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Color enhancing effect of carboxylic acids on anthocyanins

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Abstract

The color enhancing effect of aromatic and aliphatic carboxylic acids on anthocyanin-glucosides was investigated in this work. The color of black rice anthocyanin-rich fraction was successfully improved after the addition of aromatic acids (*p*-coumaric acid, ferulic acid, and sinapic acid), aliphatic acid (lipoic acid) and tannic acid (as a complex acid). The addition of carboxylic acids showed an enhancing and stabilizing effect on the color of black rice juice during storage, which was observed through changes in CIELAB and spectrophotometric parameters. Sinapic, ferulic, and *p*-coumaric acids enhanced the color of black rice juice the most, with the highest efficiency for the dimethoxylated cinnamic acid derivate (sinapic acid). New anthocyanin-derived pigments were first isolated through silica gel TLC and identified using Maldi-Mass spectrometry at m/z values of 613, 659, 673, and 639, respectively, for vinylpyranoanthocyanins formed after adding coumaric, ferulic, sinapic, and lipoic acids. Based on these experimental results, the addition of carboxylic acids in an anthocyanin-rich fraction can be used as an efficient tool for their color enhancement and stability.

Keywords: Anthocyanin-rich fraction; Carboxylic acids; Bathochromic shift; Hyperchromic effect; Vinylpyranoanthocyanins; Color enhancement and stability; Black rice

1. Introduction

Food color has a significant impact by being one of the major factors used by consumers to take the purchasing decision because it influences human behavior (Temares 2001). Anthocyanins are an important group of natural pigments. They are widely distributed in the nature and are responsible of attractive red, purple and blue colors of many flowers, fruits and vegetables. From the viewpoint of the global awareness on the health benefits and attractive color of the pigments they contain, the attractive red or blue color of food products, like strawberry jams, blueberry yoghourt or raspberry jellies is currently considered as an important quality parameter in the Japan food market.

Anthocyanins, which are highly unstable pigments, are a group of naturally occurring compounds with a practical use in food industry. However, using those coloring compounds with a strong and stable color during the processing and storage steps remains problematic. It is well-known (Goto & Kondo, 2003) that the color of anthocyanin strongly depends on the pH, appearing red in acidic, violet in neutral, and blue in basic aqueous solution. Very recently, however, reinvestigation has disclosed that the color variation and stabilization of anthocyanins in aqueous solution could be due to other phenomenon, namely self-association, co-pigmentation and intermolecular or intramolecular hydrophobic interactions between anthocyanidins (aromatic nuclei) and aromatic acids (Darias-Martin et al., 2002; Talcott, Brenes, Pires, & Del Pozo-Insfran, 2003). In this work, novel anthocyanins resulted from the chemical modification of native anthocyanins using various carboxylic acids have shown various colors in the acidic range of the pH, supporting previous statement.

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Based on the above considerations, research on the chemistry of colors based on intermolecular interactions between anthocyanins and carboxylic acids was performed in applied level, in order to increase the π–π electrons system of this pigment, to enhance their color and improve their stability. To reach this goal, carboxylic acids (aliphatic or aromatic), in particular lipoic acid, *p*-coumaric acid, ferulic acid, sinapic acid, and tannic acid have been used as color enhancing agents of anthocyanins in this work.

2. Materials and methods

2.1. Color enhancers

The color enhancers used in this study were aromatic (*p*-coumaric acid, ferulic acid, sinapic acid), aliphatic (lipoic acid), and complex (tannic acid) compounds. The chemical structures of these compounds are given in Fig. 1.

2.2. Preparation of anthocyanin-rich fraction from black rice

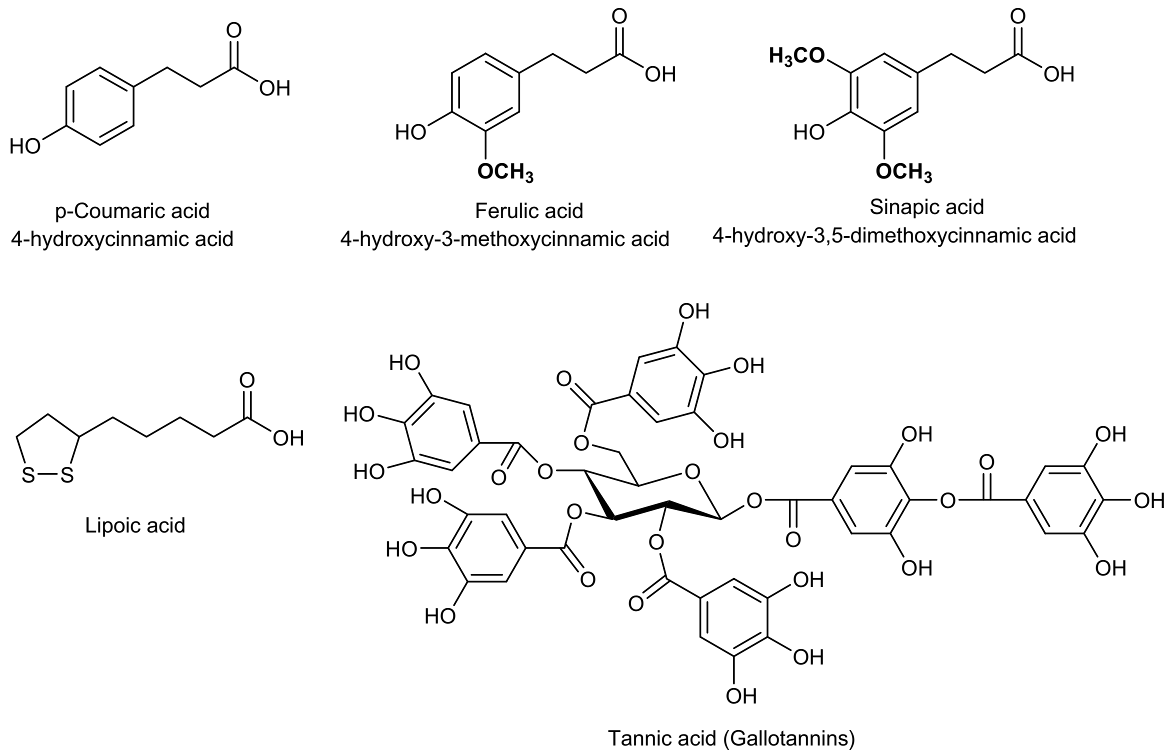
Black rice flour (1 g) was extracted with acidic MeOH (10 mL) containing 1% HCl to fully release anthocyanins from the vacuolar inclusions. Extracting solvent was removed under reduced pressure and the residue was dissolved in water to make a solution with an absorbance value less than 1.2. The pH of anthocyanin-rich solution was adjusted at 3.5 using 0.1 N HCl. The anthocyanin-rich fraction of black rice was then ultrasonicated during 45 min to break down microbial cell walls before adding carboxylic acids.

2.3. Addition of carboxylic acids into the anthocyanin-rich fraction

In order to enhance the color of the anthocyanin-rich fraction of black rice, carboxylic acids (2, 4, and 6 mg/mL in 1 mL) were first converted into their respective chlorides by reacting them with the Lewis base ($AlCl_3$) (1 mL, 2 mg/mL) for 3 h in a sealed test tube. Then, carboxylic acids (in their chloride form, 2 mL) were added individually into the anthocyanin-rich fraction (3 mL), followed by a vortex-mixing and an incubation during 1 h at room temperature. After incubation, enhanced solutions were subjected to UV–Vis spectrophotometric and CIELAB measurements. Then, enhanced juices were stored at 6 °C in sealed tubes and the color stability and enhancement of the anthocyanin-rich fraction was followed using spectrophotometric, CIELAB, and Maldi-mass measurements as described below.

2.4. Spectrophotometric measurements

Absorption spectra of enhanced juices were recorded using a UV–Vis spectrophotometer, scanning the visible



p-Coumaric acid
4-hydroxycinnamic acid

Ferulic acid
4-hydroxy-3-methoxycinnamic acid

Sinapic acid
4-hydroxy-3,5-dimethoxycinnamic acid

Lipoic acid

Tannic acid (Gallotannins)

Fig. 1. Structures of carboxylic acids: aromatic (*p*-coumaric acid, ferulic acid, sinapic acid), *aliphatic* (lipoic acid), and *complex* (tannic acid) *compounds*. Within aromatic carboxylic acids, compounds were selected based on the number of methoxyl groups attached to the cinnamic acid aglycon, with zero for *p*-coumaric acid, one for ferulic acid, and two for sinapic acid.

range of radiations from 400 to 800 nm. The change of the maximum absorbance (A_{max}) at varying wavelengths (λ_{max}) was observed. This revealed a possible *hyperchromic effect* (ΔA_{max}) and *bathochromic shift* ($\Delta\lambda_{max}$), resulting from the formation of a more longer chromatophore (via an intermolecular interaction between the carboxylic acid and the anthocyanin).

2.5. CIELAB measurements

CIELAB parameters were determined using the D-65 diffused illumination of a Minolta Chroma CR-210 Colorimeter. The measured parameters were L^* (lightness), a^* (redness), and b^* (yellowness). The three measured color parameters were converted into C^* (chroma), h (hue angle), and ΔE (total color change) using following equations:

(1) $C^* = [(a^*)^2 + (b^*)^2]^{1/2}$
(2) $h = \arctan(b^*/a^*)$
(3) $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

2.6. Maldi mass spectroscopic analysis

After their isolation through a silica gel TLC using buthanol:acetone:water (3:1:1, v/v) as a mobile phase, Maldi mass spectroscopy was applied to the analysis of the newly formed pigments. Mass spectrum was taken by operating in the positive laser ionization mode.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was conducted using SPSS (version 10, 1999, SPSS Inc., Chicago, IL) to compare CIELAB parameters in different treatments, and values were given as means ± SD. Significant ($P < 0.05$) differences between means of three replicates were identified using Tukey's test.

3. Results and discussion

3.1. Spectral characteristics as affected by the addition of carboxylic acids

The color enhancement of anthocyanin-rich fraction of black rice using aromatic (*p*-coumaric, ferulic, and sinapic acids), aliphatic (lipoic acid) and complex (tannic acid) acidic compounds improved the color of the black rice anthocyanin-rich fraction, resulting in an enhancement of the absorbance and generally, a shift to the higher λ_{max} values of the newly formed pigments (vinylpyranoanthocyanins). In the first experiment, the color of anthocyanins was enhanced by adding various concentrations (2, 4, and 6 mg/mL) of carbocylic acids. This resulted in an inverse correlation, by means, the higher was the concentration of carboxylic acids, the lower were the bathochromic and hyperchromic shifts (Fig. 2). In detail, non-enhanced anthocyanin-rich fraction (the control) had 530

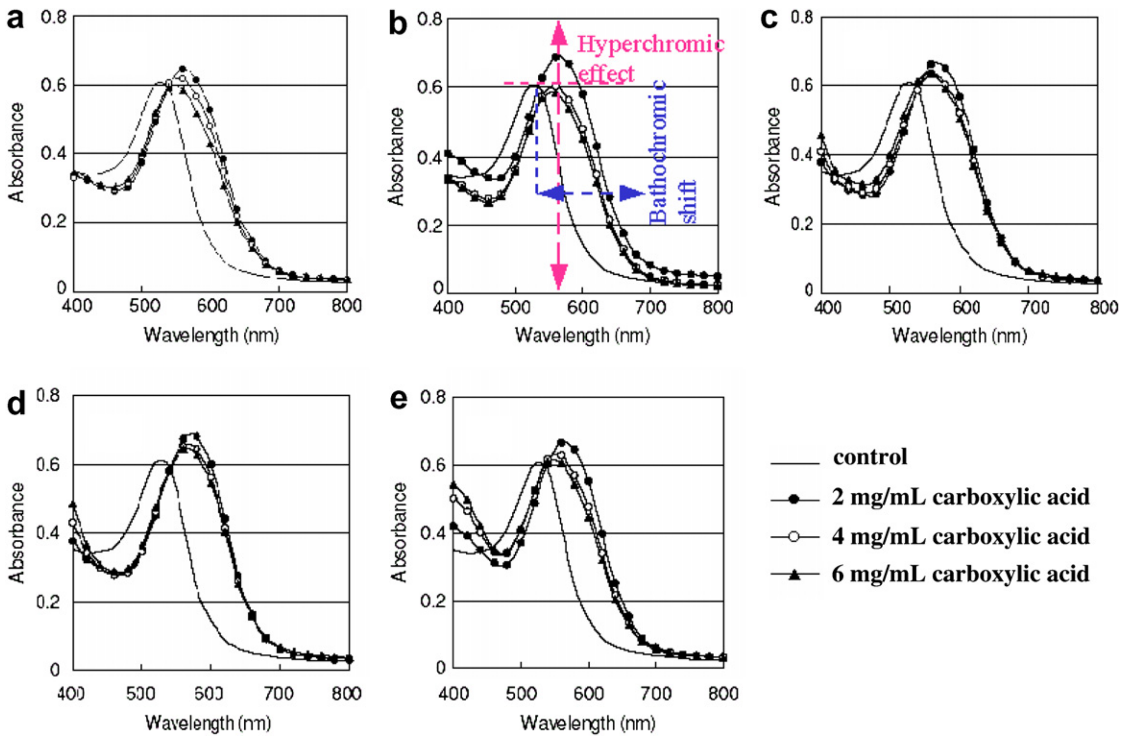


Fig. 2. Effect of various concentrations of carboxylic acids on the VIS spectral characteristics of the anthocyanin-rich fraction of black rice. The addition of carboxylic acids was done by using increasing concentrations of acrboxylic acids (2, 4, 6 mg/mL). An inverse correlation was observed between increasing concentrations and the resulting λ_{max} and A_{max} values. Juice enhanced with (a), *p*-coumaric acid; (b), ferulic acid; (c), sinapic acid; (d), lipoic acid; and (e), tannic acid.

and 0.622, respectively, as λ_{max} and A_{max} values, whereas those of solutions enhanced with *p*-coumaric acid (560 and 0.643; 553 and 0.623, 545 and 0.601), ferulic acid (565 and 0695; 557 and 0.606; 554 and 0.588), sinapic acid (566 and 0.667; 559 and 0.633; 557 and 0.637), lipoic acid (571 and 0.691; 567 and 0.658; 564 and 0.647), tannic acid (565 and 0.669; 551.5 and 0.634; 532 and 0.601) as values of λ_{max} and A_{max} following various concentrations of respective carboxylic acids (2, 4, and 6 mg/mL). These results showed a decreasing tendency of both λ_{max} and A_{max} when increasing concentrations of carboxylic acids were added to the anthocyanin-rich fraction and could be explained as follows: using high concentrations, carboxylic acids could not be completely converted to the chloride form (more electrophile), thus the non-converted compound could react as a nucleophile to form their related dimers (less reactive to anthocyanins) prior to their addition in the black rice juice.

Fig. 3 shows the spectral characteristics of the anthocyanin-rich fraction of black rice, as affected by the addition of carboxylic acids (the λ_{max}, expressed as bathochromic or hypsochromic effect, and the A_{max}, expressed as hyperchromic or hypochromic shift). The observed hyperchromic shift of anthocyanin-rich fraction is due to the increase in the π–π electrons system (chromophore), responsible of the absorption in the VIS radiations range. This explanation is in accordance with the below suggested intermolecular interactions. The first proposed reaction mechanism (Fries rearrangement) allows the substitution of the chromophore in the 4 or 6 position of the chromane ring of anthocyanin-glucosides (Grossman, 2003), whereas the second (Michaelis addition) leads to the substitution of chromophore at the 4 or 5 position.

The addition of carboxylic acids in the anthocyanin-rich fraction of black rice improved the juice color during 4 weeks of storage and the color enhancement measured as the shift of the λ_{max} of the absorption spectra (bathochromic shift) during storage was time-dependent. The longer the storage was, the higher the color enhancement effect became. The effect of adding carboxylic acids on the λ_{max} showed, except for the non-enhanced solution, a shift to higher values with different extents. The non-enhanced solution considered as zero bathochromic shift at the beginning of the storage had demonstrated, after four weeks of storage, a hypsochromic shift (shift to lower values of the λ_{max}) of −0.6%. In contrast, samples enhanced

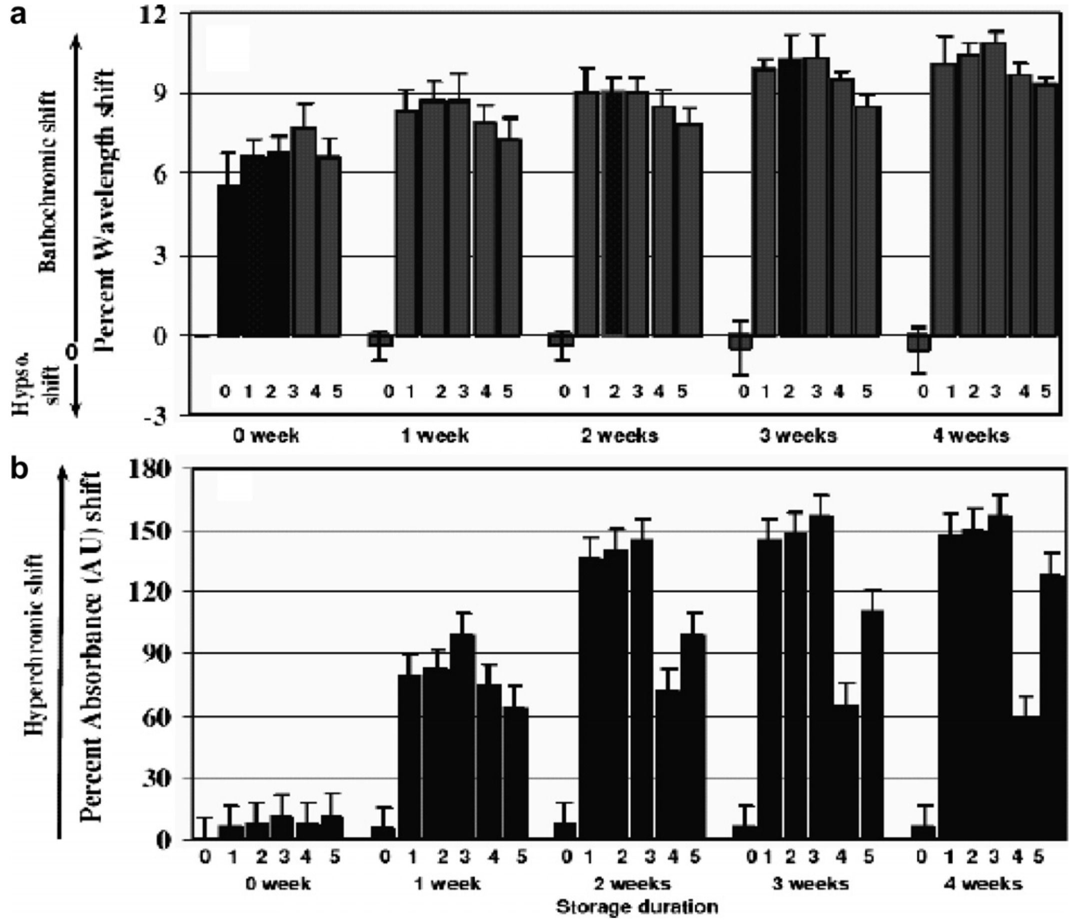


Fig. 3. Effect of the addition of carboxylic acids on the visible spectral characteristics of anthocyanin-rich solutions of black rice. (a) expressed as bathochromic or hypsochromic shifts, (b) expressed as hyperchromic shift, with (1) Non-enhanced juice, and enhanced with (2) *p*-Coumaric acid, (3) Ferulic acid, (4) Sinapic acid, (5) Lipoic acid, and (6) Tannic acid.

with carboxylic acids increased their $\lambda_{\max}$ with a bathochromic shift from 5.6% to 10.0% for *p*-coumaric acid, 6.6 to 10.4% for ferulic acid, 6.8% to 10.9% for sinapic acid, 7.7% to 9.7% for lipoic acid, and 6.6% to 9.3% for tannic acid, respectively, during storage.

Both non-enhanced and enhanced anthocyanin-rich solutions increased their values of $A_{\max}$ (hyperchromic shift) with different extents. Non-enhanced solution showed a hyperchromic shift of 0–5.7%, whereas enhanced solutions demonstrated significant increment of their $A_{\max}$: from 5.4% to 147.0%, 7.2% to 150.0%, 10.9% to 156.0%, 7.6% to 59.0%, 11% to 128.0% as $A_{\max}$ shift of solutions enhanced, respectively, with *p*-coumaric acid, ferulic acid, sinapic acid, lipoic acid, and tannic acid at the beginning and after four weeks of storage. The hyperchromic effect of treated samples is explained by the increase in the chromophore resulting from the formation of vinylpyranoanthocyanins, whereas that of non-enhanced sample might be attributed to a possible intermolecular association between molecules of anthocyanin leading to the formation of dimers during the storage of the samples.

All five carboxylic acids used in this work enhanced juice color during storage. That enhancement was more efficient with sinapic acid (a dimethoxylated carboxylic acid) from the seventh day of storage and thenceforth. Carboxylic acids with or without aromatic nuclei and different substituents in the aromatic skeleton of the molecule significantly affects the anthocyanin color enhancement and stability.

3.2. Effect of the carboxylic acids addition on the hue (*h*)

Among samples tested, only the non-enhanced solution remained in the red color region with a slight increase of the hue value from 36 ± 0.0 to 38 ± 0. Immediately after addition of carboxylic acids to the anthocyanin-rich fraction and during storage, a significant increment of the hue values of enhanced solutions ranging from 287 ± 3.0 to 311 ± 2.0, 295 ± 0.9 to 313 ± 2.0, 298 ± 1.0 to 335 ± 3.0, and 297 ± 2.0 to 302 ± 3.0, respectively, for *p*-coumaric, ferulic, sinapic, and tannic acids, with the highest increment of the hue value for the dimethoxylated phenolic acid (sinapic acid) as shown in Table 1. This increment of the hue value strongly attests the length increasing of the π–π electrons system in the structure of the newly formed anthocyanins. In contrast, a decrease of the hue value was observed in the aliphatic carboxylic acid (lipoic acid) from 308 ± 2.0 to 238 ± 2.0, which was thought to be due to the fact that the novel pigment derived from this compound was not stable during storage, then could lead to a reversible reaction generating in part both the native anthocyanins and lipoic acid. The hue values of enhanced solutions were higher than that of non-enhanced, and showed purple, bluish and vivid blue colors as shown in Table 1 and Fig. 5. This result supports the present work which demonstrated the color enhancing ability of carboxylic acids having the same carbon skeleton and substituted by various number of methoxyl residues.

3.3. The metric chroma (C^*) as affected by the addition of carboxylic acids

The metric chroma correlates with the saturation of the color (Gonnet, 1999). The C^* values in the beginning and at the end of storage are shown in Table 1. Chroma of original plain juice decreased during storage from 32.1 ± 1.2 to 22.9 ± 1.1 and the same tendency was observed for all enhanced solution, from 23.6 ± 1.1 to 11.6 ± 0.6, 25.0 ± 1.0 to 11.7 ± 0.2, 19.8 ± 2.6 to 12.8 ± 0.8, 20.7 ± 0.5 to 11.8 ± 1.0, 21.3 ± 2.5 to 19.6 ± 1.4, respectively, for *p*-coumaric, ferulic, sinapic, lipoic, and tannic acids. These data indicates that the addition of carboxylic acids significantly decreased the value of chroma 2 h after acylation and during storage, leading to less vivid colors.

3.4. The total color difference (ΔE) as affected by the addition of carboxylic acids

The total color difference, which is a combination of the changes of the three components (*chroma, hue, and lightness*) was the greatest with the dimethoxylated aromatic acid (sinapic acid). The monomethoxylated and non-methoxylated aromatic acids demonstrated similar change of the color difference values from 5.4 ± 0.1 to 13.9 ± 1.5, 5.4 ± 1.2 to 12.7 ± 1.3, and 5.6 ± 0.3 to 18.4 ± 0.5, respectively, for *p*-coumaric, ferulic, and sinapic acids. The total color difference of tannic acid was the highest at the begin-

Table 1
Chroma (C^*), hue (h), and color difference (ΔE) of enhanced anthocyanin-rich fraction of black rice

Juice + carboxylic acid	Chroma (C^*)		Hue (h)		Color difference (ΔE)	
Time (days)	0b	28	0b	28	0b	28
Black rice juice	2.1 ± 1.2e	22.9 ± 1.1.b	36 ± 0.0a	38 ± 0.1a	–	3.0 ± 0.7a
+ *p*-Coumaric acid	23.6 ± 1.1c	11.6 ± 0.6a	287 ± 2.6b	311 ± 1.8d	5.4 ± 0.1b	13.9 ± 1.5c
+ Ferulic acid	25.0 ± 1.0d	11.7 ± 0.2a	295 ± 0.9c	313 ± 1.5c	5.4 ± 0.1b	13.9 ± 1.5c
+ Sinapic acid	19.8 ± 2.6a	12.8 ± 0.8a	298 ± 1.3d	335 ± 2.9f	5.6 ± 0.3c	18.4 ± 0.5d
+ Lipoic acid	20.7 ± 0.5b	11.8 ± 1.0a	308 ± 1.7e	238 ± 1.9b	3.6 ± 1.0a	12.5 ± 0.6c
+ Tannic acid	21.3 ± 2.5bc	19.6 ± 1.4c	297 ± 2.3d	302 ± 3.2c	8.1 ± 0.9c	10.5 ± 1.0b

Chroma, hue, and color difference are expressed as means ± standard deviation. b means measurements done immediately after adding carboxylic (carb.) acids. For each assay, carboxylic acids were added at the concentration of 2 mg/mL and all measurements were taken in triplicates, $n = 3$. The same letters in the same column are not significantly different ($P = 0.05$).

ning, though its increment was not so significant as compared to the three aromatic carboxylic acids. In contrast, lipoic acid (the aliphatic carboxylic acid) had lower color change at the beginning but the increment of this value was significantly higher than that of tannic acid as shown in Table 1. A value of $\Delta E = 1$ is assumed as a basis for a color difference noticed by human eye (Gonnet, 1998). Using the ΔE, the color stability is appreciated by the increase or no change of its value. For all enhanced juices, the ΔE was higher than that of the non-enhanced one, with the highest value for sinapic acid.

3.5. Changes in the mass spectral characteristics and proposal of the chemical pathway

To make the evidence of the formation of novel pigments after adding carboxylic acids, newly formed compounds were isolated using Silica gel TLC and their mass spectra were measured. The new anthocyanin derived from pigments in black rice juice was detected at *m*/*z* values of 613, 659, 673, and 639, respectively, for the juices enhanced by *p*-coumaric, ferulic, sinapic and lipoic acids.

Fig. 4 shows the mass spectra of the new pyrano-anthocyanin molecules isolated and detected from enhanced juice indicating that novel pigments were formed and giving a clear explanation to the origin of the color enhancement resulted from this chemical modification of black rice anthocyanins. The mass spectral data given in this work are related to the cyanidin aglycone, which is the major anthocyanin in black rice. Peonidin-3-glucoside being the minor pigment in the black rice and further in diluted anthocyanin-rich fraction, thus, derived novel pigment was not isolated and consequently their mass spectra were not determined in this work.

On the basis of these experimental results, aromatic acids and especially methoxylated phenolic acids were the efficient enhancing agents with the highest color enhancement ability. Among aromatic acids, sinapic acid (the dimethoxylated compound) was more efficient in enhancing the color of juice than ferulic (monomethoxylated) and *p*-coumaric acid (non-methoxylated). Thus, this observation leads to postulate that the higher the methoxylation level of anthocyanin (for the same aglycon) is, the higher the color enhancement and stability effect becomes.

Fig. 4 shows molecular ions of new vinylphenols derived from the reaction with *p*-coumaric acid (*m*/*z* 613), ferulic acid (*m*/*z* 659), sinapic acid (*m*/*z* 673), and lipoic acid (*m*/*z* 639), respectively. This is a clear evidence of the formation of new pigments in enhanced juices. Schwarz, Wabnitz, and Winterhalter (2003) suggested the formation of anthocyanin–vinylphenol adducts in red wines through enzymatic decarboxylation, then probably, with regards to the mass characteristics of isolated new pigments, similar compounds were also formed into the juice after chemical modification of

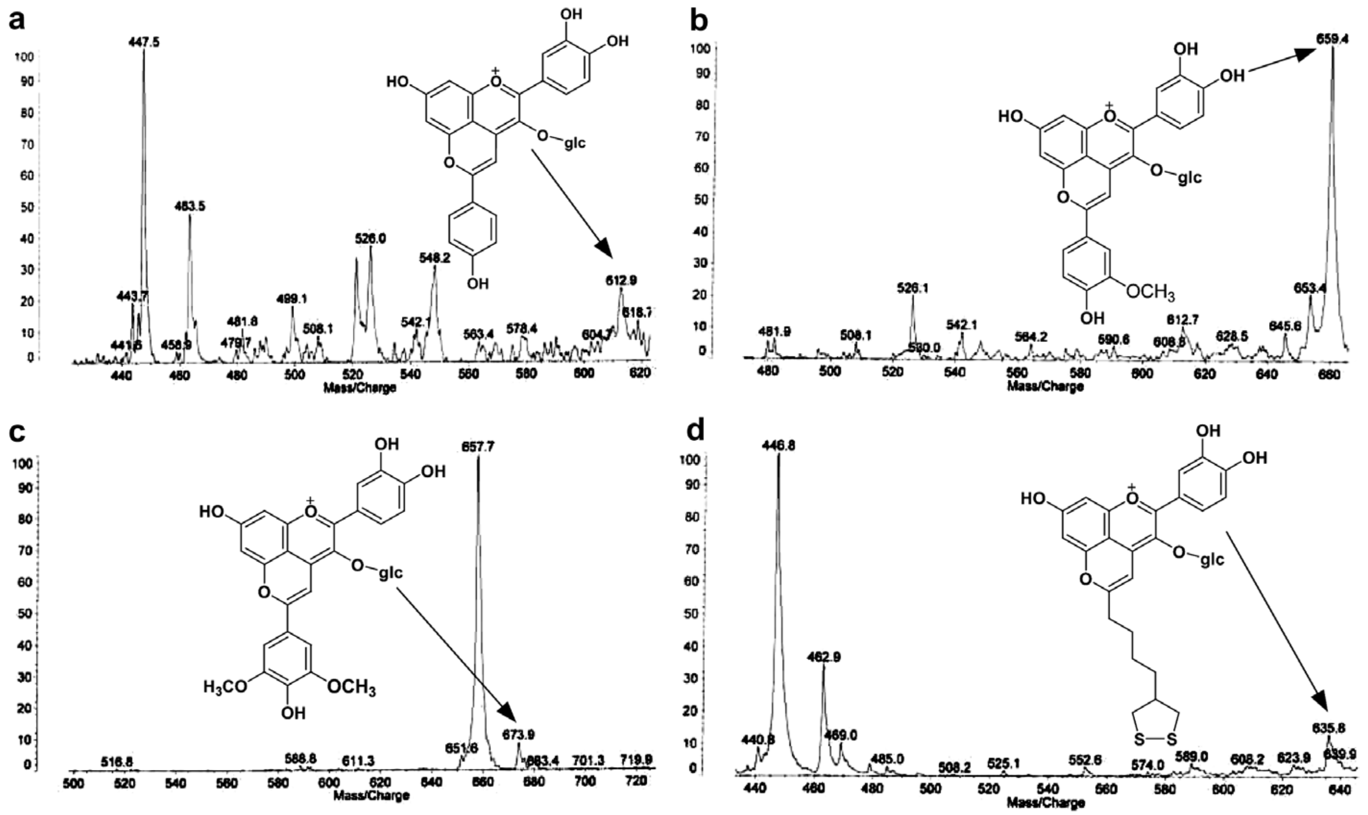


Fig. 4. Full scan mass spectra and molecular structures of cyanidinbased pyranoanthocyanin. Vinylphenolanthocyanins after addition of carboxylic acids: *p*-coumaric acid (*m*/*z* 613), ferulic acid (*m*/*z* 659), sinapic acid (*m*/*z* 673), and lipoic acid (*m*/*z* 639).

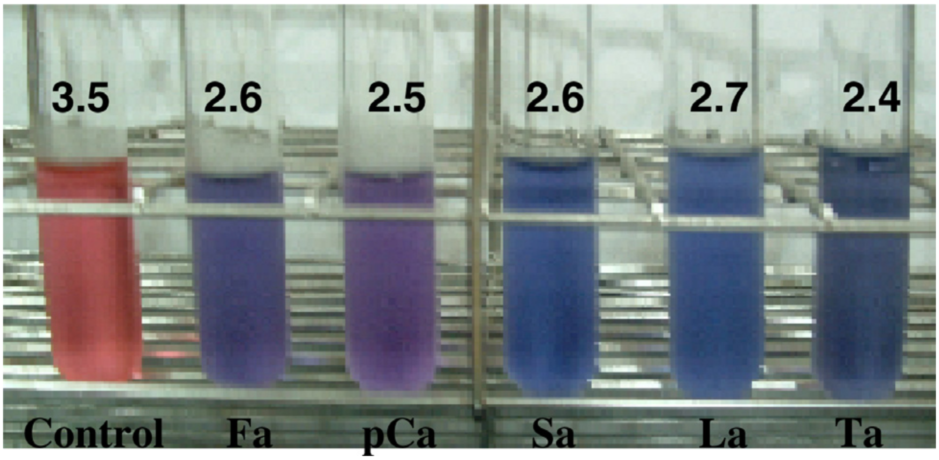


Fig. 5. Various colors of anthocyanin-rich solution of balck rice as affected by the addition of carboxylic acids. With pCa, p-coumaric acid; Fa, ferulic acid; Sa, sinapic acid; La, lipoic acid, and Ta, tannic acid. The control (O) used in this assay was the non-enhanced anthocyanin-rich fraction. Values above each image show the pH of the enhanced solution immediately after adding carboxylic acids.

the black rice anthocyanin-rich fraction. Although the mechanism of the chemical modification was not elucidated in the present work, these results support the hypothesis and finding of color enhancing effect of carboxylic acids on anthocyanin. As Harborne (1967) reported that the acyl groups mainly occurs at the positions of C-3 and C-6 of monosaccharide, therefore, proposed mechanisms of the chemical modification of anthocyanin-glucosides are reported in this work as a hypothesis of the next investigation. The first chemical pathway is related to the sugar acylation, followed by the *Fries rearrangement*: $AlCl_3$ catalyzes the conversion of aryl esters (resulted from sugar acylation) to aryl alkyl ketones. The second pathway lies with the *Michaelis addition*, which proceeds through the formation of vinylphenols via a following serie of reactions; Michaelis addition, cyclization, dehydrogenation, decarboxylation, oxidation, and aromatization. Among the two supposed pathways, the latter seems to be more evident according to the proposed structure of newly formed pigments.

Both of the above cited mechanisms can be elucidated using ^{1}H NMR but were not investigated in the present work and that study belongs to the next challenge.

4. Conclusion

The present experimental results suggest that anthocyanin, a less stable pigment, can be converted into vinylphenols (a more stable compound) via chemical modification (Michaelis addition) resulting in the enhancement of their color and stability. The chemical modification of anthocyanins studied in this experiment improved the color of the anthocyanin-rich fraction of black rice by stabilizing and enhancing their spectrophotometric and CIELAB parameters. This chemical modification resulted also in the formation of vinylpyranoanthocyanins, which possessed higher coloring properties, stability and stronger colors as compared to their corresponding non-modified ones.

The color enhancement of black rice anthocyanin-rich fraction can be regarded as a promising tool of improving unstable color of anthocyanin-glucosides by adding carboxylic acids into the medium.

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