International Journal of Sciences
(ISSN 2305-3925)
http://www.ijSciences.com

Research Article
Volume 2, Issue July 2013

STABILITY EVALUATION OF ANTHOCYANIN EXTRACTED FROM PROCESSED GRAPE RESIDUES

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Abstract: Anthocyanins are extremely important phenolic compounds due to their antioxidant potential. They are used as natural colorants in food industry, but upon the industrial processes, they show low stability. Grape and grape-made products are a source of several phenolic compounds and the residues proceeding from wine making may display a large amount of these compounds. The aim of this work was to evaluate the stability of anthocyanins extracted from grape residues industrially processed for wine making. For the evaluation, the residues of grapes Bordô and Isabel, which were extracted with ethanol 70% acidified to pH 2.0, were used. For stability analysis, the application of organic compounds co-pigments, such as caffeic, ferulic and p-coumaric acids at 0.5; 0.8; 1:1 (p/v) concentrations, was evaluated. The acid that showed higher stability was p-coumaric acid at a 1:1 (p/v) concentration, with 84.37% of retention time and half-life of 1,634 days. The use of these organic acids as stabilizers in solution increased anthocyanins useful lifetime.

Keywords: Vitis labrusca L. - Anthocyanins - Co-pigments - Organic Acids

Introduction

Anthocyanins are natural pigmented phenolic compounds responsible for blue, purple and red colors in flowers, fruits, leaves and stems. These compounds have an antioxidant potential, acting as singlet oxygen reducers in lipidic oxidation reactions and metal chelation, a large amount of properties, such as pharmacological, anti-allergenic, anti-arteriogenic, anti-inflammatory, anti-microbial, anti-thrombotic and cardioprotective and vasodilator effects (PUUPPONEN-PIMIÃ et al., 2001; MANACH et al., 2005). Most substances responsible for coloring belong to the flavonoids class. The classification of the flavonoid type present in a plant extract is based initially on the study of solubility and coloring reaction properties. Grape is a source of several phenolic compounds in high concentrations and the byproducts and residues proceeding from wine making processes, mostly, may maintain reasonable amounts of these compounds.

Free anthocyanins are rarely found in plants and they occur together with glycosylated sugars that stabilize the molecule. Sugars that are usually linked to anthocyanins are glucose, galactose, rhamnose and arabinose. R1 and R2 groups vary according to Figure 1, where it can be noticed that R3 and R4 groups are usually hydroxyls or glycolic (mostly, glycoside). In nature, anthocyanins occur as monoglycoside (glucose linked to position 3) or diglycoside (glucose linked to positions 1 and 3) (FRANCIS, 2000).

Figure 1. Basic structure of an anthocyanin (FRANCIS, 2000).

Anthocyanin sugars are acylated by p-coumaric, ferulic, caffeic, p-hydroxibenzoic, sinapinic, malonic, acetic, succinic and malic acids. Copigmentation reaction may be the main molecular interaction mechanism involved in color and astringency variations during wine production and aging (MAZZA, 1995). The increase in stability occurs because the pigment competes with water and interacts with anthocyanin, making complexes with colorful forms and modifying the pigment nature (GRIS et al., 2007). The phenolic acids of cinnamic series are found in grape combined with tartaric acids as monoesters. In Figure 2, it can be observed the representation of cinnamic acid, from which the phenolic acids of this series derive, being them ferulic, p-coumaric and caffeic acids.

Figure 2. Cinnamic acids structure (BALASUNDRAM, SUNDRAM and SAMMAN, 2006).

If we consider anthocyanin coloration only as a function of pH, we are led to believe that plants should not be colored, once the natural pH in plants, in most cases, is between neutral and slightly acid. In this pH scope, most anthocyanins are not colored.

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However, it is observed that anthocyanins are always associated with colored parts in plants, pointing out that these substances must be stabilized by uncommon physicochemical factors. The presence of compounds named co-pigments may be one of the aforesaid factors. Non-anthocyanic flavonoids, alkaloids, amino acids and nucleotides may act as co-pigments and even anthocyanin itself may act co-pigmented to another anthocyanin. There may be three basic anthocyanin stabilizing mechanisms: with or without intramolecular co-pigmentation, intermolecular co-pigmentation and self-association together with anthocyanidin, flavonoid or aromatic acid and sugar molecules. Anthocyanin solutions that are much diluted show an increase in coloration, when co-pigmented with rutin, but this increase in absorbance in slowly reduced as anthocyanin concentration increases. The lowest anthocyanin availability for co-pigmentation with rutin may be associated with kation flavilium by means of anthocyanin self-association, when they are present in higher concentrations.

Anthocyanin extraction is the first step to determine its content in any kind of plant tissue and residues. Anthocyanins are located in the vacuoles of hypodermic cells, close to the surface and the extraction procedure usually involves the use of acid solvents that denature the cellular tissue membrane and dissolve the pigments, simultaneously (WROLSTAD; GIUSTI, 2001).

The aim of this work was to evaluate the stability of anthocyanins extracted from agroindustrial residues of grape used in wine making processes, varieties Bordô and Isabel, upon the application of co-pigments, such as caffeic, p-coumaric and ferulic acids.

EXPERIMENTAL STEP

Sampling

The residue of industrially processed grapes (*Vitis labrusca* L.), varieties Isabel (80%) and Bordô (20%) was collected in Cooperativa Agroindustrial dos Viticultores de Marialva (COAVITTI), in the municipality of Marialva, located in the north of Paraná state (latitude 23º29’06” S and 51º29’31”W). The material collection was carried out in January 2011, after a separation process in the wine making fermentation tank. The residue was pressed, in order to take off the wine excess, in a mechanical press and afterwards it was put in dark-colored (black) plastic bags with 0.5mm density and stored at -18ºC for further analyses.

Extract preparation

Extract obtention was carried out from 100g of grape residue sample, homogenized with 200 mL of extracting solution (70 mL of ethanol 70% and 30 mL of HCl 0.1%, pH 2.0), for two minutes in a blender, and left to rest for twelve hours at 4ºC in a beaker covered with parafilm and aluminum foil for protection against light incidence. Afterwards, a filtration was made, transferring the content to a 250mL volumetric flask and completing it with an extracting solution (Ju and Howard, 2003).

An aliquot of 2.0mL was taken from the stock solution at 4±0.5ºC to a volumetric flask of 25mL, completing the volume with extracting solution and leaving it at room temperature and in the dark for two hours. The extracting solution was used as blank. The extract absorbance reading was made in an UV-Vis spectrometer (Hitachi, mod. 2001) at 535nm. To determine the concentration of anthocyanin (total anthocyanin mg/100 g) the expressions from Equations 1 and 2, which provides the simplified calculation for its determination, were used (VANNI, KWIATKOWSKI, CLEMENTE, 2009).

$$FD = VEB / VA \times VS \quad (1)$$

$$AT\ (mg / 100g) = A \times FD / E^{1\%}_{1cm} \quad (2)$$

Where:

FD = Dilution factor

VEB = Gross extract volume (250 mL)

VA = Volume of the extraction aliquot used for the dilution in extracting solution (2 mL).

VS = Volume of the solution used for the extract dilution (25 mL)

AT = Total Anthocyanin (mg) in 100g of sample.

A = Diluted extract absorbance in the maximum absorption wave length.

$E^{1\%}_{1cm}$ = 98.20; Molar absorptivity coefficient for a mixture of purified anthocyanins from the processed grape extract

Evaluation of degradation parameters

The calculations of degradation speed constant (K), half-life ($t^{1/2}$) and percentage of color retention (%R) (GRIS et al., 2004) are used to analyze the anthocyanin pigments degradation as a function of time. Pigment degradation speed and ($t^{1/2}$) can be calculated according to Equations 3 and 4, respectively (VANINI; KWIATKOWSKI; CLEMENTE, 2009).

$k.t = -2.303x \log At_x / At_o$ (3)

$t^{1/2} = 0.693 / k$ (4)

Where:

At_x = Absorbance in relation to the final time of the experiment,

At_o = Absorbance in time zero, initial time of the experiment,

K = Speed Constant (hs^{-1}),

t = Time (days, hours, minutes, seconds),

$t^{1/2}$ = half-life time.

The percentage of color retention (%R), related to time, can be calculated by the absorbance readings, using Equation 5 (VANINI, KWIATKOWSKI, CLEMENTE, 2009).

$\%R = At_x / At_o \times 100$ (5)

Where:

%R = Percentage of color retention,

At_x = Absorbance as a function of the experiment final time,

At_o = Absorbance in time zero, initial time of the experiment.

Results and Discussion

The stability of anthocyanins extracted from grape residues of wine making, added with p-coumaric acid, at 25ºC and with absence of light can be seen on Figure 3. The increase in stability can be observed in all concentrations, when compared to the control treatment. It occurs due to the fact that the co-pigment competes with water and interacts with anthocyanins, creating complexes with colored forms and modifying the co-pigment nature. It can also be observe that the increase in absorbance (hyperchromatic effect) with higher intensity on concentration 1.0:1.0 (p/v), and with lower intensity in the control.

Figure 3. Anthocyanin stability, in the processed grape extract, added with p-coumaric acid, at a 25ºC, with absence of light. A = Control; B = 0.5:1.0; C = 0.8:1.0; D = 1.0:1.0 (p/v) p-coumaric acid:anthocyanin extract.

The presence of caffeic acid in the molecule increases anthocyanin stability and the existence of interaction between the pelargonidin chromophore and caffeil groups of anthocyanins extracted from petals of *Pharbits nil*, cultivars purple-red (Dangles, Saito and Brouillard, 1993). An increase in absorbance values (hyperchromatic effect) as well as a batochromatic displacement, usually between 5 and 20 nm or more, in the maximum absorption length, with treatments with caffeic acid.

Figure 4. Anthocyanin stability in processed grape extract with caffeic acid, at 25ºC, with absence of light. A = Control; B = 0.5:1.0; C = 0.8:1.0; D = 1.0:1.0, caffeic acid:anthocyanin extract (p/v).

The pigment/co-pigment complex created is dependent on both concentrations as the co-pigment/anthocyanin relation increases. It can be observed that there was an increase in absorbance values on the first day, with a hyperchromatic effect. It occurs due to the absorption coefficient that increases the colored molecules concentrations on the maximum absorption wave lengths, in treatments with and without caffeic acid (Figure 4). After the seventh day, there was a decrease in absorbance values in relation to the control. It can be explained by a local reduction on the chromophore flavilium polarity, caused by its involvement with the co-pigment, by means of a hydrophobic association. After this period, the samples showed constant absorbance linearity upon the addition of caffeic acid and the experiment lasted until day 400.

As for the experiment with caffeic acid addition, the concentration 0.5:1.0 (p/v) showed higher color retention (83.62%) and half-life time was 1,552 days, being observed that upon the addition of caffeic acid, the absorbance values were higher than the control (Figure 4). Darias-Martins et al. (2001) reported that after adding an amount of caffeic acid to wine, there was an increase of 60% in the absorbance values after 90 days. It shows that caffeic acid provides higher stability, increasing the concentration of anthocyanins. Tests T2 and T3 showed lower color retention.

Figure 5. Anthocyanin stability in processed grape extract with ferulic acid, at 25ºC, with absence of light. A = Control; B = 0.5:1.0; C = 0.8:1.0; D = 1.0:1.0, ferulic acid:

As for co-pigmentation with ferulic acid, the control treatment showed higher absorbance values, when compared to the others, with retention time of 81,06% and half-life time of 1,322 days. The treatment with the same acid at a 0.5:1.0 concentration showed a retention time of 19.48% and half-life time of 169 days. Upon the addition of ferulic acid, at a concentration of 0.5:1.0 (p/v) there was slow anthocyanin degradation, thus having a batochromatic effect (Figure 5). The retention time was 11.44% and half-life time was 129 days upon the addition of ferulic acid at a concentration of 0.8:1.0 (p/v) and there was slow anthocyanin degradation,

thus having a batochromatic effect. Upon the addition of ferulic acid at 1.0:1.0 (p/v) there was also higher anthocyanin degradation, when compared with the control treatment. The retention time was 22.84% and half-life time was 161 days. Regarding the other acids used in this study, ferulic acid showed the lowest retention time and the lowest half-life time, in days.

In Table 1, the retention time numbers and half-life values for anthocyanin extract of processed grape residues are displayed.

Table 1. Retention time and half-life of anthocyanin extracted from grape residues (varieties Bordo and Isabel) of wine making.

Treatment T9 had the best color retention time in anthocyanin extract of processed grape residues, with 84.37% of color retention and half-life time of 1,634 days, indicating that the addition of p-coumaric acid at a 1.0:1.0 (p/v) concentration provides higher stability.

Conclusions

Out of the used acids, the one which least degraded the anthocyanins and kept the best stability in the extract of processed grape residues were p-coumaric and caffeic acids. Thus, it is possible to extract anthocyanins and, upon the addition of organic acids, increase its half-life time, in order to avoid fast anthocyanin degradation.

Acknowledgement

To COAVITTI - Cooperativa Agroindustrial dos Viticultores de Marialva, Paraná, Brasil, for providing the samples.

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Table 1. Retention time and half-life of anthocyanin extracted from grape residues (varieties Bordo and Isabel) of wine making.

Treatments	Added Concentration (p/v)	Retention Time (R%)	Half-life time (t ½) in days
C1	0.0:0.0	81.06	1.322
T1	0.5:1.0	83.62	1.555
T2	0.8:1.0	67.00	718
T3	1.0:1.0	68.31	727
T4	0.5:1.0	19.48	169
T5	0.8:1.0	11.74	129
T6	1.0:1.0	22.84	191
T7	0.5:1.0	62.50	592
T8	0.8:1.0	74.24	906
T9	1.0:1.0	84.37	1.634

C1- control; treatments T1; T2; T3 - caffeic acid; T4; T5; T6 - ferulic acid; T7; T8; T9 - p-coumaric acid.

Figure 1. Basic structure of an anthocyanin (FRANCIS, 2000).

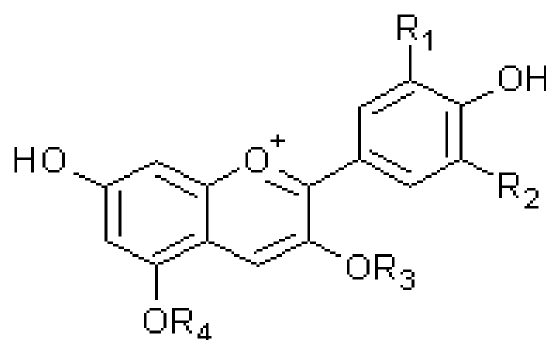


Figure 2. Cinnamic acids structure (BALASUNDRAM, SUNDRAM and SAMMAN, 2006).

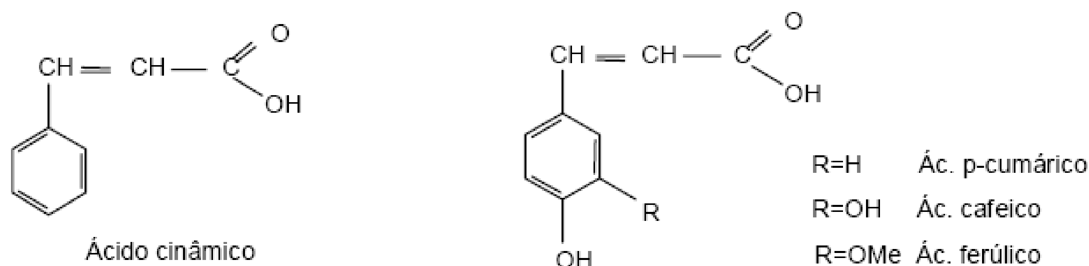
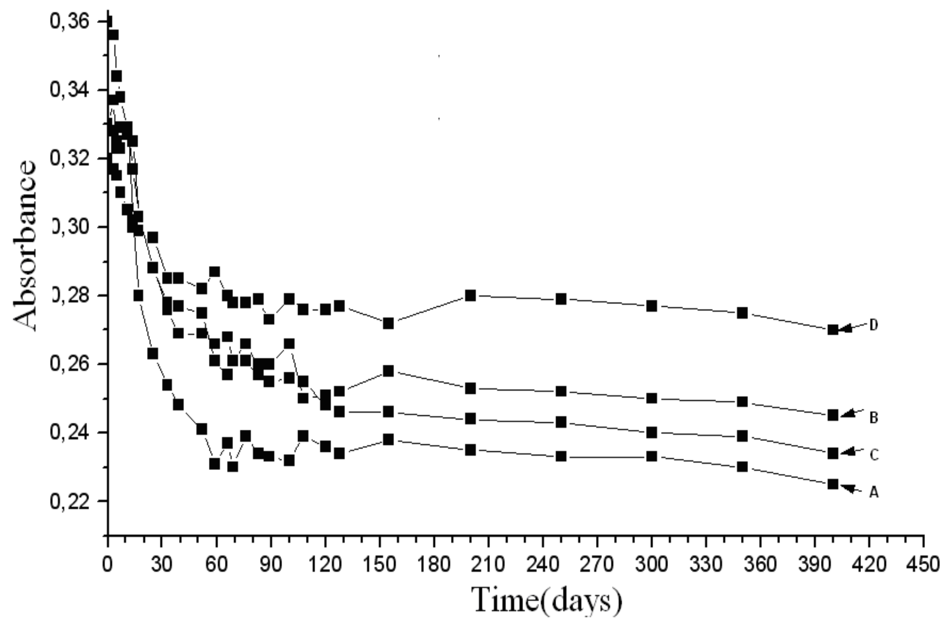


Figure 3. Anthocyanin stability, in the processed grape extract, added with p-coumaric acid, at a 25ºC, with absence of light. A = Control; B = 0.5:1.0; C = 0.8:1.0; D = 1.0:1.0 (p/v) p-coumaric acid:anthocyanin extract.



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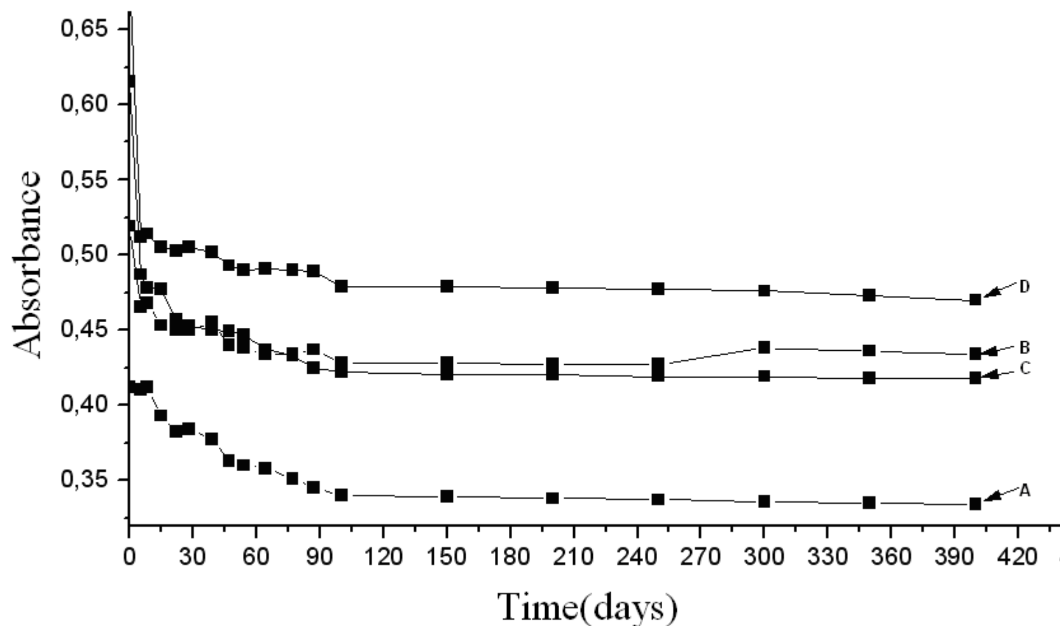

Figure 5. Anthocyanin stability in processed grape extract with ferulic acid, at 25ºC, with absence of light. A = Control; B = 0.5:1.0; C = 0.8:1.0; D = 1.0:1.0, ferulic acid:anthocyanin extract (p/v).