Research Article

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Increase in colour stability of pomegranate juice against 5-hydroxymethylfurfural (HMF) through copigmentation with phenolic acids

Meltem Türkyılmaz,[a*] Fatmagül Hamzaoğlu,[b] Rumeysa Betül Arı Çiftci[c] and Mehmet Özkan[c]

Abstract

BACKGROUND: Anthocyanins are responsible for both attractive colour of pomegranate juice (PJ) and its health-promoting effects against cancer and coronary heart disease. However, 5-hydroxymethylfurfural (HMF) at some concentrations causes anthocyanin degradation. The present study aimed to reduce the degradation of PJ anthocyanins as a result of HMF at various concentrations (0–20 mg L^{-1}) through phenolic acid [PA; ferulic (FA), gallic (GA) and caffeic acids (CA)] copigmentation during storage at 20 °C.

RESULTS: A strong correlation ($r = 0.872$) was found between anthocyanin degradation rate and HMF concentration in PJ without PA addition. An increase in HMF concentration during storage caused faster (< 32%) anthocyanin degradation. However, PA addition reduced (< 60 times) the HMF formation rate. The lowest HMF formation rates (0.07–0.28 day^{-1}) were determined in PJ with added GA. Although GA caused an important increase in content of cyanidin-3-glucoside (16–42%), which is major PJ anthocyanin, against HMF at all concentrations, CA (15%) and FA (28%) increased cyanidin-3-glucoside content against 10 mg of HMF L^{-1}. FA maintained its protection effect against the highest HMF concentration (20 mg of HMF L^{-1}), but CA lost its protection effect. Generally, FA increased stabilities of hyperchromic effect (HE) (9.6–27.7%) and colour density (CD) (57.1–74.3%) at all HMF concentrations, although CA increased HE stability (19.8–37.7%) in the presence of 10 and 20 mg of HMF L^{-1}. Interactions of 'all individual anthocyanins-FA' and 'delphinidin-based anthocyanins-GA/CA' resulted in copigmentation.

CONCLUSION: FA addition was recommended to increase CD and HE for PJ containing HMF between 3.1–5.6 mg L^{-1}, whereas the addition of GA was recommended to increase anthocyanin stability for PJ containing 12.0 mg of HMF L^{-1}.

Keywords: anthocyanin stability; colour density; copigmentation; ferulic acid; caffeic acid; gallic acid; HMF

INTRODUCTION

In the food and beverage market, a stable and attractive colour has become more important day by day and plays an important role in the preferences of consumers. Indeed, previous studies have indicated that the colour of foods also affects the perception of food characteristics such as odor, texture and flavour.[1,2] Unfortunately, foods are affected by the processes applied in production and storage and, as a result, significant losses are observed in natural pigments that are some of the bioactive compounds in foods.

Bioactive compounds in pomegranates[3] show antioxidant, antimicrobial,[4] anti-carcinogenic and anti-inflammatory properties.[5] Pomegranates that have an intense red–violet colour resulting from anthocyanins among their bioactive compounds are generally processed in fruit juice or juice concentrate. As a result of the heating applied during the production, anthocyanins, which are extremely sensitive to heat, are degraded and significant losses occur in the attractive colours of pomegranate juice (PJ) and its concentrate. However, the heat applied in a fruit/vegetable juice production [such as blanching (95 °C for 2 min), mashing heating (95 °C for 4 min) and pasteurization (85 °C for 2 min)] and storage of the product not only have a direct effect on the degradation of anthocyanins, but also indirectly cause the degradation of anthocyanins. For example, intermediate products of Maillard reaction or sugar decomposition products

* *Correspondence to: M Türkyılmaz, Institute of Food Safety, Ankara University, Diskapi, 06110 Ankara, Turkey. E-mail: mturkyilmaz@ankara.edu.tr and meltemturkyilmaz@gmail.com*

a *Institute of Food Safety, Ankara University, Ankara, Turkey*

b *Department of Gastronomy and Culinary Arts, Ardeşen Faculty of Tourism, Recep Tayyip Erdogan University, Rize, Turkey*

c *Department of Food Engineering Faculty of Engineering, Ankara University, Ankara, Turkey*

formed during storage can also have a degrading effect on anthocyanins. Among these products, 5-hydroxymethylfurfural (HMF) and furfural are some of the most important products that have degradative effect on anthocyanins. Thus, the necessity of taking precautions to protect anthocyanins from the degradative effects of HMF during heating and storage have emerged.

In the literature, there are many studies investigating the effect of HMF on anthocyanins. However, there are conflicting results about effect of HMF. For example, Calvi, & Francis[6] reported that HMF formed under heating and storage conditions did not have a negative effect on anthocyanins. By contrast, most studies[7-11] demonstrated that even very low concentrations of HMF have a degradative effect on anthocyanins. Indeed, a study conducted in blackberry[11] reported that even 0.012 M HMF increased the degradation of cyanidin-3-glucoside. The reason for the degradation of anthocyanins in the presence of HMF was attributed to the reaction between furfural and the two hydroxyl groups of the B ring in the anthocyanin molecule.[11] Moreover, Debicki-Pospišil *et al.*[11] reported that the reaction between furfural and cyanidin-3-glucoside was possible only with the aglycon of cyanidin-3-glucoside (i.e. cyanidin). Furthermore, Es-Safi *et al.*[12] revealed by HPLC-electrospray ionization-mass spectrometry analysis that condensation reaction between cyanidin-3-glucoside and HMF led to various oligomeric bridged compounds, which finally precipitate. Because of these the contradictory results, the effect of different concentrations (5, 10 and 20 mg L^{-1}) of HMF on PJ anthocyanins was determined in the present study. Moreover, the effect of the added phenolic acids (PAs) [gallic (GA), caffeic acid (CA) and ferulic (FA)] as copigments on the anthocyanin degradation resulting from HMF was demonstrated for the first time.

Many studies examining the effect of copigmentation in various fruit juices are available in the literature.[13-15] However, no study has investigated the effect of copigments (PAs) in the presence of HMF at various concentrations in fruit juices or concentrates during storage. In the present study, the effects of HMF at various concentrations (5, 10 and 20 mg L^{-1}) and various copigmentation sources (GA, CA and FA) added to PJs were revealed for the first time. Increasing the colour intensity of products containing intense anthocyanins such as PJ and preserving the stabilities of anthocyanins are of great importance during storage. Therefore, it is hoped that both increasing the colour intensity and ensuring colour stability will provide a great advantage to the producers of PJ and its concentrate.

MATERIALS AND METHODS

Chemicals and reagents

The PAs (GA, CA and FA), cyanidin 3-*O*-glucoside, cyanidin 3,5-*O*-diglucoside and HMF were all obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Standards of pelargonidin 3-*O*-glucoside and pelargonidin 3,5-*O*-diglucoside were from Extrasynthese (Genay, France) and the standard of delphinidin-3-*O*-glucoside was from Polyphenols Laboratories AS (Sandnes, Norway). The solvents for HPLC and other reagents (analytical grade) were all obtained from Merck Co. (Darmstad, Germany). Sep-Pak C_{18} cartridges used for the purification of anthocyanins, phenolics and HMF were purchased from Waters Co. (Milford, MA, USA). Ultrapure water (18.2 MΩ.cm) was daily prepared and used in all analyses (Millipore Simplicity UV, Molsheim, France).

PJ production

Pomegranates (*Punica granatum* L., cv. Hicaznar) were obtained from the Alata Horticultural Research Institute (Erdemli, Mersin, Turkey) and immediately processed into juice in the fruit juice pilot plant at Ankara University. Pomegranates were first washed thoroughly in a bubble washing machine (İnoksen, Ankara, Turkey) in cold tap water for 10 min by applying an airflow of 3.5 m^3 min^{-1}. The washed pomegranates were then passed under the sprayed water to avoid second pollution. After discarding the damaged fruits, the top and bottom of the pomegranate husks were removed with a sharp stainless steel knife to prevent microbial contamination. Then the pomegranates were cut into quarters (132.8 kg) that were pressed on a-rack-and-cloth-press (Bucher-Guyer, Niederweningen, Switzerland) by gradually increasing the pressure up to a maximum of 15 MPa (150 bar) within 30 min. The amount of extracted juice was 41.6 kg and juice yield was 31.3% (w/w). To prevent the flow of excessive tannins into juice, the juice yield was aimed to be around 30%. The resulting juice was filtered through muslin cloth to remove the coarse particles and then transferred into 1.5 L polyethylene terephthalate bottles without clarification. The juice samples were stored at −25 °C (MDF-U731M; Panasonic Healthcare Co. Ltd, Tokyo, Japan) until the copigment additions.

Addition of copigments and HMF into PJs and storage

The juice samples were thawed in a temperature-controlled incubator (MIR 153; Sanyo Co. Ltd, Osaka, Japan) at 4 °C overnight. Because the highest hyperchromic effect (HE) was found at the ratio of 1:5 (anthocyanin: PA, w/w), the anthocyanin:copigment ratio was selected as 1:5 (w/w). Once the PAs (FA, CA and GA) were added into PJ samples at a ratio of 1:5, the juice samples were sonicated [Elmasonic S 180 (H); Elma Schmidbauer GmbH, Singen, Germany] at 30 °C for 45 min to completely dissolve the PAs. These PAs were selected because of their different chemical structures and properties. The reason for these selections are explained in detail in the Results and Discussion section. After sonication, HMF was added at three different concentrations allowed by various Institutions and Organizations [5 mg L^{-1}, The International Federation of Fruit Juice Processors; 10 mg L^{-1}, The Association of the Industry of Juices and Nectars; and 20 mg L^{-1}, European Union] to the PA added PJ samples. The juice sample without the addition of PAs and HMF was taken as the control sample. Moreover, the samples with added PAs were also evaluated as the control group of the samples with added PAs and HMF. The same sonication was also applied to the control sample. The PAs and HMF containing juice samples were transferred into 100 mL glass jars and the lids were tightly closed. To prevent microbial spoilage during storage, the juice samples in glass jars were pasteurized in a water-bath (WB 14; Memmert GmbH + Co. KG, Schwabach, Germany) at 85 °C for 2 min once the cold point reached to 85 °C, followed by a thermocouple thermometer (Digi-Sense Model No: 91100-50; Cole-Palmer Instrument Co., Vernon Hills, IL, USA). Right after pasteurization, the juice samples were promptly cooled to room temperature in a tank filled with icy-water and then stored in the temperature-controlled incubator (MIR 253) at 20 °C for 5 weeks. The storage time was chosen based on the reduction of two half-lives (75%) in major anthocyanin content in the control group.[16] The following analyses were carried out in PJ samples containing PAs and HMF every week during storage.

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Physiochemical analyses

An automatic digital refractometer (Rx-7000α; Atago, Tokyo, Japan) was used to measure total soluble contents of the juice samples and the results were expressed in Brix (°Bx, g/100 g). Turbidities were determined by a turbidimeter (HACH 2100N). The pH values of the samples were measured potentiometrically with a pH meter (SevenCompact pH/Ion S220; Mettler-Toledo, Schwerzenbach, Switzerland). Prior to pH measurements, 4 mL of PJ was diluted with 16 mL of ultrapure water (Millipore Simplicity UV) and the diluted juice was mixed thoroughly on a magnetic stirrer (RCT basic; IKA, Staufen, Germany). Titratable acidity of the juice samples was determined according to the method outlined by IFU[17] and the results were expressed as 'g anhydrous citric acid per 100 mL juice.' Prior to physiochemical analyses, the temperature of juice samples was brought to 20 °C in the temperature-controlled incubator (MIR 153).

Total monomeric anthocyanins

Total monomeric anthocyanins contents of the juice samples were measured using the pH-differential method according to the method described by Giusti and Wrolstad.[18] Juice samples were first filtered through the 0.45-µm polyvinylidene fluoride (PVDF) filter (Sartorious AG, Goettingen, Germany) to remove the haze. On 0.5-mL filtered juices, 9.5 mL of 0.025 M potassium chloride (pH 1.0) and 9.5 mL of 0.4 M sodium acetate buffer solutions (pH 4.5) were added. The mixtures were then mixed on a vortex (Vortex Genius 3; IKA) and left at room temperature for 15 min to bring the equilibrium. The absorbances were read at 512 nm ($\lambda_{visible\ max}$) for anthocyanins and 700 nm for haze correction on a UV-visible double-beam spectrophotometer (Evolution 201; Thermo Fisher Scientific Inc., Cambridge, UK) using 1-cm path length disposable cuvettes (Brand Gmbh, Wertheim, Germany) at room temperature. The blank consisted of ultrapure water. The anthocyanin concentration of the juice samples was proportional to the difference in the absorbance values at $\lambda_{visible\ max}$ of anthocyanin pigments at pH 1.0 and 4.5. Monomeric anthocyanin contents of the juice samples were expressed as cyanidin-3-glucoside equivalents using a molecular weight of 445.2 g mol^{-1} and an extinction coefficient of 26 900 L mol^{-1} cm^{-1}.

Bathochromic and HEs, and polymeric colour and colour density (CD)

The possible copigmentation was followed primarily by the increases in bathochromic effect and HE. A shift from the maximum wavelength (λ_{max}) in the absorption of anthocyanins was defined as bathochromic effect and an increase in an absorbance value at λ_{max} as HE (ΔA_{max}, ABS). These effects were expressed as a percentage. Prior to spectrophotometric measurements, the juice samples were diluted at the ratio of 1:20 (PJ:0.025 M potassium chloride buffer solution, pH 1.0) and left at room temperature for 15 min to reach equilibrium. To support the occurrence of possible copigmentation of anthocyanins, the percentage polymeric colour and CD of the juice samples were also determined using the bisulfite bleaching method as outlined by Giusti and Wrolstad.[18] The polymeric colour includes only brown and does not include colour as a result of monomeric anthocyanins. Although monomeric anthocyanins are degraded by metabisulfite, polymeric anthocyanins resist bleaching by metabisulfite. Absorbances of two juice samples (i.e. one treated with water and the other with potassium metabisulfite) were measured at 420 nm (brown colour), 497 nm (anthocyanin colour) and 700 nm (haze correction) via the spectrophotometer (Evolution 201) using 1-cm path length disposable cuvettes (Brand Gmbh) at room temperature. Ultrapure water was used as the blank.

HPLC analyses

Anthocyanin profile

The extraction and purification of anthocyanins were carried out according to the method described by Lee et al.[19] The extracted anthocyanins were loaded onto a Sep-Pak C-18 cartridge (100 mg, 0.25 mL; Waters Co.) that was placed on a vacuum manifold system (Waters Co.) for uniform purification. The extraction and purification of anthocyanins are detailed in Türkyılmaz *et al.*[20] The separation and identification of anthocyanins in PJ samples was carried out using HPLC (model 1200 series; Agilent, Waldbronn, Germany) with a binary pump, a photo diode array detector, a thermostatted auto sampler, a degasser and a thermostatted column compartment. For the processing of the chromatographic data, model 1200 series ChemStation rev. B.02.01 software (Agilent) was used. Anthocyanins were seperated on a reverse-phase C18 column (250 × 4.6 mm, 5 µm) (Phenomenex Inc., Los Angeles, CA, USA) equipped with a C18 guard column (4 × 3 mm, 5 µm) (Phenomenex Inc.) at a flow rate of 1 mL min^{-1}, sample injection volume of 50 µL, elution time of 15 min and column temperature of 25 °C. Separation of anthocyanins was performed by the linear gradient program outlined by Skrede et al.[21] with minor modifications: from 0% to 12% A for 10 min, from 12% to 22% for 10 min and holding at 22% A for 5 min. Solvent A was acetonitrile (100%, v/v) and solvent B was a mixture of *O*-phosphoric acid, acetic acid, acetonitrile and water (1:10:5:84, v/v/v/v). The chromatograms of anthocyanins were recorded at 520 nm. Individual anthocyanins were identified by comparing retention times and the absorption spectra of unknown peaks with the external reference standards. Quantification of anthocyanins were carried out using calibration curves (r^2 = 0.9875–0.9999) for external standards with at least six data points.

Phenolic profile

The phenolics were purified using the C18 cartridge (Waters Co.) as reported previously.[22] After filtering through the 0.45-µm PVDF filter (Sartorious AG) into amber coloured auto sampler vials, the purified and filtered extract was directly injected into HPLC (model 1200 series; Agilent). The same column and guard column as described above for the Anthocyanin profile were used for the separation of phenolics. The flow rate was 1 mL min^{-1}, sample injection volume was 50 µL, elution time was 85 min and the column temperature was 25 °C. Separation of phenolics was performed by the linear gradient program outlined by Skrede et al.[21] with minor modifications: from 0% to 12% A for 10 min, from 12% to 22% for 10 min and holding at 22% A for 5 min. Solvent A was was acetonitrile (100%, v/v) and solvent B was dilute formic acid (1%, v/v). The chromatograms of phenolics were recorded at 280 nm for catechins, 320 nm for PAs and 360 nm for flavonoids. The details of the analysis were reported in our previous study.[23]

HMF content

HMF was also purified on the Sep-Pak C-18 cartridge (Waters Co.) using a vacuum manifold system (Waters Co.). As for the anthocyanin and phenolic profile determinations, the activation of cartridge was carried out with 5 mL of ethyl acetate, then 5 mL of methanol (containing 0.01% HCl, v/v) and finally 2 mL of aqueous 0.01% HCl (v/v). Next, 1 mL of PJ was loaded onto the activated

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cartridge. Upon loading, the interfering organic acids and sugars were eluted from the column. The extract containing HMF was filtered through the 0.45 μm PVDF filter (Sartorious AG) into amber coloured auto sampler vials. The purified and filtered extract was then directly injected for HPLC (model 1200 series; Agilent). The same column and guard column as described above for the Anthocyanin profile were used for the separation of HMF. The flow rate was 0.7 mL min^{-1}, sample injection volume was 50 μL, elution time was 30 min and the column temperature was 25 °C. Separation of HMF was performed by isocratic elusion with a methanol:water mixture (10:90, v/v). Water contained 1% acetic acid (v/v). The chromatograms of HMF were recorded at 285 nm. Identification of HMF was carried out by comparing retention times and absorption spectra of unknown peak with the external reference standard and quantified using external standard calibration curve of HMF (0–40 mg L^{-1}, $r^2 = 0.9999$) that contained seven data points.

Recovery assays for HMF analysis. Recoveries were determined by adding a known amount of the external standard of HMF to the samples before extraction of HMF. For recovery assays, 5 mL of a sample was weighed into test tubes, and the external standards were spiked into the samples at the same or half of the concentration in original sample. Recovery assays were carried out in duplicate. The amount extracted for each spiked level was calculated from the calibration curve of HMF standard. Recoveries and reproducibilities were calculated according to the equations given by Li *et al.*[24] The amounts of HMF in the samples were calculated taking into account of recovery values.

Statistical analysis

The results from anthocyanin content, phenolic content, polymeric colour and CD were subjected to one-way analysis of variance. The concentration of HMF and PAs, as well as storage time, were considered as the main effect. All analyses were replicated twice. MSTAT, version 22 (SPSS, Chicago, IL, USA) statistical software was used to determine the effects of HMF and various PAs on colour and phenolic contents. Duncan's multiple range test at a significance of $P < 0.05$ was run to determine the statistical differences among means.

RESULTS AND DISCUSSION

pH and titratable acidity of PJs with added copigments and HMF

The pH and titratable acidity of the control group were 3.33 and 1.41 g per 100 mL, respectively (data not shown). The addition of HMF at various concentrations (0, 5, 10 or 20 mg L^{-1}) or various copigments (FA, GA and CA) caused a slight change in titratable acidity (1.45–1.61 g per 100 mL) of the juice samples. The change in the juice sample with added HMF was completely HMF concentration-dependent. The addition of 5 mg L^{-1} HMF did not make any difference in pH and titratable acidity ($P > 0.05$), wheeras 10 (1.58 g per 100 mL) and 20 (1.64 g per 100 mL) mg L^{-1} HMF addition caused a slight but statistically significant increase in titratable acidity ($P < 0.05$). Similarly, among copigments, FA (1.59 g per 100 mL) and GA (1.61 g per 100 mL) also resulted in some increase in titratable acidity ($P < 0.05$). However, HMF and copigments did not affect the pH of the juice samples ($P > 0.05$). This showed that the effects of added HMF and copigments on anthocyanins of PJ were pH-independent.

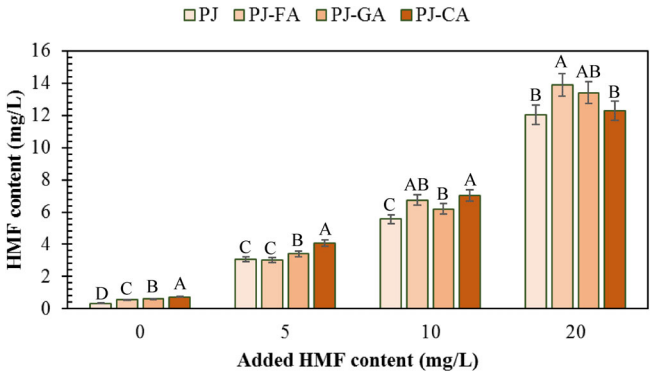

Figure 1. HMF contents of the PJ samples with added PAs and HMF before storage. PJ, pomegranate juice; PJ-FA, pomegranate juice with added ferulic acid; PJ-GA, pomegranate juice with added gallic acid; PJ-CA, pomegranate juice with added caffeic acid.

The changes in HMF contents of the PJs

Before storage, the PJ without HMF and PA addition (control group) contained 0.3 mg of HMF L^{-1} (Fig. 1). When the PAs were added, HMF contents of the samples significantly increased ($P < 0.05$). The highest HMF content was detected in the PJ-CA (0.7 mg L^{-1}), followed by PJ-GA (0.6 mg L^{-1}) and PJ-FA (0.5 mg L^{-1}) (Fig. 1). These findings can be attributed to the promoting effect of PAs on HMF formation. Indeed, a previous study[25] showed that a PA can increase both the formation of 3-deoxyglucosone (3-DO), which is a precursor of HMF, and the conversion of 3-DO to HMF. Moreover, a strong positive correlation ($r = 0.918$) was found between the contents of PA and 3-DO,[26] which indicated the significant contribution of PAs on HMF formation. This contribution results from di-hydroxyphenyl and carboxyl groups in phenolics.[25] Thus, the changes in the effects of the PAs investigated in the present study on HMF formation might have resulted from the differences in PA chemical structures.

CA, FA and GA are also known as 3,4-dihydroxycinnamic acid, trans-4-hydroxy-3-methoxycinnamic acid and 3,4,5-trihydroxyb enzoic acid, respectively. Considering the chemical structures of the PAs and all results of the present and previous studies,[25,26] dihydroxyphenyl group in CA led to HMF formation in a higher amount before storage. Moreover, the results of the present study showed that the promotion effect of PAs on HMF formation reduced when the number of hydroxyphenyl group was higher (as in GA) or lower (as in FA) than 2. However, the inference needs to further corroboration by advanced analytic techniques (i.e. HPLC-mass spectrometry and NMR).

The present study investigated, not only the effect of PA addition on HMF formation in the samples without HMF addition, but also their effects in the samples with added HMF at various concentrations. In the samples with added HMF, less HMF content than added to the samples was detected after pasteurization (Fig. 1). For example, HMF content of the PJ without PA addition was detected as 3.1 mg L^{-1} in the sample with 5 mg of HMF L^{-1} added. The reduction in HMF content can be attributable to two reasons. First, HMF is an intermediate product of Maillard reaction or caramelization and it participates in the formation of some brown coloured compounds. Therefore, absorbance (ABS) values at 420 nm (A_{420}, browning index) of the samples were determined to reveal the browning effect of HMF added to the samples. The A_{420} for the control group was

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0.89 and the addition of 5 mg of HMF L^{-1} did not change the A_{420} (0.90) of the sample ($P > 0.05$, data not shown). These results revealed that the added HMF did not transform the brown coloured compounds. Second, the reduction in HMF content after addition is a result of added HMF changing from free form to bound form. When the changes in the contents of individual phenolic (Fig. 2D,E) and anthocyanin (Fig. 3B) of the PJ before and after the addition of 5 mg of HMF L^{-1} were investigated, the results indicated that pelargonidin-3,5-diglucoside (4.5%), α-punicalagin (4.2%) and delphinidin-3-glucoside (3.0%) contents reduced after the addition of 5 mg of HMF L^{-1}. Thus, HMF could have bounded to these anthocyanins or the phenolic. Indeed, the addition of 5 mg of HMF L^{-1} led to an increase in CD (5.6%) of the sample (Fig. 4B). These results also strengthened the presence of interaction between anthocyanins and HMF before storage. A detailed discussion about this interaction is provide below in the section on 'The changes in anthocyanins of the PJ samples during storage and their relationship with copigmentation'.

Similar results were also determined in the samples with added 10 and 20 mg of HMF L^{-1}. HMF contents of the PJs without PA addition but with added 10 and 20 mg of HMF L^{-1} were detected as 5.5 and 12.0 mg L^{-1} after pasteurization (Fig. 1). In the samples with added PA and HMF (5, 10 and 20 mg L^{-1}), HMF contents

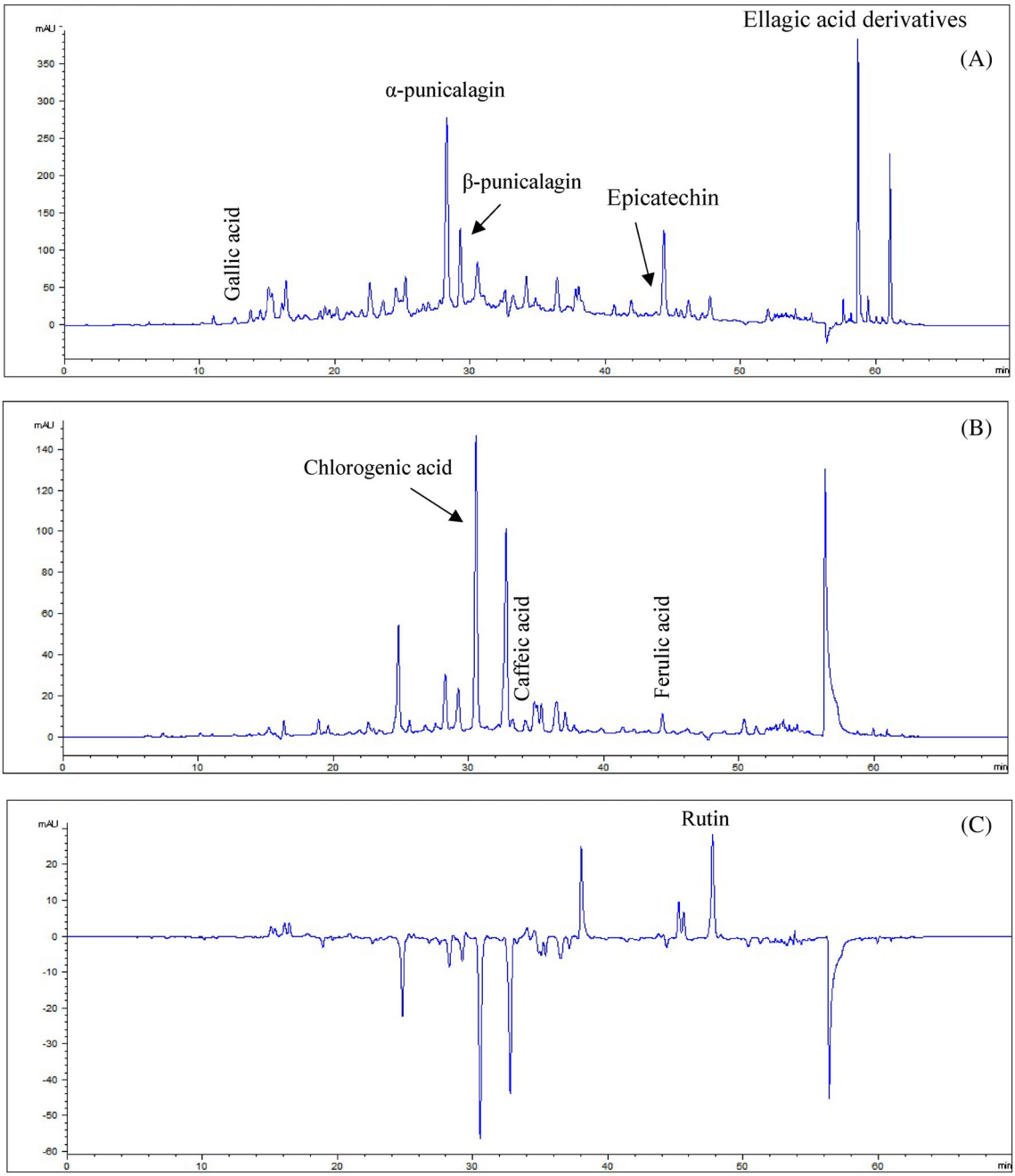

Figure 2. Phenolic profile (A–C) of PJ and changes in individual phenolics (D, E) of the PJ samples with added PAs and HMF before storage. PJ, pomegranate juice; PJ-FA, pomegranate juice with added ferulic acid; PJ-GA, pomegranate juice with added gallic acid; PJ-CA, pomegranate juice with added caffeic acid.

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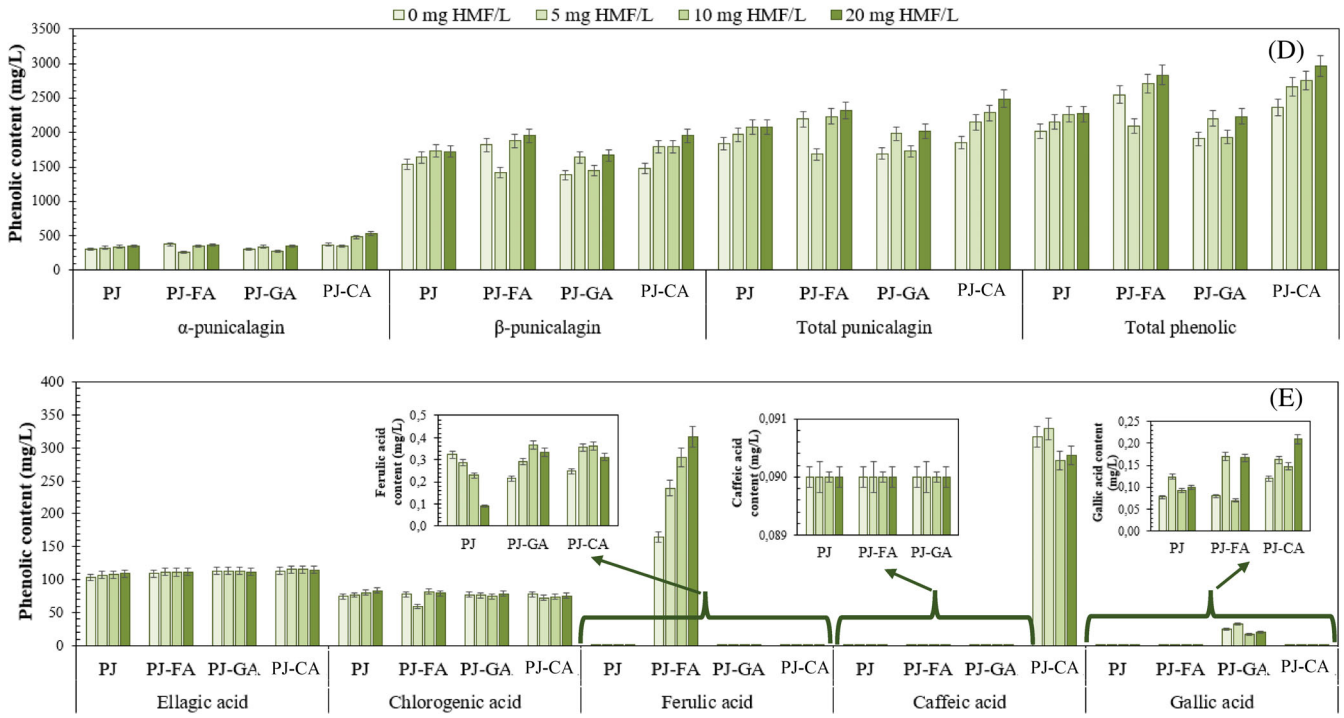

Figure 2 (Continued)

were 3.0–4.1, 6.2–7.0 and 12.3–13.9 mg L^{-1}, respectively, after pasteurization (Fig. 1). Similar to the samples with added PA but without HMF addition (Fig. 1), PA addition resulted in higher content of HMF in the PJs with added HMF. Moreover, PA addition into the samples with added 5, 10 or 20 mg of HMF L^{-1} reduced (by up to 18.9%) the A_{420} values. These results showed that PA addition prevented the formation of brown coloured compounds in the presence of HMF higher than 3.1 mg L^{-1} in the PJ. Therefore, we suggest the addition of PA to the PJ to prevent browning when the juice contains HMF higher than 3.1 mg L^{-1}. The preventive effect of PA on brown colour formation can be attributable to its altering effect on the formation of Maillard reaction products. For example, previous studies indicated that FA and GA inhibited the formation of pyrazine and pyridine derivatives[27] and FA also controlled the formation of Maillard reaction products.[28] However, these studies did not investigate their effects on HMF formation. The present study revealed that FA, GA and CA increased HMF formation before storage, but this situation changed during storage.

During storage, HMF contents of all samples increased. After storage, HMF contents of the samples with added 5, 10 and 20 mg of HMF L^{-1} reached to 4.7, 6.8 and 14.2 mg L^{-1}, respectively (data not shown). The HMF formation rate generally reduced in PJ-FA ($r = -0.869$) and PJ-CA ($r = -0.755$), as well as the control group ($r = -0.773$), as the content of HMF that was added to PJ increased (Table 1). However, the lowest HMF formation rate among the samples was found in the PJ-GA (Table 1), which was up to 60 times lower than that in control group (Table 1). This may be because GA is derived from benzoic acid, which is a primary catalyst in the aerobic oxidation of HMF into 2,5-diformylfuran.[29] In this reaction, the higher electron density of the aromatic ring with carboxyl of benzoic acid provides a significant contribution to the aerobic oxidation of HMF into 2,5-diformylfuran.[29] As in all chemical reactions, substrate and catalyst concentrations in this reaction are also important parameters for the reaction to take place. The lowest HMF formation rate in the PJ-GA was found in the juice sample that with added 5 mg of HMF L^{-1} (Table 1). The result may also be attributed to the order of reaction kinetic for HMF formation. Because the HMF formation followed first-order reaction kinetics, the increase in HMF concentration also increased the rate of HMF formation.

Finally, unlike GA, FA and CA are derived from cinnamic acid. In the light of the results of the preennt study, it can be assumed that when a PA, which was added to PJ, is a derivation of cinnamic acid, mono-hydroxy and/or methoxyphenyl groups in the PA would cause faster HMF formation during storage compared to the PA with a di-hydroxyphenyl group (in contrast to before storage). Indeed, the HMF formation rate in the PJ-CA, which has a di-hydroxyphenyl group, was up to 1.9 times lower than that in PJ-FA (Table 1). Thus, the PA with a di-hydroxyphenyl group caused more HMF formation in PJ during pasteurization, whereas the PA with mono-hydroxy and/or methoxyphenyl groups led to more HMF formation during storage.

In conclusion, when the production of PJ with low HMF is aimed for during storage, we recommend adding GA to PJ. In the case where cinnamic acid addition is preferred, the cinnamic acid should have a di-hydroxyphenyl group to reduce the HMF formation during storage.

The changes in phenolic profiles of the PJs

β-punicalagin, α-punicalagin, total punicalagin, chlorogenic acid, CA, GA, FA and the ellagic acid contents of the juice samples were determined by HPLC (Fig. 2A–C). In PJs with added HMF (without added PAs), the content of β-punicalagin, which was the major tannin of PJ, generally increased with the increase in HMF content

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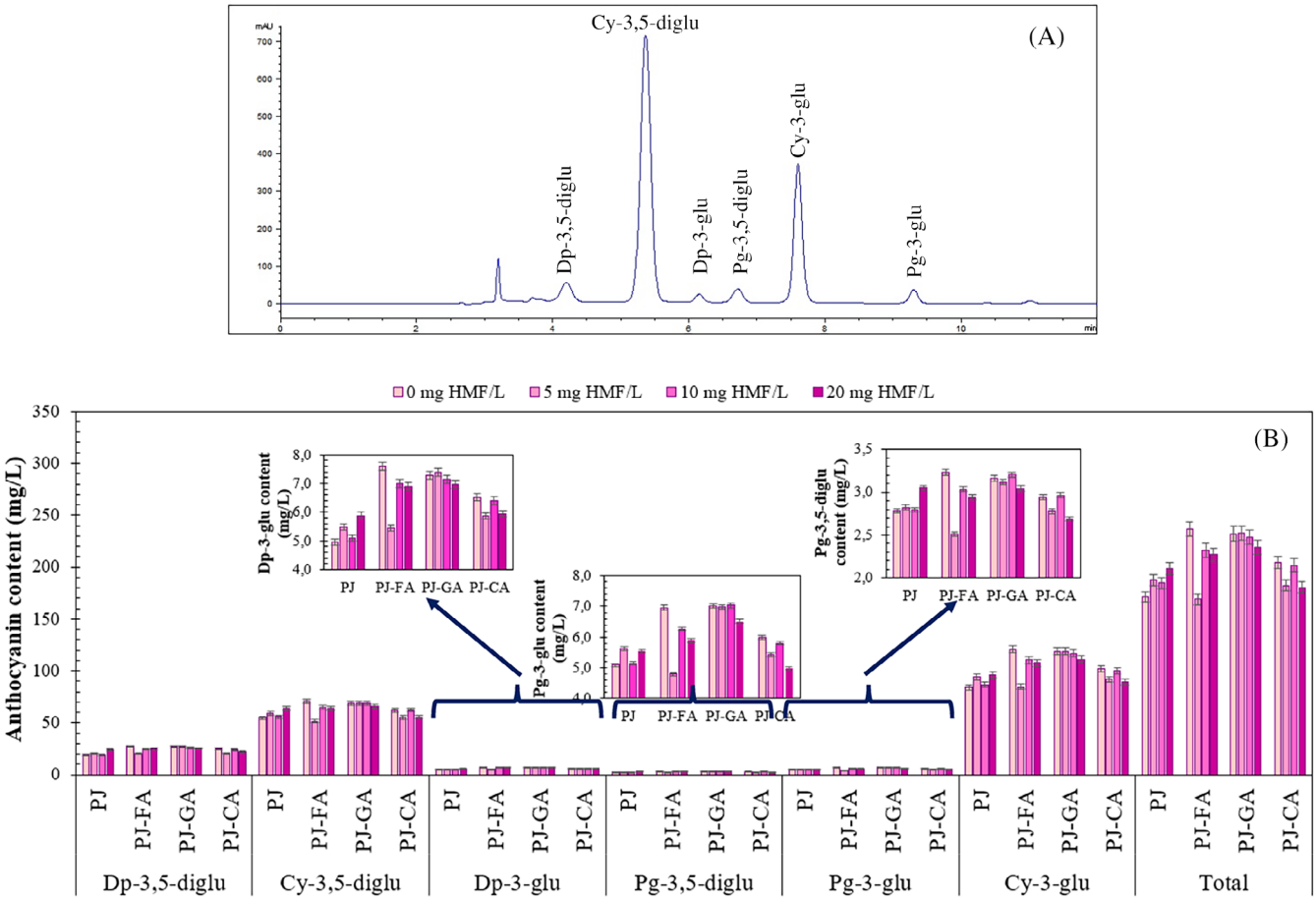

Figure 3. Anthocyanin profile (A) of PJ and changes in total and individual anthocyanins (B) in the PJ samples with added PAs and HMF. Cy-3-glu, cyanidin-3-glucoside; Cy-3,5-diglu, cyanidin-3,5-diglucoside; Dp-3-glu, delphinidin-3-glucoside; Dp-3,5-diglu, delphinidin-3,5-diglucoside; Pg-3-glu, pelargonidin-3-glucoside; Pg-3,5-diglu, pelargonidin-3,5-diglucoside; PJ, pomegranate juice; PJ-FA, pomegranate juice with added ferulic acid; PJ-GA, pomegranate juice with added gallic acid; PJ-CA, pomegranate juice with added caffeic acid.

($r = 0.925$) (Fig. 2D). Two reasons could account for the increase. First, the increase in β-punicalagin content may be a result of the competition between HMF and β-punicalagin. Some competing reactions involving HMF and tannins, which were well described by Es-Safi *et al.*,[30] can occur during food processing and storage. Because of the competition, the participation of β-punicalagin in a reaction might have been prevented by HMF. This might also have caused a relative increase in the amount of β-punicalagin in PJ. HMF can also interact with tannins such as punicalagins by dimethylene ether bridges.[31] However, the increases in β-punicalagin content of the PJs (without PA addition) despite the addition of HMF showed that this interaction did not occur in PJ. If the interaction between HMF and β-punicalagin had occurred, the contents of both compounds would have decreased.

The other reason for an increase in β-punicalagin content may be the interaction between FA and anthocyanins that can also interact with β-punicalagin. By contrast to the changes in β-punicalagin content, FA content of the PJs (without PA addition) caused a reduction as the HMF concentration increased ($r = -0.903$) (Fig. 2E). The reduction might have resulted from the interaction between individual anthocyanins and FA in the presence of HMF. Indeed, the interaction between phenolics and anthocyanins was accelerated in a media containing aldehydic derivatives, such as HMF.[30] The changes in the content of individual anthocyanins were also investigated and it was found that FA addition led to a reduction in all individual anthocyanins in the presence of HMF (Fig. 2E), which also strengthened the assumption about the interaction between FA and anthocyanins. Thus, it appeared that FA interacted with anthocyanins and this interaction prevented the interaction between anthocyanins and β-punicalagin. As a result of the prevention, β-punicalagin content might have increased. Moreover, this effect of FA may be dependent on HMF concentration because FA led to an increase in β-punicalagin content especially in case of adding 10 and 20 mg of HMF L^{-1}.

Unlike β-punicalagin and FA, the addition of HMF at various concentrations did not have a significant effect on CA content of the PJs (without PA addition) ($P > 0.05$). Thus, it was considered that there was no relationship between HMF and CA contents. However, in the PJ-CA, the added HMF at 10 and 20 mg L^{-1} concentrations reduced the CA content, which indicated that the relationship between CA and HMF contents might also be dependent on the concentrations of both compounds. Indeed, some studies indicated that the addition of CA into milk or model systems reduced HMF formation as a result of blocking of ε-amino groups of lysine, which is a primary substrate of the Maillard reaction.[32,33] Because PJ also contains lysine (15–52 mg L^{-1}),[34] the addition of CA reduced the HMF formation

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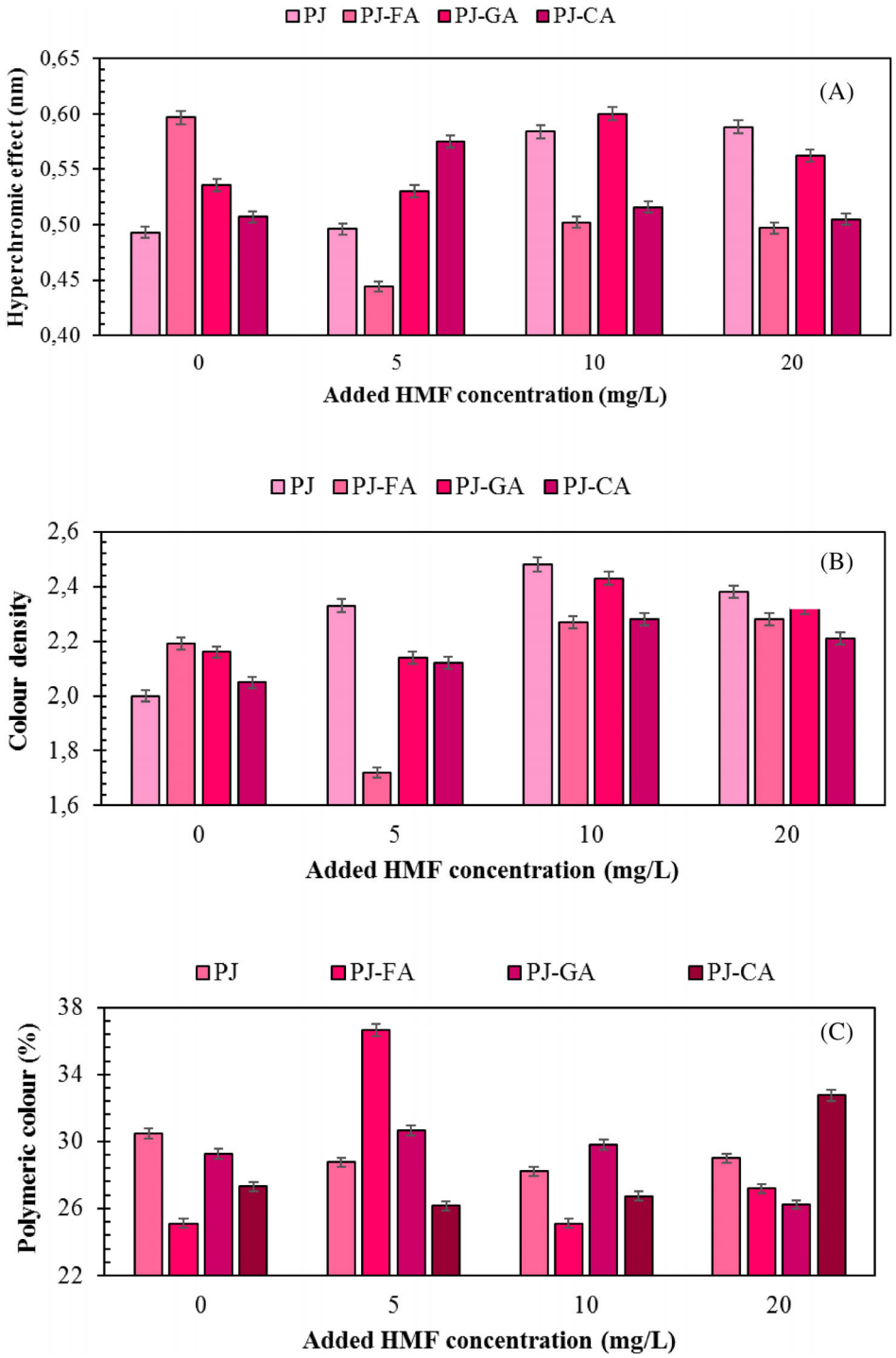

Figure 4. Changes in hyperchromic effect (A), colour density (B) and polymeric colour (C) in the PJ samples with added PAs and HMF. PJ, pomegranate juice; PJ-FA, pomegranate juice with added ferulic acid; PJ-GA, pomegranate juice with added gallic acid; PJ-CA, pomegranate juice with added caffeic acid.

rate compared to the PJ without PA addition. However, unlike previous studies, the results of the present study also revealed that the addition of 10 or 20 mg of HMF L^{-1} into PJ reduced CA content. As a result of the reduction in the content of CA, this PA lost its HE (Fig. 4A). Thus, the effect of HMF on CA could be different from that on FA. The reason for the reduction in CA content depending on HMF content should be examined in future studies. Moreover, when the effect of CA addition on the content of β-punicalagin was investigated, it was seen that β-punicalagin content increased after CA addition in the samples with added 10 and 20 mg of HMF L^{-1}. This may be indirectly a result of the relationship between HMF and CA. For example, HMF may have stimulated CA for the interaction rather than that of β-punicalagin.

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Table 1. Kinetic data for the degradation of anthocyanins, reductions in hyperchromic effect and colour density and increases in HMF and polymeric colour of PJ samples during storage

Added HMF concentration (mg L^{-1})		Samples			
		PJ (Control)	PJ-FA	PJ-GA	PJ-CA
HMF formation					
0	$k \times 10^2$[a]	7.37	5.16	0.12	3.94
5	$k \times 10^2$	1.91	2.79	0.07	0.97
10	$k \times 10^2$	0.97	0.53	0.28	0.39
20	$k \times 10^2$	0.78	0.48	0.21	0.44
Delphinidin-3,5-diglucoside degradation					
0	$-k \times 10^2$	2.07	6.56	4.38	4.10
	$t_{1/2}$[b]	33.5	10.6	15.8	16.9
5	$-k \times 10^2$	2.81	3.45	4.38	3.34
	$t_{1/2}$	24.7	20.1	15.8	20.7
10	$-k \times 10^2$	3.25	4.54	3.62	3.96
	$t_{1/2}$	21.3	15.3	19.1	17.5
20	$-k \times 10^2$	3.43	4.81	3.59	4.17
	$t_{1/2}$	20.2	14.4	19.3	16.6
Cyanidin-3,5-diglucoside degradation					
0	$-k \times 10^2$	2.93	6.29	4.68	4.35
	$t_{1/2}$	23.7	11.0	14.8	15.9
5	$-k \times 10^2$	3.52	3.43	4.72	3.73
	$t_{1/2}$	19.7	20.2	14.7	18.6
10	$-k \times 10^2$	3.73	4.58	4.26	4.31
	$t_{1/2}$	18.6	15.1	16.3	16.1
20	$-k \times 10^2$	3.92	4.74	3.89	4.47
	$t_{1/2}$	17.7	14.6	17.8	15.5
Delphinidin-3-glucoside degradation					
0	$-k \times 10^2$	3.64	6.93	5.23	5.04
	$t_{1/2}$	19.0	10.0	13.2	13.7
5	$-k \times 10^2$	4.45	3.78	5.62	4.49
	$t_{1/2}$	15.6	18.3	12.3	15.4
10	$-k \times 10^2$	4.33	5.11	5.16	4.97
	$t_{1/2}$	16.0	13.6	13.4	13.9
20	$-k \times 10^2$	4.79	5.30	4.56	5.48
	$t_{1/2}$	14.5	13.1	15.2	12.6
Pelargonidin-3,5-diglucoside degradation					
0	$-k \times 10^2$	2.07	3.71	3.11	2.86
	$t_{1/2}$	33.5	18.7	22.3	24.0
5	$-k \times 10^2$	2.19	2.03	3.06	2.44
	$t_{1/2}$	31.6	34.1	22.6	28.4
10	$-k \times 10^2$	2.35	2.90	3.02	2.83
	$t_{1/2}$	29.5	23.9	22.9	24.5
20	$-k \times 10^2$	2.65	2.90	2.58	2.67
	$t_{1/2}$	26.2	23.9	26.8	26.0
Cyanidin-3-glucoside degradation					
0	$-k \times 10^2$	4.77	8.77	6.26	6.13
	$t_{1/2}$	14.5	7.90	11.1	11.3
5	$-k \times 10^2$	5.73	4.77	6.70	5.67
	$t_{1/2}$	12.1	14.5	10.3	12.2
10	$-k \times 10^2$	5.73	6.50	6.20	6.22
	$t_{1/2}$	12.1	10.7	11.2	11.1
20	$-k \times 10^2$	6.10	6.50	5.41	7.05
	$t_{1/2}$	11.4	10.7	12.8	9.83
Pelargonidin-3-glucoside degradation					
0	$-k \times 10^2$	4.95	10.1	6.84	6.79
	$t_{1/2}$	14.0	6.86	10.1	10.2
5	$-k \times 10^2$	6.08	6.26	7.35	6.56
	$t_{1/2}$	11.4	11.1	9.43	10.6

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Table 1. Continued

Added HMF concentration (mg L^{-1})		Samples			
		PJ (Control)	PJ-FA	PJ-GA	PJ-CA
10	$-k \times 10^2$	6.38	7.46	6.86	6.73
	$t_{1/2}$	10.9	9.29	10.1	10.3
20	$-k \times 10^2$	6.79	7.78	6.15	7.65
	$t_{1/2}$	10.2	8.91	11.3	9.06
Total anthocyanin degradation					
0	$-k \times 10^2$	3.68	7.48	5.44	5.18
	$t_{1/2}$	18.8	9.27	12.7	12.4
5	$-k \times 10^2$	4.47	4.10	5.64	4.63
	$t_{1/2}$	15.5	16.9	12.3	15.0
10	$-k \times 10^2$	4.61	5.39	5.14	5.16
	$t_{1/2}$	15.0	12.9	13.5	13.4
20	$-k \times 10^2$	4.84	5.62	4.61	5.60
	$t_{1/2}$	14.3	12.3	15.0	12.4
Reduction in hyperchromic effect					
0	$-k \times 10^2$	2.44	3.64	3.18	2.83
	$t_{1/2}$	28.4	19.0	21.8	24.5
5	$-k \times 10^2$	2.30	2.10	2.92	3.59
	$t_{1/2}$	30.1	33.0	23.7	19.3
10	$-k \times 10^2$	3.43	2.69	3.71	2.86
	$t_{1/2}$	20.2	25.8	18.7	24.2
20	$-k \times 10^2$	3.48	2.76	3.64	2.53
	$t_{1/2}$	19.9	25.1	19.0	27.4
Reduction in colour density					
0	$-k \times 10$	0.82	0.78	0.64	0.78
	$t_{1/2}$	8.45	8.89	10.8	8.89
5	$-k \times 10$	0.97	-	0.74	0.93
	$t_{1/2}$	7.15	-	9.37	7.45
10	$-k \times 10$	0.85	0.54	0.79	0.95
	$t_{1/2}$	8.15	12.8	8.77	7.30
20	$-k \times 10$	1.06	0.61	0.71	0.94
	$t_{1/2}$	6.54	11.4	9.76	7.37
Formation of polymeric colour					
0	$k \times 10$	1.87	2.40	2.21	2.12
5	$k \times 10$	1.95	1.66	2.01	2.24
10	$k \times 10$	1.94	2.58	2.08	1.99
20	$k \times 10$	1.94	2.12	2.43	1.61

Abbreviations: PJ, pomegranate juice; PJ-FA, pomegranate juice with added ferulic acid; PJ-GA, pomegranate juice with added gallic acid; PJ-CA, pomegranate juice with added caffeic acid.
[a] k was expressed as 'day^{-1}'.
[b] $t_{1/2}$ was expressed as 'days.'

Similar to CA, GA content was also reduced with additions of 10 and 20 mg of HMF L^{-1}. Thus, the effect of GA addition on β-punicalagin content was also similar to CA addition. Moreover, the changes in the contents of total punicalagin and total polyphenols were similar to that in β-punicalagin content. However, the contents of ellagic and chlorogenic acids were not generally affected by the addition of either HMF or PAs. The interaction between these PAs and anthocyanins and also the prevention effect of added PAs on the interaction between anthocyanins and phenolics in PJ were investigated in the section on 'The changes in anthocyanins of the PJ samples during storage and their relationship with copigmentation.' In the light of the results of the study, we recommend adding FA and CA to PJ in the presence of HMF to protect phenolics in PJ, thereby producing PJ with a higher phenolic content (Fig. 2D,E).

The changes in anthocyanins of the PJ samples during storage and their relationship with copigmentation

Total monomeric anthocyanin content of the control group was 171.1 mg L^{-1} prior to storage. By contrast to expectations, HMF, which was added to PJs at concentrations of 5, 10 and 20 mg L^{-1}, caused a significant increase (up to 16%) in the anthocyanin content of PJ (without PA addition) (Fig. 3B). These increases may have been resulted from two different reasons. The first reason might be the antioxidant activity of HMF.[35] The stability of anthocyanins will increase in the presence of an antioxidant such as

HMF because the stability of anthocyanins against oxidation is very low. The second reason might be the copigmentation effect of HMF that resulted in an increase in anthocyanin stability and CD of PJ samples. Indeed, an increase in HMF content led to an increase in HE ($r = 0.842$) (Fig. 4A), which is one of the most significant indicators of copigmentation, and CD ($r = 0.637$) (Fig. 4B) before storage. Thus, the changes in HE and CD resulted from the copigmentation effect of HMF. However, this effect was temporary and reversed during storage.

During storage, there was a regular decrease in the anthocyanin contents of the control group and the samples with added HMF, and this decrease was in accordance with the first-order reaction kinetics. The calculated kinetic parameters for the degradation of anthocyanins are presented in Table 1. Anthocyanin degradation rates (k) of the PJs with added 5, 10 and 20 mg of HMF L^{-1} were −0.0447, −0.0461 and −0.0484 day^{-1}, respectively, whereas that of control group was −0.0368 day^{-1}. The k values clearly revealed that antioxidative and/or copigmentation effects of HMF were not sufficient to protect the anthocyanins during storage and the rate of anthocyanin degradation also increased as the HMF concentration increased ($r = 1.000$). Generally, all individual anthocyanins in PJ also degraded faster when HMF concentration increased (Table 1). Thus, the degrading effect of HMF on pomegranate anthocyanins is directly dependent on the HMF concentration.

The PAs, which were added to PJ, led to a very significant reduction (up to 60 times) in the HMF formation rate compared to the control group ($k = 0.0737$ day^{-1}) (Table 1) because the PAs react with reactive carbonyl species and thus alleviate the formation of Maillard reaction products such as HMF[36] during storage. Therefore, the PAs were assumed to cause a reduction in the degradation of anthocyanins resulting from the interaction with HMF. However, similar to the effect of HMF, addition of PAs also caused a faster reduction [$k = (-0.0518) - (-0.0748)$ day^{-1}] in anthocyanins (Table 1). All reductions in the contents of anthocyanins do not necessarily mean the degradation of anthocyanins. When the anthocyanins interact with a copigment such as PAs, their contents also decrease.[37] Indeed, the changes in HEs of the samples with PAs indicated copigmentation. The HEs (Fig. 4A) and CDs (Fig. 4B) of the samples with added PAs were up to 21% and 13%, respectively, higher than that of control group. As a result, the reductions in anthocyanin contents of the samples with added PAs stemmed from copigmentation rather than degradation because the CD and HE would have also reduced if degradation was the reason for the reduction in anthocyanin content.

We also determined the changes in individual anthocyanins of the juice samples to show which PA had copigmentation effect on which individual anthocyanin. 3-glucosidic and 3,5-diglucosidic forms of cyanidin, delphinidin and pelargonidin were determined in PJ samples (Fig. 3A). The major anthocyanin in all samples was cyanidin-3-glucoside, followed by cyanidin-3,5-diglucoside, delphinidin-3,5-diglucoside, delphinidin-3-glucoside, pelargonidin-3-glucoside and pelargonidin-3,5-diglucoside, respectively (Fig. 3B). All PAs led to a significant increase (up to 44%) in cyanidin-3-glucoside content ($P < 0.05$, Fig. 3B). Although GA protected cyanidin-3-glucoside against HMF at all concentrations, protection effects of FA and CA were dependent on HMF concentration (Fig. 3B). Both CA (15%) and FA (28%) caused an important increase in cyanidin-3-glucoside content against 10 mg of HMF L^{-1}. However, FA maintained its protection effect against the highest HMF concentration (20 mg of HMF L^{-1}), whereas CA lost its protection effect (Fig. 3B). HMF and PAs, which were added into PJ, led to similar changes in the contents of cyanidin-3-glucoside on the other individual anthocyanins (Fig. 3B).

In the juice sample with added PA, the correlations between the changes in all individual anthocyanin contents and HE were investigated to determine which individual anthocyanins led to an increase in HE. When FA was used as a PA, strong correlations ($r = 0.898$–0.939) between HE and all individual anthocyanins were detected. Moreover, CD in these samples also was higher than in control group after storage. Furthermore, the stabilities of HE and CD also increased (up to 74%) in PJ-FA in the presence of HMF (Table 1). Thus, we recommend adding FA to PJ becasuse this PA increased the stabilities of CD and HE in the presence of HMF, regardless of its concentration.

Unlike PJ-FA, only delphinidin-3-glucoside ($r = 0.689$–0.693) and delphinidin-3,5-diglucoside ($r = 0.600$–0.607) gave rise to an increase in HE in the PJ-GA and PJ-*CA*. Especially, when 20 mg of HMF L^{-1} was added to PJ, GA increased HE by 10% after storage. In addition, this PA caused a significant enhancement in the stabilities of CD (33%) and all individual anthocyanins (up to 11%) (Table 1). These findings also revealed that the effects of PAs resulted from copigmentation. Therefore, when PJ contains 12.0 mg of HMF L^{-1} (the sample with added 20 mg of HMF L^{-1}), GA addition to PJ is also recommended to increase CD and HE of PJ during storage.

As mentioned before, HMF concentration changed the effect of the PAs. When the PAs were added into PJs with added 5 mg of HMF L^{-1}, the addition of only FA resulted in a significant increase in the stabilities of total anthocyanin content (8%) (Table 1) and HE (15%) (Table 1). This increase resulted from the increases in the stabilities of cyanidin-3-glucoside (16%), delphinidin-3-glu (14%), pelargonidin-3,5-diglucoside (10%) and cyanidin-3,5-diglucoside (3%) by FA (Table 1). In addition, FA also increased total punicalagin contents of the PJs (Fig. 2D). Because punicalagins are the phenolics showing the highest antioxidant activity in PJ,[38] the stability of the individual anthocyanins might have also been increased by the antioxidant activity of these phenolics. Similarly, in the PJ with added 10 mg L^{-1}, FA also increased the stabilities of HE (22%) and CD (36%), whereas CA increased the stability of only HE (17%) (Table 1). By contrast to FA and CA, the addition of only GA resulted in a significant increase in the stabilities of total anthocyanin (5%) (Table 1) and HE (26%) (Table 1) when the PAs were added into PJs with added 20 mg of HMF L^{-1}. GA also increased the stabilities of all individual anthocyanins, except for delphinidin-3,5-diglucoside and cyanidin-3,5-diglucoside (Table 1). This may be a result of the reduction effect of GA on the HMF formation rate. Moreover, GA also showed higher HE during 7 days of storage (data not shown). Thus, the interaction of all individual anthocyanins (except for delphinidin-3,5-diglucoside and cyanidin-3,5-diglucoside) with GA led to copigmentation. Nevertheless, delphinidin-3-glu ($r = 0.988$, $P < 0.05$) and cyanidin-3-glucoside ($r = 0.987$, $P < 0.05$) showed the highest correlations with HE. As a result, total anthocyanin stability in this sample increased during storage because of the reduction in the negative effect of HMF on anthocyanins and the copigmentation effect resulting from the addition of GA. Thus, we recommend GA addition to increase anthocyanin stability in PJ containing HMF at approximately 12.0 mg L^{-1}, whereas we recommend FA addition if the PJ contains 3.1 and 5.6 mg of HMF L^{-1}.

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CONCLUSIONS

All added PAs increased HE and CD of PJ before the addition of HMF into PJ. Although strong correlations were found between the contents of all individual anthocyanins and HE in PJ-FA, only delphinidin based anthocyanins gave rise to an increase in HE in PJ-GA and PJ-CA. The stability of colour enhancement effect of these PAs was also investigated after the addition of HMF. Although the addition of PAs into PJ promoted the formation of HMF before storage, these additions resulted in a significant reduction (up to 60 times) in the rate of HMF formation during storage. GA was the primary PA that decreased the HMF formation rate at most. The added PAs were able to reduce the negative effects of HMF on the anthocyanins and/or colour of PJ resulting from the reducing effect of the PAs on HMF formation rate and their copigmentation effect. However, the effect of the PAs was dependent on HMF concentration. Therefore, if a PA is added into PJ containing 3.1 mg of HMF L^{-1}, FA should be preferred to increase the stabilities of total anthocyanins and HE. Moreover, FA can be preferred to increase CD and HE in the PJ with 5.6 mg of HMF L^{-1}. However, when the PJ contains 12.0 mg of HMF L^{-1}, GA addition to PJ is recommended to increase CD and HE of PJ during storage. Future studies should investigate the effects of phenolic extracts from natural sources for which the major phenolic acids are FA and/or GA on PJ anthocyanins in the presence of HMF at various concentrations with respect to low-cost production.

AUTHOR CONTRIBUTIONS

MT was responsible for the study conceptualization, methodology and data evaluation and writing the manuscript. MÖ was responsible for writing and editing. FH was responsible for all of the HPLC analyses. RBAÇ was responsible for the spectrophotometric analyses.

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DATA AVAILABILITY STATEMENT

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

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