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Spectrophotometric study of the stability of anthocyanins from Cabernet Sauvignon grape skins in a model system

Estudo espectrofotométrico da estabilidade de antocianinas de cascas de uvas Cabernet Sauvignon em sistema modelo

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Summary

Gallic acid was evaluated as copigment for an anthocyanin crude extract of Cabernet Sauvignon grape skins. Furthermore, comparison of the stability of the grape anthocyanins and their interaction with gallic acid were investigated when stored under different conditions of pH (3.0 and 4.0), temperature (4 ± 1 °C and 29 ± 2 °C), in the presence of light (2,500 lumens) and in its absence, with or without gallic acid, in both a model system and in a real rehydratation beverage. The results revealed that pH, temperature and the presence of light significantly affected the stability of anthocyanins ($p < 0.05$). The highest half-life values for anthocyanin and percent color retention were reached when the samples were maintained at pH 3.0, temperature of 4 ± 1 °C and in the absence of light. In the model system, gallic acid (2:1, w:v) did not affect significantly ($p > 0.05$) the stability of anthocyanins. In a rehydratation beverage system, addition of gallic acid decreased the half-life time and percentage color retention of the anthocyanins.

Key words: *Anthocyanins; Cabernet Sauvignon grapes; Crude extract; Gallic acid; Model system; Stability.*

Resumo

O ácido gálico foi avaliado como copigmento para o extrato bruto de antocianinas de casca de uvas Cabernet Sauvignon. Além disso, nossas investigações foram direcionadas para comparar a estabilidade das antocianinas de uvas e sua interação com o ácido gálico sob diferentes condições de estocagem de pH (3,0 e 4,0), temperatura (4 ± 1 °C e 29 ± 2 °C), sob a presença (2.500 lúmens) e ausência de luz, com ou sem a presença de ácido gálico, em um sistema modelo e em sistema real de uma bebida de rehidratação. Os resultados revelaram que o pH, a temperatura e a presença da luz afetaram significativamente a estabilidade das antocianinas ($p < 0.05$), diminuindo sua estabilidade. Os melhores resultados de tempo de meia vida das antocianinas, e de percentagem de retenção de cor foram observados quando as amostras foram mantidas em pH 3,0, temperatura de 4 ± 1 °C e no escuro. Em sistema modelo, o ácido gálico (2:1, p:v) não afetou significativamente ($p > 0.05$) a estabilidade das antocianinas. Em sistema de bebida de rehidratação, a adição de ácido gálico diminuiu a percentagem de retenção de cor e o tempo de meia vida das antocianinas.

Palavras-chave: *Antocianinas; Uvas Cabernet Sauvignon; Extrato bruto; Ácido gálico; Sistema modelo; Estabilidade.*

1 Introduction

The color is an important factor influencing consumers' acceptability of the food. The replacement of synthetic by natural colorants as food additives has substantially increased (MAZZA and MINIATI, 1992; YOUDIM et al., 2000). Anthocyanins are considered as potential substitutes of synthetic colorants owing to the bright and attractive colours they confer to food and are approved as food colorants (BRASIL, RDC 382, 1999). Another favourable aspect of anthocyanins is their contribution to the antioxidant properties of certain foods; thus there is considerable interest in their health effects (WANG et al., 2000; YOUDIM et al., 2000; HAGIWARA et al., 2001; KUSKOSKI et al., 2004). However, the manufacturing and processing of berry products can lead to deterioration of anthocyanins and consequently the colour of the product. The colour of carbonated grape beverages resulted in only 30% loss of the pigments when kept in the dark, against 50% with exposure to light, but having otherwise equal storage conditions. Furtado et al. (1993) found that during the light induced degradation, formation of the same final products as for thermal degradation was observed, however, the kinetic pathway of the degradation reaction was different involving the excitation of the flavylium cation.

Anthocyanins are nevertheless colorful in nature and their colored forms must be strongly stabilized by other natural components, the so-called copigments, existing in the cells of flowers, fruits and berries (BROUILLARD, 1982). A wide range of different molecules has been found to act as copigments. The most common structurally related with copigment compounds are flavonoids, and other polyphenols, alkaloids, amino acids and organic acids (BROUILLARD et al., 1989). As well as resulting in highest absorbance values (hyperchromic shift) certain copigments lead to a bathochromic shift in the wavelength at which the maximum absorbance was observed, providing a blue-purple tone in an otherwise red solution (BOULTON, 2001). Copigmentation can be a valuable, natural tool for improving the colour of anthocyanin rich food products, the colour of which can be stabilized and enhanced by the addition of different plant extracts rich in copigments. It has been observed already that copigmentation is more intense in strawberry (*Fragaria virginiana*) and chokeberry (*Aronia arbutifolia*) than purified anthocyanin molecules of these juices (WILSKA-JESZKA and KORZUCHOWSKA, 1996). This indicates that several other components of the juice material play a role in the copigmentation phenomenon than just one added copigment molecule.

A variety of health promoting products obtained from by-products of the grape and wine industry has been introduced to the market (GÓMEZ-PLAZA et al., 2006). Anthocyanin-rich extracts from fruit and vegetables are interesting, especially when they can be obtained from otherwise waste materials such as the grape pomace from the winemaking industry. Gómez-Plaza et al. (2006) evaluated the stability and antioxidant power from grape pomace extracts from different vinification 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), verifying that the pomace of a rosé wine vinification process exhibited the highest concentration of anthocyanins and highest colour intensity, together with an important antioxidant capacity. Although the extracts obtained from the method with highest maceration had the greatest thermal and light stability, the chromatic characteristics and stability of the extracts suggest that they could be used for colouring acidic foods, and especially those that are held at low temperatures for a limited length of time before consumption. Given their antioxidant capacity, these by-products could be interest for use as both health ingredients and technologically functional compounds. Since Cabernet Sauvignon (*Vitis vinifera* L.) is the most widely grown wine grape in Santa Catarina State (Brazil), its residue could be another potential source of anthocyanins as a natural food colorant. The use of a crude extract, which does not depend on high cost processes of purification, could be an alternative to use of this industrial residue as source of anthocyanins for food applications. Gallic acid is an organic acid that is present in a wide variety of plants, being present in tea, red wine, fruits, beverages and various medicinal plants. They are known to have anti-inflammatory, antimutagenic and anticancer effects (GICHNER et al., 1987; KROES et al., 1992; INOUC et al., 1995). Until now, gallic acid has been evaluated as copigment only on the stability of purified pigments, but not on the stability of anthocyanins in crude extracts. This study aimed at evaluating the anthocyanin stability from crude extracts of Cabernet Sauvignon (*Vitis vinifera* L.) grape skins as well as the possible effect of added gallic acid on the stability of anthocyanins in a model solution and in a real beverage system, under different conditions of temperature, pH, presence or absence of oxygen and light.

2 Material and methods

The study was carried out with red grapes (*Vitis vinifera* L.) of Cabernet Sauvignon cultivars from the *Empresa de Pesquisa e Extensão Agropecuária de Santa Catarina* (EPAGRI), *Estação Experimental de São Joaquim* (1400 m asl), Brazil. Gallic acid (3,4,5-trihydroxybenzoic acid) (Aldrich Chemical Co., Milwaukee, WI) was used to evaluate the copigmentation reaction. All the other reagents were qualified for analysis as well.

2.1 Anthocyanin extraction and quantification

Grapes from the 2002 harvest were washed in running water and then in water containing 200 mg.L^{-1} of

sodium hypochlorite. The skin of grapes were separated from the pulp, blanched in boiling water for 2 min and frozen at -18 ± 1 °C in polythene bags. One hundred grams of grape skins were left to macerate overnight in the dark, in a beaker with 400 mL of a mixture of ethanol:1.5 N HCl (85:15), at 4.0 ± 1 °C (LEES and FRANCIS, 1972). The crude extract obtained was filtered through a nylon filter and the skin remains washed with 100 mL of the extracting solvent. A second vacuum filtration was carried out in Whatman (N. 2) filter paper; the extract concentrated to 50% of the ethanol initial volume and evaporated under vacuum (Büchi Rotaevaporator-R-144) at 35 °C. The concentrated extract was finally filtered through a 0.45 μm Millipore filter, kept in an amber flask at 4.0 ± 1 °C and used for analysis in the experiments. The pH value of the crude extract was 1.32. The content of total anthocyanins was determined according to the Lees and Francis (1972) method, using ε = 98.2 (FULEKI and FRANCIS, 1968).

2.2 Kinetic study

The concentration of gallic acid to be added to the anthocyanins (weight/volume ratios w/v) was determined from the total solid content of the crude extract, which had been previously determined by drying 3 mL of anthocyanin crude extract at 105 °C until constant weight was reached (AOAC, 1998). The initial total soluble solids-to-volume ratios values obtained were tested in the model system (1:1, 2:1; 3:1 and 4:1, w:v ratios).

Control samples (model system without gallic acid) and test samples (model system with gallic acid) were prepared as follows: the crude extract was transferred to volumetric flasks of 25 mL, added of 0.05% (w:v) potassium sorbate to prevent microbial growth (BRASIL, 1987) and the volume completed with a model system of citrate buffer (0.1 M citric acid – sodium citrate; Merck), at pH 3.0 and 4.0 to obtain absorbance values between 1-1.5 at 520 nm (absorption maximum). The pH meter (MP 220 meter – Metler – Toledo) was calibrated with pH 7.0 and 4.0 buffer solutions (Nalgon). After agitation for 30 min, the solutions were transferred to flasks with lids (25 mL) and allowed to stand for 2 h to reach equilibrium (FULEKI and FRANCIS, 1968). Each experiment was carried out with two repetitions (in triplicate), maintaining the screw-cap tubes with test and control samples under the following conditions: in the presence of oxygen (from air), at 4 ± 1 °C and 29 ± 2 °C, pH 3.0 and 4.0, under fluorescent lamp (2,500 lumens) and in the dark (flasks wrapped in aluminium foil). The first-order reaction rate constants (k) and half-life time ($t_{1/2}$) values were calculated according to Kirca and Cemeroglu (2003) (Equations 1a and 1b):

$$\ln (A_t/A_0) = -k.\ t \quad (1a)$$

$$t_{1/2} = -\ln 0.5.\ k^{-1} \quad (1b)$$

where,

t = time (h), A_t= Absorbance at time t, A_0 = Absorbance at time zero.

Taking into account the composition and pH of commercialized rehydration beverages, control samples (rehydration beverage without gallic acid) and test samples (rehydration beverage with 2:1, w:v of gallic acid) were prepared aiming at final absorbance values close to 1.0. They were prepared with sucrose, glucose, sodium chloride, sodium citrate and potassium mono-phosphate diluted in 1000 mL of deionised water, obtaining a final pH of 3.05 (FALCÃO et al., 2004). All samples were agitated for 30 min, transferred to flasks with lids and let to rest for 2 h. After, they were submitted to thermal treatment to simulate the process of elaboration of rehydration beverages in the food industry (63 °C for 5 min) and cooled at room temperature (29 ± 2 °C) (FALCÃO et al., 2004).

The absorption spectra of samples were monitored by UV-Vis absorption spectrophotometry in the visible wavelength range from 400 to 700 nm, at regular time intervals, until 60% or more of the pigments were degraded. A Hitachi U2010 spectrophotometer (Tokyo, Japan) fitted with a 10 mm optical path quartz cell was used in this monitoring. After reaching equilibrium in the dark (2 h), readings were taken at the anthocyanin maximum wavelength ("zero time" absorbance) for the model system. For the rehydration beverage system, "zero time" readings were carried out after the samples had been cooled, to consider the effect of the process (63 °C for 5 min). Distilled water was used as blank in all experiments (GIUSTI et al., 1999). The colour retention percentage values in beverage system were calculated according to Katsaboxakis et al. (1998) (Equation 2):

$$(\text{Abs at time t}/\text{ Abs at time zero}).100 = \%\text{Colour Retention} \quad (2)$$

2.3 Data analysis

In the model system the experiment was conducted using a randomised 2^4 factorial design, with duplicate trials (factors: treatment, pH, temperature and presence of light, two levels) were studied by means of an analysis of variance (ANOVA). For the rehydration beverage system the experiment was conducted entirely at random, with two repetitions, using a factorial 2^2 design (factors: treatments and temperature, in two levels). All the statistic analyses were carried out using the using Statistica 6 (2001) (StatSoft Inc., Tulsa, OK, USA).

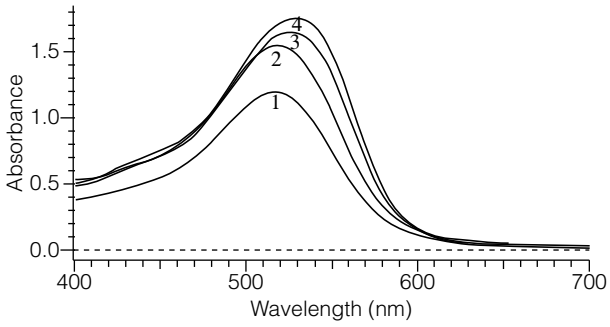
3 Results and discussion

The approximate content of total anthocyanins in the crude extract of Cabernet Sauvingon grapes was calculated to be 237 mg.100 g^{-1} of grape skins, which was 33 mg less than recently reported for *V. labrusca* grapes (270 mg.100 g^{-1} of grape skins) (BORDIGNON-LUIZ et al.,

2007). The content of total solid of the crude extract was calculated as 13.3 mg.mL^{-1}. Different proportions of gallic acid were analysed at random in relation to the total solids of the anthocyanin crude extract to verify possible copigmentation reactions (Figures 1 and 2). The control samples exhibited a bathochromic shift ($\Delta\lambda$ = 5 nm) with an increase in pH, in agreement with the literature (BARANAC et al., 1997). Increases in the volume ratio gallic acid:anthocyanin increased the shift in the maximum absorption wavelength (bathochromic effect) and the anthocyanin absorbance values (hyperchromic effect), suggesting an interaction between the grape crude extract anthocyanins and the gallic acid at pH 3.0 and 4.0 (Table 1). The occurrence of bathochromic and hyperchromic shifts, which confirm the copigmentation interaction of anthocyanins with phenolic compounds, has been reported in several studies (DANGLES and BROUILLARD, 1992; DAVIES and MAZZA, 1993, BARANAC et al., 1996; BARANAC et al., 1997; DIMITRIC-MARKOVIC et al., 2000; BOULTON, 2001; BORDIGNON-LUIZ, et al., 2007; GRIS et al., 2007). For the purpose of comparison, the study of anthocyanin stability with gallic acid as function of time was carried out using the ratio of 2:1 (w:v) of gallic acid:anthocyanin crude extract, in order to achieve similar final absorbance values at pH 3.0 and 4.0 (Figure 1).

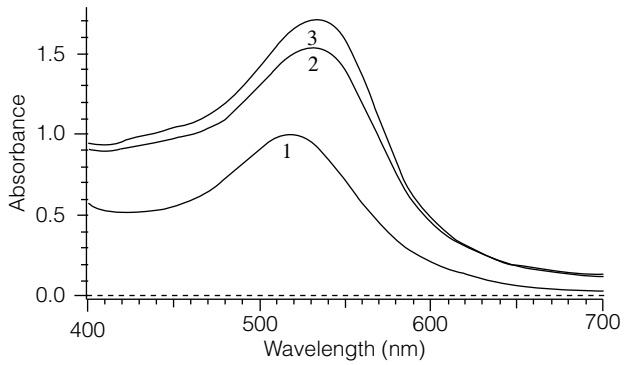
3.1 Kinetic study in a model system

The 2^4 Factorial model was considered well adjusted with a R^2 = 0.987 correlation coefficient. Through the analysis of variance (ANOVA) performed on the half-life values of the samples, it was possible to verify that the factors of pH, temperature and presence of light significantly influenced the anthocyanin stability. The addition of gallic acid at a ratio of 2:1 (w:v) showed no significant effect ($p > 0.05$) on the half-life of the anthocyanins (Table 2). This result is in disagreement with that obtained by Miniati et al. (1992), who verified an increase in stability of diglucosides malvin, pelargonidin and cyanidin added with gallic acid and quercetin as copigments. This can be justified by the fact that a crude extract of Cabernet Sauvignon grape anthocyanins was used and not the purified pigments. Possibly the proportion of gallic acid used was not sufficient to maintain the anthocyanins more stable than the control samples over time. Comparison of the different storage temperatures used showed that samples maintained at 4 ± 1 °C exhibited longer half-life values. This result is in agreement with the literature. Normally, a temperature increase causes a logarithmic increase in anthocyanin destruction (MARKAKIS, 1982); this can



pH 3.0, T (°C) 20 ± 2°
1 = control λ = 515 nm
2 = gallic acid: crude extract 2:1 λ = 520 nm
3 = gallic acid: crude extract 3:1 λ = 530 nm
4 = gallic acid: crude extract 6:1 λ = 530 nm

Figure 1. Effect of gallic acid addition at maximum absorption wavelength for anthocyanins from grape skins at pH 3.0 at T (°C) = 20 ± 2 (λ = 520 nm). 1 = control (anthocyanins without gallic acid) 2, 3 and 4 = test (anthocyanins: gallic acid in concentrations 2:1; 3:1 and 6:1, w:v, respectively).



pH 4.0, T (°C) = 20 ± 2°
1 = control λ = 520 nm
2 = gallic acid: crude extract 2:1 λ = 530 nm
3 = gallic acid: crude extract 3:1 λ = 535 nm

Figure 2. Effect of gallic acid addition at maximum absorption wavelength for anthocyanins from grape skins at pH 4.0 at T (°C) = 20 ± 2 (λ = 520 nm). 1 = control (anthocyanins without gallic acid); 2 and 3 = test (anthocyanins: gallic acid in concentrations 2:1 and 3:1, w:v, respectively).

Table 1. Gallic acid concentration values and anthocyanin crude extract volumes used in the model system experiment at pH 3.0 and 4.0.

pH	Gallic acid:crude extract ratio (w:v) in model system	Crude extract in buffer solution (vial 25 mL)*	Δλ (nm)	ΔA (%)
3.0	2:1 (26.6 mg.mL^{-1})	0.8 mL	5	20
3.0	3:1 (39.9 mg.mL^{-1})	0.8 mL	10	37
3.0	6:1 (79.8 mg.mL^{-1})	0.8 mL	15	46
4.0	2:1 (26.6 mg.mL^{-1})	1.8 mL	10	54
4.0	3:1 (39.9 mg.mL^{-1})	1.8 mL	15	71

Δλ = batochromic shift (nm). ΔA= hyperchromic shift percentage (%).*Values used to obtain initial absorbance ~ 1.000.

occur during the storage and stocking of foods (MAZZA and MINIATI, 1993). According to Brouillard and Dubois (1977) the mechanism of anthocyanin degradation by heat probably occurs due to the opening of the flavylium cation ring, followed by conversion to the chalcon form, which is colorless and is an irreversible degradation.

In the presence of light, the anthocyanins degraded more quickly than when maintained in the dark, at both pH values and this result is in accord with the literature (FURTADO et al., 1993; KATSABOXAKIS et al., 1998).

The best results for half-life and colour retention percentages were observed for samples stored at a temperature of 4 ± 1 °C in the dark. Under certain storage conditions, the control samples showed a significantly greater stability than those with added gallic acid (Table 2). Darias-Martín et al. (2001), while experimenting with red wines, verified that catequin added as a copigment in the pre-fermentation phase initially conferred an increase in colour intensity, but after 90 days storage, the copigment provoked a reduction in sample absorbance values in relation to the control. The reactions involved in the reduction of anthocyanic pigment stability by the addition of a copigment require further investigation in order.

Increase of the pH from 3.0 to 4.0 contributed to anthocyanin degradation, thereby reducing the pigments half-life. It is known that pH increase disturbs the chemical equilibrium of anthocyanins in aqueous solution. Changes in the acid-alkali equilibrium, quickly transform the coloured form of the flavylium cation into the quinoidal base through proton loss, though the reaction is only possible in the presence of a free hydroxyl group (MARKAKIS, 1982; MAZZA and MINIATI, 1993). These compounds are thermodynamically unstable and are transformed into the hemiacetal and chalcone compounds through the flavylium cation form (BROUILLARD and DELAPORTE, 1977).

The results of the variance analysis of the factors under study: pH, temperature, presence of light and presence of gallic acid, indicated that significant interactions did occur ($p < 0.05$). The pH factor interacted with temperature ($p = 0.0035$) and with the presence of light ($p = 0.0013$), in relation to the sample half-life values; anthocyanins were shown to be more stable at pH 3.0, at a temperature of 4 ± 1 °C and in the dark, presenting longer half-life values under these conditions. When maintained at pH 4.0, temperature of 29 ± 2 °C and in the presence of light, the samples exhibited the shortest half-life values.

3.2 Kinetic study in a rehydration beverage system

Aiming at evaluating the applicability of anthocyanins in foods as a natural colour, a stability study was conducted on a real food system, such as a rehydration beverage. The stability of the anthocyanins in the beverage system was evaluated for 217 h. Figure 3 shows the anthocyanin absorbance values over time for control and test samples.

The colour of the rehydration beverage was considered satisfactory even after 217 h. The 2^2 factorial model was considered well adjusted with a correlation coefficient of $R^2 = 0.989$. Through variance analysis it was possible to verify that the factors temperature and treatments significantly interfered in anthocyanin half-life values ($p < 0.05$) and that only the temperature interfered significantly with the colour retention percentages ($p = 0.0008$). The statistics

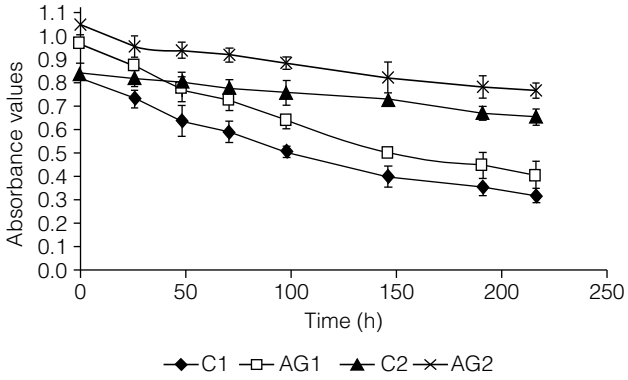


Figure 3. Absolute absorbance values of anthocyanins with and without gallic acid in beverage system over time. C1 = control at 29 ± 2 °C; AG1 = test (2:1, w:v) at 29 ± 2 °C; C2 = control at 4 ± 1 °C; AG2 = test (2:1, w:v) at 4 ± 1 °C.

Table 2. Half-life values of model system samples without (control) and with (test) gallic acid (2:1, w:v) under different storage conditions.

Storage conditions			k (h^{-1})		Half-life (h)*	
pH	T (°C)	Light/dark	Control samples	Test samples	Control samples	Test samples
3.0	4 ± 1	D	0.0002	0.0001	3,734 ± 200	4,103 ± 24
3.0	4 ± 1	L	0.0013	0.0009	616 ± 9	863 ± 21
3.0	29 ± 2	D	0.005	0.0005	1,953 ± 60	1,537 ± 97
3.0	29 ± 2	L	0.0036	0.0036	164 ± 10	179 ± 14
4.0	4 ± 1	D	0.0003	0.0003	2,533 ± 180	2,477 ± 208
4.0	4 ± 1	L	0.0009	0.0009	621 ± 64	637 ± 17
4.0	29 ± 2	D	0.0006	0.0005	1,756 ± 234	1,256 ± 31
4.0	29 ± 2	L	0.0024	0.0034	247 ± 2	205 ± 7

*Average of two repetitions in triplicate. L = under presence of light (2,500 lumens); D = in the dark.

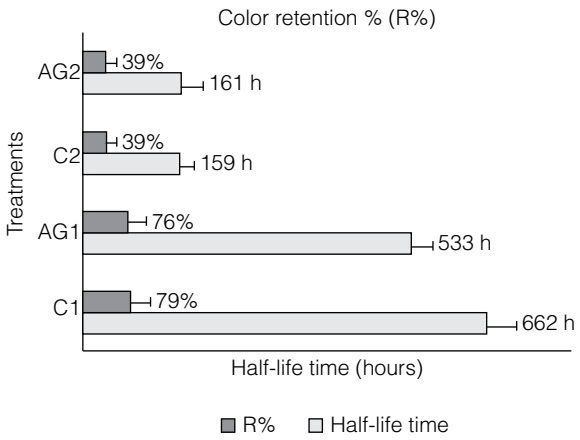


Figure 4. Half-life time and colour retention percentage (R%) values of a rehydration beverage system without (C) and with (AG) gallic acid. C1 = control at 4 ± 1 °C; AG1 = test at 4 ± 1 °C (gallic acid 2:1, w:v); C2 = control at 29 ± 2 °C; AG2 = test at 29 ± 2 °C (gallic acid 2:1, w:v).

results also indicated that significant interactions occurred between treatments and temperature ($p = 0.0010$). In the time interval evaluated, gallic acid added at the ratio of 2:1 (w:v) showed no significant effect on the colour retention percentage ($p = 0.8099$). To the half-life values for samples maintained at 29 ± 2 °C, the presence of gallic acid conferred no increase, but rather a significant reduction of half-life values at 4 ± 1 °C ($p = 0.0059$) (Figure 4).

4 Conclusions

The findings of this work showed that although interaction between the anthocyanins of the crude extract and added gallic acid has occurred, such interaction did not confer any efficient protection in the form of colour stability of the anthocyanins in a crude extract of Cabernet Sauvignon grape skins, either in a model or in a rehydration beverage system, at the ratios selected for the study. In the latter system (samples at 4 ± 1 °C), addition of gallic acid actually reduced the half-life of anthocyanins. In general, the factors, temperature, pH and the presence of light, significantly interfered with anthocyanin stability ($p < 0.05$), diminishing their colour stability. Maximum colour stability was reached when the samples were maintained at pH 3.0, at 4 ± 1 °C, and in the dark.

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