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ELHAM NIKKHAH

MASOUD KHAIAMY

REZA HEIDARY

ALI SORAYAIE AZAR

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The effect of ascorbic acid and H_2O_2 treatment on the stability of anthocyanin pigments in berries

Elham NIKKHAH[1,*], Masoud KHAIAMY[1], Reza HEIDARY[1], Ali Sorayaie AZAR[2]

[1]Department of Biology, Faculty of Science, Urmia University, IRAN

[2]Islamic Azad University, Maragheh Branch, IRAN

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Abstract: Anthocyanins are natural pigments widely distributed in nature. Anthocyanin pigment molecules are a subclass of flavonoids. They are responsible for the red, purple, and blue colors observed in many flowers, fruits, and vegetables. Fruits and berries are the main sources of anthocyanins in nature. Anthocyanins are thought to contribute to the nutritive value of fruits and berries due to their antioxidative, anti-carcinogenic, anti-inflammatory, and anti-angiogenic properties. Anthocyanins can also improve the nutritional value of processed foods by preventing the oxidation of lipids and proteins. As such, identification of agents that can affect the stability of anthocyanins and the protective effect of anthocyanins is very important. In the present study anthocyanin pigment was extracted from 3 different berries (Morus nigra L., Morus alba var. nigra, and Fragaria L.). After soaking and wetting in ethanol (1% acidified), the extracted anthocyanin pigments were exposed to 3 different concentrations of ascorbic acid (AA) (10%, 25%, and 50%) and H_2O_2 (9.31, 18.61, and 27.92 mmol/L). Six groups of anthocyanin solutions were refrigerated and kept in darkness for 63 days, and every 3 weeks anthocyanin absorbance was recorded at 526 nm. AA absorbance decreased relative to the blank in all the treated samples. These results indicate the destructive effect of AA on anthocyanins. In the samples treated with H_2O_2 anthocyanin degradation increased and the intensity of color decreased as the concentration of H_2O_2 increased.

Key words: Anthocyanin, ascorbic acid, H_2O_2, degradation, berries

Dutlarda anthosiyanin pigmentlarinin stabilitesi üzerine askorbik asit ve H_2O_2 davranışının etkisi

Özet: Anthosiyaninler, doğada geniş çapta dağılmış doğal pigmentlerdir. Anthosiyanin renk molekülleri flavonoidlerin bir alt sınıfıdır. Bunlar birçok çilek, meyve ve sebzelerde kırmızı, mor ve mavi renklerden sorumludur. Meyve ve çilekler doğada anthosiyaninlerin ana kaynağıdır. Anthosiyaninlerin anti-oksidatif, anti-kanserojen, anti-inflamatuvar ve anti-anjiyogenetic bakımından meyve ve çileklerin sağlığa katkıda bulunduğu düşünülmektedir. Anthosiyaninler aynı zamanda gıda ürünlerinde lipit ve proteinlerin oksidasyonunu engelleyerek işlenmiş yiyeceklerin besin değerini artırabilmektedir. Böylece bazı etkenlerin tanımı, bunların anthosiyaninin stabilitesi ve anthosiyanin kararlılığında etkili olması bakımından çok önemlidir. Bu çalışmada anthosiyanin pigment üç farklı çilekden (*Morus nigra* L., *Morus alba* var. *nigra* ve *Fragaria* L.) ekstre edildi. Etanolde (% 1 asidite) emdirilerek ve daha sonra ıslatılarak ekstre edilen anthosiyan pigmentler askorbik asit (% 10, % 25 ve % 50) ve H_2O_2 (9,31, 18,61 ve 27,92 mmol/L) nin üç faklı konsantrasyonuna maruz bırakıldı. Anthosiyaninin altı çözeltisi karanlıkta ve 63 gün ve her hafta soğutucuda tutuldu, anthosiyanin miktarı 526 nm kaydedildi. Tamamıyla, AA durumunda tüm örneklerde absorbans miktarı blankla bağlantılı olarak azaldı. Bu sonuçlarla anthosiyanine askorbik asidin yıkıcı etkisi gösterildi. H_2O_2 durumunda, H_2O_2 'nun yüksek konsantrasyonunda, anthosiyanin miktarı azalması yüksektir ve renk yoğunluğu düşüktür.

Anahtar sözcükler: Anthosiyanin, askorbik acid, H_2O_2, indirgeme, dut

Introduction

Anthocyanins belong to flavonoid groups and are responsible for the attractive colors—ranging from red to blue—of flowers and fruits (1). Interest in the field of anthocyanin chemistry has been generated by restricted and limited use of synthetic dyes as food ingredients. Because of low toxicity, anthocyanins have great potential as food coloring and have replaced synthetic red dyes. Recently, anthocyanins have been reported to have pharmacological effects, such as lowering the atherogenic index (2), and lowering triglyceride and free fatty acid levels (3). Moreover, Kamei et al. reported that anthocyanins are more effective in inhibiting the growth of tumor cells than other flavonoids (4).

Nonetheless, many commercial limitations exist for the use of anthocyanin extracts in food products, including low stability, which is influenced by pH, temperature, oxygen, light, polymeric forms, and concentration, and the presence of phenolic compounds and some chemical structures (5). Some anthocyanins are more stable than others, depending on their molecular structure. One example is malvidin, a major anthocyanin in grapes that is more stable than other anthocyanins due to dimethyl oxylation of the molecule (6). Hydroxylation of organic acids, in most cases, makes anthocyanins more stable molecules (7,8). Anthocyanins have a C6C3C6-skeleton typical of flavonoids. They are glycosylated polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium cation, i.e. the flavylium cation. The main constituent of anthocyanins is aglycone, the flavylium cation, which contains conjugated double bonds responsible for absorption of light at around 500 nm, causing the pigments to appear red to the human eye. Aglycones are referred to as anthocyanidins, which are usually penta- (3,5,7,3′,4′) or hexa-substituted (3,5,7,3′, 4′,5′).

Currently, 22 different anthocyanidins are known, but only 6 are commonly used in foods. The most important anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin. These aglycones differ in the number of hydroxyl and methoxyl groups in the B-ring of the flavylium cation (5). One of the general methods for combating oxidation and increasing the nutritional value of food products is the use of fruit juices with ascorbic acid (AA), but studies have shown that the presence of AA has a negative effect on anthocyanin stability, leading to the mutual degradation of these compounds (9-11). Additionally, hydrogen peroxide (H_2O_2) has been used in foods and food packaging materials for various purposes in many European countries for over 30 years. H_2O_2 is the most commonly used packaging sterilant in aseptic processing systems (12). As berries contain large amounts of anthocyanins and transformation of these pigments into other forms by variety of agents, such as enzymes, oxidation, light, temperature, and some chemicals, during storage causes their color to change from red to brown, which has a negative impact on the appearance of products and diminishes their usefulness, the aim of the present study was to investigate the effects of AA and H_2O_2 on the color appearance and color stability of fruit juice.

Materials and methods

Sample preparation

Samples of berries were obtained locally. These samples included *Morus nigra, Morus alba* var. *nigra*, *and Fragaria* L. The berries were washed with distilled water and kept frozen at −18 °C until use. The experiments were performed in 2007 at the biochemistry lab of Urmia University, Iran.

Extraction of anthocyanins

Extraction was carried out according to Chiriboga and Francis (13). Briefly, after taking the samples out of the freezer, they were left at room temperature for 30 min to defrost. Then 1000 g of each sample was placed in a mixer and after adding ethanol solvent was mixed for 10 min. Next, the products were filtered in a Büchner funnel vacuum and Whatman filter paper (grade 1); the remains of each mixture left on the filter paper was again washed with the above-mentioned solvent and filtered. The quantity of solvent used was sufficient to completely remove all color from the

berries, leaving a clear liquid. Then the filtered product was placed in a balloon container within a vacuum evaporator at 35 °C to separate the ethanol-acid solvent. The balloon container was separated from the vacuum evaporator and distilled water was added to dissolve the concentrated extract that formed at the bottom of the balloon container. The product was then transferred to a 1000-mL container, brought to the volume of 1000 mL using distilled water, and was centrifuged at 8000 rpm. The supernatant was separated and kept for further analysis.

Treatment with ascorbic acid (AA)

To examine the effect of AA, 3 concentrations of AA (10%, 25%, and 50%) were selected. Anthocyanin extracts (90 mL) were poured into 3 groups of test tubes, each with 3 replicates. At first, the pH of the anthocyanin extracts was regulated with a pH meter (pH 2). Subsequently, different concentrations of AA were added to the anthocyanin extracts. The 3 groups of anthocyanin extracts were refrigerated and kept in darkness for 63 days, and every 3 weeks the anthocyanin absorbance at 526 nm was recorded. The selected doses and wavelength were selected according to Duangmal et al. (14).

Treatment with hydrogen peroxide (H_2O_2)

To examine the effect of H_2O_2, 3 concentrations (9.31, 18.61, and 27.92 μmol/L) were selected. Anthocyanin extracts (90 mL) were poured into 3 groups of test tubes, each with 3 replicates. At first, the pH of the anthocyanin extracts was regulated with a pH meter (pH 2). Subsequently, different concentrations of H_2O_2 were added to the anthocyanin extracts. The 3 groups of anthocyanin extracts were refrigerated and kept in darkness for 63 days, and every 3 weeks anthocyanin absorbance at 520 nm was recorded. The selected doses and wavelength were selected according to Özkan et al. (15).

Statistical analysis

Statistical analysis of the data was performed with ANOVA using Microsoft SAS. All experiments were repeated 3 times.

Results and discussion

The effect of ascorbic acid (AA)

The results of statistical analysis show that during the 63-day experimental period the greatest value of absorbance was at zero time, whereas the lowest absorbance was linked to secondary time in *Morus nigra,* and third time in *Morus alba* var. *nigra* and *Fragaria* L. (Figure 1). These results highlight the destructive effect of time on the stability of anthocyanins. In the case of concentration, there was a significant difference between the primary concentration and other concentrations. In total, the greatest value of absorbance occurred with zero concentration (blank) and the lowest absorbance occurred with the primary concentration. Nonetheless, as the AA concentration increased absorbance gradually increased relative to the primary concentration. In all the samples absorbance decreased relative to the blank; therefore, results of this study suggest that AA had a destructive effect on the anthocyanins.

Other studies have shown that AA plays various roles in color stability (16,17). Poei-Langston and Wrolstad observed that addition of AA to a model system of anthocyanins resulted in the loss of pigment stability (11). Skrede et al. also reported that AA caused a decrease in pigment stability (18). Moreover, Marti et al. reported that decomposition of anthocyanin accelerated in the presence of AA (11,19). Duangmal et al. reported that fortification with vitamin C (AA) reduced the half-life values of pigments. They have observed that vitamin C promoted anthocyanin degradation (14). Duangmal et al. reported marked destruction of anthocyanins and vitamin C in Mao juice during storage. Statistical analysis showed that mean anthocyanin absorbance in the presence of various concentrations of AA significantly differed (14). The presence of AA was observed to have a negative impact on anthocyanin stability, leading to the mutual degradation of these compounds (9,10,20).

The results of the present study are in accord with all of the above-mentioned studies. These results

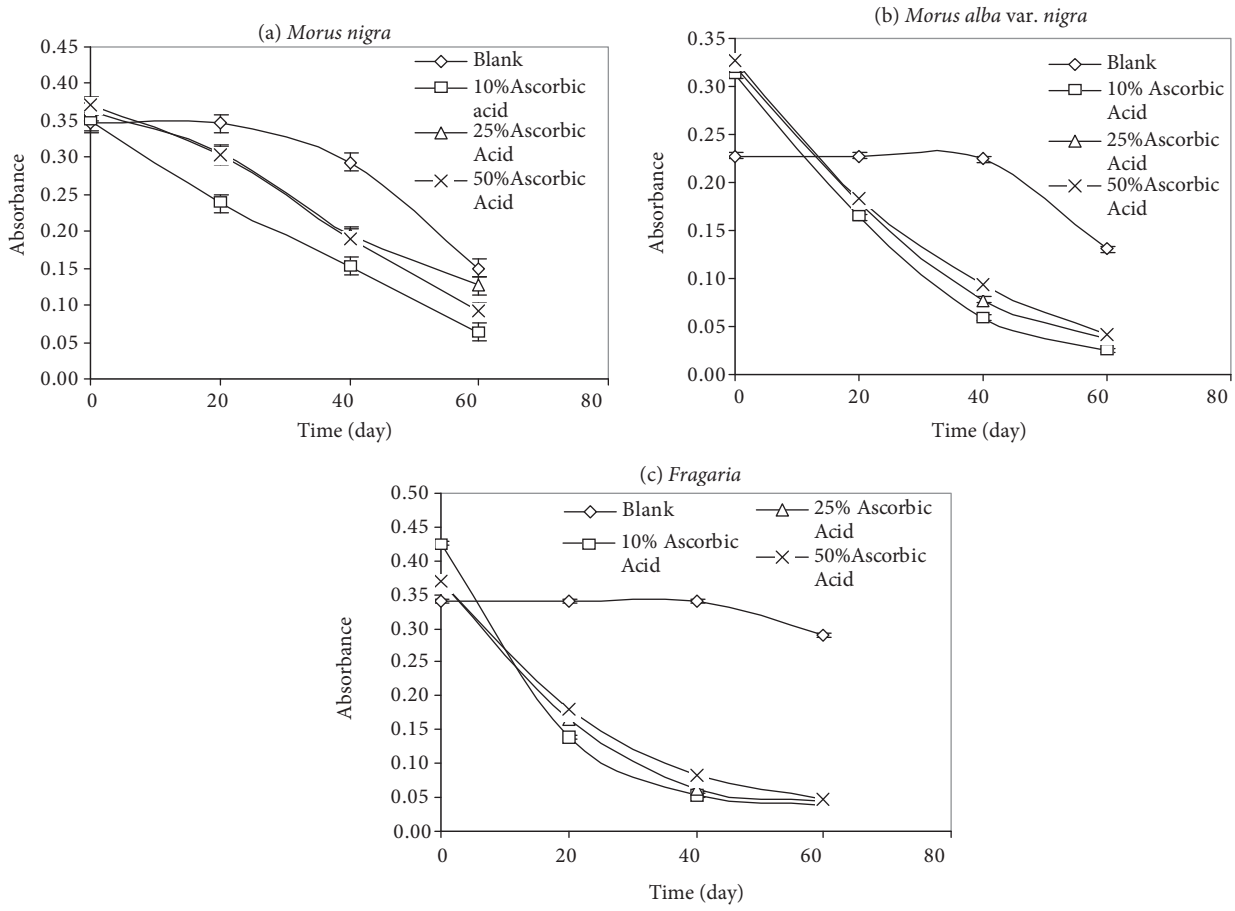

Figure 1. Changes in the anthocyanin content of extracts treated with AA during storage at 0, 21, 42, and 63 days. According to the 3 graphs, anthocyanin absorbance decreased as the concentration of AA increased, relative to the blank (the mean ± SE of 3 measurements).

might have been due to AA's enhancing polymer pigment formation and bleaching anthocyanin pigments. Direct condensation between anthocyanins and AA has been suggested as a mechanism for anthocyanin degradation (11). The mechanism proposed by Jurd (21) for the degradation of anthocyanins in the presence of AA, which was subsequently supported by Poei-Langston and Wrolstad (11), is the direct condensation of AA on the carbon 4 of the anthocyanin molecule, causing the loss of both. On the other hand, according to Iacobucci and Sweeny, the loss of anthocyanin color in the presence of AA occurs due to oxidative cleavage of the pyrylium ring by a free radical mechanism in which AA acts as a molecular oxygen activator and produces free radicals (22). One of these free radicals is H_2O_2. Formation of H_2O_2 due to AA oxidation affects anthocyanin stability (16,17) and leads to a decrease in red color (9). As AA and its derivatives are used in many foods (including fruit juices) to improve their nutritional quality and to prevent enzymatic browning reactions (23), and because of its potent antioxidant capacity by acting as a singlet oxygen quencher (24), use of this substance in some cases is inevitable. Nonetheless, there are some methods for decreasing AA's negative effects on anthocyanin stability. Shrikhande and Francis reported that the presence of flavonol exerts a protective effect with respect to the degradation of anthocyanins in the presence of AA—probably by competing with

anthocyanins in preference for condensation reactions (25)—and also observed that the stability of acylated anthocyanins increases in the presence of AA (26). It seems that AA protects anthocyanins from enzymatic degradation (17). Sapers and Simons (27), and Sapers et al. reported that the stability of acylated anthocyanins increases in the presence of AA and that AA protects anthocyanins from enzymatic destruction (17).

The effect of H_2O_2

In the present study higher concentrations of H_2O_2 increased anthocyanin degradation and decreased the intensity of color. The results of statistical analysis show that during the 63-day study period, absorbance in the 3 groups of anthocyanins (each with a different concentration of H_2O_2) was significantly different, as in the above-mentioned reports. Based on our statistical analysis, there were significant differences between the means of various times, from 0 to 3. With time, absorbance and anthocyanin content gradually decreased. This result was observed in 3 samples (Figure 2). Moreover, there were significant differences between various concentration means and H_2O_2 concentrations. Anthocyanin degradation increased and, as a result, absorbance decreased. In all samples the highest level of absorbance was at zero concentration and the lowest was at the 3rd concentration (27.92 mmol/L).

One of the chemical agents that affect the stability of anthocyanins is H_2O_2. The deleterious effect of

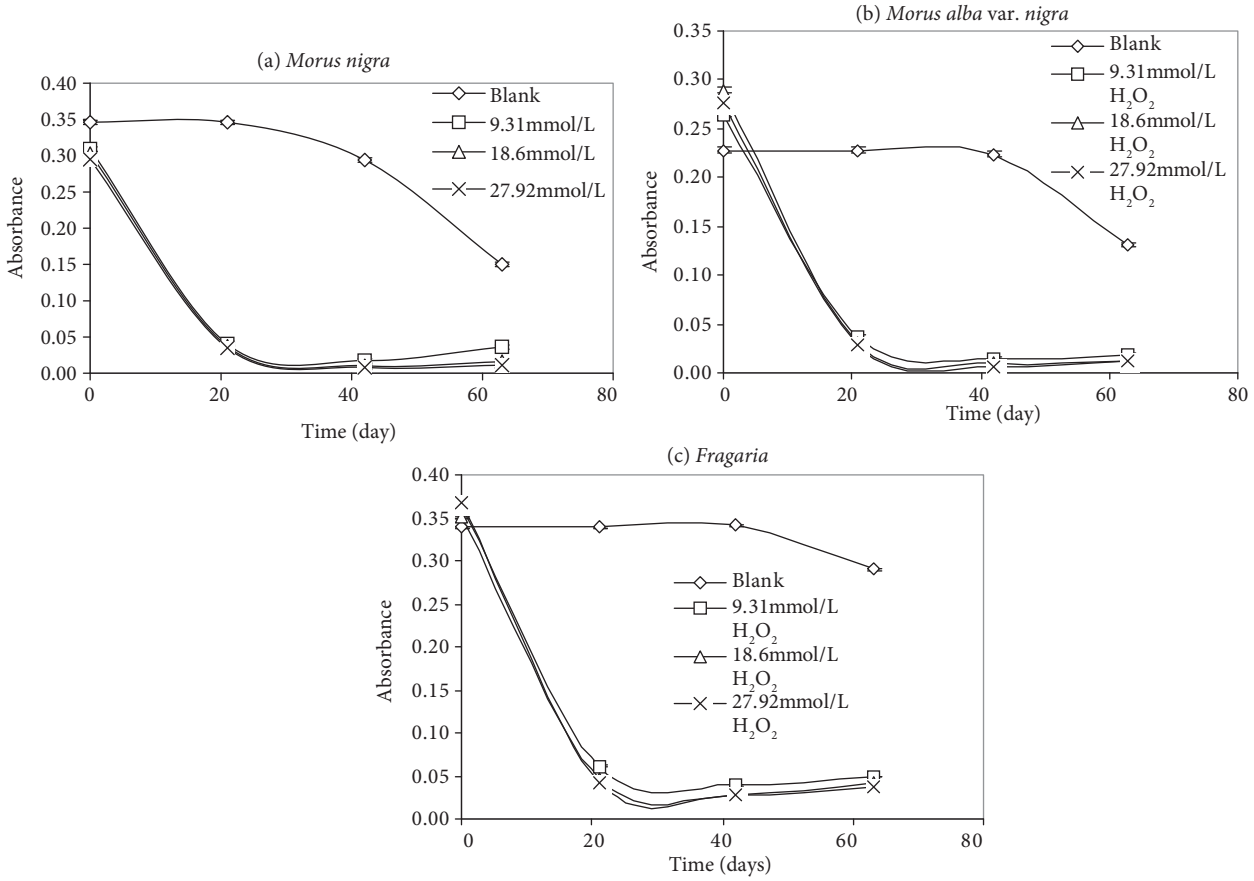

Figure 2. Changes in the anthocyanin content of extracts treated with H_2O_2 during storage at 0, 21, 42, and 63 days. Based on the 3 graphs, anthocyanin absorbance decreased as the concentration of H_2O_2 increased, relative to blank (mean ± SE of 3 measurements).

H_2O_2 on anthocyanin stability in fruit juices is well known. Degradation of anthocyanins in the presence of H_2O_2 has been demonstrated in strawberry (29), sour cherry juice (30), and in orange, grape, and pomegranate juice (31). Different sensitivities to various concentrations of H_2O_2 have been reported (27). Sapers and Simmons observed rapid decolonization of strawberry, raspberry, and cherry anthocyanins in the presence of H_2O_2. They used H_2O_2 as a surface sterilizer in sweet cherries, raspberries, and strawberries, and observed rapid bleaching of anthocyanins. Statistical analysis shows that the concentrations of anthocyanins in the presence of various H_2O_2 concentrations were significantly different, and in the presence of high concentrations of H_2O_2 destruction of anthocyanins was rapid, as was observed in the present study (27).

The susceptibility of anthocyanins to H_2O_2 has been known for a long time. Sondheimer and Kertesz were among the first to investigate the kinetics of anthocyanin degradation by H_2O_2 in both strawberry juice and in a pure solution of the major strawberry anthocyanin (pelargonodin-3-glucoside) (29). According to their research, oxidative degradation of anthocyanins occurs in 2 steps: an initial reversible reaction with the formation of anthocyanin-H_2O_2 adduct, followed by a slower irreversible one. It was shown that the decomposition and dissociation products of H_2O_2 are responsible for the oxidation and subsequent degradation of phenolic compounds (27,28). In fact, De et al. reported that the °OH radical is the main reactive species to cleave the benzene ring in phenolic compounds, and degrades the substrate into CO_2 and H_2O (32). Von Elbe and Schwartz reported that quinines, formed by the oxidation of phenols, also have deleterious effects on anthocyanins (33). Thus, 2 factors can primarily affect the degradation of anthocyanins by H_2O_2 in fruit juices, which generally contain copious amounts of phenolic compounds: (a) the amount of free radicals and the HOO anion formed by the decomposition and dissociation of H_2O_2, respectively; and (b) the amount of quinones formed by the H_2O_2-catalyzed oxidation of phenolic compounds (33).

Conclusion

Attractive color is one of the most important sensory characteristics of fruit and berry products; however, the red color of berry products is unstable and susceptible to degradation. Maintaining a strong and stable color in berry wines and juices is problematic during processing and storage. In the present study we observed that there was marked destruction of anthocyanins in berry juices containing H_2O_2 during storage. Destruction of anthocyanins in berry juices containing AA was also observed, but with less intensity. As such, aseptic systems should be frequently controlled to ensure the effective removal of residual H_2O_2 from food contact surfaces and fortification of aseptically packed anthocyanin-rich fruit juices with AA should be avoided or carried out very carefully.

Corresponding author:
Elham NIKKHAH
Department of Biology,
Faculty of Science,
Urmia University,
IRAN
E-mail: tu8084@yahoo.com

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