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오디에서 열처리와 자외선 조사가 Anthocyanin-Polyphenol Copigment Complex의 안전성에 미치는 효과

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Effects of Temperature and UV Irradiation on Stability of Anthocyanin-Polyphenol Copigment Complex in Mulberry Fruits

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ABSTRACT : Anthocyanin and polyphenolic compounds present in fruits of mulberry (*Morus alba* L.) were determined and the influence of temperature and UV irradiation on stability of the anthocyanin-copigment complex were investigated. The copigmentation substance selected in non-anthocyanin fraction from mulberry for the study included: phenolic acid (hydroxybenzoic acid) and flavonoid (quercetin-3-*O*-β-D-glucopyranoside). The copigmentation effect increased with the copigment content. UV irradiation had a stronger degradation effect on the copigmentation complex than heating at 80℃. The non-anthocyanin fraction of mulberry and isolated flavonoid (quercetin-3-*O*-β-D-glucopyranoside) from mulberry fruit predominated over other copigment substances.

Key Words **:** Mulberry, Anthocyanin, Copigmentation, Stability

INTRODUCTION

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom (Harborne, 1998; Shahidi and Naczk, 2004). They are produced in most higher plants such as blackberry, red and black raspberries, nectarines peaches, blueberries, bilberries, cherries, currants, pomegranates, ripe gooseberries, onion, red radish, red soybeans, purple corn, basil, blood orange, elderberries, red cabbage, fennel, red lettuce, grapes, red-skinned potato and purple sweet potato (Eder, 2000; Prior, 2004; Shahidi and Naczk, 2004). Anthocyanins can be found in all parts of the plants. Though aggregate mostly in flowers and fruits (Brouillard, 1988), they also present in leaves, stems and storage organs (Delgado-Vargas and Paredes-López, 2003). So far, anthocyanins have not been broadly used in foods and beverages, since they are not as stable as synthetic dyes. Nevertheless, anthocyanins are highly unstable and easily susceptible to degradation. The stability of anthocyanins is influence by pH, temperature, presence of enzymes, light, oxygen, structure and concentration, and the presence of complexing compounds such as other flavonoids, phenolic acids, protein, metals and minerals (Markakis, 1982).

Recent investigations (Davies and Mazza, 1993; Mazza and Brouillard, 1987, 1990) have suggested that the molecular copigmentation of anthocyanins with other compounds (copigments) is the main colour-stabilizing mechanism in plants. Copigment alone is usually colourless, but when added to an anthocyanin solution it greatly enhances the colour of the solution. A copigment may be one of flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals, and anthocyanins themselves (Mazza and Brouillard, 1990). Anthocyanin copigmentation gives brighter, stornger and more stable

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colour than those presented by anthocyanins alone. In food science, copigmentation is considered an important interaction, as colour is one of the quality factors strongly affecting consumer acceptance of food. Copigments have electron-rich π systems, which are able to associate with the comparatively electron-poor flavylium ion. This association provides protection from the nucleophilic addition of water on the flavylium ion (Mazza and Brouillard, 1987). The attack by water converts the flavylium ion into the colourless pseudobase, which consequently results in the loss of colour. The complexation of a copigment with an anthocyanin causes a hyperchromic effect (ΔA), means an increase in colour intensity, where the λ_{max} of the absorption spectrum increase, and as a bathochromic shift ($\Delta\lambda$) consist of a wavelength of maximum absorbance (Chen and Hrazdina, 1981; Mazza and Miniati, 1993).

Various substances, such as chlorogenic acid (Brouillard *et al*., 1989; Brouillard *et al*., 1991; Dangles *et al*., 1992; Wilska-Jeszka and Korzuchowska, 1996), rutin (Baranac *et al*., 1996), quercetin (Baranac *et al*., 1997) have been identified as good copigments. Some of these copigments are expensive or synthetic substances and further studies are required to find their natural cheap equivalents.

In previous studies, mulberry (*Morus alba*) fruits have been reported to exhibit a variety of biological activities, such as anti-thrombotic (Yamamoto *et al*., 2006), antioxidant (Bang *et al*., 2007; Naderi *et al*., 2004), antimicrobial (Choi *et al*., 2009; Takasugi *et al*., 1979), anti-inflammation (Kim and Park, 2006) and neuroprotective effects (Kang *et al*., 2006). These activities are generated by anthocyanins, and a group of naturally being phenolic compounds that are responsible for the color of mulberries. Mulberry fruit is rich in anthocyanins and can be considered as a potential source for production of a natural red food colorant. The fruits of mulberry are known to contain cyanidin-3-glucoside and cyanidin-3-rutinoside are the major anthocyanins (Liu *et al*., 2004) and a number of flavone derivatives in very high concentration (Lugasi and Takacs, 2002; Bang *et al*., 2007) and the highest total phenolic content was observed in mulberry (1515.9 ± 5.7 ㎎ gallic acid equivalents (GAE)/100 g fresh matter (FM)) among other berry species (Lin and Tanga, 2007). The hydroxybenzoic acid, myricetin and quercetin glucoside are the most predominant among flavone derivatives (Ryszard *et al*., 2005)

The objectives of this research were to firstly, study the effects of copigmentation substance from non-anthocyanin fraction of mulberry fruits on the colour stability mulberry anthocyanin, secondly to investigate the copigments impact of phenolic acid (para-hydroxybenzoic acid) and flavonoid (quercetin-3-*O*-β-D-glucopyranoside) prepared from the fruits of mulberry on colour stability of mulberry anthocyanin. Finally, The stability of colorants was investigated at pH 2.5, 3.5, and 4.5 under heating and UV irradiation in the presence and absence of copigment.

MATERIALS AND METHODS

1. Extraction from mulberry fruits

Mulberry fruits (*Morus alba* L.) at a commercially mature stage were obtained from the HyeJeon College, Hongsung, Korea. Fruits were selected according to uniformity of shape and color. The fruits were freeze dried (PVTF 200K, Ilshin Lab Co, Korea) then stored at –20℃ for further studies. Only the edible portion of the fruit was retained, and the inedible seed discarded. A composite, from approximately 200 g of fully ripe fruits, was used in these trials. Replications were made from these de-seeded fruit by macerating with a known volume of ethanol aqueous solution (1 : 15 w/v, fruit/60%EtOH ratio) adjusted to pH 3.5 with citric acid for 24 h at 4℃, and subsequently filtered through Whatman #1 filter paper using Büuchner funnel. The colorant extract was washed by mixture solution consist of ethylether : hexane (1 : 6 V/V) twice. Residual ethylether and hexane were removed from the anthocyanin extract by using a rotary evaporator at 40℃ under vacuum condition using Buchi Labortechnik AG (CH-9230, Switzerland).

2. Fractionation of antocyanin and non-anthocyanin

Colorant extract of mulberry was passed through Amberlite IRC-50 cation exchange resin column. Anthocyanins was adsorbed onto the column, other polyphenol compounds, sugar, acids, and other water-soluble compounds were eluted with more than 2 volume of distilled water until the wash water was clear. These eluted extracts were partitioned into two polyphenolic fractions using ethyl acetate, which separated most phenolic acids and flavonoids (non-anthocyanin fraction) from the remaining aqueous fraction (anthocyanin fraction). The reamined materials (anthocyanin fraction) was then eluted with acidified ethanol (0.5% (v/v) of hydrochloric acid) until there was no color

in the eluent. The eluent was concentrated on a rotary evaporator under reduced pressure at 40℃ and the resulting concentrate was lyophilized to form a pigment powder.

3. Phytochemical Analysis

The HPLC analysis of anthocyanins was performed as described by Konczak-Islam *et al*., (2003). The pigment solutions were filtered through a 0.45 ㎛ syringe-driven filter unit (Millipore Corporation Bedford, Mass). The HPLC system consisted of two LC-10AD pumps, SPD10A detector, CTO-10AS column oven, DGV-12A degasser, SIL-10AD autoinjector, and SCL-10A system controller (Shimadzu, Japan) equipped with Luna (3 ㎛ C18(2), 4.6 ㎜ × 100 ㎜, Phenomenex, Calif) column at 35℃. The following solvents in water with a flow rate of 1 ㎖/min were used: A (1.5% phosphoric acid) and B (1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile). The elution profile was a linear gradient elution with 25%-5% solvent B in solvent A for 100 minutes. The chromatograms were monitored at 530 ㎚ and recorded, and quantified using a cyanidin 3-glucoside standard (Polyphenols Laboratories AS, Sandnes, Norway).

Major flavonoids and phenolic acids present in non-anthocyanin fraction from mulberry colorant extract were separated by HPLC using modified chromatographic conditions of Talcott and Lee (2002). Separations were performed on a 250 ㎜ × 4.6 ㎜ i.d. Acclaim 120-C18 column (Dionex, Sunnyvale, CA) with a C18 guard column. Mobile phases consisted of water (phase A) and 60% methanol (phase B) both adjusted to pH 2.4 with phosphoric acid. A gradient solvent program ran phase B from 0 to 30% in 3 min, 30-50% in 5 min, 50-70% in 17 min, 70-80% in 5 min, and 80-100% in 5 min and held for 10 min at a flow rate of 0.8 ㎖/min. Polyphenolics were identified by spectroscopic interpretation, retention time, and comparison to authentic standards (Sigma Chemical Co., St. Louis, MO).

4. Isolation and Characterization of major polyphenol compounds from non-anthocyanin fraction in colorant extract of mulberry

Ten gram of the non-anthocyanin fraction, soluble in H_2O : MeOH (7 : 3, v/v), was applied to a Sephadex LH-20 column (procedure A), and chromatographed in a stepwise gradient with H_2O : MeOH (7 : 3/0 : 10, v/v). 200 ㎖ of each combination of solvents were eluted through the column and fractions of 50 ㎖ were collected and 38 fractions were obtained. The fractions eluted with H_2O:MeOH (7 : 3, v/v) from procedure A (38.4 ㎎) were re-chromatographed on Sephadex LH-20 (procedure B) with H_2O:MeOH (8 : 2), as mobile phase, to obtain phenolic acd, para-hydroxy benzoic acid (5.5 ㎎, Pouchert and Behnke, 1993). Those fractions, eluted from procedure A with H_2O:MeOH (6 : 4, 5 : 5, 4 : 6, v/v) were added together (150 ㎎) and re-chromatographed on Sephadex LH-20 (procedure C) with H_2O:MeOH (6 : 4, v/v) to obtain the flavonoid, quercetin-3-*O*-β-D-glucopyranoside (7.1 ㎎, Markham *et al*., 1978). Isolated polyphenolic compounds obtained and analysed by TLC plate on DC-Plastikfolien cellulose F (Art.5565) and Silica gel 60 F_{254} with TBA (*t*-BuOH-acetic acid-water (3 : 1 : 1, v/v/v, solvent A)and 6% acetic acid(solvent B) as solvents system. Two compounds were identified by a combination of spectroscopic methods (^{1}H, ^{13}C NMR, EI- and FAB MS) and comparison with the literature data.

5. Color stability and effect of copigmentation substance from non-anthocyanin fraction from mulberry colorant extract

For study the effects of copigmentation substance from non-anthocyanin fraction containing phenolic acid (*p*-hydroxybenzoic acid) and flavonoid (quercetin-3-*O*-β-D-glucopyranoside) of mulberry fruits on the colour stability mulberry anthocyanin and was investigated stability of colorant solutions with and without copigment under heating, storage and UV irradiation. In case, For thermal stability, colorant solutions (7 ㎖) with and without copigment substance from non-anthocyanin fraction containing phenolic acid (*p*-hydroxybenzoic acid) and flavonoid (quercetin-3-*O*-β-D-glucopyranoside) in flask tubes with screw caps were placed in a water bath at 80℃. Each tube was used for one spectral measurement only, so as to minimize the contact with oxygen.

For UV stability, colorant solutions with and without copigment (7 ㎖) in glass open cuvettes, were placed under UV light (LB 301.1 BAKMED lamb, 253.7 ㎚, 2.1 ㎽/㎠). Absorption spectra of the anthocyanin solutions, with and without copigment, were recorded in the visible wavelength range from 450 to 600 ㎚. These spectra were measured using a UV-1650 UV - VIS spectrophotometer (Shimadzu, Tokyo, Japan) in a 10-㎜ pathlength cell, with buffer

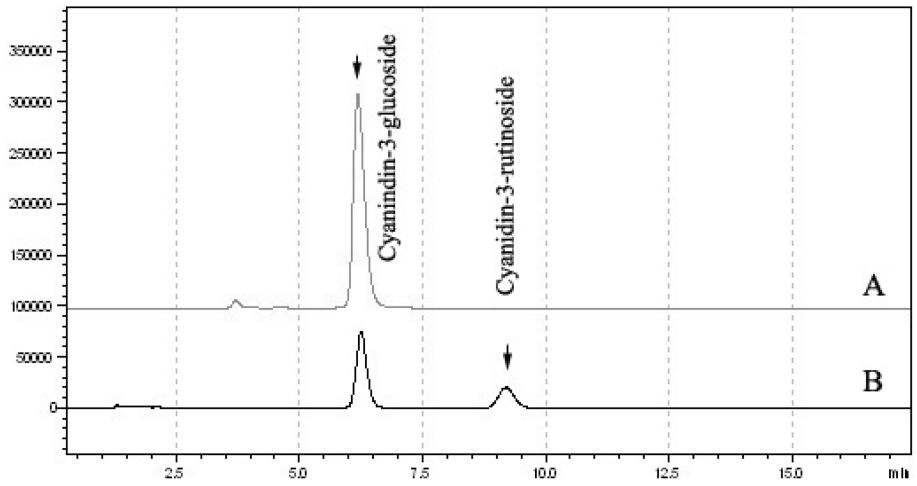

Fig. 1. HPLC chromatogram of (A) isolated cyanidin-3-glucoside from mulberry and (B) anthocyanin fraction extracted from mulberry fruits monitored at 530 ㎚.

solutions as a reference. Buffer solutions of three different pH values were prepared by mixing sodium citrate (6 g/ℓ) and 50% citric acid. All experiments were repeated in triplicate.

RESULTS AND DISCUSSION

1. Anthocyanin and polyphenolic characterization of mulberry extract

Due to recurrent issues associated with the instability of anthocyanins during processing and storage, the food industry is constantly looking for novel, inexpensive and stable sources of pigments. Anthocyanins present in mulberry fruits may offer a new source of these pigments; however, their stability has yet to be determined. Furthermore, the characterization of the major polyphenolic compounds in mulberry and their overall contribution to the copigmentation capacity has not been previously investigated. Therefore, this study examined the polyphenolic composition and its copigmentation effect in anthocyanin stability of mulberry under a variety of experimental conditions.

Fig. 1 shows a typical HPLC chromatogram of anthocyanin fraction extracted from mulberry fruits, canidin-3-glucoside and cyanidin-3-rutinoside are the major anthocyanins. The anthocyanin concentrations detected in mulberry fruits included cyanidin-3-glucoside (1247 ± 29 ㎎/㎏), and cyanidin-3-rutinoside (440.0 ± 3.1 ㎎/㎏). Spectrophotometric determinations of total anthocyanin content of mulberry fruits (2,021 ± 72 ㎎/㎏, data not shown) also revealed major differences in pigment colour intensities. Additional differences between spectrophotometric and chromatographic measurements might have originated from copigmentation reactions among anthocyanins and other non-anthocyanin polyphenolics naturally present in mulberry fruits, which are known to enhance visual colour and result in higher estimates of total anthocyanin contents in spectrophotometric assays (Wilska-Jeszka and Korzuchowska, 1996).

Non-anthocyanin polyphenolics of mulberry fruits (Fig. 2) included a diversity of phenolic acids and flavonoids. Phenolic acids detected in non-anthocyanin fraciton included protocatechuic, *p*-hydroxybenzoic, vanillic, syringic and ferulic acids, with p-hydroxybenzoic acid being predominantat concentrations 23 ㎎/㎏ (compound I). The predominant flavonoid present in mulberry fruits was quercetin-3-*O*-ß-D-glucopyranoside at concentrations 412 ㎎/㎏ (compound II).

Compound I was yellow amorphous powder, EI-MS : $[M]^+$ *m/z* 138, $[M\text{-}OH]^+$ *m/z* 121 and $[M\text{-}COOH]^+$ *m/z* 93, ^{1}H-NMR (400 MHz, d, acetone-d_6) : 6.91 (2H, d, J = 8.6 Hz, H-3,5), 7.90 (2H, d, J = 8.6 Hz, H-2,6), ^{13}C-NMR (100 MHz, δ, acetone-d_6) : 116.29 (C-3,5), 121.45 (C-1), 133.05 (C-2,6), 161.64 (C-4), 167.22 (C-7). All data were identical with that of *p*-hydroxybenzoic acid.

Compound II was yellow amorphous powder, mp 231-232 ℃, FAB-MS *m/z* $[M+H]^+$ 465, ^{1}H-NMR (400 MHz, δ, CD_3OD-d_4) : 3.23~3.73 (5H, *m*, H-2", 3", 4", 6"), 5.25 (1H, *d*, J = 7.4 Hz, H-1"), 6.19 (1H, *d*, J = 2.0 Hz, H-6), 6.38

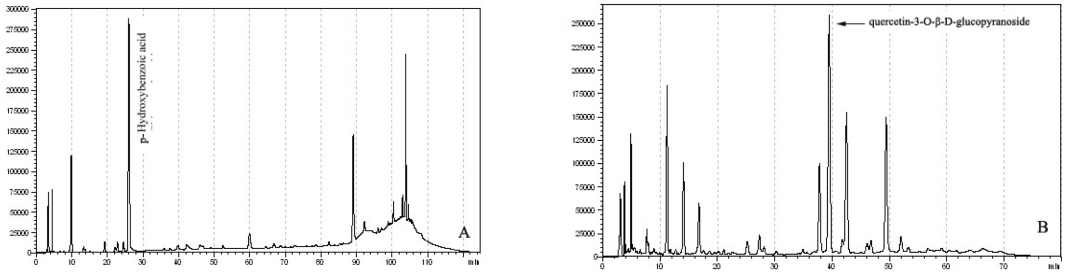

Fig. 2. HPLC chromatogram of (A) phenolic acids at 280 ㎚ and (B) flavonoids monitored at 360 ㎚ present in non-anthocyanin fraction form mulberry fruits.

Table 1. Influence of one hour heating at 80℃ on bathochromic shift and the hyperchromic effect in solution of copigments and anthocyanin fraction from mulberry at pH3.5‡.

Anthocyanin	Copigment¶	ΔA		Δλmax	
		Before	After heating	Before	After heating
C3G§	NAF	0.548	0.422	23.2	20.9
	HBA	0.004	0.002	2.4	2.2
	QgluPy	0.231	0.203	8.2	7.0
	HBA + QgluPy	0.325	0.224	11.3	9.8
	Chloro	0.006	0.003	2.5	2.2

‡ΔA and $\Delta\lambda_{max}$ are changes of wavelength and absorbance of visible maximum at 525 ㎚ upon addition of copigments; §C3G: Cyanidin 3-glucosie; ¶Species of copigment substance, NAF: non-anthocyanin fraction of mulberry (containing polyphenolic compounds excepted antocyanin); HBA: Hydrobenzoic acid; QgluPy: quercetin-3-*O*-β-D-glucopyranoside; Chloro : Chlorogenic acid

(1H, *d*, *J* = 2.0 Hz, H-8), 6.86 (1H, *d*, *J* = 8.5 Hz, H-6'), 7.58 (1H, *dd*, *J* = 2.1, 8.5 Hz, H-6'), 7.71 (1H, *d*, *J* = 2.1 Hz, H-2'). ^{13}C-NMR (100 MHz, δ, CD_3OD-d_4) : 62.58 (C-6"), 71.24 (C-4"), 75.75 (C-2"), 78.14 (C-5"), 78.41 (C-3"), 98.74 (C-8), 99.91 (C-6), 104.35 (C-1"), 105.72 (C-10), 116.04 (C-2'), 117.60 (C-5'), 123.21 (C-6'), 123.23 (C-1'), 135.65 (C-3), 145.92 (C-3'), 149.87 (C-4'), 158.48 (C-9), 159.04 (C-2), 163.06 (C-5), 166.01 (C-7), 179.51 (C-4). All data were identical with that of quercetin-3-*O*-*β*-D-glucopyranoside

2. Anthocyanin-polyphenolic compounds copigment complex in mulberry fruits

Anthocyanin concentration was constant, 3×10^{-4} at all pH values. Hydroxybenzoic acid (HBA) concentration was 3×10^{-4} M, quercetin-3-*O*-β-D-glucopyranoside, 3×10^{-4} M (QgluPy) or 3×10^{-2} M ($2 \times$ QgluPy) and non-anthocyanin fraction from the fruits of mulberry fruits (*Morus alba* L.), 6×10^{-4} (calculated as quercetin-3-*O*-β-D-glucopyranoside). The hyperchromic effect means an increase in colour intensity while the bathochromic shift consists of a shift of the wavelength of maximum absorbance. The hyperchromic effects on the main cyanidin absorption peak manifest copigmentation of these molecules, in the buffer solutions under investigation. Because the cyanidin concentration was constant in each solution, it seems obvious that the magnitude of the bathochromic and hyperchromic effects depended on the concentrations of the copigments.

Absorption spectra of cyanidin and spectra of the copigments formed with adding non-anthocyanin fraction of colorant extract from mulberry fruits at the pH 3.5 buffer solutions. Molar ratios of the components in the copigment were 1 : 1, 1 : 2, and 1 : 3 (cyanidin/non-anthocuyanin fraction). The magnitude of copigmentation effect increased with copigment content. For the solution with the lowest concentration of the copigment (1 : 1), ΔA and $\Delta\lambda_{max}$ amounted to 0.07 and 15.8 ㎚, respectively (Fig. 3). For the solution with the highest concentration of copigment (1 : 3) an increase in ΔA and $\Delta\lambda_{max}$ was measured 7.8 and 1.5-fold, respectively (Table 1). Several types of chemical groups were observed to induce anthocyanin copigmentation. Among those, flavonones, aurones, and flavonols show the most significant color modifications, including chroma and lightness (Delgado-Vargas and Paredes-López, 2003). The

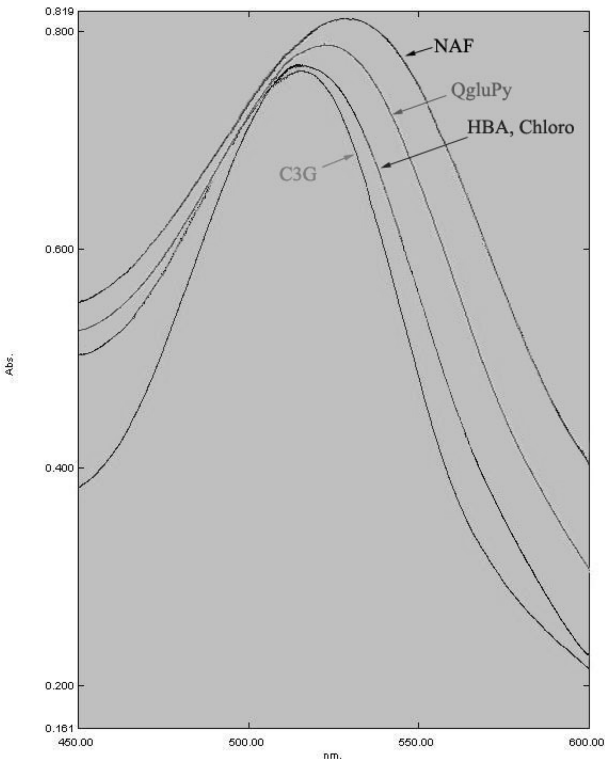

Fig. 3. The absorption of spectra of cyanidin-3-glucoside, without and with copigments[‡] from mulberry polyphenol compounds (more ration 1 : 1) at pH 3.5.

[‡] Species of copigment substance, NAF: non-anthocyanin fraction of mulberry (containing polyphenolic compounds excepted antocyanin); HBA: Hydrobenzoic acid; QgluPy: quercetin-3-*O*-β-D-glucopyranoside; Chloro : Chlorogenic acid

absorption spectra of cyanidin with and without copigments at pH 3.5. As can be seen in Table 1, the bathochromic shift and hyperchromic effects are the highest ($\Delta A = 0.548$, $\Delta\lambda_{max} = 23.2$ ㎚) with complexes of cyanidin with non-anthocyanin fraction of colorant extract and anthocyanin fraction from mulberry fruits. With other complexes, these effects are lower and amount to $\Delta A = 0.231$, $\Delta\lambda_{max} = 8.2$ ㎚ for quercetin-3-*O*-β-D-glucopyranoside; $\Delta A = 0.006$, $\Delta\lambda_{max} = 2.6$ ㎚ for chlorogenic acid; and $\Delta A = 0.004$, $\Delta\lambda_{max} = 2.5$ ㎚ for *p*-hydroxybenzoic acid. It can be seen that the use of the non-anthocyanin fraction (Colorant extract consist of various polyphenol compound in mulberry fruits) as a new copigment appears to be the most powerful, the next is quercetin-3-*O*-β-D-glucopyranoside, while *p*-hydroxybenzoic acid and chlorogenic acid seem to be rather poor copigments as reference phenolic acid.

Waterhouse (2002) stated that the number of hydroxyl group in the flavonoid copigment affects the magnitude of copigmentation. More hydroxy group present on the copigement, the storonger the copigmentation and complex formation. In this study, number of hydroxy groups is not a reason for copigmentation in mulberry anthocyanin because cyanidin with well-known copigments, chlorogenic acid more contain hydroxy group than hydroxybenzoic acid.

3. Influence of temperature in the pigmentation effect

The color of anthocyanins is based on the fully conjugated 10-electron A-C ring system, with some contribution by the B ring as well. If that is disrupted, the color is lost as when anthocyanins are in high pH medium or bleached by bisulfite (Waterhouse, 2002). However, the structure in resonance is the cause of their instability, and consequently the groups attached to the structure (namely hydroxyl, methoxyl, glycosyl, and acyl) influence the stability substantially (Delgado-Vargas and Paredes-López, 2003). Other factors like the pH, temperature, light, presence of other phenolic compounds, enzymes, metal ions, sugars, ascorbic acid, and oxygen etc. also have significant impact on the stability of anthocyanins (Shahidi and Naczk, 2004).

Increasing time and temperature of heating resulted in changes in anthocyanin from mulberry fruits and copigment contents and the copigmentation complex, which resulted in the hyperchromic effect, and a bathochromic shift in the main absorption peaks. Changes in the bathochromic shift and in the hyperchromic effect as a function of heating at 80℃ for 1 h, at pH 3.5, and molar ratio 1 : 3, are shown in Table 1. The highest bathochromic shift was observed for cyanidin with non-anthocyanin fraction of colorant extract from mulberry fruits complex. The lowest batho-chromic shift was observed with the complex of cyanidin and chlorogenic acids and these results indicate that the phenolic acids are rather poor copigments. According to Mazza and Brouillard (1990), the interaction between the pigment and the copigment is exothermic and the tem-perature increase causes dissociation of the copigmentation complexes, giving colourless compounds, thus resulting in a loss of colour. In the investigated model solutions, a decrease of the copigmentation effect with increase of temperature was also observed (Table 1). The highest hyper-chromic effect and bathochromic shift was measured when the complex of cyanidin with non-anthocyanin fraction of colorant extract from mulberry fruits was used. The addition

Table 2. Influence of one hour UV irradiation on bathochromic shift and the hyperchromic effect in solution of copigments and anthocyanin fraction from mulberry at pH3.5‡.

Anthocyanin	Copigment¶	ΔA		$\Delta\lambda_{max}$	
		Before	After UV irradiaton	Before	After UV irradiaton
C3G§	NAF	0.548	0.687	23.2	25.7
	HBA	0.104	0.239	5.4	6.5
	QgluPy	0.231	0.376	8.2	9.7
	2 × QgluPy	0.325	0.385	11.3	13.8
	Chloro	0.006	0.119	2.5	3.9

‡ l and A are changes of wavelength and absorbance of visible maximum at 525 ㎚ upon addition of copigments; §C3G: Cyanidin 3-glucosie; ¶Species of copigment substance, NAF: non-anthocyanin fraction of mulberry (containing polyphenolic compounds excepted antocyanin); HBA: Hydrobenzoic acid; QgluPy: quercetin-3-*O*-β-D-glucopyranoside; Chloro : Chlorogenic acid

of residual copigments reduces both bathochromic shift and hyperchromic effects.

In aqueous solution, anthocyanins undergo structural transformations that are pH-dependent which had been studied and summarized by Brouillard (1982). It has been found that four major anthocyanin forms exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone. Anthocuanins show red colour only in a very limited pH range. pH less than 2. As the pH is raised kinetic competition occurs between the hydration reaction on position 2 of flavylium cation and the proton transfer reactions related to its acidic hydroxyl groups. Also incresing the pH causes a decrease of both the colour intensity and the concentration of the flavylium cation, as it is hydrated by nucleophilic attack of water, to the colourless carbinol form.

The changes in absorbance as functions of pH and UV irradiation, observed in model experiments for cyanidin in the absence and in the presence of the copigments (Fig. 4), can be interpreted by the mechanism proposed by Brouillard *et al* (1991), to describe the intermolecular copigmentation effect. After 1-h UV irradiation, an increased bathochromic shift and the hyperchromic effect were observed in all the solutions (Table 2). This was the result of a strong degradation effect of UV light on cyanidin without copigments. The cyanidin/non-anthocyanin fraction of mulberry (NAF) complex exhibited greater copigmentation magnitudes than the other complexes. As can be seen, the non-anthocyanin fraction of mulberry (NAF) containing hydrobenzoic acid and quercetin-3-*O*-β-D-glucopyranoside gave the higher than seperated application of HBA, QgluPy and 2 × QgluPy hyperchromic effects respectively.

We investigated the influence of UV irradiation on the stability of the cyanidin-copigment complex. They found that the presence of copigments in anthocyanin solutions inhibited the degradation influence of UV on anthocyanin stability. Fig. 4 shows that the absorbance at 525 ㎚ decreases with increasing irradiation time at all investigated pH values. Non-anthocyanin fraction of mulberry, used in this experiment as the copigment, prevented the UV degradation and stabilized the cyanidin better than other copigments containing isolated compounds from non-anthocyanin fraction of mulberry such as HBA and QgluPy, which resulted in the highest enhancement of absorbance at 525 ㎚, especially at pH 3.5

This result indicated that naturally being copigments in mulberry fruit products (mulberry juice, syrup, wine and Jam etc) affect color stability within a given pigment source, these naturally being copigments were shown to be key elements to decrease anthocyanin degradation during processing for food products.

As previously discussed, the nature of polyphenolic copigments from mulberry and their relative molar ratio (QgluPy < 2 × QgluPy) to anthocyanin concentration were also influential on anthocyanin stability. Isolation of polyphenolic copigments revealed not only the appreciable difference in anthocyanin stability exhibited by cyanidin-3-glucoside, major anthocaynin in mulberry but also their specific role in anthocyanin stability. This occurs when there are two aromatic ring substance in solution that have very different electron densities. The phenomenon of copigmentation is because of molecular association between pigments and other organic molecule in solution usually non-colored such as flavonoids and polyphenol, alkaloids, amino acid and organic acid. Stability of anthocyanins can be enhanced though intramolecular or intermolecular copigmentation.

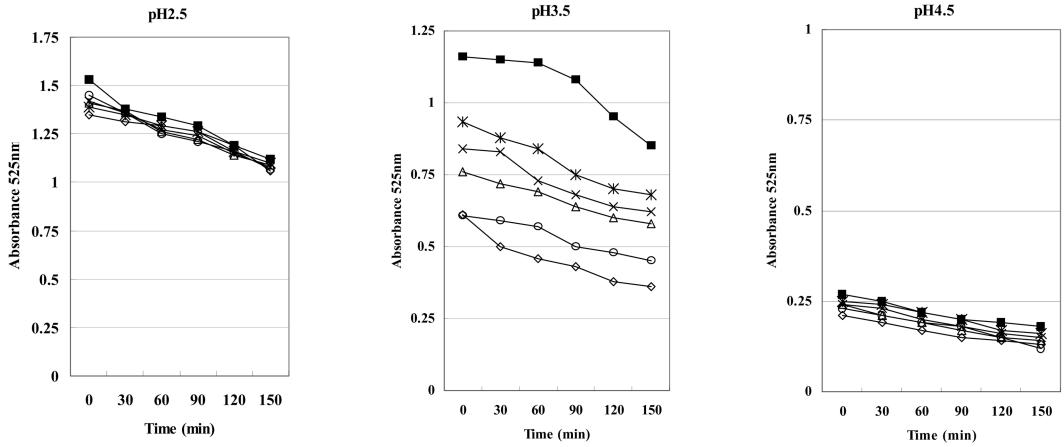

Fig. 4. Influence of time of UV irradiation and pH value (2.5-.4.5) on absorbance at 525 ㎚ of a cyanidin-3-glucoside, with and without copigments: mole ratio 1 : 3.

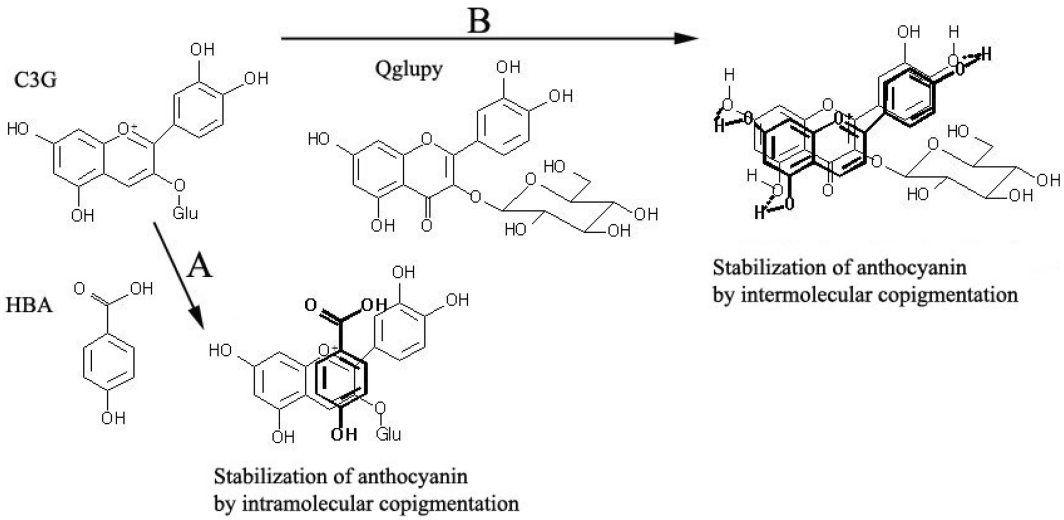

Fig. 5. Suggested mechanism of anthocyanin/*p*-hydroxybenzoic acid (A) and anthocyanin/quercetin-3-*O*-β-D-glucopyranoside (B) complexation.

Acylated anthocyanins containing two or more aromatic acyl groups may affect the color through a mechanism called intramolecular copigmentation (Mazza and Miniati, 1993; Harborne and Williams, 2001). Anthocyanins also interact with other flavonoids and related compounds to produce an increase in color intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic effect). Such a phenomenon is called intermolecular copigmentation, which can take place in acidic, neutral and even slightly alkaline aqueous solution (Mazza and Miniati, 1993; Brouillard and Dangles, 1994).

We found that cyanidin exists essentially in the flavylium form and the hyperchromic shift in the spectral maximum, observed in the presence of the copigment, is due to the interaction of the cyanidin flavylium cation with the hydroxybenzoic acid by intramolecular copigmentation manner and suggested mechanism of interaction of ralvylium (red) with electron rich phenol (Fig. 5A). In terms of the intramolecular copigmentation, a sandwich type stacking of the aromatic residue of acyl groups with the pyrylium ring of the flavylium cation decreases hydration at C-2 and C-4 positions (Mazza and Miniati, 1993).

Intermolecular copigmentation also enhances the stability through intermolecular stacking (Clifford, 2000), quercetin-3-

O-ß-D-glucopyranoside, copigmentation defined as interaction between coloured anthocyanin and colouless copigment, which this not bound covalently to the anthocyanin molecule. They attributed this effect to extensive H-bonding and ionic bonding between the negatively charged rlavonoid and the electron deficient flavylium structure. As flavylium cation (C3G) and the quinolidal base (quercetin-3-*O*-β-D-glucopyranoside) are almost planar, with efficently delocalised p-electrons, this makes the interactions between copigments and the anthocyanin much easier leading to overlapping arrangement of two molecule, protect in the coloured flavylium cation from the nucleophilic attack of the water molecule (Fig. 5B) (Williams and Hrazdina, 1979; Delgado Cargas and Peredes-Lopes, 2003). Red cabbage and purple potato extracts typically contain cinnamic acid derivatives diacylated to their anthocyanins that can simultaneously stack on both faces of the anthocyanin chromophore in a sandwich type complex and thus offer greater color stability, while black carrots contain only monoacylated moieties that can only protect one face of the pyrylium ring (Rodriguez-Saona *et al*., 1999; Boulton, 2001; Malien-Aubert *et al*., 2001).

We investigated that stabilization effect conferred by copigmentation has been attributed to hydrophobic interactions between anthocyanins (C3G) and polyphenolic compounds (hydroxbenzoic acid and quercetin-3-*O*-β-D-glucopyranoside) consequently protecting the pigment from further degradation reactions by pH and UV irradiation

Characterization of the major polyphenolic compounds present in mulberry and their contribution to the copigmenation capacity was determined for the first time. The effect of exogenously added copigments on color enhancement and stability was previously evaluated in many food systems containing isolated anthocyanins, yet the effect of naturally being copigments on color stability was not previously investigated prior to this study. The stability of mulberry anthocyanins as a new source of anthocyanin pigments was also established and can be used to determine application and functional properties of non-anthocyanin fraction from mulberry in a variety of food and nutraceutical products.

LITERATURE CITED

Bang IS, Park HY, Yuh CS, Kim AJ, Yu CY, Ghimire B, Park JG, Choung MG and Lim JD. (2007). Antioxidant activities and phenol compounds composition of extracts from mulberry (*Morus alba* L.) fruit. Korean Journal of Medicinal Crop Science. 15:120-127.

Baranac JM, Petranovic NA and Dimitric-Markovic JM. (1996). Spectrophotometric study of anthocyanin copigmentation reactions. Journal of Agricultural and Food Chemistry. 44: 1333-1336.

Baranac JM, Petranovic NA and Dimitric-Markovic JM. (1997). Spectrophotometric study of anthocyanin copigmentation reactions. 2. Malvin and the nonglycosidized flavone quercetin. Journal of Agricultural and Food Chemistry. 45:1694-1697.

Brouillard R. (1982). Chemical structure of anthocyanins. In Markakis (ed.) Anthocyanins as food colors. Academic Press, New York, USA. p. 1-40.

Boulton R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: a critical review. American Journal of Enology and Viticulture. 52:67-86.

Brouillard R and Dangles O. (1994). Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging. Food Chemistry. 51:365-371.

Brouillard R, Mazza G, Saad Z, Albrecht-Gary AM and Cheminat A. (1989). The copigmentation reactions of anthocyanins: a microprobe for the structural study of aqueous solution. Journal of the American Chemical Society. 111:2604-2610.

Brouillard R, Wigand MC, Dangles O and Cheminat A. (1991). pH and solvent effect on the copigmentation reaction of malvin with polyphenols, purine and pyrimidine derivatives. Journal of Chemical Society Perkin Transactions. 2:1235-1241.

Brouillard R. (1988). Flavonoids and flower color. In Harborne (ed.) The flavonoids: advances in research since 1980. Chapman and Hall, London, England. p. 525-538.

Chen LJ and Hrazdina G. (1981). Structural aspects of anthocyanin flavonoid complex formation and its role in plant colour. Phytochemistry. 20:297-301.

Choi SR, You DH, Kim JY, Park CB, Kim DH, Ryu J, Choi DG and Park HM. (2009). Antimicrobial activity of methanol extracts from *Cudrania tricuspidata* Bureau according to the parts harvested and time. Korean Journal of Medicinal Crop Science. 17:335-340.

Clifford MN. (2000). Anthocyanins-nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture. 80:1063-1072.

Dangles O, Wigand MC and Brouillard R. (1992). Polyphenols in plant pigmentation: the copigmentation case. Journal of Agricultural and Food Chemistry. 41:209-216.

Davies AJ and Mazza G. (1993). Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. Journal of Agricultural and Food Chemistry. 41:716-720.

Delgado-Vargas F and Paredes-López O. (2003). Anthocyanins and betalains. In Natural Colorants for Food and Nutraceutical Uses. CRC Press, Boca Raton. p. 167-219.

Eder A. (2000). Pigments in food analysis by HPLC. Nollet (ed.) Marcel Dekker, New York. p. 845-880.

Harborne JB and Williams CA. (2001). Anthocyanins and other flavonoids. Natural Product Reports. 18:310-333.

Harborne JB. (1998). Phenolic compounds in phytochemical

methods - a guide to modern techniques of plant analysis 3rd edition. Chapman and Hall, New York, USA. p. 66-74.

Kang TH, Hur JY, Kim HB, Ryu JH and Kim SY. (2006). Neuroprotective effects of the cyanidin-3-O-beta-d-glucopyranoside isolated from mulberry fruit against cerebral ischemia. Neuroscience Letters. 391:122-126.

Kim AJ and Park S. (2006). Mulberry extract supplements ameliorate the inflammation related hematological parameters in carrageenan-induced arthritic rats. Journal of Medicinal Food. 9:431-435.

Konczak-Islam I, Okuno S, Yoshimoto M and Yamakawa O. (2003). Composition of phenolics and anthocyanins in a sweet potato cell suspension culture. Biochemical Engineering Journal. 14:155-161.

Lin JY and Tanga CY. (2007). Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chemistry. 101:140-147.

Liu X, Xiao G, Chen W, Xu Y and Wu J. (2004). Quantification and purification of mulberry anthocyanins with macroporous resins. Journal of Biomedicine and Biotechnology. 5:326-331.

Lugasi1 A and Takacs M. (2002). Flavonoid aglycons in foods of plant orgin II. Fresh and Dried Fruits. Journal Acta Alimentaria. 31:63-71.

Malien-Aubert C, Dangles O and Amiot MJ. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition: Protective effects by intra- and intermolecular copigmentation. Journal of Agricultural Food Chemistry. 49:170-176.

Markakis P. (1982). Stability of anthocyanins in foods. In Markakis (ed.) Anthocyanins as Food Colors. Academic Press Inc. New York, USA. p. 163-178.

Markham KR, Ternai B, Stanley R, Geiger H and Mabry TJ. (1978). Varvone-13 NMR studies of flavonoids-III. Naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron. 34:1389-1397.

Mazza G and Brouillard R. (1987). Recent developments in the stabilization of anthocyanins in food products. Food Chemistry. 25:207-225.

Mazza G and Brouillard R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. Phytochemistry. 29:1097-1102.

Mazza G and Miniati E. (1993). Anthocyanins in fruits, vegetables and grains. CRC Press: London, U.K.

Naderi GA, Asgary S, Sarraf-Zadegan N, Oroojy H and Afshin-Nia F. (2004). Antioxidant activity of three extracts of *Morus nigra*. Phytotherapy Research. 18:365-369.

Pouchert CJ and Behnke J. (1993). The Aldrich Library of ^{13}C- and ^{1}H-NMR Spectra. Aldrich Chemical company. p. 1116.

Prior RL. (2004). Absorption and metabolism of anthocyanins: potential health effects in phytochemicals-mechanisms of action. In Meskin *et al* (ed.) Phytochemicals: mechanism of action. CRC Press, Boca Boton. p. 1-19.

Rodriguez-Saona LE, Giusti MM and Wrolstad RE. (1999). Color and pigment stability of red radish anthocyanins and red-fleshed potato anthocyanins in juice model systems. Journal of Food Science. 61:688-694.

Ryszard Z, Marian N and Jaroslaw N. (2005). Phenolic acid profiles in some small berries. Journal of Agricultural Food Chemistry. 53:2118-2124.

Shahidi F and Naczk M. (2004). Contribution of phenolic compounds to flavor and color characteristics of foods in phenolics in food and nutraceuticals. CRC press, Boca Boton. p. 443-461.

Takasugi M, Nagao S, Masamune T, Shirata A and Takahashi K. (1979). Structures of moracins E, F, G, and H, new phytoalexins from diseased mulberry. Tetrahedron Letter. 20: 4675-4678.

Talcott ST and Lee J. (2002). Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. Journal of Agricultural and Food Chemistry. 50:3186-3192.

Waterhouse AL. (2002). Wine Phenolics. Annals of the New York Academy of Sciences. 957:21-36.

Williams M and Hrazdina G. (1979). Anthocyanins as food colorants: Effect of pH on the formation of anthocyanin-rutin complex. Journal of Food Science. 44:66-68.

Wilska-Jeszka J and Korzuchowska A. (1996). Anthocyanins and chlorogenic acid copigmentation-influence on the colour of strawberry and chokeberry juices. Zeitschrift für Lebensmittel-Untersuchung und-Forschung. 203:38-42.

Yamamoto J, Naemura A, Ura M, Ijiri Y, Yamashita T and Kurioka A. (2006). Testing various fruits for anti-thrombotic effect: I. Mulberries. Platelets. 17:555-564.