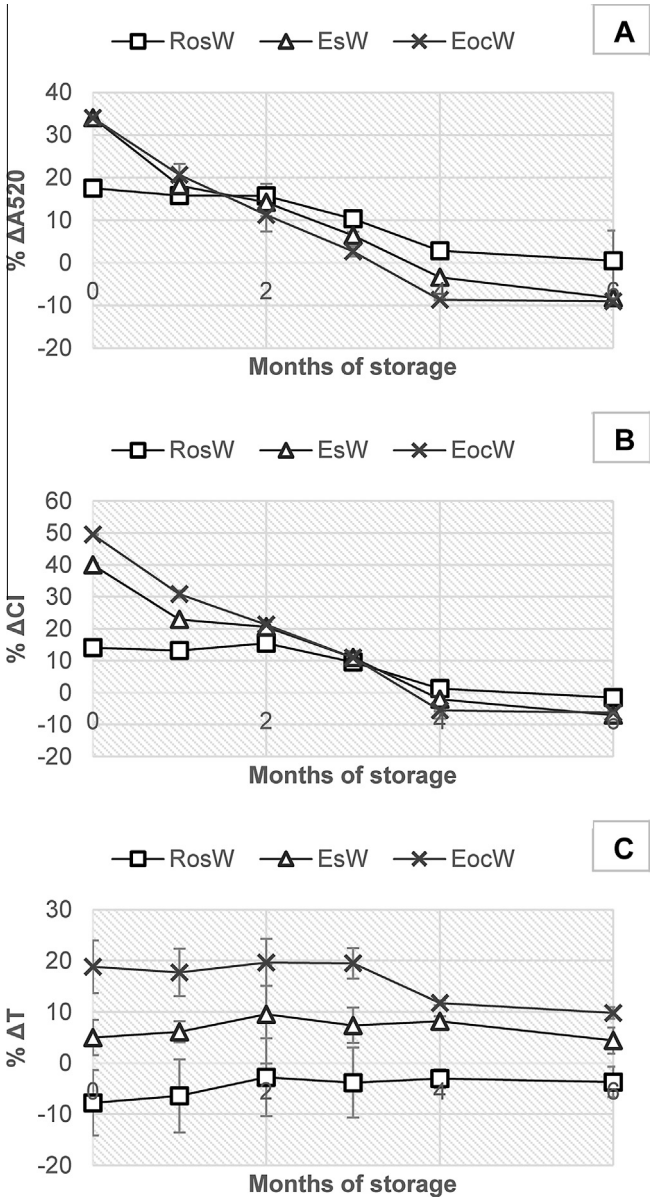

Fig. 3. The % change of A_{520} (A), CI (B) and T (C) of samples with added cofactors compared to CW during storage.

$$Monomeric\ anthocyanins + Cofactors \rightleftharpoons Copigmented\ anthocyanins \quad (1)$$

$$Monomeric\ anthocyanins + Proanthocyanidins \rightarrow Polymeric\ pigments \quad (2)$$

A decline in monomeric anthocyanins concentration due to polymerization reactions changes the chemical equilibrium between monomeric and copigmented anthocyanins, enabling the latter to dissociate so as to increase the concentration of monomers (Eq. (1)). However, since the one-way polymerization reactions (Eq. (2)) evolve at a slow rate, compared to the fact that most copigments are formed/dissociate rapidly, an initially higher concentration of copigments appears not able to change the rate of polymerization.

3.2.2. Effect on wine color

The CI of CW increased from 0.533 to 0.866 during 6 months of storage. This increase is attributed to the reactions between flavan-3-ols and anthocyanins. Three different mechanisms have been postulated: (1) reactions between anthocyanins (+) and flavan-3-ols (−) that lead to A+ → F− adducts. These molecules are colorless and turn red when oxidized. (2) Reactions between flavan-3-ols (+) and anthocyanins (−) that lead to F+ → A− adducts, where anthocyanins (−) correspond to the carbinol base and adducts formed are theoretically colorless, but are rapidly dehydrated into a stable colored form. This reaction is completely independent of the oxidation conditions. (3) Acetaldehyde mediated reactions of flavan-3-ols and anthocyanins, where acetaldehyde is formed by oxidation of ethanol and these reactions lead to A–F adducts in which the flavonoid units are linked through a methylmethine bond (Bimpilas et al., 2015; Cheynier, 2006). Since wine samples in the present study were stored under modified atmosphere and chemical oxidation phenomena were avoided, the formation of F–A adducts via the 2nd reaction is assumed to predominate over the other two. These adducts generate, upon dehydration, colored flavylium chromophores that enhance color expression. (Hayasaka & Kennedy, 2003; Jackson, 2008; Ribéreau-Gayon et al., 2006).

The differences of color parameters (ΔA_{520}, ΔCI, ΔT) for RosW, EsW and EocW versus CW are presented in Fig. 3. ΔA_{520} and ΔCI are following the same trends, a fact attributed to the high % contribution of A_{520} in fresh wine. During the first 3 months of storage, the positive difference of RosW, EsW and EocW is profound, with EsW and EocW presenting the highest values, in accordance to the results presented in Section 3.1.4. These results depict the contribution of the enhanced copigmented anthocyanin content of the enriched samples, for the time period that % polymeric anthocyanins contribute less than 60% of the total color. However, there is a decreasing trend of ΔA_{520} and ΔCI with storage time, which leads to values close to 0% for all samples after 6 months. The statistical analysis indicated no significant differences between CW and wines with added cofactors in terms of A_{520} and CI, after 3 months of storage.

On the other hand, ΔT remained practically constant during storage of the samples where the plant extracts were added. The respective values after 6 months were: 4.4% for EsW; 13.2% for EocW; while the negative deviation for RosW was close to zero, −3.7%.

4. Conclusions

Rosmarinic acid and ethanolic extracts from *O. vulgare* or *S. thymbra* that were rich in hydroxycinnamic acids proved efficient to promote copigmentation of anthocyanins and consequently increase the color of fresh Merlot wine. The increase of red color was higher by the addition of either extract, compared to rosmarinic acid, a fact attributed to the flavonoid content of the extracts, in addition to hydroxycinnamic acids. Flavonoids were also responsible for the increase in hue, observed when either extract was added to wine, as they are yellow pigments with absorbance at 420 nm. During storage of wine, a decrease in monomeric and copigmented anthocyanins and an increase in the polymerized analogs was observed, as expected. The samples with added cofactors retained higher percentages of copigmented anthocyanins and higher color intensity, compared to the control wine, up to 3 months, while afterwards, differences were not statistically significant. Polymerized anthocyanins increased with the same rate in all samples, showing that the initial higher concentration of copigments appears not able to change the rate of polymerization.

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References

Álvarez, I., Aleixandre, J. L., García, M. J., Lizama, V., & Aleixandre-Tudó, J. L. (2009). Effect of the prefermentative addition of copigments on the polyphenolic composition of Tempranillo wines after malolactic fermentation. *European Food Research and Technology, 228*, 501–510.

Bimpilas, A., Tsimogiannis, D., Balta-Brouma, K., Lymperopoulou, T., & Oreopoulou, V. (2015). Evolution of phenolic compounds and metal content of wine during alcoholic fermentation and storage. *Food Chemistry, 178*, 164–171.

Bloomfield, D. G., Heatherbell, D. A., & Nikfardjam, M. P. (2003). Effect of *p*-coumaric acid on the color in red wine. *Mitteilungen Klosterneuburg, 53*, 195–198.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture, 52*, 67–87.

Cheynier, V. (2006). Flavonoids in wine. In Ø. M. Andersen & K. R. Markham (Eds.), *Flavonoids: Chemistry, biochemistry and applications* (pp. 273–299). Boca Raton: CRC Taylor and Francis Group.

Darias-Martin, J., Carrillo, M., Diaz, E., & Boulton, R. B. (2001). Enhancement of red wine colour by pre-fermentation addition of copigments. *Food Chemistry, 73*, 217–220.

Dobrei, A., Poiana, M. A., Sala, F., Ghita, A., & Gergen, I. (2010). Changes in the chromatic properties of red wines from Vitis vinifera L. cv. Merlot and Pinot Noir during the course of aging in bottle. *Journal of Food, Agriculture & Environment, 8*, 20–24.

Drosou, C., Kyriakopoulou, K., Bimpilas, A., Tsimogiannis, D., & Krokida, M. (2015). A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products, 75*, 141–149.

Favre, G., Peña-Neira, Á., Baldi, C., Hernández, N., Traverso, S., Gil, G., & González-Neves, G. (2014). Low molecular-weight phenols in Tannat wines made by alternative winemaking procedures. *Food Chemistry, 158*, 504–512.

Figueiredo-González, M., Cancho-Grande, B., & Simal-Gándara, J. (2013a). Garnacha Tintorera-based sweet wines: Chromatic properties and global phenolic composition by means of UV–Vis spectrophotometry. *Food Chemistry, 140*, 217–224.

Figueiredo-González, M., Cancho-Grande, B., & Simal-Gándara, J. (2013b). Evolution of colour and phenolic compounds during Garnacha Tintorera grape raisining. *Food Chemistry, 141*, 3230–3240.

Glories, Y. (1984). The colour of red wines. Part 2. Measurement, origin and interpretation. *Connaissance de la Vigne et du Vin, 18*, 253–271.

Gómez-Míguez, M., González-Manzano, S., Escribano-Bailón, M. T., Heredia, F. J., & Santos-Buelga, C. (2006). Influence of different phenolic copigments on the color of malvidin 3-glucoside. *Journal of Agricultural and Food Chemistry, 54*, 5422–5429.

González-Manzano, S., Dueñas, M., Rivas-Gonzalo, J. C., Escribano-Bailón, M. T., & Santos-Buelga, C. (2009). Studies on the copigmentation between anthocyanins and flavan-3-ols and their influence in the colour expression of red wine. *Food Chemistry, 114*, 649–656.

González-Manzano, S., Santos-Buelga, C., Dueñas, M., Rivas-Gonzalo, J. C., & Escribano-Bailón, T. (2008). Colour implications of self-association processes of wine anthocyanins. *European Food Research and Technology, 226*, 483–490.

Hayasaka, Y., & Kennedy, J. A. (2003). Mass spectrometric evidence for the formation of pigmented polymers in red wine. *Australian Journal of Grape and Wine Research, 9*, 210–220.

He, F., Liang, N. N., Mu, L., Pan, Q. H., Wang, J., Reeves, M. J., & Duan, C. Q. (2012). Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression. *Molecules, 17*, 1571–1601.

Hermosín Gutiérrez, I., Sánchez-Palomo Lorenzo, E., & Vicario Espinosa, A. (2005). Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars, Cabernet Sauvignon, Cencibel, and Syrah. *Food Chemistry, 92*, 269–283.

Ivanova, V., Vojnoski, B., & Stefova, M. (2012). Effect of winemaking treatment and wine aging on phenolic content in Vranec wines. *Journal of Food Science and Technology, 49*, 161–172.

Jackson, R. S. (2008). Chemical constituents of grapes and wine. In R. S. Jackson (Ed.), *Wine science: Principles and applications* (pp. 270–331). San Diego: Academic Press.

Janicsák, G., Máthé, I., Miklóssy-Vári, V., & Blunden, G. (1999). Comparative studies of the rosmarinic and caffeic acid contents of Lamiaceae species. *Biochemical Systematics and Ecology, 27*, 733–738.

Kouri, G., Tsimogiannis, D., Bardouki, H., & Oreopoulou, V. (2007). Extraction and analysis of antioxidant components from Origanum dictamnus. *Innovative Food Science & Emerging Technologies, 8*, 155–162.

Li, Y. G., Tanner, G., & Larkin, P. (1996). The DMACA–HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture, 70*, 89–101.

Liao, H., Cai, Y., & Haslam, E. (1992). Polyphenol interactions. Anthocyanins: Co-pigmentation and colour changes in red wines. *Journal of the Science of Food and Agriculture, 59*, 299–305.

Petersen, M., & Simmonds, M. S. (2003). Rosmarinic acid. *Phytochemistry, 62*, 121–125.

Rein, M. J., & Heinonen, M. (2004). Stability and enhancement of berry juice color. *Journal of Agricultural and Food Chemistry, 52*, 3106–3114.

Ribéreau-Gayon, P., Glories, Y., Maujean, A., & Dubourdieu, D. (2006). Phenolic Compounds. In P. Ribéreau-Gayon, Y. Glories, A. Maujean, & D. Dubourdieu (Eds.), *Handbook of enology. The chemistry of wine stabilization and treatments* (Vol. 2, pp. 141–203). Hoboken: John Wiley & Sons.

Rustioni, L., Bedgood, D. R., Failla, O., Prenzler, P. D., & Robards, K. (2012). Copigmentation and anti-copigmentation in grape extracts studied by spectrophotometry and post-column-reaction HPLC. *Food Chemistry, 132*, 2194–2201.

Tsimogiannis, D., Samiotaki, M., Panayotou, G., & Oreopoulou, V. (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC–MS/MS. *Molecules, 12*, 593–606.

Vaadia, M., 1997. Copigmentation in varietal wines from Napa Valley (Master of Science Thesis). Davis: University of California.

Waterhouse, A. L. (2005). Determination of total phenolics. In R. E. Wrolstad, T. E. Acree, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith, & P. Sporns (Eds.), *Handbook of food analytical chemistry* (pp. 463–471). Hoboken: John Wiley & Sons.