WFL Publisher
Science and Technology
Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.11 (1): 36-39. 2013 *www.world-food.net*

Influence of organic acids on the stability of anthocyanins extracted from residues of grape processing

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Received 6 September 2012, accepted 18 January 2013.

Abstract

Anthocyanins are regarded as the most important phenolic compound due to their antioxidant potential, being the most important phenolic compounds used as natural colorant in food industry. In this work, grape (varieties 'Bordeaux' and 'Isabel') residues from wine industry were used for anthocyanin extraction and organic acids were applied in order to study their effect on anthocyanin stability. Separation of anthocyanin fractions was made by HPLC. Among the samples, there was balance period of 10 min. The eluent flow must be 1 ml min^{-1} and the wavelength used was 525 and 530 nm. In the grape residues (mixture has 20% Bordeaux and 80% Isabel), the minimum concentration of anthocyanins was 43.2 mg/100 g and the maximum concentration was 51.1 mg/100 g. The main anthocyanins found were delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside. The organic acids that better influenced the stability of anthocyanins were p-coumaric and caffeic acids.

Key words: Copigment, grape 'Bordeaux', grape 'Isabel' , caffeic acid, ferulic acid, p-coumaric acid.

Introduction

Anthocyanins (from Greek anthos, flower, and Kianos, blue) are pigments responsible for the blue and purple colors and all shades of red, found in flowers, fruits, leaves and stems of plants, these pigments being visible to the human eye. The antioxidant potential of phenolic compounds acts as reducer of oxygen in lipid oxidation reactions, metal chelation, and it also presents a wide range of properties such as pharmacologic, antiallergenic, antiatherogenic anti-inflammatory, antimicrobial, antithrombotic and cardio protective effects of vasodilators [7, 11]. In natural products, most of the substances responsible for color belong to the class of flavonoids. The classification of the flavonoids, present in a plant extract is based initially on the study of solubility properties and coloration reactions. Grape is a source of high concentrations of various phenolic compounds and most of winemaking by-products can hold considerable amounts of mainly phenolics, which belong to the group of flavonoids. Free anthocyanins are rarely found in plants, occurring with glycosylated sugars that stabilize the molecule. The frequency of glycosylation occurs at position 3, the second occurs at position 5, but it can also occur at position 7, 3 ', 4' and 5 ' (Fig. 1) [3]. Glucose, arabinose, xylose, rhamnose, galactose (or disaccharides that are consisted of these sugars) and fructose are the most common sugars linked to anthocyanidins, occurring as monoglucosides, diglucosides and glycosylated triglycerides in the form of aglycone.

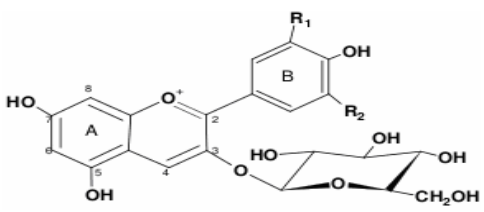

Figure 1. Anthocyanin structure [3].

Anthocyanin sugars are acylated by p-coumaric, ferulic, caffeic, p-hydroxybenzoic, sinapic, malonic, acetic, succinic and malic acids. The co-pigmentation reaction is probably the main mechanism of molecular interaction involved in variations of color and astringency during wine production and aging [6]. The increased stability is due to the fact that the pigment competes with water and interacts with the anthocyanins, thus, interacting with colored forms and changing the nature of the pigment [4].

In experiments with red wine, Darias-Martin [2] observed the effect of the addition of caffeic acid and catechin in grape must during the pre-fermentation. After 90 days, the authors found that caffeic acid caused a 60% increase in the absorbance values of wine, while the wine added with catechin showed absorbance values lower than the control samples.

The cultivation of grapes at low altitudes does not seem to favor the biosynthesis of monoglucosidic anthocyanins in grape skins, if compared with those grown at higher altitudes. In some varieties, temperatures above 35°C strongly diminish the accumulation of anthocyanins and the lack or excess of moisture tends to decrease its content [8].

Materials and Methods

Samples of processed grape (*Vitis labrusca*) residues, a mixture of varieties 'Bordeaux' and 'Isabel', were obtained at COAVITI Cooperativa agroindustrial dos viticultores de Marialva (Agribusiness Cooperative of Viticulturists from Marialva), northern Parana, Brazil, at latitude (23°29'06" S and 51°29'3" W), 2008 harvest. The samples were packed in coolers and transported to the Laboratory of Food Biochemistry/UEM, where they were packed in 0.5 mm plastic bags, and stored at -18±0.5°C for later analysis.

Extract preparation: A 100 g sample of the processed grape mixture was weighed in a beaker, in which 200 ml of an extraction solution (70 ml of 70% ethanol and 30 ml of HCl 0.1% at pH 2.0) was added[5]. The sample underwent mechanical stirring for 2 min and it was left to stand overnight, under refrigeration at 4±1°C and it was covered with aluminium foil to prevent from degradation of anthocyanins. In the first filtering the crude extract was filtered using nylon cloth to remove the thickest part and the residue was washed several times with 25 ml aliquots of extracting solution to complete a volume of 250 ml. A second vacuum filtration was made using a 0.45 µm membrane (Millipore, Bedford, MA) and the sample was stored at 4.0±1°C in amber flasks for later analysis. During the experiments the extract value was kept at pH 2.0.

Determination of total anthocyanins: In order to evaluate the total anthocyanins, 2 ml was removed from the extract at 4±1°C and transferred to a 25 ml volumetric flask, and the volume was completed with the extraction solution (70 ml of ethanol 70% and 30 ml of HCl 0.1% at pH 2.0). It was left to rest for two hours; readings were made by UV-Vis absorbance at the maximum absorption wavelength. To calculate total anthocyanins mg/100 g, the Equation 1 was used, it represents the simplified calculation for the determination of anthocyanins according to Vanini *et al.*[12].

$$FD = VEO/VA \times VS$$

$$AT\ (mg/100\ g^{1}) = A \times FD / E^{1\%}_{1cm} \quad (1)$$

where FD dilution factor, VEO volume of original crude extract (250 ml), VA volume of the aliquot used for dilution with the extracting solvent (2 ml), VS volume of the solvent used for dilution (25 ml), AT total anthocyanins (mg/100 g of sample), A absorbance of the diluted extract at the wavelength of maximum absorption and $E^{1\%}_{1cm} = 98.2$; coefficient of molar absorption.

Study of the stability of anthocyanins with organic acids: To determine the concentration of caffeic, p-coumaric and ferulic acids to be added to the anthocyanins, the amount of total solids in the stock solution was calculated by drying 3 ml of the solution at 105°C [1]. The result (mg/ml) was used as initial parameter for the addition of acids in different proportions weight/volume (w/v). Control samples (without addition of acids) and sample tests (with addition of acids in the proportions (0.5: 1, 0.8:1, 1.0:1 w/v) were prepared. The solutions were prepared in 25 ml flasks, and absorbance readings were made by UV-Vis, after 2 h rest, without light and heat. These readings were held for 400 alternate days.

Separation of anthocyanin fractions by HPLC: Finngan Surweyor PDA Plus Detector was the equipment used, from Termo Scientific, with LiChrospher RP-18 (5 µm) column of 250 x 4 mm ID. Solvent A (water; formic acid (90:10)) and solvent B (methanol; acetonitrile; water; formic acid (22.5:22.5:45:10)). The gradient starts 100% of A from 0 to 1 min ; after 1 to 30 min, 0% to 30% in B in A, to make the linear gradient, and 30 to 60 min 30% B in A. Among the samples, there is a balance period of 10 min. The eluent flow must be 1 ml min^{-1} and the wavelength used was 525 and 530 nm.

Results and Discussion

In order to identify anthocyanins in various foods and plants, two procedures can be used: a direct comparison, when there is availability of stock material and an indirect comparison, if there is no stock material. In this case, a careful comparison with literature data is acceptable. Although errors may occur when using the indirect comparison, its use is justified not only due to the lack of standards and the laborious procedure for the identification but also due to the difficulty to obtain anthocyanin standards and their high price.

The UV-Vis spectrophotometry is a very important auxiliary tool for the determination of anthocyanins in which the absorption spectra show the wavelength of maximum absorption of anthocyanins. To determine total anthocyanins in the stock solution, a scan was performed to observe their maximum molecular absorption. The value found was $\lambda = 535$ nm.

Muñoz-Espada *et al.* [10] found some values of the present study using grape peel (bagasse) of cultivar 'Concord' and obtained 330 mg/100 g of total anthocyanins. The literature reports that the amount and composition of anthocyanins present in grape bagasse are different according to species, cultivar, maturity and climatic conditions [9, 10]. The content of anthocyanins in red grapes varies from 30 to 750 mg/100 g of the ripe fruit. For Concord grapes, the values ranged from 61 to 112 mg/100 g, while for wine grapes, such as 'Pinot Noir','Cabernet Sauvignon' and 'Vincent', the averages of anthocyanin concentrations varied from 33.92 to 439 per mg/100 g. Mattivi *et al.* [9] recently found average concentrations of anthocyanins of 215, 234, 179, 115, and 99 mg/100 g to 'Cabernet Sauvignon', 'Syratt', 'Cabernet France', 'Merlot' and 'Pinot Noir' wine grapes, respectively.

Samples were tested at 25°C and in the absence of light, with extracting solution (70 ml of ethanol 70% and 30 ml of HCl 0.1% at pH 2.0); in the form of flavylium cation, which shows the red color at a lower pH. The red color of anthocyanins is lost at pH above 4.0. For general application the pH between 1.0 and 3.0 provides more stability to anthocyanins. At high pH, this cation is converted into other colorless species. Table 1 shows that the minimal concentration of anthocyanins in Bordeaux grape was 180.8 mg/100 g, and the maximum concentration 211.4 mg/100 g, and for Isabel grapes, the minimum concentration was 8.9 mg/100 g and the maximum 12.3 mg/100 g. In the grape mixture (20% of 'Bordeaux' and 80% of 'Isabel'), the minimum concentration of anthocyanins was 43.2 mg/100 g, and the maximum concentration was 51.1 mg/100 g. The minimum concentration of anthocyanins obtained from the processed grape residue was 23.1 mg/100 g and the maximum concentration was 28.2 mg/100 g. The recovery of anthocyanins in processed grapes was 52.50% in relation to that amount of the mixture, which was 48.70 mg/100 g.

Table 2 shows the results of the concentrations added in the extract of anthocyanins with the three organic acids, their retention time and half-life.

In Fig. 2, the experiment of the stability performed in UV-Vis spectrometer in absorbance mode, in the stock solution of grapes processed at pH 2.0 is displayed. The conditions were: temperature 25°C, absence of light and two hours rest. Readings were made during 400 alternate days.

In treatment A , control at pH 2.0 without addition of ferulic acid, an increased absorption was found, when compared to the others with a retention time of 81.06% and a half-life of 1,322 days, while B had a retention time of 19.48% and half-life of 169 days when added ferulic acid at a concentration of 0.5:1 (w/v), bathochromic effect. In treatment C, the retention time was 11.44%

Table 1. Average of total anthocyanins in processed grapes and grape extract (n=3).

Tests	Bordeaux grape (grape) mg/100g±δ	Isabel grape (grape) mg/100g±δ	Mixture (20%Bordô grape 80% Isabel grape). mg/100g±δ	Processed grape extract mg/100g±δ
1	206.2±2	10.0±2	49.2±2	24.2±2
2	200.7±2	9.5±2	47.7±2	26.3±2
3	180.8±2	8.9±2	43.2±2	23.1±2
4	210.0±2	11.4±2	51.1±2	28.2±2
5	211.4±2	10.7±2	50.8±2	27.2±2
6	200.0±2	12.3±2	49.8±2	23.2±2
7	197.0±2	12.2±2	49.1±2	27.3±2
	Average 200.8	Average 9.2	Average 48.7	Avarege 25.6

Expressed as average and standard deviation, n = number of repetitions, δ = δ standard deviation.

Table 2. Treatments with organic acids: their retention time and half-life.

Treatments	Concentration added (w/v)	Retention time (R%)	Half life time (t ½).days
C1	0 : 0	81.06	1.322
T1	0.5:1	83.62	1.555
T2	0.8:1	67.00	718
T3	1.0 :1	68.31	727
T4	0.5:1	19.48	169
T5	0.8:1	11 .74	129
T6	1.0 :1	22.84	191
T7	0.5:1	62.50	592
T8	0.8:1	74.24	906
T9	1.0 :1	84.37	1.634

C-1 Without treatment, with treatment T1, T2, T3 (caffeic acid), T4, T5, T6 (ferulic acid), T7, T8, T9 (p-coumaric acid).

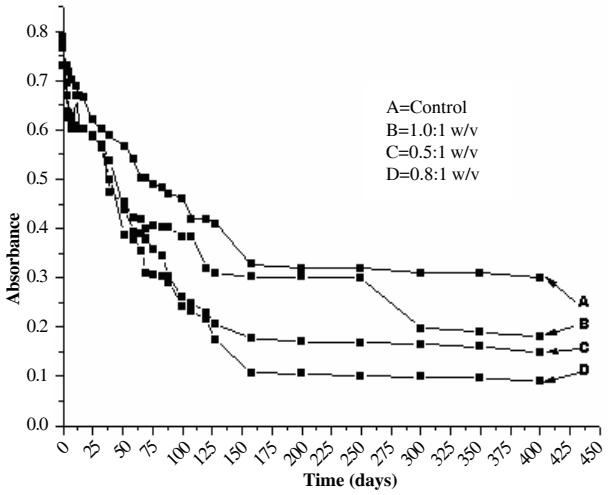

Figure 2. Stability of anthocyanins in the grape extract processed with ferulic acid, at 25ºC in the dark. (λ= 535 nm).

and the half-life was 129 days with the addition of ferulic acid at a concentration of 0.8:1 (w/v), bathochromic effect, while in D, there was a retention time of 22.84% and the half life of 161 days, with the addition of ferulic acid at a concentration of 1.0:1 (w/ v). A higher degradation occurred, when compared to the control.

In the treatment with p-coumaric acid, the control, with no addition and in the treatments the amounts of 0.5:1; 0.8:1 and 1.0:1 (w/v)were added; the readings were made at 25ºC and in the absence of light.

Acylation with cinnamic acid (p-coumaric acid) showed considerable effects on color characteristics of all derivatives. The nature of the sugar and the different positions in which cinnamic acid binds to sugar are factors sufficiently able to offer characteristics of different colors to the anthocyanins molecule. Copigmentation reaction stabilizes anthocyanins in their colorful forms retarding the normal hydration reaction. Thus, it is assumed that the copigmentation is a selective process of the colored forms. The increase in stability occurs, the copigment competes with water and interacts with anthocyanins, complexing with colorful forms and changing the copigment nature (Fig. 3); it also occurs an increase in the absorbance value (hyperchromic effect) with higher intensity in control, without addition of p-coumaric acid on the first day, and in relation to the addition of p-coumaric acid.

The addition of p-coumaric acid (0.8:1 w/v) was not significant to the control and in relation to other concentrations the absorbance values were nearly equal, and stabilizing. The experiment was carried out for 400 days. The C1 control, with its color retention time of 81.06%, a half-life of 1322 days, and with the addition of organic acid had the best color retention time with the T9 treatment. It showed 84.37% and the half-life of 1,634 days, showing that the stability with the addition of p-coumaric acid was in the concentrations (1.0:1 w/v). It had better stability between the concentrations T7 and T8, as shown previously.

The presence of caffeic acid in the molecule increases the

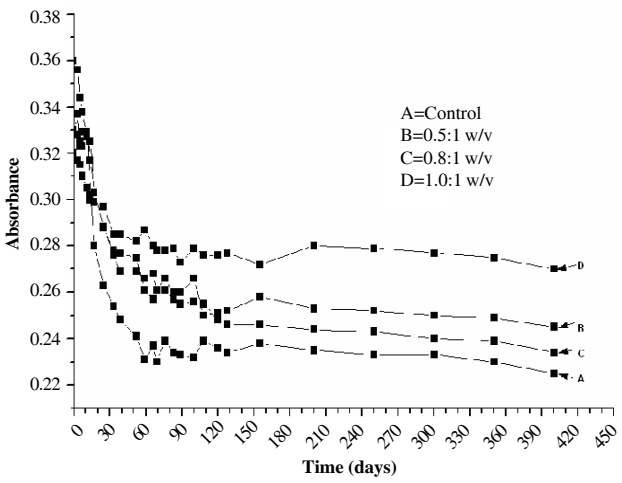

Figure 3. Stability of anthocyanins in the grape extract processed with p-coumaric acid, at 25ºC in the dark. (λ = 535 nm).

stability of anthocyanins and the existence of interaction between the pelargonidin chromophore and caffeic groups from anthocyanins that were extracted from *Pharbitis nil* petals (red-purple cultivars). There is an increase in the absorbance values (hyperchromic effect) and a bathochromic displacement, usually between 5 and 20 nm or more in maximum length absorption, with treatments using caffeic acid.

The pigment/copigment complex formed depends on the concentration of both. As the relationship copigment/ anthocyanins increases, there is a raise in the absorbance of the samples with a hyperchromic effect on the first day, because of

the absorption coefficient, increasing concentrations of the color molecules at the wavelength of maximum absorption in the treatments with and without addition of caffeic acid in the concentrations of 0.5:1, 0.8:1 and 1.0:1 (w/v) as shown in Fig. 4.

After the seventh day there was a decrease in absorbance compared to the control. This fact can be explained due to a local reduction in the flavylium chromophore polarity caused by its

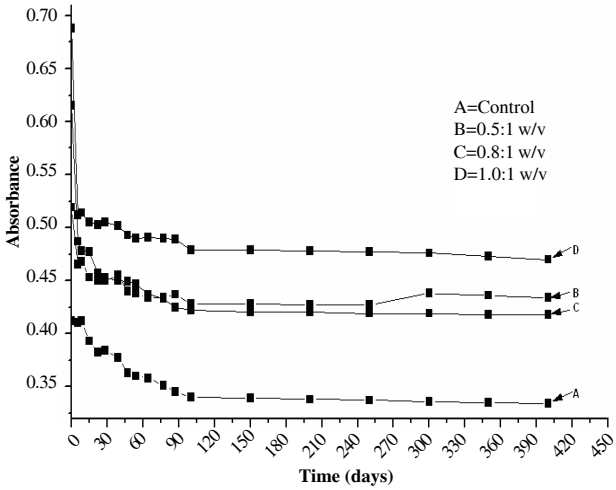

Figure 4. Stability of anthocyanins in the grape extract processed with caffeic acid at 25°C in the dark. (λ = 535 nm).

involvement with the copigment through hydrophobic associations. Afterwards, the samples had constant absorbance linearity after the addition of caffeic acid and the experiment was carried on up to 400 alternate days.

The experiment with caffeic acid at a concentration of 0.5: 1 (w/v) had the highest color retention of 83.62%, and a half life of 1552 days. It was observed that with the addition of caffeic acid, the absorbance values were higher than the control. It was shown by Darias-Martin [2] that by adding an amount of caffeic acid to wine at the end of 90 days, a 60% increase in the absorbance values occurred in the wine. It shows that caffeic acid has greater stability, increasing the anthocyanins concentrations. T2 and T3 tests had the lowest color retention.

Separation of processed grape extract fractions by HPLC: To determine the mixture of processed grape residue, various samples at -18°C were removed from the freezer, comprising 80% of 'Isabel' and 20% of 'Bordeaux' grapes. The extract was filtered under vacuum with 0.45 µm Millipore paper [5] and analyzed by HPLC, as the chromatogram shows in Fig. 5.

The main anthocyanins found in processed grape extract from the mixture of 'Bordeaux' and 'Isabel' varieties were: 1 delphinidin 3-glucoside, 3 cyanidin 3-glucoside, 4 petunidin 3-glucoside, 7 peonidin 3-glucoside and 10 malvidin 3-glucoside. Other peaks were not determined due to lack of standards.

Conclusions

It was shown that the content of total anthocyanins in grape processing residue can be a source to obtain these compounds, which are used as colorants in food industry.

The organic acids that better influenced the stability of anthocyanins were p-coumaric acid and caffeic acid.

The following anthocyanins were identified in the residue:

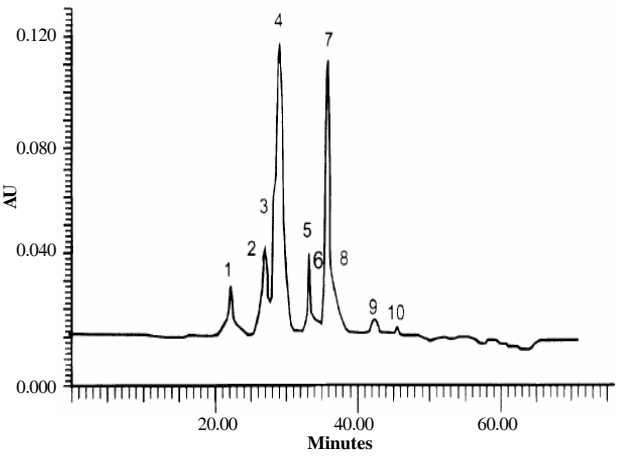

Figure 5. Chromatogram of anthocyanins in the processed grape extract. Anthocyanins isolated, peak 1- delphinidin 3-glucoside, peak 3- cyanidin 3-glucoside, peak 4 - petunidin 3-glucoside, peak 7- peonidin 3-glucoside, peak,10- malvidin 3-glucoside.

delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside.

Acknowledgements

To Coavitti - Cooperativa agroindustrial dos viticultores de Marialva - (Agribusiness Cooperative of Viticulturists from Marialva), Parana, Brazil.

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