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Comparison of chromatic properties, stability and antioxidant capacity of anthocyanin-based aqueous extracts from grape pomace obtained from different vinification methods

Encarna Gómez-Plaza *, Angeles Miñano, Jose María López-Roca

Departamento de Tecnología de Alimentos, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30071 Murcia, Spain

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Abstract

The chromatic properties of anthocyanin-based aqueous extracts obtained from grape pomace from different vinifications methods (one rosé vinification, 6 h of skin contact time and three red wine vinifications, with 4, 8 and 12 days of skin contact time) have been studied. Differences due to the vinification method from which the pomace was obtained were clearly observed. Extracts from rosé vinifications had the highest content of anthocyanin compounds (measured both spectrophotometrically and by HPLC methods) although the total phenol content did not differ significantly between the different pomace extracts. As regards the stability of the different aqueous extracts under different pH, temperature and light conditions, only small differences were observed. Also, the antioxidant capacity of the extracts was evaluated and no significant differences were found between them; a better correlation being found between antioxidant capacity and estimated total phenol content than with anthocyanin content.
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Keywords: Anthocyanins; Colorants; Grape pomace; Stability; Antioxidant capacity

1. Introduction

Anthocyanins are of interest in the food, cosmetic and pharmaceutical industries as they can be used as substitutes for synthetic colorants and antioxidants. Natural plant colorants are in high demand by the food industry to replace synthetic dyes. However, replacing synthetic dyes is a challenging task because they tend to show greater stability with respect to light, oxygen, temperature and pH, among other factors (Cevallos-Casals & Cisneros-Zevallos, 2004). Although anthocyanins have a high potential for use as natural colorants due to their attractive colors and innocuousness (Giusti & Wrolstad, 2003; Pazmino-Duran, Giusti, Wrolstad, & Gloria, 2001), they do present stability problems. The color and stability of anthocyanin pigments are dependent on several factors, including structure, concentration, pH, temperature, light, presence of copigments, metallic ions, enzymes, etc.

Another favourable aspect of anthocyanins is that they contribute greatly to the antioxidant properties of certain foods (Einbond, Reynertson, Luo, Basile, & Kennelly, 2004) and so there is considerable interest in the possible health effects of these compounds (Chidambara Murthy, Singh, & Jayaprakasha, 2002; Kahkonen, Heinamaki, Ollilainen, & Heinonen, 2003), and a variety of health-promoting products obtained from by-products of grape and wine industry have been introduced to the market.

Anthocyanin-rich extracts from fruit and vegetables are interesting as food colorants, especially when they can be obtained from otherwise waste materials (Clifford,

* Corresponding author. Tel.: +34 968 367323; fax: +34 968 364147.
E-mail address: encarnag@um.es (E. Gómez-Plaza).

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2000). For example, the grape pomace from the winemaking industry. Anthocyanins accumulate primarily in the skins of grape berries and are extracted into the must and wine during skin contact period. However, the extraction of pigments from grape skins prior to, during or following fermentation is far from complete, typically reaching only 30–40% (Singleton & Esau, 1969; Van Balen, 1984). Therefore, grape pomace potentially constitutes a very abundant and relatively inexpensive source of anthocyanins (Malien-Aubert, Dangles, & Amiot, 2001). Grape pomace, obtained during wine production, consists of pressed skins and seeds, representing as much as 20% of the original weight of the grapes. Anthocyanins are probably the most valuable components of grape pomace and a large number of methods for their extraction have been reported (Mazza, 1995).

When the objective is obtaining colorant or antioxidant products for the food industry, water and/or hydroalcoholic extractants are usually preferred (Cacace & Mazza, 2002; Ju & Howard, 2003; Prodanov, Domínguez, Blazquez, Salinas, & Alonso, 2002), although the regulations of some countries only permit water to be used as the extracting solvent (Giusti & Wrolstad, 2003). Particularly, the extraction with sulphited water has been extensively used in the extraction of anthocyanin-type pigments from fruit and vegetables. An extensive review of methods used can be found in Francis (1989) and Mazza (1995).

When using solvent extraction for the isolation of anthocyanins, the extraction procedure is not selective and a number of other plant constituents may be co-extracted along with the desired pigments (Dupuy, Combe, & Salgues, 1980). Sugars, for example, may lead to Maillard type browning reactions and their decomposition products may increase the anthocyanin degradation rate (Cevallos-Casals & Cisneros-Zevallos, 2004). To combat this problem, purification by solid phase extraction (SPE) with different adsorbents is a relatively simple method, allowing the elimination of polar, non-phenolic impurities in one step (Coutinho, Quadri, Moreira, & Quadri, 2002; Kohler, José, Quadri, Quadri, & Moreira, 2002). Kraemer-Schafhalter, Fuchs, and Pfenhauser (1998) tested several resins and, among them, Amberlite XAD-7 permitted a good separation of anthocyanins.

When selecting the grape pomace source, not only the pigment yield, but also the color quality imparted by the structural modifications of anthocyanins and accompanying matrix compounds should be taken into consideration (Stintzing & Carle, 2004). Based on that, we have studied the chromatic characteristics, stability and antioxidant capacity of four different grape pomaces, obtained from one rosé wine vinification and three different red wine vinifications. Although it seems clear that the winemaking technique will have an effect on the characteristics of the grape pomace, a fact assumed by several authors (Larrauri, Ruperez, & Saura Calixto, 1996; Schieber, Stintzing, & Carle, 2001), the effect of the vinification method on the chromatic characteristics, stability and antioxidant power of the aqueous extracts obtained from these pomaces has not previously been reported.

2. Materials and methods

2.1. General

All the extraction experiments were carried out with *Vitis vinifera* L. (Monastrell variety) grape pomace. The pomace was separated from the liquid (must or wine) after pressing in a pneumatic press and was kept at −18 °C until analysis. The grape pomace was obtained from four different vinifications carried out in 100 l tanks: rosé vinification (6 h of skin contact time), and three red wine vinifications (4, 8 and 12 days of maceration, M4, M8 and M12, respectively). All vinifications were made with the same lot of grapes.

Three hundred grams of grape pomace were placed in a 2 l glass container and extracted for 72 h with 900 ml of extractant (sulphited water (1 g/l) at 60 °C), with occasional stirring. After this time, the aqueous extract was separated from the grape pomace and centrifuged to eliminate impurities. Experiments were made in triplicate.

The aqueous extract was passed through an Amberlite XAD-7 column (Fluka, Germany) for purification purposes. The adsorbent (45 g) was soaked in water and undersized particles were removed with the supernatant. The slurry was packed in an Extrelut NT 20 column (Varian, CA) and washed with 100 ml of distilled water before the aqueous extract was passed through. Anthocyanins were retained in the column, which was then washed with 500 ml of distilled water to wash out water-soluble impurities (the absence of sugars was checked with a hand refractometer). The adsorbed pigments were eluted using 120 ml of absolute ethanol. The ethanolic extract was concentrated under vacuum at 30 °C until dryness and the dry residue redissolved in 100 ml of water. After that, the samples were centrifuged to eliminate impurities and the pH of the supernatant was adjusted to three, so all the chromatic determinations are done at the same pH. The absence of anthocyanins bound to SO_2 was checked by measuring the absorbance at 520 nm of the samples before and after adding 35 μl of 10% acetaldehyde to 3 ml of extract diluted 1:10 with water and observing that no differences in absorbance was detected.

2.2. Chromatic determinations

Absorbance measurement were done, diluting when necessary, using a UV–Vis spectrophotometer (Helios

Alpha Thermospectronic, Thermo Electronic Co., USA) with 0.2 cm or 1 cm path length glass cells, and all absorbance values were corrected to 1 cm path length.

Absorbances at 280 and 365 nm and the determination of polymeric pigments were performed using a method adapted from that of Levengood and Boulton (2004). Absorbances at 280 nm (A_{280}) and 365 nm (A_{365}) were measured for the estimation of total phenol content and the flavonol content, respectively, using standard solutions of chlorogenic acid and quercetin for quantification. Polymeric pigments (PPC) were evaluated by measuring the A_{520} of the sample after addition of SO_2 for decoloration of monomeric anthocyanins.

Color intensity (as the sum of absorbances at 420, 520 620 nm) and brown index (expressed as the absorbance ratio at 420 nm by that at 520 nm) were determined according to Glories (1984). Total anthocyanins (mg/l) were measured spectrophotometrically following the method described by Cayla, Cottereau, and Renard (2002).

CIELab characteristics of anthocyanin-containing solutions were determined in the samples, diluted 1:10, by measuring the transmittance of the wine every 10 mm from 380 to 770 nm, using the D_{65} illuminant and a 10° observer, with 0.2 cm path length glass cells. The spectrophotometer has the necessary software to calculate the CIELab parameters directly. The chroma (C) and hue angle ($H°$) were calculated by the formulae $C = (a^{*2} + b^{*2})^{1/2}$ and $H° = (\tan^{-1} a^*/b^*)$.

Monomeric anthocyanins were determined by direct injection of the extract into a Waters 2690 liquid chromatograph (Waters, PA, USA), equipped with a Waters 996 diode array detector and a Licrochart RP-18 column (Merck, Darmstadt, Germany), 25 × 0.4 cm, 5 μm particle size, using as solvents water plus 4.5% formic acid (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 1.5 ml/min. Elution was performed with a gradient starting with 10% B to reach 15% B at 25 min, 21% B at 65 min, and then became isocratic for 15 min. Chromatograms were recorded at 520 nm. Identification and quantification (using malvidin-3-glucoside chloride (Extrasynthèse, Genay, France) as external standard) were previously described (Gómez Plaza, Gil Muñoz, López Roca, & Martínez, 2000).

2.3. Stability tests

Color stability was studied at three different pH values (3, 4, 5), at three different temperatures (10, 25, and 38 °C) and in daylight or dark conditions. Colorant concentration was adjusted as to develop an initial A_{520} of 0.5 (±0.05) at pH 3. Colorant solutions (10 ml) were put in flask tubes closed with screw caps. Each tube was used for one spectral measurement only so as to minimize contact with oxygen.

2.4. Antioxidant capacity

The total antioxidant potential of samples was determined using a ferric reducing antioxidant power (FRAP) assay, a simple direct test of antioxidant capacity that measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII–tripyridyltriazine compound from the colorless oxidized FeIII form by the action of electron donating antioxidants. The procedure described by Katalinic, Milos, Modun, Music, and Boban (2004) was followed. The working FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer at pH 3.6 with 2.5 ml of 10 mM TPTZ in HCl 40 mM and 2.5 ml of 20 mM ferric chloride. Freshly prepared FRAP reagent was warmed to 37 °C and a reagent blank reading was taken at 593 nm (M1). Subsequently, 10 μl of sample and 30 μl of deionized water was added to the FRAP reagent. Absorbance measurements were monitored during 8 min. Because there was little decrease in absorbance between 4 and 8 min we used its value after 4 min for further calculations. The samples were incubated at 37 °C throughout the monitored period. The change in absorbance between the final reading selected (4 min) and the M1 readings were selected for calculations of FRAP values. Standard curves were prepared using a FeII solution of known concentration. The results were corrected for dilution.

2.5. Statistical data treatment

Significant differences among wines and for each variable were assessed with analysis of variance (ANOVA). This statistical analysis was performed using Statgraphics 2.0 Plus (Statistical Graphics Corp., USA).

3. Results and discussion

3.1. Effect of the different vinification method on the chromatic properties of the grape pomace extracts

Fig. 1 shows a chromatogram of the extracts and Fig. 2 shows the monoglucoside content of the different samples and the sum of monoglucosides. Malvidin-3-monoglucoside was the predominant monoglucoside in all the samples and only traces of acylated derivatives were detected. The highest concentration of anthocyanin monoglucosides was found in the aqueous extract obtained from the rosé pomace and the profile of the distribution of the different monoglucosides was the closest to that described for Monastrell grapes, with cyanidin-3-monoglucoside being found in a relatively large proportion (Fernandez-Lopez, Hidalgo, Almela, & López-Roca, 1992). In the other extracts (M4, M8 and M12), this monoglucoside was found in lower

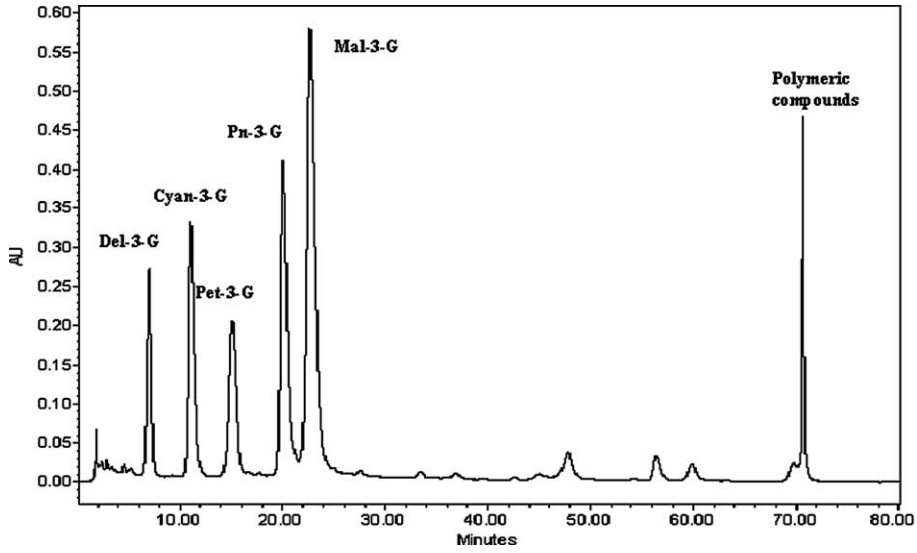

Fig. 1. Chromatogram of M8 extract (Del-3-G: Delphinidin-3-glucoside; Cyan-3-G: cyanidin-3-glucoside; Pet-3-G: petunidin-3-glucoside; Pn-3-G: peonidin-3-glucoside; Mal-3-G: malvidin-3-glucoside).

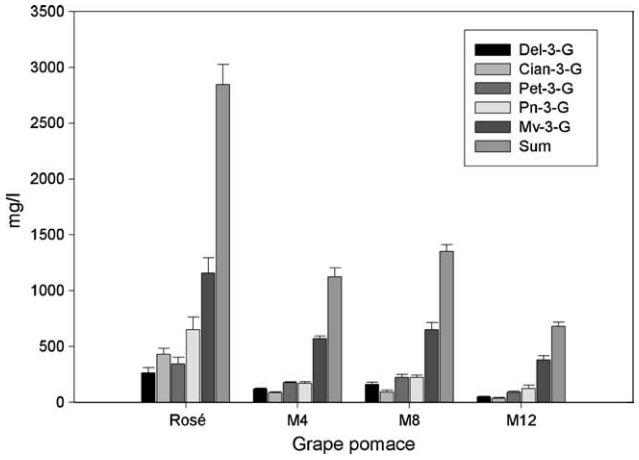

Fig. 2. Concentration of the anthocyanin monoglucosides in the aqueous extract depending on the vinification method (Del-3-G: Delphinidin-3-glucoside; Cyan-3-G: cyanidin-3-glucoside; Pet-3-G: petunidin-3-glucoside; Pn-3-G: peonidin-3-glucoside; Mal-3-G: malvidin-3-glucoside; Rosé: extracts from rosé vinification; M4, M8 and M12: extracts from pomace from red wine vinification after 4, 8 or 12 days of skin contact time).

proportion. Fernandez-Lopez, Almela, Muñóz, Hidalgo, and Carreño (1998) established that the proportion of cyanidin-3-glucoside decreased from grapes to wine perhaps because of its instability. García-Beneytez, Revilla, and Cabello (2002) also stated that cyanidin-3-glucoside was present in grapes but not in wines or skins after red wine vinification.

The results of the spectrophotometrical analyses are shown in Table 1. Luminosity (L^*) was lower in the grape pomace extract from the rosé vinification and the highest value was found in M12 extracts. Chromaticity and hue angle were quite similar among the studied extracts.

The color intensity was higher in rosé pomace extracts, at double the values of the red wine pomace extracts, with no significant differences between the M4, M8 and M12 extracts. The highest brown index was found in the M12 extracts. Polymeric pigment values were higher in the M8 and M12 extracts, while lower and similar values were found for the rosé and M4 extracts. No significant differences were found in total phenols for the different extracts.

The flavonol content was also higher in the rosé extract. These compounds are mainly located in the skin of the grape berries and their concentration in wines increase with maceration time (Gil-Muñoz, Gómez-Plaza, Martínez, & López-Roca, 1999), decreasing at the same time in the skins.

The concentration of total anthocyanins, expressed in mg/l, is higher than the sum of the different monoglucosides (measured by HPLC) but this is due to the quantification method, based on spectrophotometric measurements. When measuring absorbance at 520 nm for the determination of total anthocyanins, not only free anthocyanins are measured but also all the polymeric compounds that absorb at this wavelength. The concentration of anthocyanins, when expressed as g/kg of grape pomace ranged from 1.4 for rosé pomace to 0.5 in the M12 extract. Prodanov and Salinas (1999) found yields of 0.64–1.18 g/kg in Tempranillo pomace and Muñoz, Sepulveda, and Schwartz (2002) found 495 mg/l in Carmenere grape skins extracted with sulphited water. Thus, we have obtained a good recovery of anthocyanins.

The pomace extract resulting from rosé vinification would be the best source of anthocyanins and the one with the highest color intensity. M4 and M8 extracts had similar results in color intensity, anthocyanin con-

Table 1
Chromatic characteristics of the aqueous extracts depending on the vinification method

	Rosé	M4	M8	M12
*L**	20.6a	32.6b	34.4b	39.7c
*C**	62.4a	65.3ab	69.4b	67.6b
*H**	32.6b	25.3a	31.7b	30.2b
Color intensity	102.4b	51.2a	52.8a	42.1a
Brown index	0.4a	0.6ab	0.4a	0.7b
Total phenols[a]	131.9a	136.3a	138.2a	123.5a
Flavonol content[b]	919.7c	683.0b	617.9ab	515.5a
Polymeric pigments[c]	4.2a	4.4a	5.8b	5.3b
Anthocyanins[d](mg/l)	4051.5c	2244.2b	2457.3b	1466.6a
Anthocyanins[e] (g/kg)	1.3c	0.75b	0.82b	0.49c

Different letter within the same row means significant differences ($p < 0.05$) according to a LSD test.
Rosé: extracts from rosé vinification; M4, M8 and M12: extracts from pomace from red wine vinification after 4, 8 or 12 days of skin contact time.
[a] Expressed as mg/l of chlorogenic acid.
[b] Expressed as mg/l of quercetin.
[c] Absorbance units.
[d] Expressed as mg/l of malvidin-3-glucoside.
[e] Expressed as g of malvidin-3-glucoside per kg of pomace.

centration and flavonol content. It seems that from day 4 to day 8 of maceration during vinification there is a low degree of extraction of anthocyanins and phenolic compounds from skins. Several studies have shown that the maximum pigment extraction in must/wine is reached during the first days of alcoholic fermentation and further skin contact time has no effect on increasing pigment concentration (Nagel & Wulf, 1979; Ribereau-Gayon, 1982; Somers, 1980; Van Balen, 1984). The evidence suggests that an equilibrium based on adsorption–desorption is established between the concentrations of anthocyanins in the wine and the cellular concentration in the skin tissue, and that, when this equilibrium is attained, no more anthocyanins can be extracted from skins to wine, which could be the reason for the similar concentration of anthocyanins in the M4 and M8 extracts. It should be assumed that grape pomace after 12 days of maceration had a more degraded structure due to the several days of maceration in a hydroalcoholic medium (wine). Therefore, the extraction of skin tannins was facilitated and thus more polymeric compounds and pigments that absorb at 420 nm were extracted. Moreover, during the extraction and processing of this pomace, a proportion of the monomeric anthocyanins might become degraded and/or condensed with other flavonoids to form polymeric pigments (Bridle & Timberlake, 1997). All these facts led to the M12 extract to present the highest brown index values and polymeric pigment content, and the lowest anthocyanin concentration and color intensity.

3.2. Stability of the different aqueous extracts

The results of the stability test (Table 2) showed that there was little difference in the stability of the colorant solutions according to the vinification method, the behaviour of the extracts being very similar in the different conditions, although M12 had a slightly higher thermal stability than the other treatments (the absorbance values decreasing to 65–58% of that of the initial solutions, compared with the other extracts, that experienced a larger decrease in absorbance) and rosé extracts had the lowest brown index in the different conditions. As temperature increases the stability decreases. At a storage temperature of 10 °C, the A_{520} did not decrease with time, there was even a slight increase, whereas at 25 °C the decrease ranged between 10% and 17% and at 38 °C this decrease was found to be between 35% and 49%. The increase in A_{520} observed after 15 days at 10 °C could be due to a temperature effect since as temperature increases the dissociation of copigmented forms is favoured and may actually cause a loss in the color due to copigmentation (Boulton, 2001). The effect of low temperatures was also observed by Fossen, Cabrita, and Andersen (1998), one sample containing cyanidin-3-glucoside, after 60 days at 10 °C retaining at least 90% of its color.

The samples exposed to daylight showed a more pronounced decrease in A_{520} than those kept in the dark, reaching the difference of 15% at 38 °C in rosé extracts.

The M4 extract was also studied at three different pHs (3, 4 and 5) at 25 °C and in daylight. The initial A_{520} of the M4 extract at pH 4 and 5 was only 53% and 25% of that at pH 3, respectively. The decrease in absorbance readings as pH increased was due to the hydration of the red flavylium cation to yield the colorless carbinol form. However, the stability, measured as the percentage of decrease in A_{520} at the pH considered after 15 days, reached similar values to those found at pH 3.

Anthocyanins are very unstable molecules and their stability can be increased with copigmentation.

Table 2
Effect of pH, temperature and light on the stability of the different aqueous extracts

Colorant	pH	Temperature	Light	Days	BI	$\%A_{520}$
Rosé	3			0	0.34	100
Rosé	3	10	No	15	0.31	105
Rosé	3	25	No	15	0.33	90
Rosé	3	25	Yes	15	0.35	81
Rosé	3	38	No	15	0.55	51
Rosé	3	38	Yes	15	0.55	45
M4	3			0	0.35	100
M4	3	10	No	15	0.36	102
M4	3	25	No	15	0.42	83
M4	3	25	Yes	15	0.44	79
M4	3	38	No	15	0.74	55
M4	3	38	Yes	15	1.32	44
M4	4	25	Yes	0	0.75	100(53)[a]
M4	4	25	Yes	15	0.54	93
M4	5	25	Yes	0	0.68	100(25)[a]
M4	5	25	Yes	15	0.94	81
M8	3			0	0.37	100
M8	3	10	No	15	0.38	103
M8	3	25	No	15	0.42	90
M8	3	25	Yes	15	0.44	88
M8	3	38	No	15	0.70	58
M8	3	38	Yes	15	0.74	54
M12	3			0	0.45	100
M12	3	10	No	15	0.46	102
M12	3	25	No	15	0.52	89
M12	3	25	Yes	15	0.55	84
M12	3	38	No	15	0.82	65
M12	3	38	Yes	15	0.93	58

Rosé: extracts from rosé vinification; M4, M8 and M12: extracts from pomace from red wine vinification after 4, 8 or 12 days of skin contact time.
[a] The values between parentheses represent the decrease of A_{520} respect to the A_{520} of the extract at pH 3.

Considering the lack of acylated anthocyanins in our samples, intramolecular copigmentation will not occur and intermolecular copigmentation must have played a major role in the stabilization of these aqueous extracts. As Malien-Aubert et al. (2001) stated, copigmentation is more efficient in samples displaying higher copigment/pigment ratios. Aqueous fruit extracts with a high anthocyanin content contain mixtures of different compounds that may serve as copigments for intermolecular association with anthocyanins (Cevallos-Casals & Cisneros-Zevallos, 2004). If we calculate the copigment/pigment ratio (flavonol/anthocyanins) of our extracts we could see how the M12 extract had the highest ratio and this extract was the one exhibiting the highest thermal and light stability. It has been stated that copigments from the flavonol subgroups, which strongly absorb damaging UV radiations, must provide additional protection against photodegradation of the colored forms (Malien-Aubert et al., 2001). Eiro and Heinonen (2002) also showed that malvidin-3-monoglucoside alone lost color quickly after 55 days at room temperature and daylight and copigment addition increased anthocyanin color stability.

3.3. Antioxidant capacity

Reactive oxygen species (ROS) are constantly generated in vivo for physiological purposes and often over-produced in pathological conditions resulting in oxidative stress (Guo et al., 2003). Several methods have been developed to evaluate the total antioxidant activity of foods and derivatives. One method commonly used is the FRAP assay, which has found increased applications in the evaluation of antioxidant components of dietary polyphenols (Aruoma, 2003). It can be used to measure the total reducing capability of antioxidants. The FRAP assay treats the antioxidants in the samples as reductants in a redox-linked colorimetric reaction, is simple and easily standardized. Some studies have found that the results of the FRAP assay correlate highly with the measurements of antiradical efficiency and the hydroxyl-free radical scavenging activity (Arnous, Makris, & Kefalas, 2002), with the oxygen radical antioxidant capacity (ORAC) (Prior & Cao, 1999; Tsai, McIntosh, Pearce, Camden, & Jordan, 2002), and with DPPH scavenging ability (Katalinic et al., 2004), so this method can be used for the quick evaluation of antioxidant capacity of grape pomace.

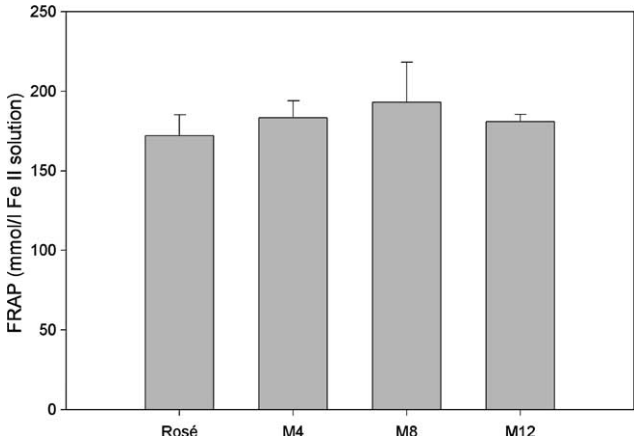

Fig. 3. FRAP activity (mean and standard deviation) in the different extracts.

The results are shown in Fig. 3. No significant differences were found in antioxidant capacity between the different extracts and higher correlation was found between FRAP activity and total phenol content ($r^2 = 0.4$, at 95% significance level) than with anthocyanin content ($r = 0.03$, with no statistical significance). Katalinic et al. (2004) also found that FRAP activity correlated with total phenols in red wine samples. Although several authors have indicated the antioxidant properties of anthocyanins (Kahkonen & Heinonen, 2003; Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002), other phenols may be contributing to the antioxidant power in pomace extracts. Also, synergistic effects among the phenolic compounds present in the extracts, as observed for catechin-malvidin (Rosseto et al., 2002) could occur.

As a conclusion, the aqueous extract obtained from the pomace of a rosé wine vinification process presented the highest concentration of anthocyanins and highest color intensity together with an important antioxidant capacity. Although the extracts obtained from the most macerated grapes (M12 extracts) had a slightly higher thermal and light stability, they also presented the lowest anthocyanin concentration and highest browning index, characteristics that made them less suitable for colorant purposes. The chromatic characteristics and stability of the extracts suggest that they could be used for coloring acidic foods, and especially those that are going to be kept at low temperatures for a limited time before consuming. Given their antioxidant capacity, they may present interest for use as health supplements and nutraceutical compounds.

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