



Investigating the Effect of Amino Acid Addition on the Colour Values of Black Mulberry (*Morus nigra* L.) and Orange Juice (*Citrus sinensis* L. Osbeck) Blend

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Abstract

This study investigated the effects of orange juice (OJ) addition and amino acids with different chemical structures on anthocyanin stability in black mulberry juice (BMJ), and to identify their roles in copigmentation. BMJ (0.65% acidity) was blended with OJ to increase acidity (1.34%). Five amino acids (aspartic acid (Asp), phenylalanine (Phe), valine (Val), asparagine (Asn), and lysine (Lys)) were added to the BMJ: OJ system. Changes in total monomeric anthocyanins (TMA), colour density, polymeric colour ratio, anthocyanin profile, amino acid profile, and ascorbic acid content were evaluated. All amino acids reduced TMAs (up to 5.4%). Asp promoted copigmentation by increasing $A_{\max}$ and colour density, while Phe, Val, and Asn induced condensation, and LYS accelerated anthocyanin degradation. Asp, Phe, and Asn reduced browning and polymeric colour formation through amino acid: o-quinone interactions or PPO inhibition via amino acid: Cu(II) chelation. In contrast, Lys underwent Maillard reaction, increasing browning and polymeric colour ratio. Asp, Phe, Val, Asn, and Lys formed chelates with Cu(II) and Fe(III) ions, increasing ascorbic acid stability. In contrast, Val combined with phenolic compounds reduced the protective effect on ascorbic acid. As a result of these reactions, the amino acid-added samples had lower free amino acid content than added (10.3–93.5%). The data suggest that copigmentation occurs only in Asp with an acidic side chain, suggesting van der Waals forces or electrostatic interactions between negatively charged Asp and positively charged anthocyanins in BMJ and BMJ: OJ media. Therefore, amino acids with acidic side chains are recommended as copigments.

Keywords Black mulberry juice · Anthocyanins · Aspartic acid · Copigmentation · Colour stability

Introduction

Black mulberry is a concentrated source of bioactive compounds (minerals, phenolic compounds, vitamins, and anthocyanins) responsible for its characteristic red colour [1]. These beneficial compounds contribute to the anti-carcinogenic, anti-inflammatory, anti-diabetic, and anti-aging properties of black mulberries [2–6]. However, despite its considerable health benefits, black mulberry juice is less popular than other fruit juices when consumed alone

because of its low acidity. Although it is most commonly consumed as syrup, molasses, jam, or marmalade, it is also consumed as a beverage by mixing it with higher-acid fruit juices in Turkey [7]. Similarly, oranges are rich in bioactive compounds. It is also one of the most preferred fruits in the form of fruit juice. Orange juice (OJ) is rich in bioactive compounds such as ascorbic acid, other water- and fat-soluble vitamins, phenolics, organic acids, and carotenoids, which impart antioxidant, anti-inflammatory, and anti-carcinogenic properties and have been linked to reduced risks of cardiovascular disease and kidney stone formation [8–11].

Colour is one of the most significant factors influencing consumer food preference. The attractive red colour of black mulberry juice (BMJ) is attributed to anthocyanins, specifically cyanidin-3-glucoside and cyanidin-3-rutinoside [12]. However, fruit juice processing stages have a detrimental effect on anthocyanins; therefore, strategies need to

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be developed to increase their stability during these stages. One method of increasing the resistance of anthocyanins to processing steps and increasing colour density is copigmentation [13]. Copigmentation is an interaction between anthocyanins and organic compounds. This interaction between anthocyanins and organic compounds can be achieved using various methods, including intramolecular, intermolecular, metal complex, or self-assembly approaches. Intramolecular copigmentation occurs when anthocyanins form covalent bonds with copigments. In this type of copigmentation, the copigment becomes a part of the anthocyanin. Intermolecular copigmentation occurs through weak interactions (hydrogen bonding, electrostatic interactions etc.) between anthocyanins and copigments. In self-association, the copigment is the anthocyanin itself, whereas the metal complex occurs between anthocyanins and metals such as iron and aluminum [14, 15]. This type of copigmentation is not preferred in the food industry because it indicates metal complex migration [16].

Organic compounds used as copigments include phenolic compounds, organic acids, alkaloids, anthocyanins, and amino acids [17]. Among these organic compounds, the copigmentation effect of phenolic compounds has been extensively studied [18]. Although the copigmentation effect of amino acids on anthocyanins has been less extensively studied than that of phenolics, there is growing interest in this area. Gao et al. examined the influence of amino acids (glutamate, tryptophan, proline, tyrosine, and Phe) on the copigmentation of aronia anthocyanins in the presence of ascorbic acid. They reported that 0.3% tryptophan yielded the highest improvement in stability [19]. Cheng et al. investigated the effect of whey protein isolate on anthocyanin stability of black mulberry anthocyanin extract [20]. The study found that whey protein isolate improved the stability of anthocyanins. In a study investigating the effect of different copigments (five phenolic acids, two flavonoids, and three amino acids (Asp, methionine, tryptophan)) on the colour stability of anthocyanin extract obtained from *Rhododendron arboreum*, it was found that amino acids increased the colour density of anthocyanins, but did not increase the anthocyanin stability against light, temperature, and oxygen [17]. Türkyılmaz et al. examined the role of Asp, proline, and Val in anthocyanin stability during the thermal treatment of pomegranate-orange juice mixtures [21]. The results showed that all three amino acids increased the stability of anthocyanins (up to 4.7 times), especially at 90 °C. This study is the first to investigate the copigmentation effect of the amino acids Asp, Phe, Val, Asn, and Lys on BMJ and a BMJ: OJ blend. In addition, this study is the first to investigate the effect of ascorbic acid on the copigmentation between black mulberry anthocyanins and amino acids.

Materials and Methods

Chemicals and Reagents

Amino acids (Asp, Phe, Val, Asn, and Lys) and cyanidin-3-glucoside were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). The solvents used in all HPLC and spectrophotometric analyses were purchased from Merck Co. (Darmstadt, Germany). Sep-Pak C18 cartridges used to purify the anthocyanins were purchased from Waters Co. (Milford, MA, U.S.A.). Ultrapure water (18.2 MΩ.cm), used in all HPLC and chemical analyses, was freshly prepared using a Millipore water purification system (Millipore Simplicity UV, Molsheim, France).

BMJ and OJ Production

Black mulberries (*Morus nigra*) were obtained from Antalya (Türkiye) and processed into juice in the juice pilot plant at Ankara University Pressing was carried out in a rack and cloth press (Bucher-Guyer Ltd., Niederweningen, Switzerland) with a yield of 53%. Oranges (*Citrus sinensis*) were obtained from Mersin (Türkiye) and subjected to manual pressing, resulting in a 49% (w/w) yield. Both juices were filtered through 7 layer of muslin cloth. The resulting juices were not clarified, transferred directly into one-litre PET bottles and stored at a temperature of −25 °C (MDFU731M, Panasonic Healthcare Co. Ltd., Tokyo, Japan) until copigment addition.

Adding Copigments and OJ to BMJ

Asp, Phe, Val, Asn, and Lys were selected as copigments because of their distinct chemical structures. Phe features an aromatic and hydrophobic structure, Val an aliphatic structure, Lys a basic structure, Asp an acidic and hydrophilic structure, and Asn an amide structure. Amino acids were initially added to BMJ to allow copigmentation to occur. For this purpose, three distinct anthocyanin: amino acid ratios (1:1, 1:2, and 1:3, w: w) were tested in this study. The highest copigmentation effect was observed at a 1:3 anthocyanin: amino acid ratio. Amino acids were subsequently added to BMJ at this ratio and sonicated (Elmasonic S 180 (H), Elma Schmidbauer GmbH, Singen, Germany) at 30 °C for 15 min to dissolve the amino acids. This ratio was determined with reference to the Turkish Food Codex Communiqué on Fruit Juices and certain similar products. According to the communiqué, the fruit juice ratio in black mulberry nectar should be at least 40% (w: w). The prepared samples were pasteurised in a water bath (Memmert WB 14, Germany) at 85 °C for 2 min.

Total Monomeric Anthocyanin (TMA) Content

The TMA content in BMJ: OJ blends was determined using the pH-differential method, described by Giusti et al. [22]. The juice samples were passed through a 0.45 µm PVDF membrane filter (Sartorius AG, Goettingen, Germany). The filtrates were then diluted (1:50) in two buffer systems: 0.025 M potassium chloride at pH 1.0 and 0.4 M sodium acetate. After a 15-min incubation at room temperature, absorbance readings were recorded at 510 nm ($\lambda_{\max}$ for anthocyanins) and 700 nm (turbidity correction) using 1-cm disposable cuvettes (Brand GmbH, Wertheim, Germany) on a ThermoScientific Evolution 201 double-beam spectrophotometer (Cambridge, UK). Ultrapure water was used as the blank. TMA was determined as cyanidin-3-glucoside equivalents using the following Eq. (1).

$$\text{Total monomeric anthocyanin content (mg/L)} = \frac{A \times M_w \times DF \times 1000}{\epsilon \times l} \quad (1)$$

Where

$A = (A_{\lambda_{\max}} - A_{700})_{\text{pH } 1.0} - (A_{\lambda_{\max}} - A_{700})_{\text{pH } 4.5}$; M_w : molecular weight of cyanidin-3-glucoside (445.2 g/mol); DF : dilution factor (50); ϵ : molar absorptivity of cyanidin-3-glucoside (26,900 L·cm⁻¹·mol⁻¹); l : pathlength (1 cm).

Bathochromic and Hyperchromic Effect

The main indicators of copigmentation are the increase in bathochromic and hyperchromic effects. An increase in the wavelength ($\lambda_{\max}$) corresponding to the maximum absorbance indicates the bathochromic effect, and the increase in the maximum absorbance ($A_{\max}$) indicates the hyperchromic effect. After 15 min of incubation, the samples were diluted 1:9 with water (v: v), and the wavelengths were scanned in the range of 450–600 nm. The values of hyperchromic effect ($A_{\max}$) and bathochromic effect ($\lambda_{\max}$) were determined using a UV-VIS spectrophotometer (ThermoScientific Evolution 201).

Colour Density (CD), Polymeric Colour (PC) and Polymeric Colour Ratio (PCR)

CD, PC and PCR values were determined using the bisulfite bleaching method described by Giusti et al. [22]. A 1 mL of BMJ: OJ blend was diluted with water to 50 mL. Then, it was mixed with water for CD and with potassium metabisulfite (20%) (2.8:0.2) for PC. After equilibration at room temperature for 15 min, absorbance values were determined at 510 nm ($\lambda_{\max}$) (anthocyanin), 420 nm (browning) and 700 nm (turbidity) nm. Ultrapure water was used as the blank. CD, PC and PCR were calculated using the following Eqs. (2–4).

$$CD = [(A_{420} - A_{700}) - (A_{510} - A_{700})] \times DF \quad (2)$$

$$PC = [(A_{420} - A_{700}) - (A_{510} - A_{700})] \times DF \quad (3)$$

$$PCR = (PC/CD) \times 100 \quad (4)$$

Where DF : dilution factor (50).

HPLC Analyses

Anthocyanin Profile

Anthocyanins were extracted and purified following the procedure reported by Lee et al., which was later adapted by Türkyılmaz et al. [23, 24]. Solid-phase purification was conducted using C18 cartridges (100 mg, 0.25 mL, Waters Co.) connected to a vacuum manifold to ensure consistent extraction. Identification of individual anthocyanins was carried out with an Agilent 1200 series HPLC-DAD system (Waldbronn, Germany), equipped with a binary pump, PDA detector, thermostatted autosampler, degasser, and column oven. Data acquisition and processing were performed using ChemStation software (Agilent 1200, rev. B.02.01). Anthocyanin separation was performed according to the method described by Skrede et al. with slight modifications, employing a C₁₈ reversed-phase column (250×4.6 mm, 5 µm; Phenomenex, USA) fitted with a C₁₈ guard column (4×3 mm, 5 µm) [25].

The mobile phase consisted of solvent A (acetonitrile) and solvent B (o-phosphoric acid, acetic acid, acetonitrile, water; 1:10:5:84, v/v/v/v). The gradient program was as follows: 0–10 min, 0–12% A; 10–20 min, 12–22% A; followed by a 5-min hold at 22% A. The flow rate was 1 mL/min, injection volume was 50 µL, column temperature was 25 °C, and detection wavelength was 520 nm. Quantification was conducted using a cyanidin-3-glucoside calibration curve ($R^2 = 0.9995$) with at least six data points, and sample dilution factors were considered.

Amino Acid Profile

The amino acid profiles of BMJ and BMJ: OJ samples enriched with different amino acid were determined using the HPLC method described by Hermosin et al. and modified by Türkyılmaz et al. [21, 26]. For analysis, the amino acid-added samples were diluted with borate buffer (sodium tetraborate–HCl, pH 9.00) at a ratio of 0.5:24.5 (v/v), whereas the control sample was diluted 2:3 (v/v). Subsequently, 10 µL of diethyl ethoxymethylenemalonate (DEMM) was added, and derivatised on a magnetic stirrer (IKA-Werke RT10P, Staufen, Germany) at 50 °C for 50 min. The derivatised mixtures were filtered through a 0.45 µm PVDF filter

(Sartorius AG), transferred into 2 mL amber vials, stored at 4 °C for two days, and then injected into HPLC (Agilent 1200 series). Amino acids were separated on C₁₈ column (250 × 4.6 mm, 5 µm) fitted with a C₁₈ guard column (Waters X-Bridge, Ireland). The mobile phase consisted of solvent A (100% acetonitrile) and solvent B (2.5 mM acetate buffer, pH 5.8). Gradient elution was performed as follows: 1% A for 8 min, 1–16% A for 5 min, 16–18% A for 0.5 min, 18% A for 3.5 min, 18–22% A for 3 min, 22–32% A for 12 min, 32% A for 7 min, and finally, a return to 1% A within 11 min. The chromatographic conditions included a flow rate of 0.9 mL/min, a 50 min elution time, a 50 µL injection volume, column temperature of 16 °C, and detection at 280 nm wavelength. Amino acids were identified by comparing the retention times with external standards. Quantification was performed using calibration curves for each external standard [Asp ($R^2 = 0.9998$), Phe ($R^2 = 0.9999$), Val ($R^2 = 0.9998$), Asn ($R^2 = 0.9999$), and Lys ($R^2 = 1.0000$)] each constructed from five concentration levels. The results were expressed in mg/L.

Ascorbic Acid Analysis

The ascorbic acid content in the BMJ samples was determined using the HPLC-DAD procedure described by Türkyılmaz et al. [21]. For analysis, 1 mL of each BMJ: OJ blend was diluted to 50 mL with a metaphosphoric acid solution (2.5%, w/v). The diluted samples were filtered through a 0.45 µm PVDF membrane (Sartorius AG), transferred into 2 mL amber HPLC vials, and injected immediately. The same Agilent 1200 series HPLC system mentioned in the “2.7.1 Anthocyanin profile” section was used to detect and quantify ascorbic acid in the juice samples. Separation was achieved using a C18 reversed-phase column (250 × 4.6 mm, 5 µm) fitted with a C18 guard column (Phenomenex Inc.). A 2% KH₂PO₄ solution (pH 2.4, adjusted with o-phosphoric acid) was used as the mobile phase, and the analysis was carried out isocratically for 15 min at a flow rate of 0.5 mL/min. The injection volume and column temperature were set

to 10 µL and 30 °C, respectively, and the wavelength was set to 244 nm. The identification of the ascorbic acid peak was based on comparison of retention times and UV spectra obtained with the DAD detector. Quantification was performed using the linear equation of a seven-point calibration curve (0–50 mg/L, $R^2 = 0.9999$), with the appropriate dilution factor applied for each sample.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., USA). Results were evaluated using one-way analysis of variance (ANOVA). Significant differences between the means were determined using Duncan's multiple range test ($p < 0.05$). All experimental analyses were conducted at least in duplicate. Statistically significant differences are indicated by lowercase superscript letters in the figures.

Results and Discussion

Effect of Asp on BMJ and BMJ: OJ Anthocyanins

Among the amino acids added as copigments to BMJ and BMJ: OJ samples, Asp was the only amino acid with an acidic side chain (Table 1). The addition of Asp to BMJ and BMJ: OJ samples resulted in a decrease in TMA levels by 2.75 and 2.12%, respectively ($p < 0.05$) (Fig. 1A). The reduction in TMA levels in fruit juices with added copigments is a predictable result. Sendri et al. investigated the copigmentation effect of phenolic compounds and amino acids on anthocyanins obtained from *R. arboreum* [17]. In general, both phenolic compounds and amino acids reduced the amount of TMA. Asp, methionine and tryptophan were used in this study, and the most significant reduction in TMA levels was observed with the addition of Asp. The reduction in TMA levels observed in the BMJ and BMJ: OJ samples with the addition of Asp may be attributed to either the

Table 1 Structural and electrostatic classification of the investigated amino acids at BMJ* pH conditions [29]

Amino Acid	Side-Chain Structure and Classification	Isoelectric Point (pI)	Charge State of Side Chain at BMJ pH (~4.2)
Asp	-CH ₂ -COOH (Acidic/Polar)	2.77	Negative -CH ₂ -COO ⁻
Phe	-CH ₂ -C ₆ H ₅ (Aromatic/Non-polar)	5.48	Neutral
Val	-CH-(CH ₃) ₂ (Aliphatic/Non-polar)	5.96	Neutral
Asn	-CH ₂ -CONH ₂ (Neutral/Polar)	5.41	Neutral
Lys	-CH ₂ -CH ₂ -CH ₂ -CH ₂ -NH ₂ (Basic/Polar)	9.74	Positive -CH ₂ -CH ₂ -CH ₂ -CH ₂ -NH ₃ ⁺

*BMJ: Black mulberry juice, Asp: Aspartic acid, Phe: Phenylalanine, Val: Valine, Asn: Asparagine, Lys: Lysine

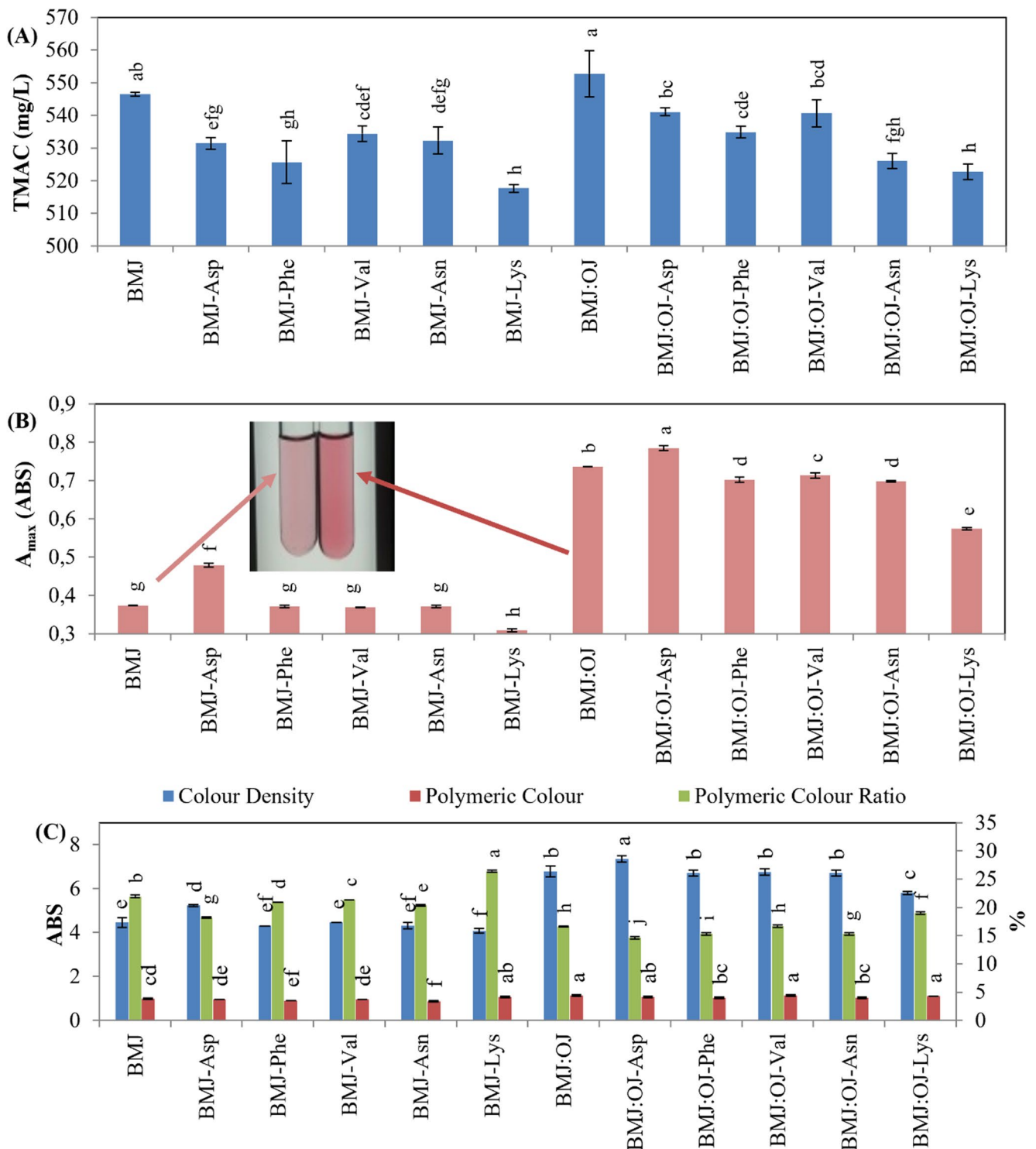


Fig. 1 Evaluation of anthocyanin and colour characteristics in black mulberry juice and blends. ^{a-h} Different superscript indicates statistically significant difference ($p < 0.05$). (A) Total monomeric anthocyanin content (TMAC, mg/L), (B) Maximum absorbance (A_{max} , ABS), (C) Colour density (CD, ABS), Polymeric colour (PC, ABS) and Polymeric colour ratio (PCR, %). BMJ: black mulberry juice, BMJ-Asp: Aspartic acid-added BMJ, BMJ-Phe: Phenylalanine-added BMJ,

BMJ-Val: Valine-added BMJ, BMJ-Asn: Asparagine-added BMJ, BMJ-Lys: Lysine-added BMJ, BMJ: OJ: Black mulberry juice: orange juice blend, BMJ: OJ-Asp: Aspartic acid-added BMJ: OJ, BMJ: OJ-Phe: Phenylalanine-added BMJ: OJ, BMJ: OJ-Val: Valine-added BMJ: OJ, BMJ: OJ-Asn: Asparagine-added BMJ: OJ, BMJ: OJ-Lys: Lysine-added BMJ: OJ

degradation of TMA or the copigmentation of anthocyanins with Asp, resulting in the loss of their monomeric structure. To understand this phenomenon, it is essential to study the change in TMA levels in relation to other parameters such as CD, $A_{\max}$ and PCR (Fig. 1A-C). When examining the effect of OJ on the amount of TMA, it was observed that the amount of TMA increased from 546.5 to 552.7 ($p > 0.05$) in the BMJ sample and from 531.4 to 541.0 ($p < 0.05$) in the BMJ-Asp sample with the addition of OJ (Fig. 1A). OJ is a rich source of antioxidant compounds, including organic acids and phenolic compounds. This antioxidant activity may have protected the anthocyanins in BMJ from the degradative effects of ultrasound and pasteurisation.

When the $A_{\max}$ values of the BMJ and BMJ: OJ samples were examined, it was observed that the $A_{\max}$ value increased from 0.3095 to 0.4785 (27.8%) in the BMJ sample with the addition of Asp (Fig. 1B). In the BMJ: OJ sample, although not as strong as in BMJ, an increase in the $A_{\max}$ value was observed, rising from 0.7365 to 0.7845 (6.52%). This change in the $A_{\max}$ value of the TMA content in BMJ supports the hypothesis of copigmentation between Asp and BMJ anthocyanins (Fig. 1A, B). Studies have indicated that these copigmentation processes occur through the formation of weak non-covalent intermolecular interactions, such as hydrogen bonding, and electrostatic interactions [27]. Amino acids contain oxygen and nitrogen atoms that can form hydrogen bonds with anthocyanins. Unlike other amino acids, Asp has an additional carboxyl group (Table 1). The increased number of carboxyl groups enhances the binding capacity of amino acids via hydrogen bonding [28]. Furthermore, Asp is hydrophilic and negatively charged at the pH of BMJ (4.19), suggesting potential copigmentation with positively charged BMJ anthocyanins through hydrogen bonds or electrostatic interactions [29].

When the effect of OJ on $A_{\max}$ was investigated, a 96.7% increase was observed in BMJ, whereas a 63.9% increase was noted in the BMJ-Asp sample (Fig. 1B). The addition of both OJ and Asp resulted in a 109.5% increase in this value. The addition of OJ to the BMJ and BMJ-Asp samples resulted in a 1.81% increase in TMA content (Fig. 1A). The notable increase in $A_{\max}$ can be attributed to the presence of phenolic compounds in OJ, which can engage in copigmentation with anthocyanins. The Valencia variety of oranges used in this study is known to be rich in apigenin, isosacuranetin, and ferulic acid, especially hesperidin and naringin [30]. In a study investigating the effects of different phenolic compounds (hesperidin, catechin, and gallic acid) on the stability of cranberry anthocyanins, hesperidin containing extracts had the highest amount of anthocyanins [31]. Similarly, the effect of various phenolic compounds and amino acids (naringin, catechin, rosmarinic acid, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, Asp,

methionine, and tryptophan) on *Rhododendron arboreum* anthocyanins showed that naringin had the most pronounced copigmentation effect [17]. In parallel with the increase in $A_{\max}$, there was a corresponding increase in CD ($r = 0.998$), whereas the PCR value decreased ($r = -0.913$). The addition of Asp to the BMJ and BMJ: OJ samples resulted in the highest CD values (5.23 and 7.35 ABS, respectively) and the lowest PCR values (18.19 and 14.63%, respectively) (Fig. 1C). The change in CD and PCR values with the addition of Asp can be attributed to the fact that Asp prevents browning while providing copigmentation (Fig. 1C). Asp exerted an inhibitory effect on PPO activity. Wu et al. showed that Asp decreased PPO activity in fresh wet noodles by lowering the pH and forming a chelate with Cu(II) [32]. Asp also reacts with browning products and converts them to light-coloured products. In the present study, Asp may have decreased PPO activity until pasteurisation and converted browning products into lighter-coloured products after pasteurisation.

Cyanidin-3-glucoside (cy-3-glu, 872 mg/L) and cyanidin-3-rutinoside (cy-3-rut, 950 mg/L) were identified in BMJ samples (Fig. 3A). There was no significant change in the cy-3-glu, cy-3-rut, or total anthocyanin content in the BMJ samples with the addition of Asp ($p > 0.05$, Fig. 2A). In the BMJ: OJ sample, the concentration of cy-3-glu was 2.3% ($p < 0.05$) higher than that in the BMJ sample. Similarly, to TMA, OJ maintained a stable anthocyanin content throughout the ultrasonication and pasteurisation processes.

To investigate the change in ascorbic acid content in OJ, OJ was diluted at the same ratio as the BMJ: OJ samples. While OJ had an ascorbic acid content of 565.4 mg/L, the BMJ: OJ samples had lower ascorbic acid contents ranging from 527 to 543 mg/L (Fig. 2B). Martí et al. investigated the changes in ascorbic acid and anthocyanin levels in pomegranate juice with added ascorbic acid [33]. In the ascorbic acid added pomegranate juice, complete degradation of ascorbic acid was observed within four days. In contrast, in the model system prepared with distilled water, this period was 12 days at 25 °C and one month at 5 °C. Poei-Langston et al. observed that the degradation of ascorbic acid in model systems was due to condensation between anthocyanins and ascorbic acid [34]. In addition, ascorbic acid degradation is catalysed by the minerals Cu(II) and Fe(III) [35]. BMJ contains both minerals [36]. Therefore, the mineral content of BMJ may have caused greater degradation of ascorbic acid in the BMJ: OJ mixture than in OJ. When the ascorbic acid content of the BMJ: OJ and amino acid-added BMJ: OJ samples was analysed, the amino acid-added samples generally had higher levels of ascorbic acid. A study by Imer et al. investigated the effect of amino acids on the stability of ascorbic acid under Cu(II) catalysis and found that the rate of ascorbic acid degradation increased

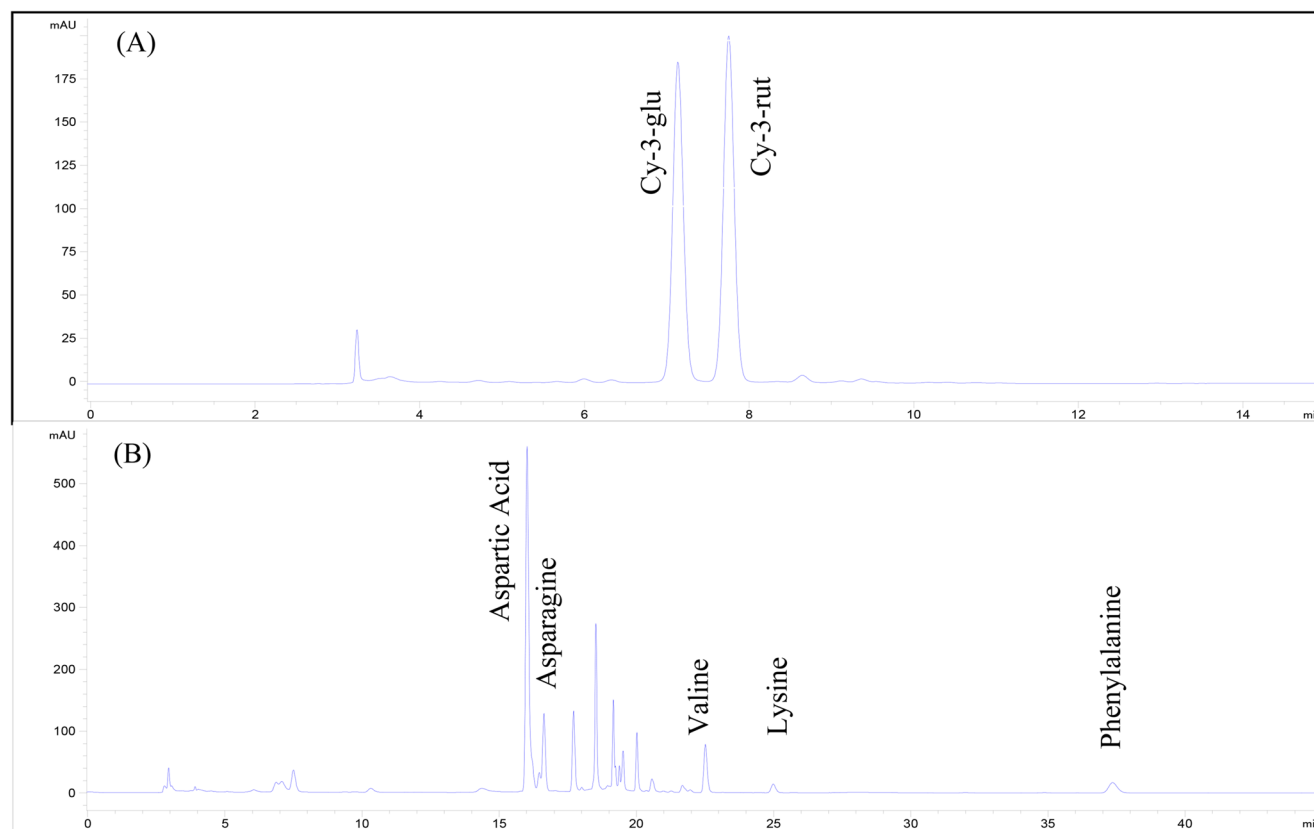


Fig. 2 Anthocyanin profile of black mulberry juice (BMJ) (A) and amino acid profile of amino acid-added BMJ mixture (B)

with increasing Cu(II) concentration ($0.785\text{--}6.28 \times 10^{-7} M$), whereas it decreased with increasing amino acid concentration ($0.1\text{--}2.0 \times 10^{-3} M$) [37]. This was attributed to the formation of a chelate between the amino acids and Cu(II) ions. In the present study, the formation of chelates of amino acids with Cu(II) and Fe(III) ions may have slowed the degradation of ascorbic acid. In this study, Asp showed the least significant increase in ascorbic acid stability (Fig. 2B). When the effects of similar amino acids (Asp, Phe and Asn) were investigated by Imer et al., they found that Asp provided the least significant stability enhancement [37]. In addition, the PCR in the BMJ: OJ samples with amino acid addition was lower than that in the control sample. This may be due to the slow formation of brown pigment caused by the slow degradation of ascorbic acid.

Considering the anthocyanin content (4198 mg/L) and dilution (40% BMJ ratio) of the BMJ, 1679 mg/L of amino acids were added to BMJ and BMJ: OJ samples. However, none of the samples to which amino acids were added showed this level of free amino acids (109–1505 mg/L, Table 2). Amino acids can undergo copigmentation or condensation with anthocyanins, Maillard reactions with reducing sugars or chelate formation with minerals in the BMJ: OJ blend [21, 37–39]. The changes observed in the $A_{\max}$ value, ascorbic acid level, and PCR value suggest that Asp

may have undergone copigmentation with anthocyanins or formed chelates with minerals such as Cu(III) and Fe(III) (Figs. 1B and C and 2B).

Effect of Phe, Val, and Asn on BMJ and BMJ: OJ Anthocyanins

Phe, Val, and Asn, chosen as copigments, have neutral side chains. These amino acids have aromatic and hydrophobic (Phe), aliphatic (Val), and amide (Asn) side chains (Table 1). The addition of these amino acids significantly reduced TMA levels in both the BMJ and BMJ: OJ samples ($p < 0.05$, Fig. 1A). The TMA content of the BMJ sample was 546.5 mg/L. In comparison, the TMA levels of the samples containing Phe, Val and Asn were 525.5, 534.4, and 532.3 mg/L, respectively. While this value was 552.7 mg/L in the BMJ: OJ samples, the addition of these neutral amino acids resulted in a decrease to 534.8, 540.6, and 526.0 mg/L (Fig. 1A). For an accurate evaluation of the reduction in TMA levels (2.19–4.83%) compared to that in the control samples, it is essential to take the $A_{\max}$ value into account. In the BMJ samples, these neutral amino acids did not show a statistically significant increase in the $A_{\max}$ value compared to the control sample ($p > 0.05$, Fig. 1B). In BMJ: OJ samples, all three amino acids resulted in a statistically

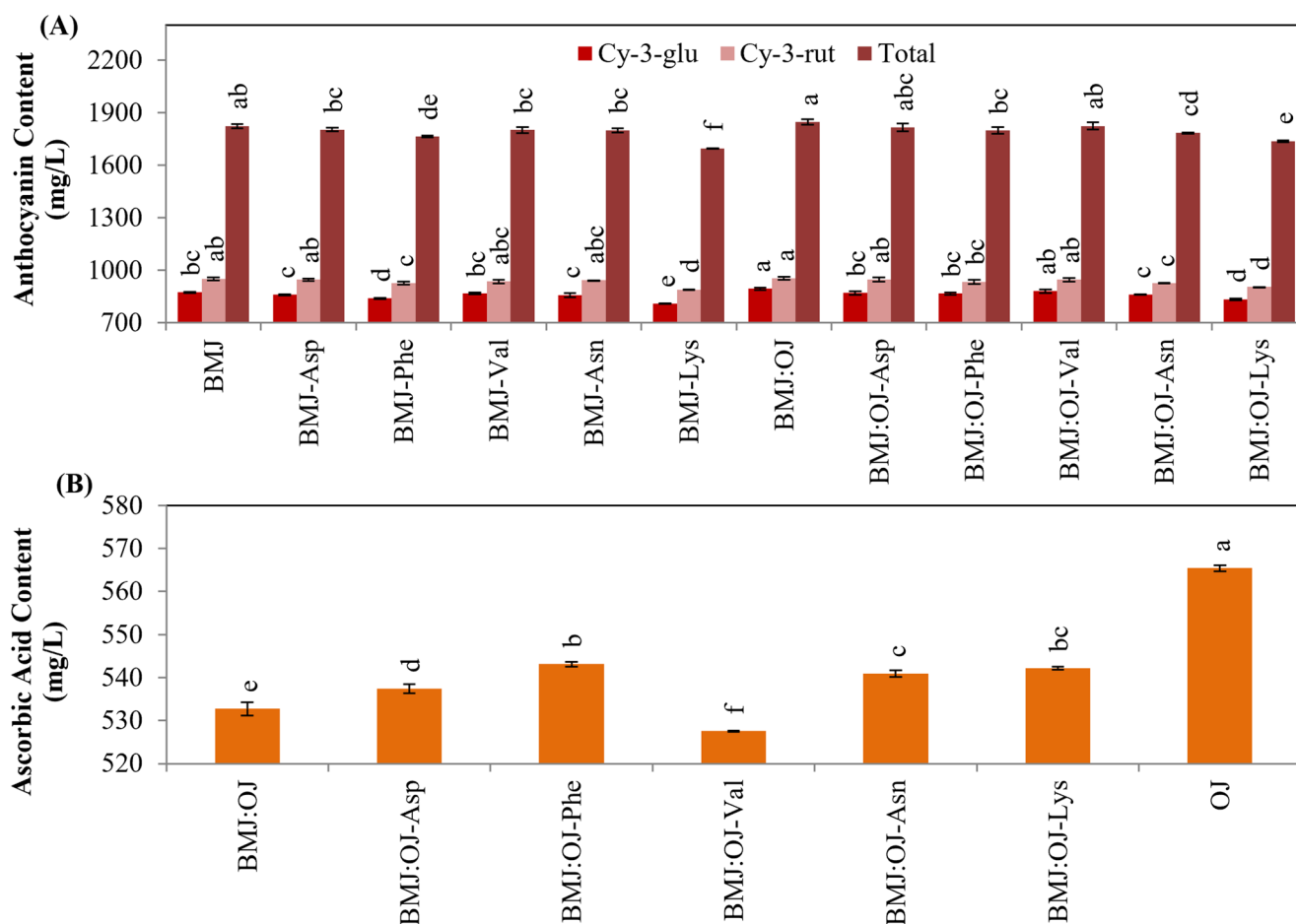


Fig. 3 Individual anthocyanin content (mg/L) of amino acid-added black mulberry juice (BMJ) and black mulberry juice: orange juice blend (BMJ: OJ) samples (A), ascorbic acid content (mg/L) of amino acid-added BMJ: OJ and orange juice (OJ) (B). ^{a–f} Different superscript indicates statistically significant difference ($p < 0.05$). BMJ: black mulberry juice, BMJ-Asp: Aspartic acid-added BMJ, BMJ-Phe: Phenylalanine-added BMJ, BMJ-Val: Valine-added BMJ, BMJ-Asn: Asparagine-

added BMJ: BMJ-Lys: Lysine-added BMJ, BMJ: OJ: Black mulberry juice: orange juice blend, BMJ: OJ-Asp: Aspartic acid-added BMJ: OJ, BMJ: OJ-Phe: Phenylalanine-added BMJ: OJ, BMJ: OJ-Val: Valine-added BMJ: OJ, BMJ: OJ-Asn: Asparagine-added BMJ: OJ, BMJ: OJ-Lys: Lysine-added BMJ: OJ, OJ: Orange juice, Cy-3-glu: Cyanidin-3-glucoside, Cy-3-rut: Cyanidin-3-rutinoside

significant decrease in $A_{\max}$ value ($p < 0.05$). In addition, these three amino acids did not significantly change the CD values in either the BMJ or BMJ: OJ samples compared to the control samples. Considering the $A_{\max}$ and CD values, these three amino acids did not cause copigmentation in the BMJ anthocyanins ($p > 0.05$, Fig. 1B, C). In this case, the observed reduction in the amount of TMA may be attributed to either the degradation of anthocyanins or the formation of colourless 4-substituted flav-2-enes as a result of anthocyanin-amino acid condensation [38]. However, it should be noted that the formation of these condensation products was indirectly inferred from spectrophotometric data. While spectrophotometric data strongly suggest the formation of condensation, this indirect inference should be validated in future investigations using more advanced analytical tools, such as HPLC-MS, or through model system analysis.

In the BMJ samples, there was no statistically significant change in the levels of cy-3-glu and cy-3-rut with the addition of Val (865.5 and 934.3 mg/L) and Asn (857.7 and 941.3 mg/L) compared to the control samples (872.7 and 950.1 mg/L) ($p > 0.05$). Conversely, the amount of these anthocyanins decreased with the addition of Phe (838.2 and 924.4 mg/L) ($p < 0.05$, Fig. 2A). The BMJ: OJ sample showed no statistically significant change in the levels of cy-3-glu and cy-3-rut upon the addition of Val ($p > 0.05$, 878.9 and 944.7 mg/L), compared to the control sample (892.5 and 953.0 mg/L). However Asn (859.6 and 924.7 mg/L) and Phe (864.9 and 932.4 mg/L) caused a decrease in the levels of these anthocyanins ($p < 0.05$). The observed change in $A_{\max}$ value of the samples containing OJ suggest that cy-3-glu and cy-3-rut anthocyanins may undergo condensation with Asn and Phe, representing an indirect inference based on spectrophotometric data.

Table 2 Free amino acid content (means $\pm$ SDs, mg/L) in control (black mulberry juice (BMJ*)), black mulberry juice: orange juice blend (BMJ: OJ), amino acid-added BMJ and amino acid-added BMJ: OJ samples

Control	BMJ	BMJ: OJ
Asp	9.78 $\pm$ 0.06	140.78 $\pm$ 0.11
Phe	-	-
Val	2.14 $\pm$ 0.01	2.61 $\pm$ 0.02
Asn	57.90 $\pm$ 0.23	86.82 $\pm$ 0.40
Lys	1.48 $\pm$ 0.00	7.62 $\pm$ 0.73
Amino acid-added samples [†]	Amino acid-added BMJ	Amino acid-added BMJ: OJ
Asp	1252.94 $\pm$ 5.57	706.54 $\pm$ 2.67
Phe	1358.39 $\pm$ 1.88	312.26 $\pm$ 0.98
Val	830.24 $\pm$ 2.12	108.95 $\pm$ 0.00
Asn	1134.68 $\pm$ 1.57	452.42 $\pm$ 0.39
Lys	1505.27 $\pm$ 1.78	394.49 $\pm$ 1.08

*BMJ: Black mulberry juice, BMJ: OJ: black mulberry juice: orange juice blend, Asp: Aspartic acid, Phe: Phenylalanine, Val: Valine, Asn: Asparagine, Lys: Lysine

[†] The measured free amino acid amounts of Asp, Phe, Val, Asn, and Lys-added BMJ and BMJ: OJ separately are given. These values correspond to the addition of each amino acid to its respective sample

The amount of ascorbic acid in the Phe (543.1 mg/L) and Asn (540.9 mg/L) added samples was lower than that of OJ (565.4 mg/L), but higher than that of the control sample (532.7 mg/L), similar to the samples containing Asp ($p < 0.05$, Fig. 2B). The Val -added sample had a lower ascorbic acid content than the control sample (527.5 mg/L, $p < 0.05$). In a study conducted by Türkyılmaz et al., proline increased the rate of ascorbic acid degradation in a mixture of pomegranate juice and OJ [21]. The study interpreted this as a consequence of the reaction of amino acids with phenolic compounds such as catechins and tannins, resulting in a reduction in the protective effect of these phenolic compounds on ascorbic acid. Valine is a non-polar and aliphatic amino acid, similar to proline. Due to this structural characteristic, valine may have interacted with the hydrophobic moieties of phenolic compounds. A study investigating *Campomanesia xanthocarpa* phenolics and ovalbumin demonstrated that valine residues within the ovalbumin structure interacted with six different phenolic compounds (hesperidin, rutin, epigallocatechin gallate, quercetin, protocatechuic acid, and syringaldehyde) [40]. Among these phenolics, hesperidin, rutin, quercetin, and protocatechuic acid are also natively present in the OJ and BMJ media utilized in our study [41, 42]. The hydrophobic interaction between valine and these protective phenolic compounds in the BMJ: OJ medium may have reduced the antioxidant protective shield of the phenolic compounds over ascorbic acid and accelerated the degradation of ascorbic acid.

The amounts of free Phe (1358 and 312.2 mg/L), Asn (1134 and 452.4 mg/L) and Val (830.2 and 180.9 mg/L) in the BMJ and BMJ: OJ samples were considerably lower than the amount added (1679 mg/L), as in the Asp-added samples (Table 2). The potential reactions of amino acids in BMJ and BMJ: OJ blend are described in “3.1 Effect of Asp on BMJ and BMJ: OJ anthocyanins”. As previously explained, these reactions suggest that neutral amino acids undergo condensation with BMJ anthocyanins in BMJ and BMJ: OJ media. Unlike Asp, these amino acids remain neutral under BMJ medium and may fail to establish hydrogen bonds or electrostatic interactions with the positively charged BMJ anthocyanins. Furthermore, while none of the three amino acids caused any change in CD in either BMJ or BMJ: OJ samples, the decrease in PCR with the addition of these three amino acids in BMJ samples and with the addition of Phe and Asn in BMJ: OJ samples showed that browning was prevented in these samples ($p < 0.05$, Fig. 1C). Ali et al. investigated the inhibitory effects of different amino acid concentrations on enzymatic browning in potatoes [43]. The amino acids Phe and Val, which were also used in present study, inhibited enzymatic browning at concentrations of 10 and 100 mM, respectively, due to the reaction of these amino acids with *o*-quinones to release colourless adducts. Furthermore, the amount of free amino acids in BMJ: OJ samples was significantly lower than that observed in BMJ samples ($p > 0.05$, 60.1–86.9%). This may be because OJ contains Cu(II) or Fe(III) minerals and phenolic compounds with which Val, Phe, and Asn can form complexes.

Effect of Lys on BMJ and BMJ: OJ Anthocyanins

Lys is the only amino acid with a basic side chain among those used for copigmentation in the BMJ and BMJ: OJ samples (Table 1). The charges of the side chains of the amino acids added as copigments had a slight effect on the pH of the BMJ and BMJ: OJ samples. The pH values of the BMJ and BMJ: OJ samples (4.19 and 3.61, respectively) were lower in the medium containing Asp with an acidic side chain (3.92 and 3.54, respectively). However, no significant change was observed in the medium containing Phe (4.20 and 3.61, respectively), Val (4.20 and 3.63, respectively), and Asn (4.15 and 3.60, respectively) with neutral side chains. In the sample containing Lys, there was an increase in pH (pH 4.68 and 3.76, respectively), which was reflected in the $\lambda_{\max}$ value of the samples. No significant difference was observed in the $\lambda_{\max}$ value between the BMJ sample (517.3 nm) and the samples to which amino acids with acidic and neutral side chains were added (515.0–518.8 nm). Conversely, the $\lambda_{\max}$ value (522.0 nm) showed a significant increase with the addition of Lys with a basic side chain ($p < 0.05$). The effect of the

amino acids on the pH of the BMJ: OJ samples was not sufficient to cause a change in the $\lambda_{\max}$ values of the samples ($p > 0.05$). It is a known phenomenon that anthocyanins can change their chemical structure and colour, depending on the pH value [44]. Anthocyanins have four different forms; the flavylium cation (pH 1.0), quinoidal base (pH 7.0), carbinol pseudobase (pH 4.5) and chalcone (pH 4.5), depending on the pH of the medium. As the pH increases, the flavylium cation undergoes hydration, resulting in a bathochromic shift due to the reduction in the proportion of flavylium cation [45]. Similarly, Gauche et al. investigated the effect of pH on copigmentation in anthocyanin extracts from Cabernet Sauvignon grapes and found that the $\lambda_{\max}$ value increased with increasing pH (pH 1.0–4.5) ($\Delta\lambda_{\max} = 0.2\text{--}5.2\text{ nm}$) [46].

In both BMJ and BMJ: OJ, Lys -added samples had the lowest TMA (517.7 and 522.7 mg/L, respectively), $A_{\max}$ (0.309 and 0.574 ABS, respectively), CD (4.07 and 5.80 ABS, respectively), cy-3glu (807.9 and 833.1 mg/L, respectively), and cy-3-rut (887.4 and 903.8 mg/L, respectively), and the highest PCR values (26.4 and 19.0, respectively) (Figs. 1A–C and 2A). The result in TMA, $A_{\max}$, CD, and PCR values indicates that Lys does not play a role in copigmentation or condensation, but rather causes the degradation of anthocyanins and increases browning. Lys is one of the amino acids that results in the most intense Maillard browning. In a study by Kwak et al., the browning values of amino acids (Asp, glutamic acid, alanine, leucine, isoleucine, Val, proline, serine, cysteine, Phe, arginine and Lys) as a result of the Maillard reaction with different sugars were measured [39]. Among the amino acids common to the present study, Lys gave the highest browning value, followed by Phe, Val, and Asp. In addition, HMF, an intermediate product of the Maillard reaction, degrades effect on anthocyanins. In a study investigating colour changes in the presence of HMF and amino acids, HMF (up to 8.2 mg/L) increased the rate of anthocyanin degradation (14.3–37.8%) [47]. Therefore, degradation of TMA, cy-3-glu and cy-3-rut may be attributed Maillard reaction intermediates.

Among the BMJ: OJ samples, the Lys-added sample, together with the Phe-added sample, had the highest amount of ascorbic acid (542.1 mg/L) (Fig. 2B). The role of Cu(II), Fe(III) minerals and amino acids used as ligands in the degradation of ascorbic acid has been explained in detail previously. When the Lys content was analysed, the Lys-added sample exhibited the highest free amino acid content in BMJ (Table 2). However, in the BMJ: OJ sample, this amount decreased significantly by 73.8%. This strong reduction and higher ascorbic acid content, suggested that the stabilisation of ascorbic acid was achieved using Lys as a ligand.

Conclusion

In the BMJ and BMJ: OJ samples, the addition of amino acids decreased TMA by 5.4%, and cy-3glu and cy-3-rut by 7.4 and 6.7%, respectively. Asp with an acidic side chain was the only amino acid that caused copigmentation, increasing $A_{\max}$ and CD in both BMJ and BMJ: OJ samples. Amino acids with neutral side chains (Phe, Val, and Asn) likely undergo condensation, whereas Lys with a basic side chain has a degradative effect on anthocyanins. Asp, Phe, Val and Asn in BMJ and Asp, Phe, and Asn in the BMJ: OJ blend reduced browning by reacting with o-quinones or inhibiting PPO via metal chelation, whereas Lys enhanced browning through Maillard reactions. In the BMJ: OJ samples, ascorbic acid content was higher in Asp, Phe, Asn, and Lys-added samples than in the control samples. This is because amino acids act as ligands and chelate with Cu(II) and Fe(III) ions, which catalyse the oxidation of ascorbic acid. The decrease in ascorbic acid content in the Val-added sample is due to the reaction of Val with phenolic compounds, which reduces the protective effect of these phenolics on ascorbic acid. Amino acid content decreased (50.6% in BMJ; 93.5% in BMJ: OJ) due to copigmentation, condensation, PPO inhibition, metal chelation, and Maillard reactions. Overall, copigmentation occurred only with Asp, likely via hydrogen bonding and/or electrostatic interactions. Under these conditions, amino acids with acidic side chains are recommended for copigmentation.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics The study did not include trials with humans or animals.

Conflict of Interest The author declare that they have no conflicts of interest.

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