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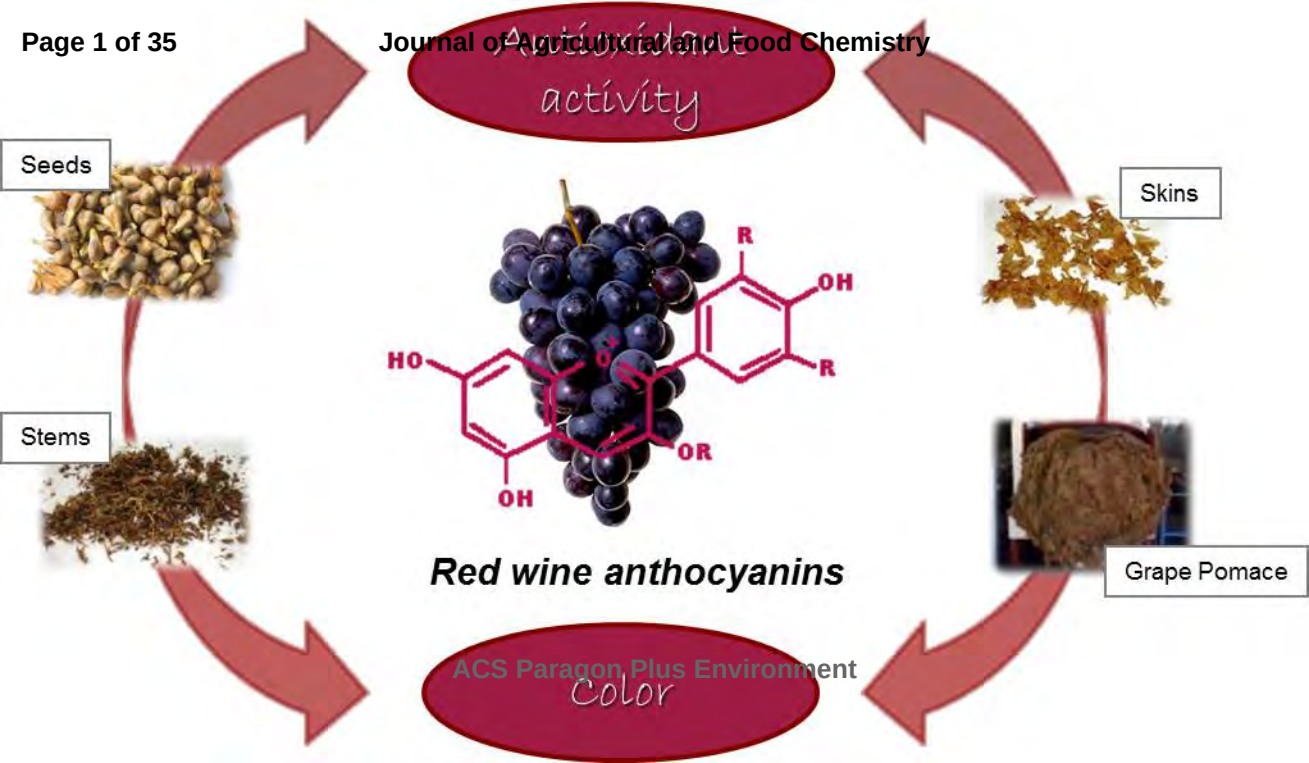
Antioxidant activity
Seeds
Skins
Stems
Grape Pomace
Red wine anthocyanins
ACS Paragon Plus Environment
Color

Comparative study of the oenological potential of different winemaking by-products: implications on the antioxidant activity and color expression of red wine anthocyanins in model solution

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Abstract

Different white winemaking by-products (pomace, skins, seeds and stems) were compared as natural sources of phenolic compounds having biological and sensory properties of oenological interest. Antioxidant and copigmentation effects of these by-products were studied in wine-like model solution. RRLC-DAD was used to establish differences on the phenolic composition and the ABTS method to compare the antioxidant activity. Spectrophotometric and colorimetric analyses were performed to asses the magnitude of copigmentation and the changes induced in the color expression of red wine anthocyanins. Antioxidant and copigmentation properties significantly varied depending on the type of by-product, which was related to their qualitative and quantitative phenolic composition. Seeds and pomace showed the highest antioxidant potential while skins and pomace led to the strongest and visually perceptible color effects on red wine anthocyanins by multiple copigmentation (darker, more saturated and vivid bluish colors). Results open the possibility of technological applications for the wine industry based on re-using winemaking by-products to improve the biological value and color characteristics of red wines.

Keywords: winemaking by-products; phenolic compounds; antioxidant activity; multiple copigmentation; anthocyanin color.

INTRODUCTION

Nowadays, an efficient management of by-products derived from the elaboration and processing of agricultural products is a necessary requirement for a sustainable food industry[1].

Focusing on the wine industry, research has consistently demonstrated the important environmental impact of the liquid and solids residues obtained from the grape vinification such as wine lees, pomaces, stems, or wastewater sludge; and the technical and economic difficulties to their elimination or transformation[2-3]. Problems associated with the management of winemaking by-products are related to their high organic loading which makes difficult their biological degradation. On the other hand, as winemaking is a seasonal activity, an intensive accumulation of residues is generated during a short period every year (grape harvesting), especially in high production regions. Under these circumstances, the European Union is becoming exigent about the preservation of water, soil, and biodiversity, and seriously promotes to wine producers regions looking for new initiatives that permit a more sustainable management and exploitation of their winemaking by-products (Council Regulation (EC) nº 491/2009).

Traditionally, winemaking by-products have been sent to distilleries for obtaining ethanol, or to be used as fertilizers or biomass. However, these activities are usually carried out by external companies representing economic costs for the wine industry[2]. In consequence, there is an increasing interest to find alternative solutions for the exploitation and valorization of those by-products, which would involve economic, social and environmental advantages[4-7]

Over the last decade, the chemical composition of winemaking by-products have been extensively investigated and was confirmed that they represent low-cost sources of many bioactive compounds, being even richer than other types of agri-food wastes[8]. Especially important at this respect are phenolic compound which have potential industrial applications (pharmaceutical, cosmetic, nutritional or agricultural) due to their strong antioxidant, anti-inflammatory, antimicrobial, or biostimulant effects[9-12]. These compounds have also interesting applications in the field of oenology not only for being responsible of the antioxidant properties of wines but also for playing a crucial role in organoleptic characteristics such as color, aroma or taste[13]. Particularly in red wines, it is well known that colorless phenolics are involved in the chemical stabilization of anthocyanin pigments by means of non-covalent interactions through intermolecular copigmentation reactions[14]. Studies carried out in model solution and focused on the application of objective color measurements have demonstrated that copigmentation cause the stabilization of the colored forms of the anthocyanins and consequently enhance their color[15,16]. Thus, copigmentation is considered a relevant interaction because obtaining wines with stable and attractive colors is a major focus for quality control purposes. Among grape components, colorless phenols including flavonoids and some phenolic acids appeared as good anthocyanin copigments, which are abundant compounds in winemaking by-products. Moreover, these compounds can act as effective oxidation substrates, which partially avoid undesirable color changes due to browning/oxidation. In fact, it has been recently reported that the addition of dehydrated waste grape skins during winemaking increased the concentration of some flavonoids in wines preventing the color loss during storage[17,18].

Despite their potential use in oenology, winemaking by-products have received little attention in comparison to other wine additives (wood chips, enzymes, enological

tannins, etc.) probably due to the difficulties related to the technical and legal concerns of its application. However, their application as natural wine additives could represents a sustainable alternative to maximize the exploitation of this valuable agricultural waste as well as to improve the quality of wines making them more competitive. In this sense, further investigations are needed to advance the knowledge of the contribution of these agricultural by-products to wine colour and colour stability.

Therefore, the main objective of this study was to compare the potential of different white winemaking by-products (pomace, seeds, skins and stems) as natural sources of antioxidants and copigments, in model solution. The information reported in this work could be useful for high production winemaking areas.

MATERIALS AND METHODS

Standards and Reagents

Gallic acid, protocatechuic, caffeic and caftaric acids, (+)-catechin (C), (–)-epicatechin (EC), quercetin, kaempferol, myricetin, sodium carbonate, potassium persulphate and tartaric acid were purchased from Sigma-Aldrich (Madrid, Spain) and malvidin-3-glucoside from Extrasynthese (Genay, France). Procyanidin dimer B1 standard was isolated in the laboratory by semipreparative HPLC[19].

2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Madrid, Spain), and HPLC grade acetonitrile was from Carlo Erba (Rodano, Italy). Folin reagent, ethanol and formic acid were obtained from Panreac (Barcelona, Spain).

Winemaking by-products and sample preparation

White winemaking by-products from Zalema grapes (*Vitis vinifera* sp.) used in this study were: pomace (PM), skins (SK), seeds (SD) and stems (ST). They were obtained from a winery located in “Condado de Huelva” Designation of Origin (south-western Spain). Zalema cultivar was selected because is a high production variety rich in phenolic compounds that represent the main and more extensively white grape cultivated in the zone.

Pomace is the main organic winemaking by-product generated from grape vinification which is constituted by a mixture of skins and pulp rests, seeds and stems. 3 kg of pomace was collected the day of harvest after Zalema grapes were pressing for winemaking.

In order to be also individually used in the experiments, particles of stems, seeds, and skins were manually separated from the pomace. All winemaking by-products were stored at -20 ºC and further freeze-dried (lyophilizer Cryodos-80, Telstar® Varian DS 102) until being extracted. The moisture contents of by-products were 50% PM, 40% ST, 30% SD and 70% SK.

The extraction of the non-anthocyanin phenolic compounds from each winemaking by-product was carried out in wine-like medium containing 5 g/L tartaric acid in 12% ethanol, buffered with 1 M NaOH to pH 3.6 and ionic strength adjusted to 0.2 M. by addition of NaCl. For this purpose, 2 g of the homogeneous lyophilised powder of samples (PM, SD; SK and ST) was individually macerated in 15 mL of wine-like medium for 12 h at room temperature (18-20 ºC), with occasional agitation and sonication. The supernatants were centrifuged (4190 *g*, 10 min) to separate out the liquid fraction containing the phenolic compounds extracted, which was filtered through 0.45 µm Millipore-AP20 filters (Bedford, MA).

The crude phenolic solution obtained from each winemaking by-product was analysed for its phenolic composition (spectrophotometric and cromatographic analysis) and antioxidant activity. Also, they were used as crude mixture of colorless phenolics (copigments) of wine anthocyanins in copigmentation experiments.

Total phenolic content

The spectrophotometric determination of the total phenolic content was performed with a Hewlett–Packard UV-vis HP8453 spectrophotometer (Palo Alto, CA, USA), using 10 mm path length glass cells and distilled water as reference.

Total phenolic content of the samples was determined using the Folin-Ciocalteu method[20]. Briefly, 0.25 mL of sample, 1.25 mL of Folin-Ciocalteu reagent, and 3.75 mL of a solution of sodium carbonate at 20% 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 take place and stabilize. Absorbance was measured at 765 nm. Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (mg GAE/L).

Phenolic composition analysis

Rapid resolution liquid chromatography (RRLC) was performed on an Agilent 1260 system equipped with a diode-array detector. Samples were filtered through to 0.45-μm pore size membrane filter and 30 μL of samples were injected in a C18 Poroshell 120 column (2.7 μm particle size, 5 cm x 4.6 mm; Agilent, Palo Alto, CA) maintained at 25 ºC. Water-formic acid (99:1, v/v) as solvent A and acetonitrile as solvent B were used, setting the flow-rate at 1.5 mL/min. The linear gradient elution was 0 min, 100% A; 5 min, 95% A and 5% B; 20 min, 50% A and 50% B; 22 min, 100% A; 25 min, 100% A. The wavelengths of detection were 280 nm (flavanols and benzoic acids), 320 nm (hydroxycinnamic acids and their tartaric esters) and 370 nm (flavonols).

Phenolic compounds were identified by their retention time, UV-vis spectra and mass spectra data in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source, and a triple quadrupole-ion trap mass analyser, as described by Jara-Palacios et al.[21]. The identification of phenolic compounds was achieved by the comparison of the retention times and mass spectra with those of the available pure standards and our date library.

The external calibration method was used for quantification, by comparing the areas with standards of gallic, protocatechuic, caffeic and *p*-coumaric acids, catechin, epicatechin, procyanidin B1, quercetin and kaempferol. Caftaric and coutaric acids were quantified using the calibration curves of caffeic and *p*-coumaric acids, respectively. Procyanidin dimers B2, B3 and B4, procyanidin B2-3-*O*-gallate, trimers and tetramer were quantified with the calibration curve of procyanidin B1. Quercetin and isorhamnetin derivatives were quantified as quercetin, and kaempferol derivates as kaempferol.

Total phenolic acids, total flavanols, total flavanol oligomers and total flavonols, were also calculated by the sum of individual phenolic acids, flavanols, flavanols oligomers, and flavonols identified, respectively. The samples were analyzed in triplicate and the results expressed as mg/L.

Antioxidant activity

The antioxidant activity was measured *in vitro* based on the ability to scavenge the $ABTS^{\bullet+}$ radical[22]. The $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (2.45 mM) in water. The mixture was kept in the dark at room temperature for 16 h before using it, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate buffered saline (PBS) at pH 7.4 to give an absorbance of 0.70 ± 0.02 at 734 nm. Then, 50 µL of each sample was mixed with 2 mL of the $ABTS^{\bullet+}$ diluted solution, vortexed for 10 s, and the absorbance was measured at 734 nm after reacting 4 min at

30 ºC. Results were obtained by interpolating the absorbance of samples on a calibration curve obtained with Trolox (30-1,000 μM). Three independent experiments in triplicate were performed for each sample and the results were expressed as Trolox-equivalent antioxidant activity (TEAC; μmols of Trolox-equivalent (TE) with the same antioxidant activity of 1 L of sample).

Copigmentation experiments

Copigmentation experiments were carried out in wine-like medium using a crude anthocyanin solution prepared from Syrah red grapes and different crude phenolic solutions (colorless copigments) obtained from white winemaking by-products.

The crude anthocyanin solution was obtained by macerating 1 g of homogeneous lyophilised powder of Syrah skins in 20 mL of wine-like medium (the same previously described), for 12 h with occasional agitation and sonication. Then, it was centrifuged (4190 *g*, 10 min) and the supernatant filtered through 0.45 μm Millipore-AP20 filters (Bedford, MA). The phenolic composition (anthocyanin pigments and other colorless monomeric phenols) of the crude anthocynin solution was analysed by HPLC following the method described in Gordillo et al.[23].

Copigmented solutions were prepared by adding separately each crude phenolic solution from winemaking by-products (PM, ST, SD, SK) to the crude anthocyanin solution at seven levels (50, 100, 200, 300, 400, 500 and 600 mg/L). The final anthocyanin concentration was the same in all cases (200 mg/L). All of the solutions (2 mL) were prepared in triplicate and equilibrated to reach the equilibrium for 2 h and stored closed in darkness at 25 ºC, after which their absorption spectra were recorded.

Colorimetric measurement

The absorption spectra (380- 770 nm) of all solutions were recorded at constant intervals (Δλ=2 nm) with a Hewlett- Packard UV-vis HP8453 spectrophotometer (Palo Alto, CA), using 2 mm path length glass cells and distilled water as a reference.

The CIELAB parameters (L*, a*, b*, C^*_{ab}, and h_{ab}) were calculated from the absorption spectra by using the original software CromaLab© [24], following the recommendations of the Commission International de L'Eclariage[25]: the 10° Standard Observer and Standard Illuminant D65.

Color difference (ΔE^*_{ab}) was determined by applying the CIE76 color difference formula. It was calculated as the Euclidean distance between two points in the three-dimensional CIELAB space defined by L*, a* and b*: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. It is assumed that color differences (ΔE^*_{ab}) higher than 3-5 units can be perceived by an average observer[26].

Copigmentation measurements

The spectrophotometric determination of the magnitude of the copigmentation was made by comparing the absorbance at 520 nm of the crude anthocyanin solution (A_0) and the absorbance at 520 nm of the same solution containing different crude phenolic mixtures from white winemaking by-products (A_c), at each concentration level and expressed as the percentage $[(A_c - A_0)/A_0]x100$[27].

The color variation due to copigmentation was also evaluated by Tristimulus Colorimetry according to the methodology described in Gordillo et al.[16], which offers an objective measurement of color because it is based on the consideration of the whole visible spectrum, and allows the real assessment of color to be obtained. Following this methodology, diverse color-difference formulas were applied in the CIELAB color space by using the scalar (L*, a*, b*) and cylindrical (L*, C^*_{ab}, h_{ab}) CIELAB color coordinates of samples. This provides a better evaluation of the quantitative and qualitative color

implications of the copigmentation, and their incidence on visual perception. The new colorimetric variables were determined as follows:

- TheTotal Color of each sample was assessed as the CIELAB color difference (ΔE^*_{ab}) applied between its color (L*, a*, and b*) with respect to distilled water (L*=100, a*=0, b*=0), as shown in Eq. (1). It represents a quantitative color attribute.

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

- The Total Color Difference induced by copigmentation was assessed as the CIELAB color difference (ΔE^*_{ab}) applied between the color of the crude anthocyanin solution (L^*_0, a^*_0, and b^*_0) and the color of the same solution copigmented with each crude phenolic mixture from white winemaking by-products (L^*_c, a^*_c, and b^*_c), as shown in Eq. (2):

Eq. (2) Total Color Difference: $\Delta E^*_{ab\ (c-0)} = [\ (L^*_c - L^*_0)^2 + (a^*_c - a^*_0)^2 + (b^*_c - b^*_0)^2\ ]^{1/2}$

- The relative contribution (%) of lightness, chroma and hue to each Total Color Difference induced by copigmentation was calculated by means of the color formulas shown in Eq. (3, 4, and 5). They represent the weight of the three color attributes that makes up the total color difference.

Eq. (3) Relative contribution (%) of lightness: $\%\Delta L = [\ (\Delta L_{c-0})^2 / (\Delta E^*_{ab\ (c-0)})^2\ ]$ x100

Eq. (4) Relative contribution (%) of chroma: $\%\Delta C = [\ (\Delta C_{c-0})^2 / (\Delta E^*_{ab\ (c-0)})^2\ ]$ x100

Eq. (5) Relative contribution (%) of hue: $\%\Delta H = [\ (\Delta H_{c-0})^2 / (\Delta E^*_{ab\ (c-0)})^2\ ]$ x100

being ΔH_{c-0} deduced as follows: $\Delta H_{c-0} = [\ (\Delta E^*_{ab\ (c-0)})^2 - ((\Delta L_{c-0})^2 + (\Delta C_{c-0})^2\ )\ ]^{1/2}$

Statistical analysis

All statistical analyses were performed using Statistica v.8.0 software[28]. Univariate analysis of variance (Tukey test) was applied to establish differences for the phenolic composition, antioxidant activity and copigmentation effects among the crude phenolic solutions from winemaking by-products (GP, SD, SK and ST). Moreover, correlations between the phenolic composition (main phenolic groups or individual phenolic compounds) and the antioxidant activity was studied by linear and multiple regressions. In all cases (differences or correlations), statistically significant level was considered at $p<0.05$.

RESULTS AND DISCUSSION

Phenolic composition

A total of 24 phenolic compounds were identified and quantified in the crude phenolic solutions from winemaking by-products by using the methodology and the chromatographic conditions previously described. The phenolic profile of samples showed the presence of several types of monomeric and oligomeric colorless phenols belonging to diverse phenolic families. The benzoic acids, hydroxycinnamic acids, flavanols, and flavonols identified were the expected, well-known, compounds usually present in grapes. It included 6 phenolic acids (gallic, protocatechuic, caftaric, caffeic, *cis*-coutaric and *trans*-coutaric acids); 2 monomeric flavanols (catechin and epicatechin), 8 oligomeric flavanols (procyanidins B1, B2, B3, B4 and B2-3-*O*-gallate, two trimers and one tetramer); and 8 flavonols (quercetin and kaempferol aglycones, and 3-glycosides conjugated forms of quercetin, kaempferol and isorhamnetin).

Table 1 summarizes the concentration for the mentioned phenolic compounds (mg/L) and the total phenolic content (mg GAE/L) of samples, showing the statistical differences found among them. In general terms, all samples presented important

298 contents of total phenolics indicating that considerable amounts of bioactive compounds
299 can be recovered from Zalema winemaking by-products. As white wines are commonly
300 elaborated by applying a shorter maceration time than red wines, the pomace obtained
301 from white vinification is not as exhausted as that from red vinification, which increase
302 its industrial value as richer agricultural by-product[18]. Nevertheless, significant
303 differences ($p<0.05$) were found for the total phenolic content among samples
304 depending on the type of winemaking by-product. The crude phenolic solution from SD
305 showed the highest total phenolic content (around two-fold the concentration of the
306 other samples), which is consistent with previous reports[29]. The higher accumulation of
307 phenolic compounds in seeds[30] but the lower extractability due to the solid cellular
308 structure[31] could explain the greater phenolic potential of SD as winemaking by-product.
309 On the contrary, significant ($p<0.05$) lower contents of total phenolics was found in the
310 crude phenolic solutions from SK and ST, indicating that they are comparatively poorer
311 sources of phenolics.

312 The chromatographic analysis showed that crude phenolic solutions from PM,
313 SK, SD, and ST had different qualitative and quantitative phenolic profile (Figure 1),
314 being the differences significant ($p<0.05$) for most of the individual compounds identified
315 (Table 1). These differences were also observed when compounds were grouped by
316 phenolic families (phenolic acids, flavanols and flavonols). Significant higher contents of
317 flavanols were found in the crude phenolic solutions from SD, which is in accordance
318 with other reports[8, 32]. The global level of flavanols was 35% higher than those found in
319 the samples from PM and 64% higher than those from SK and ST. On the other hand,
320 PM was the major source of phenolic acids, contributing to the crude phenolic solution
321 with 34% higher than ST and SD, and with 38% higher than SK. As regards the flavonol

322 contribution, SK represented the significant richest source of these compounds in
323 comparison with all other winemaking by-products studied, as expected.

324 As far as individual compounds are concerned, great variability has been
325 described in the literature about the distribution of phenolic acids in different types of
326 winemaking by-products, which is attributable to the influence of several factors as
327 grape variety, cultivation and climatic conditions, or the oenological processes applied
328 during the winemaking[33-35]. However, the qualitative profile of phenolic acids did not
329 differ much among samples. In particular, gallic acid was the main phenolic acid found
330 in the samples being transferred in significant ($p<0.05$) higher quantity by PM. Caftaric
331 acid was the second most abundant phenolic acid, being ST and PM the better sources.

332 In contrast, the group of flavonoids (flavanols and flavonols) varied considerably
333 among samples. In the case of flavanols, significant higher levels of monomeric
334 compounds were obtained from SD, mainly due to a more important contribution in
335 catechin and epicatechin. Nevertheless, Zalema winemaking by-products contributed
336 with greater quantities of oligomers than monomers to the total flavanol content (PM:
337 84%, SK: 80%, ST: 75% and SD: 70%). Among them, procyanidin B1, B4 and B2-3-*O*-
338 gallate were the most representative oligomers in all samples. Specifically, SD and PM
339 were the significant richest sources in oligomeric flavanols (98.9 and 77.3 mg/L in
340 samples, respectively), contributing to the crude phenolic solution about 2.5-fold higher
341 than SK and ST. Regarding flavonols, quercetin-3-glucoside was the predominant
342 compound in all samples. The crude phenolic solution from SK was noteworthy for
343 having significant higher levels of all flavonols compared to those from PM, ST and SD
344 (6.6% of the total phenolic contribution versus 2.3%, 1.7%, and 0.3%, respectively).

345 The individual phenolic compound present in the crude phenolic solution have
346 critical importance since each single compound can have different antiradical and

copigmentation power[36,37]. Thus, based on the results obtained, differences on the antioxidant and copigmentation properties of samples were expected as well, since different heterogeneous phenolic mixtures coexisting in competing equilibria might result in additive or suppressive effects[8,15].

Antioxidant activity

Results showed that the antioxidant activity was in accordance with the total phenolic content of each sample (Table 1). Thus, the crude phenolic solution from SD, with the highest phenolic content showed the greatest antioxidant activity (888.7 μmol TE/L) followed in decreasing order by those from GP, ST, and SK (463.3, 305.6, and 297.5 μmol TE/L, respectively).

Univariate linear regression was applied to these data in order to explore relationships between the total phenolic content and the antioxidant activity, and very strong and significant correlations (R^2 = 0.98, $p < 0.05$) were found, which indicate that Zalema winemaking by-products represent good sources of natural antioxidants with high added value.

Also a multiple regression analysis was performed to check the more influencing phenolic groups (independent variables: total phenolic acids, total flavanols and total flavonols) on the antioxidant activity (dependent variable). High multiple correlation coefficient (R^2= 0.99) and significant correlation ($p<0.05$) were obtained for the total flavanols (β= 0.98) followed by the total phenolic acids (β= -0.18).

On the other hand, two multiple regression analyses were carried out in order to determine the relative importance of individual phenolic compounds on the antioxidant activity. First, a regression analysis between antioxidant activity (dependent variable) and individual flavanols (independent variables) was performed to assess the influence

of these phenolic compounds. Results indicated good correlation (R^2=0.98) having catechin (β= 0.83), epicatechin (β= 1.01), trimer C1 (β= 1.28) and procyanidins B2 (β= 0.54) and B4 (β= 0.68) significant influence ($p<0.05$). Multiple regression analysis considering phenolic acids showed also high multiple correlation coefficient (R^2=0.99) having caffeic acid (β= -2.20), caftaric acid (β= 1.33), *trans*-coutaric acid (β= -1.20) and gallic acid (β= 1.22) more influence than *cis*-coutaric and protocatechuic acids.

Copigmentation effect

The crude anthocyanin solution obtained from Syrah skins was analyzed for its phenolic composition. Eleven anthocyans including non-acylated, acetylated and p-coumaroylated forms of the five expected anthocyanidins (delphinidin, cyanidin, petunidin, peonidin and malvidin) were identified by HPLC, which accounted for 95.8% of the total phenolic content. Also, the presence of other minor non-anthocyanin phenolic compounds was confirmed. They were mainly flavonols and phenolic acids which accounted for less than 5% of the total phenolic compounds identified. The relative proportion of each identified compound is presented in Table 2.

Figure 2 shows the magnitude of copigmentation (2a) and the Total Color (2b) of the crude anthocyanin solution containing increasing concentrations of crude phenolic solutions from Zalema winemaking by-products (PM, SK, SD and SK). Immediate intermolecular copigmentation was observed in the mixtures, which are evidenced by the hyperchromic shift of the λ_{max} of the crude anthocyanin solution (520 nm) and also by an increase of its initial Total Color (26.6 CIELAB u.). As observed, the magnitude of copigmentation differed depending on the type of winemaking by-product and the phenolic concentration applied. Stronger copigmentation effects were produced by the crude phenolic solutions from SK and PM, which induced the highest hyperchromic

shifts (from 2% to 28% and from 11% to 24%, respectively) and increases of the Total Color (from 26.6 to 34.4 and 32.5 CIELAB u., respectively). The effect was significantly ($p<0.05$) more pronounced with increasing copigment concentration. In contrast, SD and ST appeared as the less effective sources of copigments since the addition of the crude phenolics solutions at increasing levels not resulted always in progressive increases of the magnitude of copigmentation neither in the Total Color.

Our results indicate that the quantitative and qualitative profile of phenolic mixtures used as colorless copigments determined distinctive effects on the extend of multiple copigmentations, as previously reported by Gonzalez-Manzano et al.[15]. Higher relative proportions of more effective copigments as flavonols (mainly quercetin derivatives), oligomeric flavanols (mainly B-type procyanidins) or some phenolic acids in SK and PM samples could explain their greater copigmentation effect observed[36].

Also, it was confirmed that the color of the crude anthocyanin solution was improved by the crude phenolic solution from winemaking by-products, which was manifested through positive changes in the CIELAB parameters (L^*, C^*_{ab} and h_{ab}), showed in Figures 3a, 3b, and 3c). The progressive enrichment on colorless copigments resulted in decreasing lightness and hue values (L^* and h_{ab}) and increasing chroma (C^*_{ab}), which means a progressive darker and more saturated bluish color. However, important differences existed for the quantitative and qualitative color effects induced depending on the type of winemaking by-product. In this sense, crude phenolic solutions from PM and SK decreases the initial values of lightness approximately by 6% (from 86.5 to 81.3 and 81.8 CIELAB u. , respectively) and increases the chroma value by 24% and 19% (from 22.9 to 28.5 and 27.4 CIELAB u.). Concerning the qualitative attribute of color (h_{ab}), the effect was more important with crude phenolic solution from

421 PM and ST, which decreases the initial hue value approximately in 3 grades (from -
422 8.26º to -11.33º and -10.29º)

423 The Total Color Differences, $\Delta E^*_{ab\ (C-0)}$, between the crude anthocyanin solution
424 and those containing crude phenolic solution from winemaking by-products at
425 increasing concentration were calculated (Figure 4), which provide a relevant color
426 information related to visual perception. Moreover, the relative contribution of lightness
427 (%ΔL), chroma (%ΔC), and hue (%ΔH) to each color difference calculated permit us an
428 objective comparison of the colorimetric effect among the samples. Results showed that
429 the color changes were visually perceptible (ΔE^*_{ab}> 3, according to Martínez et al.[26]) for
430 the crude phenolic solution from PM at all the assayed concentrations, as well as for
431 most of those from SK. On contrast, samples from SD and ST resulted in color
432 differences lower than 3 CIELAB u. (not clearly perceptible). This fact confirm again
433 their poorer efficiency as copigments sources, which is consistent with the results
434 obtained for the magnitude of copigmentation and Total Color.

435 Regarding winemanking by-products causing perceptible color changes (SK and
436 PM), it can be observed that both samples induced similar effects independently of the
437 concentration tested. In general, the weight of the lightness and chroma modifications
438 were more marked than in hue (%ΔL=54% and 52%; %ΔC= 45% and 45.5; %ΔH= 0.1%
439 and 2%, as mean values respectively in SK and PM). This meant that for the same
440 concentration of the SK and PM crude phenolic solutions, comparable darkening and
441 greater quantity of color was induced without substantially modify the original hue.

442 In summary, white winemaking by-products from Zalema grape are rich sources
443 of phenolic compounds consisting of mainly flavanoids and phenolic acids. The
444 antioxidant activity of these compounds is related to the total phenolic content and
445 particularly to the flavanols and phenolic acids contents. The copigmentation effects in

446 model solution indicate that white winemaking by-products could improve the
447 anthocyanin color quality depending on the type of by-product used as copigment
448 source (PM, SK, SD, and ST) and the phenolic concentration applied, causing
449 perceptible color changes. Therefore, the exploitation for their potential reuse in the
450 wine industry could be of great interest, either considering the Zalema pomace or its
451 individual components (seeds, stems and skins).
452
453

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Note

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590 **FIGURE CAPTIONS**

591 **Figure 1.** RRLC chromatograms recorded at 280, 320 and 370 nm of crude phenolic
592 solution from seeds (---), skins (---), stems (---) and pomace (---). Peaks: a) 1, gallic
593 acid; 2, protocatechuic acid; 3, procyanidin B1; 4, procyanidin B3; 5, catechin; 6, trimer
594 C-C-EC; 7, tetramer; 8, procyanidin B4; 9, trimer C1; 10, procyanidin B2; 11,
595 epicatechin; 12, procyanidin B2-*O*-gallate. b) 1, caftaric acid; 2, *cis*-coutaric acid; 3,
596 *trans*-coutaric acid; 4, caffeic acid. c) 1, quercetin 3-*O*-rutinoside; 2, quercetin 3-*O*-
597 glucuronide; 3, quercetin 3-*O*-glucoside; 4, quercetin pentose; 5, kaempferol hexoside;
598 6, kaempferol 3-*O*-glucoside; 7, isorhamnetin 3-*O*-glucoside; 8, quercetin; 9,
599 kaempferol.

600 **Figure 2**. (a) Magnitude of copigmentation and (b) Total Color of the crude anthocyanin
601 solution containing increasing concentrations of crude phenolic solutions from Zalema
602 winemaking by-products (skins: SK, pomace: PM, stems: ST, and seeds: SD); mean ±
603 SD, n=3. Different letters in the same by-product mean significant differences ($p< 0.05$).

604 **Figure 3**. Changes in (a) Lightness (L*), (b) Chroma (C^*_{ab}), and (c) Hue (h_{ab}) of the
605 crude anthocyanin solution after adding increasing concentrations of crude phenolic
606 solutions from Zalema winemaking by-products (skins: SK, pomace: PM, stems: ST,
607 and seeds: SD) (mean±SD, n=3).

608 **Figure 4**. Total Color Difference induced by copigmentation ($\Delta E^*_{ab(c-0)}$) with the relative
609 contribution of lightness, chroma and hue (%ΔL, %ΔC, %ΔH). Calculated between the
610 color of the crude anthocyanin solution and after adding increasing concentrations of
611 the crude phenolic solution from Zalema winemaking by-products (skins: SK, pomace:
612 PM, stems: ST, and seeds: SD);

613

TABLES

Table 1. Mean values and standard deviations (n=3) of the phenolic composition (mg/L), Total phenolic content (mg GAE/L) and antioxidant activity (μmol TE/L), for the crude phenolic solution from winemaking by-products (PM: pomace, SK: skins, SD: seeds and ST: stems).

	PM	SK	SD	ST
Total phenolic content[a]	1138.42 ± 0.19$_a$	1049.01 ± 3.78$_b$	2575.21 ± 5.59$_c$	1000.56 ± 6.72$_b$
Total phenolic acids[b]	45.93 ± 0.43$_a$	28.16 ± 0.46$_b$	30.56 ± 0.55$_c$	31.34 ± 0.10$_d$
Total flavanols[c]	92.32 ± 1.23$_a$	50.79 ± 2.65$_b$	141.13 ± 0.76$_c$	50.54 ± 0.07$_b$
Total oligomers[d]	77.26 ± 0.37$_a$	40.91 ± 2.57$_b$	98.97 ± 0.53$_c$	37.89 ± 0.11$_b$
Total flavonols[e]	3.31 ± 0.00$_a$	5.61 ± 0.01$_b$	0.56 ± 0.01$_c$	1.46 ± 0.07$_d$
Antioxidant activity	463.29 ± 41.78$_a$	297.53 ± 15.87$_b$	888.73 ± 21.78$_c$	305.57 ± 15.42$_b$
Benzoic acids				
Gallic acid	36.13 ± 0.35$_a$	22.33 ± 0.39$_b$	30.18 ±0.04$_c$	20.12 ± 0.07$_d$
Protocatechuic acid	0.98 ± 0.19$_a$	0.41 ± 0.03$_b$	n.d.	0.15 ± 0.01$_c$
Hydroxycinnamic acids				
Caftaric acid	6.40 ± 0.01$_a$	3.44 ± 0.06$_b$	n.d.	8.67 ± 0.01$_c$
Caffeic acid	1.46 ± 0.01$_a$	1.32 ± 0.01$_b$	n.d.	1.92 ± 0.02$_c$
cis-Coutaric acid	0.26 ± 0.02$_a$	0.28 ± 0.01$_a$	0.15 ± 0.01$_b$	0.17 ± 0.02$_b$
trans-Coutaric acid	0.70 ± 0.01$_a$	0.38 ± 0.02$_b$	0.23 ± 0.02$_c$	0.31 ± 0.01$_d$
Flavanols				
(+)-Catechin (C)	7.86 ± 0.98$_a$	6.32 ± 0.04$_b$	22.03 ± 0.07$_c$	8.90 ± 0.03$_d$
(-)-Epicatechin (EC)	7.20 ± 0.25$_a$	3.55 ± 0.04$_b$	20.10 ± 0.37$_c$	3.74 ± 0.01$_d$
Procyanidin B1	25.03 ± 0.07$_a$	17.37 ± 1.20$_b$	20.51 ± 0.03$_c$	17.43 ± 0.03$_d$
Procyanidin B2	4.94 ± 0.81$_a$	4.08 ± 0.03$_a$	7.08 ± 0.07$_b$	1.46 ± 0.02$_c$
Procyanidin B3	5.22 ±0.07$_a$	3.27 ± 0.5$_b$	4.68 ± 0.05$_a$	2.52 ± 0.04$_b$
Procyanidin B4	8.05 ± 0.33$_a$	4.52 ± 0.03$_b$	11.17 ± 0.07$_c$	4.16 ± 0.02$_d$
Trimer C-C-EC	4.72 ± 0.53$_a$	2.72 ± 0.05$_b$	5.43 ± 0.05$_c$	5.50 ± 0.02$_c$
Trimer C1	4.77 ± 0.50$_a$	1.66 ± 0.03$_b$	10.80 ± 0.04$_c$	0.98 ± 0.03$_d$
Tetramer	5.80 ± 0.59$_a$	3.52 ± 0.29$_b$	9.08 ± 0.06$_c$	1.33 ± 0.02$_d$
Procyanidin B2-3-*O*-gallate	18.73 ± 0.24$_a$	3.78 ± 0.95$_b$	30.22 ± 0.26$_c$	4.52 ± 0.09$_d$
Flavonols				
Quercetin 3-*O*-rutinoside	0.17 ± 0.01$_a$	0.19 ± 0.01$_a$	0.17 ± 0.02$_a$	0.21 ± 0.07$_a$
Quercetin 3-*O*-glucuronide	0.38 ± 0.01$_a$	0.57 ± 0.01$_b$	0.15 ± 0.01$_c$	0.20 ± 0.01$_d$
Quercetin 3-*O*-glucoside	2.07 ± 0.03$_a$	3.48 ± 0.01$_b$	0.24 ± 0.04$_c$	0.86 ± 0.01$_d$
Kaempferol hexoside	0.20 ± 0.01$_a$	0.27 ± 0.01$_a$	n.d.	traces
Kaempferol 3-*O*-glucoside	0.18 ± 0.03$_a$	0.65 ± 0.01$_b$	n.d.	0.19 ± 0.01$_a$
Isorhamnetin 3-*O*-glucoside	0.15 ± 0.01$_a$	0.19 ± 0.01$_a$	n.d.	n.d.
Quercetin	0.16 ± 0.01$_a$	0.26 ± 0.02$_b$	n.d.	traces
Kaempferol	n.d.	traces	n.d.	traces

Different letters in the same row mean significant differences ($p < 0.05$) by Tukey test.
[a] as Folin-Ciocalteu method; [b,c,d,e] Sum of individual phenolic acids, flavanols, oligomers and flavonols identified.
n.d.: no detected.

Table 2. Concentration (mg/L±SD, n=3) and distribution of individual phenolic compounds (%) identified by HPLC in the crude anthocyanin solution prepared from the Syrah grape skins.

	Concentration (mg/L)	Relative proportion (%)
Total anthocyanins[a]	222.65 ± 10.03	95.8
Total phenolic acids[b]	1.97 ± 0.02	0.9
Total flavanols[c]	7.63 ± 0.97	3.3
Anthocyanins		
Delphinidin-3-glucoside	3.12 ± 0.09	4.2
Cyanidin-3-glucoside	3.12 ± 0.14	1.3
Petunidin-3-glucoside	12.42 ± 1.25	5.3
Peonidin-3-glucoside	11.79 ± 1.62	5.1
Malvidin-3-glucoside	98.32 ± 2.49	42.3
Petunidin-3-acetyl-glucoside	5.35 ± 0.71	2.3
Peonidin-3-acetyl-glucoside	8.09 ± 1.13	3.6
Malvidin-3-acetyl-glucoside	39.47 ± 1.90	16.9
Petunidin-3-*p*-coumaroyl-glucoside	2.9 ± 0.21	1.3
Peonidin-3-*p*-coumaroyl -glucoside	6.34 ± 0.36	2.7
Malvidin-3-*p*-coumaroyl -glucoside	25.13 ± 2.1	10.8
Hydroxycinnamic acids		
Caftaric acid	0.25 ± 0.01	0.1
cis-Coutaric acid	0.76 ± 0.01	0.4
trans-Coutaric acid	0.93 ± 2.1	0.4
Flavanols	traces	-
Flavonols		
Myricetin-3-glucuronide	0.96± 0.01	0.4
Myricetin-3-glucoside	0.89± 0.01	0.4
Quercetin-3-glucuronide	0.97 ± 0.03	0.4
Quercetin-3-glucoside	2.95 ± 0.02	1.3
Laricitrin-3-glucoside	0.18 ± 0.01	<0.1
Kaempferol-3-glucoside	0.14 ± 0.01	<0.1
Isorhamnetin-3-glucoside	0.92± 0.01	0.4
Syringetin-3-glucoside	0.59± 0.01	0.3

[a,b,c] Sum of individual anthocyanins, phenolic acids, flavonols identified

FIGURES

Figure 1.

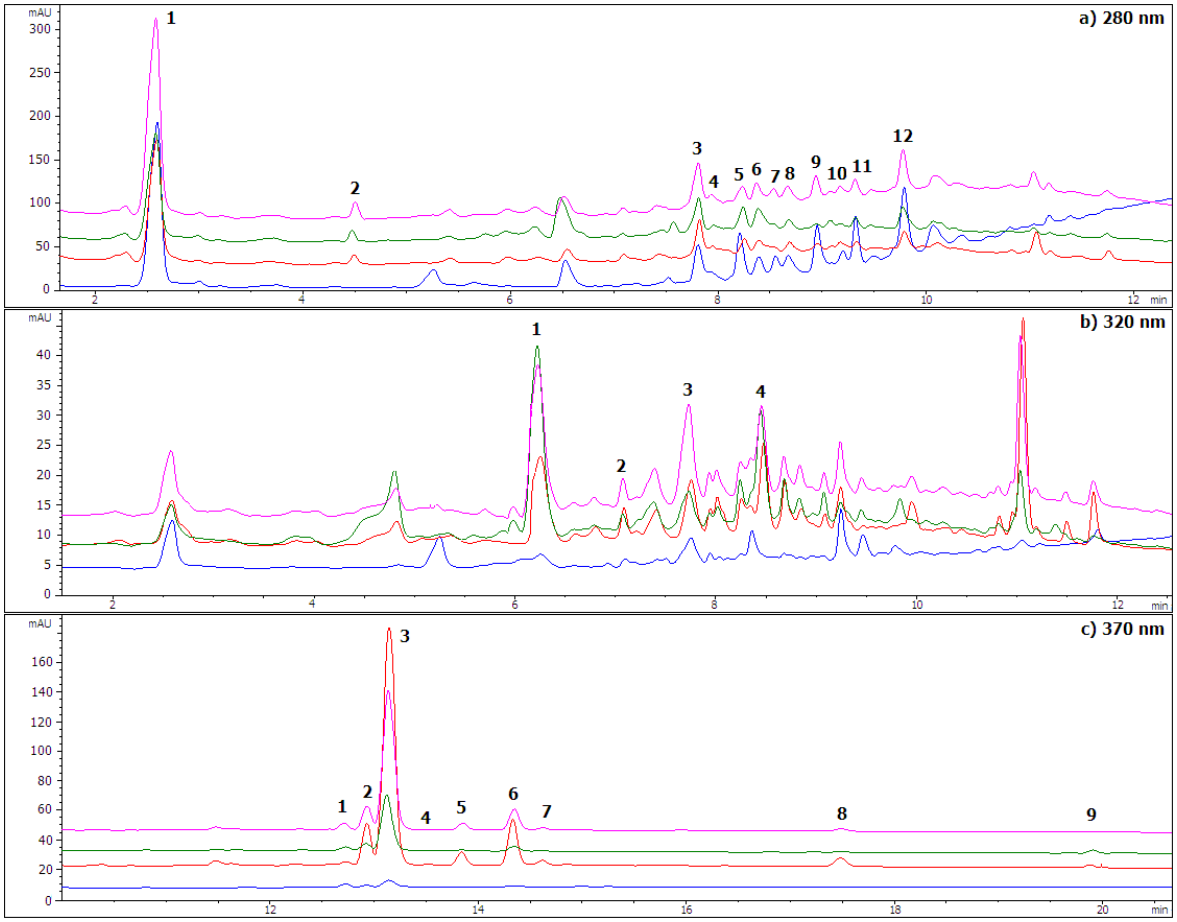


Figure 2a.

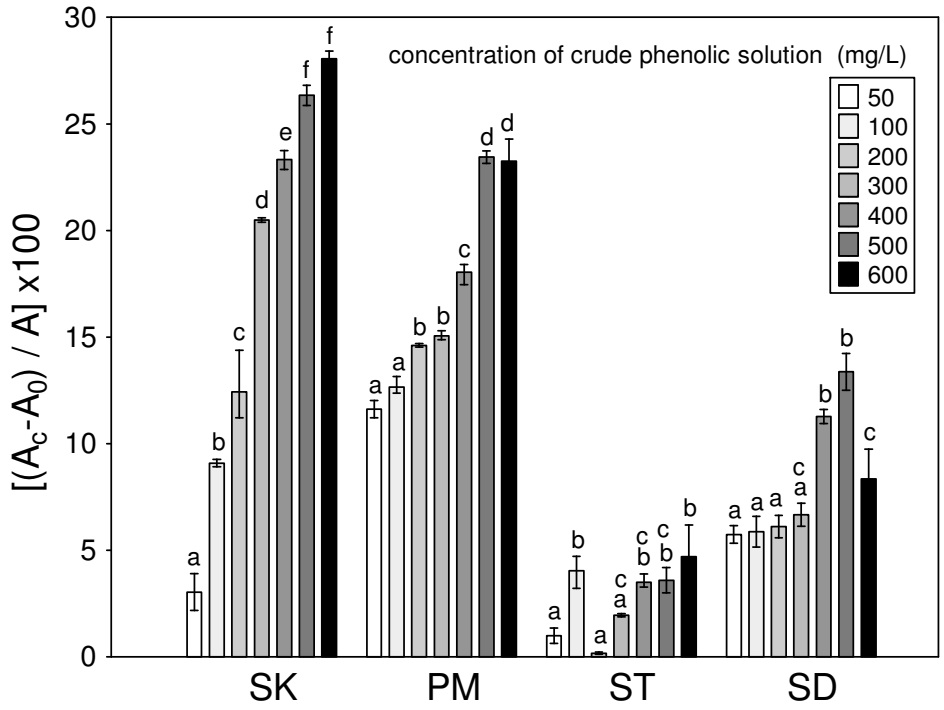


Figure 2b.

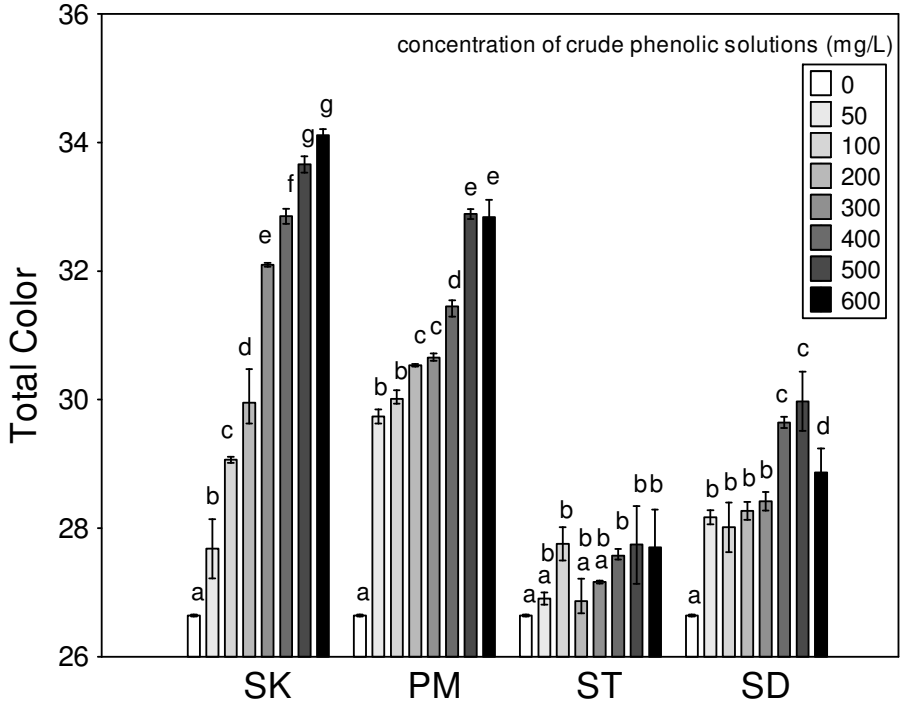
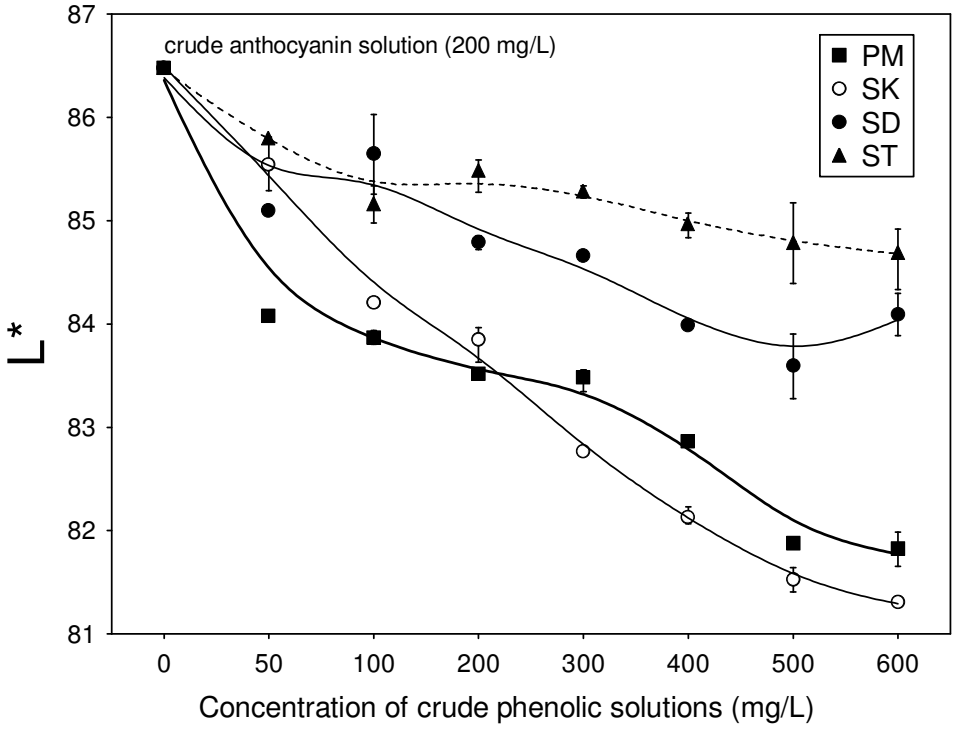


Figure 3a.



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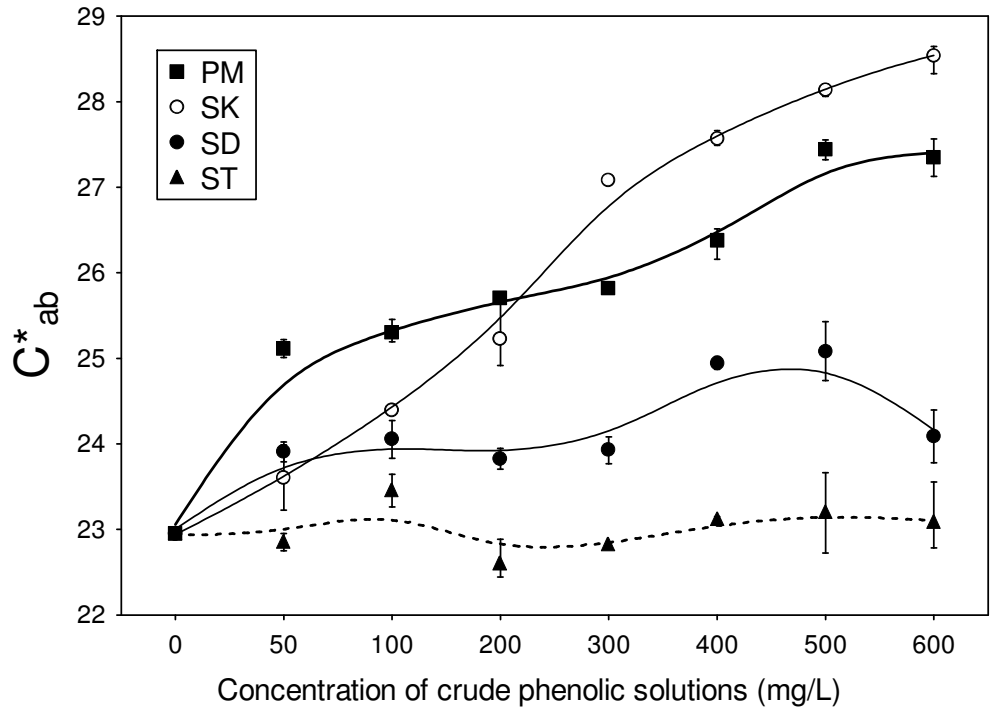

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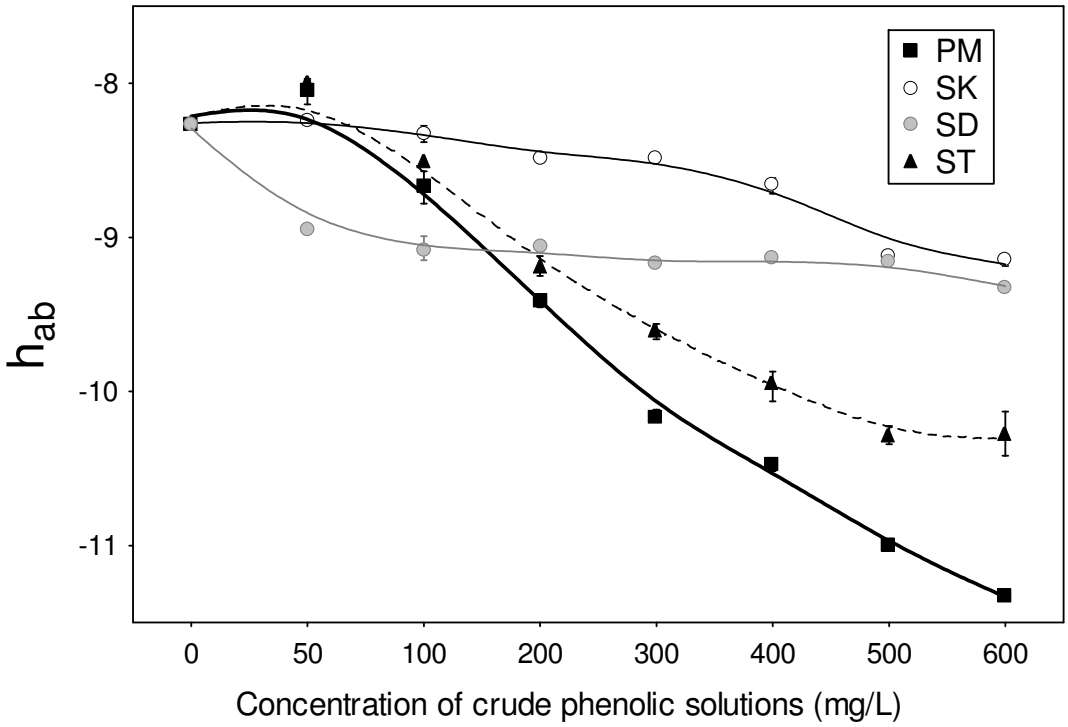
PM
SK
SD
ST
-8
-9
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0
50
100
200
300
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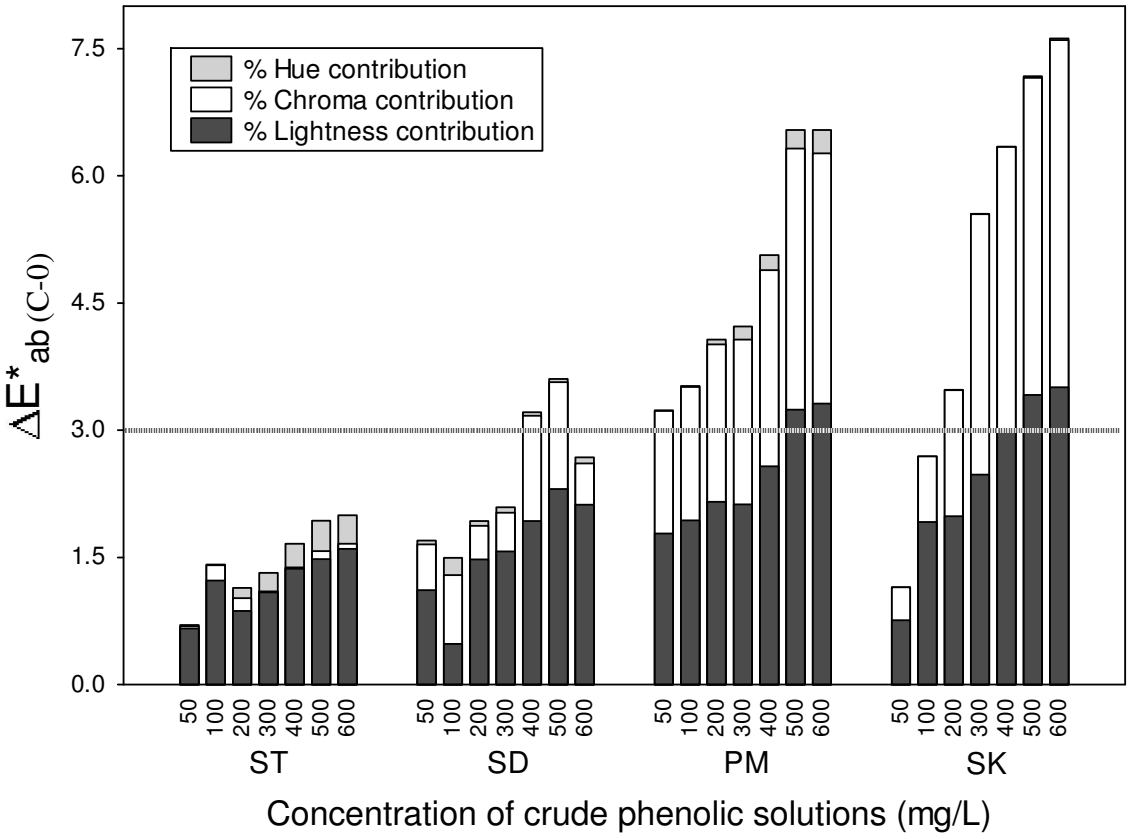
500
600
Concentration of crude phenolic solutions (mg/L)

Figure 4.


% Hue contribution
% Chroma contribution
% Lightness contribution
ΔE*ab (C-0)
7.5
6.0
4.5
3.0
1.5
0.0
50
100
200
300
400
500
600
ST
SD
PM
SK
Concentration of crude phenolic solutions (mg/L)