COLOR STABILITY OF SORGHUM 3-DEOXYANTHOCYANINS AGAINST SULFITE AND ASCORBIC ACID DEGRADATION; pH INFLUENCE

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

To my dad Mr. John W. O. Ojwang and mum Mrs. Martha A. Ojwang. Your constant motivation and faith has always given me hope. Now, this is was your dream, through me. Thank you.

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ABSTRACT

The degradation of anthocyanins by food additives like SO_2 and ascorbic acid limits their use as natural food colorants. The rare 3-deoxyanthocyanins from sorghum are relatively stable compared to other anthocyanins, but have not been investigated. The stability of apigeninidin, luteolinidin, 5-methoxyapigeninidin, 7-methoxyapigeninidin, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin, red cabbage pigment, grape blue powder and crude sorghum pigment extract against SO_2, ascorbic acid bleaching and high temperature treatment (121.1°C for 15 min) at pH 2.0, 3.0, 3.2 and 5.0 was measured in the presence (50:1 molar ratio) or absence of pyruvic acid (known to increase the stability of anthocyanins in red wine). Samples were incubated at 37°C for 5 days to synthesize the pyruvic acid adducts, and their sulfite and ascorbic acid bleaching resistance investigated at 60 ppm and 500 ppm respectively, using a Shimadzu UV-1650PC spectrophotometer for 21 days. HPLC-DAD/MS analysis confirmed the formation of the 3-deoxyanthocyanin-pyruvic acid adducts at approximately 11 – 47% conversion. Samples without pyruvic acid were the controls. Solution pH had the greatest effect on pigment stability, and SO_2 and ascorbic acid are co-pigments with 3-deoxyanthocyanin pigments in absence of pyruvic acid at pH 2.0 and 5.0, respectively.

Pyruvic acid had marginal protective influence on the stability of the 3-deoxyanthoxyanin pigments against sulfite and ascorbic acid degradation but not heat. Crude black sorghum extract was the most stable to SO_2 and ascorbic acid bleaching, with and without pyruvic acid. High temperature initiated production of new 3-deoxyanthocyanin-pyruvic acid adducts.

CHAPTER 1

INTRODUCTION

1.1. Need for research

Epidemiological evidence indicates that anthocyanin pigments and their derivatives, many found in commonly consumed fruits, cereals and vegetables, have therapeutic benefits towards various human illnesses including their usefulness in reducing risk of various circulatory disorders (Bettini and others 1985) and inflammatory diseases (Lietti and others 1976; Vincieri and others 1992; Noda 2000). They have also been shown to exhibit anticarcinogenicity (Karaivanova and others 1990); very high antioxidant capacity (Awika and others 2004b); a role in improving visual acuity (Nakaishi 2000); vasoprotective ability (Lietti and others 1976); anti-obesity, antineoplasticity (Kamei and others 1995); and have also been reported to be safe in dietary supplements (Bridle and Timberlake 1997). Despite all this evidence, their use in foods and beverages have been minimal due to their poor stability.

Due to consumer concerns over synthetic dyes, natural food colorants, particularly anthocyanins and their derivatives, have drawn increased research in the food industry (Wang and others 1997; Boyd 2000). The aesthetic role of color serves as the basis for the assessment of quality, influencing food preference, food acceptability and choice, which improve the food industry’s interest in the anthocyanins. Thus, researchers have worked to identify the relative amounts of anthocyanins from many sources that can be utilized as potential, natural commercial colorants: red cabbage (*Brassica oleraceae*) (Idaka and others 1987), black carrot (*Daucus carota* spp. *sativa*)

(Gläßgen and others 1992), grape (*Vitis vinifera*) (Wulf and Nagel 1978), black currant (*Ribes nigrum*) (Chandler and Harper 1962), purple corn (*Zea mays*) (Harborne and Self 1989), black chokeberry (*Aronia melanocarpa*) (Striegl and others 1995), black sorghum (Awika and others 2005), and many others, have been assessed in this regard.

Anthocyanins possess several advantages: they are brightly colored, especially in the red-orange region, and are water-soluble, which enhances their incorporation into aqueous food systems. They are universally associated with attractive, colorful, flavorful fruits and vegetables (Francis 1989).

Sorghums contain unique anthocyanins called 3-deoxyanthocyanins. The two principle sorghum 3-deoxyanthocyanins are apigeninidin (yellow) and luteolinidin (orange) (Nip and Burns 1969, 1971; Dykes and Rooney 2006). These rare 3-deoxyanthocyanins lack **-**OH at the carbon **3** position (Clifford 2000) and are relatively stable to pH-induced color degradation compared to other common anthocyanins and their aglycones (Sweeny and Iacobucci 1981; Gous 1989; Awika and others 2004a), which would make them good natural food colorants.

The 3-deoxyanthocyanins produce different color hues depending on the pH of the solution, ranging from yellow-orange hue in acidic solvents to orange-red hue in mildly acidic or neutral solvents. Thus, 3-deoxyanthocyanins could be of advantage in processed foods and beverages at neutral pH levels (Mazza and Brouillard 1987).

Additives like ascorbic acid are added to foods to improve the nutritional quality and to prevent enzymatic browning reactions in fruits and vegetable products (Starr and Francis 1968). Ascorbic acid also acts as a singlet oxygen quencher (Mares-

Perlman 1997; Elliot 1999; Kalt and others 1999). However, ascorbic acid degrades anthocyanins in the presence of trace amounts of iron, copper (Rababah and others 2005) and hydrogen peroxide (Özkan and others 2004). Rababah and others (2005) showed that addition of ascorbic acid to strawberry, peach and apple juices increased $L*$ (lightness) but decreased $a*$ (redness) and $b*$ (yellowness) color values significantly. On the other hand, molecular and free forms of SO_2 are used principally for their antimicrobial and antioxidant properties respectively (Amerine and others 1967). SO_2 effectively prevents ascorbic acid, vitamin A and pro-vitamin A in foods from oxidative degradation between pH 2.5 – 5.0 (Perera 2005).

Sulfur dioxide reactivity, as an electrophile, nucleophile or an acid, when added to anthocyanin-containing foods occurs at the positively charged carbon **2** and preferentially at carbon **4** positions (Berké and others 1998; Oliveira and others 2006). This decreases the color intensity of the anthocyanin pigments. However, there has been very limited research on the 3-deoxyanthocyanin compounds. Characterizing the chromatic behavior of these compounds under different pH, processing and storage conditions would widen the understanding of their chemical characteristics which is significant in the development of safe, economical and efficient natural food colors to replace the synthetic dyes such as FD&C yellow #6 and Red #40, currently used in foods.

1.2. Objectives of research

Three research objectives were established:

1. Establish stability of sorghum 3-deoxyanthocyanins against SO_2 and ascorbic acid bleaching at different pH levels relative to commercial anthocyanin pigments.
2. Determine effectiveness of pyruvic acid addition on sorghum 3-deoxyanthocyanin stability against SO_2 and ascorbic acid bleaching.
3. Determine effects of commercial sterilization conditions on sorghum pigments stability.

CHAPTER 2

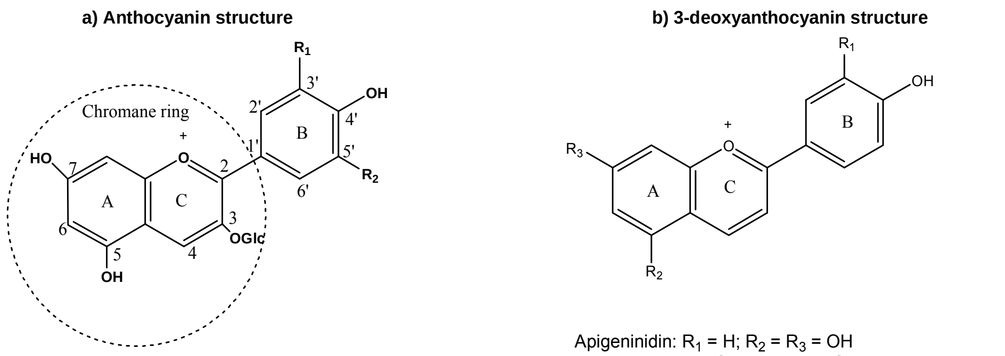
REVIEW OF LITERATURE

2.1. Background information

Flavonoids are compounds with a C6-C3-C6 skeleton that comprise the aromatic *chromane ring* (A and C rings) joined by a 3-carbon link (Harborne 1998; Prior and Wu 2006). They include *anthocyanins* (**Figure 1a**), flavonols, flavones, flavanones and flavanols. Anthocyanins, together with carotenoids and betalains, are responsible for the red, purple, and blue colors of many fruits (Schwarz and Winterhalter 2003), and flowers, leaves, stems, roots and cereal grains (Brouillard 1982; Francis 1989). Anthocyanins occur naturally in plants as glycosides, generally linked with glucose, galactose, arabinose, xylose, fructose and rhamnose (Pereira and others 1997; Chigurupati and others 2002; Mazza and others 2004). Besides chlorophyll, anthocyanins are probably the most important group of visible plant pigments in nature.

Most recent data indicate that at least 5,500 naturally occurring polyphenols, including approximately 5,000 flavonoids have been identified in nature (Yao and others 2004). Of these, over 600 *structurally* distinct anthocyanins have been separated using different methods including HPLC profiling and paper chromatography (Andersen 2001). Anthocyanins are water soluble and non-toxic (Janna and others 2006); non-mutagenic and have positive therapeutic properties (Saija 1994).

Structurally, the positive charge on the C-ring of the flavylium (2-phenylchromenylium) cation causes chemical characteristics that are different from other classes of flavonoids, especially in their oxidation states. The positive charge is principally located at carbons **2** and **4** (**Figure 1a**).



Pelargonidin: R_1 = H; R_2 = H
Cyanidin: R_1 = OH; R_2 = H
Delphinidin: R_1 = OH; R_2 = OH
Peonidin: R_1 = OCH_3; R_2 = H
Petunidin: R_1 = OCH_3; R_2 = OH
Malvinidin: R_1 = OCH_3; R_2 = OCH_3

Apigeninidin: R_1 = H; R_2 = R_3 = OH
Luteolinidin: R_1 = OH; R_2 = R_3 = OH
Apigeninidin-5-glucoside: R_1 = H; R_2 = OGlc; R_3 = OH
5-Methoxyapigeninidin: R_1 = H; R_2 = OCH_3; R_3 = OH
7-Methoxyapigeninidin: R_1 = H; R_2 = OH; R_3 = OCH_3
Luteolinidin-5-glucoside: R_1 = OH; R_2 = OGlc; R_3 = OH
5-Methoxyluteolinidin: R_1 = OH; R_2 = OCH_3; R_3 = OH
7-Methoxyluteolinidin: R_1 = OH; R_2 = R_3 = OCH_3

Figure 1: Chemical structure of (A), the six common anthocyanidins, and (B), the 3-deoxyanthocyanins.

Great stability of the anthocyanin chromophore is attributed to the *substitution* for the hydroxyl on the *para*-position of the B-ring (carbon **4'**), which increases the delocalization of the π-electrons (Pereira and others 1997). The most common substitution positions in the 2-phenylchromenylium structure are carbons **3, 5, 7, 3', 4'** and **5'** through hydroxylation, acylation or acetylation processes, which affect the reactivity of the anthocyanins (Iacobucci and Sweeny 1983a; Pereira and others 1997).

These substitution patterns are responsible for the different color parameters observed for different anthocyanin pigments, for example, the hydroxyl group at carbon **3'** is very significant in changing the color of anthocyanins from yellow-orange (e.g. strawberries, pelargonidin-based pigments) to bright red (e.g. blackberries, >80%

cyanidin 3-*O*- *β*-*D*-glucoside), and to the bluish red of young red wines (largely caused by malvidin 3-*O*-*β*-*D*-glucoside) (Schwarz and Winterhalter 2003), depending on the pH of the solution. Most pigmented plant foods contain anthocyanins.

Sorghum anthocyanins (3-deoxyanthocyanins) (**Figure 1b**) on the other hand, lack a hydroxyl group at the carbon **3** position, a unique property that increases their stability at higher pH in comparison to the common anthocyanins (Mazza and Brouillard 1987; Awika and others 2004a, b). This improves their potential as natural food colorants. Black sorghum (Tx430) variety has a higher level of 3-deoxyanthocyanins than sorghums with red pericarp (Gous 1989; Dykes and others 2005).

Other than the two major aglycones found in sorghum grains (luteolinidin and apigeninidin) (Nip and Burns 1971; Awika and Rooney 2004; Wu and Prior 2005), apigeninidin-5-glucoside and luteolinidin-5-glucoside (Nip and Burns 1969, 1971; Mazza and Miniati 1993; Wu and Prior 2005), acylated forms (Asen and others 1972; Hipskind and others 1990), and methoxylated forms like 5-methoxyluteolinidin, 7-methoxyapigeninidin and 5-methoxyluteolinidin-7-glucoside (Wu and Prior 2005), 5-methoxyapigeninidin and 7-methoxyluteolinidin (Seitz 2004), 7-methoxyapigenidin-5-glucoside (Lo and others 1996; Wu and Prior 2005) have also been identified (**Figure 1b**).

Recent interest in flavonoids, especially anthocyanins and 3-deoxyanthocyanins (from sorghums), together with their derivatives and phenolic acids stems from the fact that they have some of the strongest *physiological* effects of any plant compounds.

Many of these beneficial phenolic compounds found in fruits and vegetables are also detected in other cereal grains (e.g. corn, wheat, barley, rice, etc). In general, pigmented cereal grains (e.g. sorghum) have the highest levels of phenols and antioxidant activity among all grain categories.

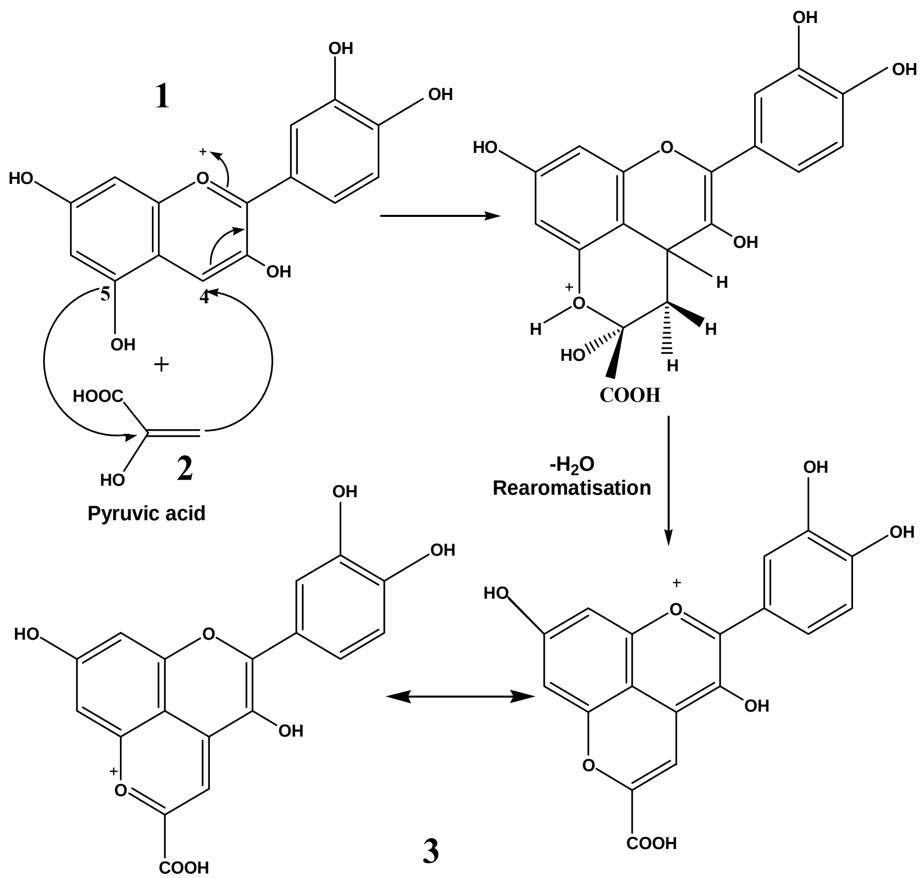


Figure 2: Mechanism for the formation of anthocyanin-pyruvic acid adducts: cyanidin, 1; pyruvic acid, 2; and cyanidin-pyruvic acid adducts, 3.

Apart from pH influence, the *intensity* and *stability* of anthocyanin pigments are also dependent on other factors like temperature, light, oxygen, enzymes, vitamin C, bisulfite, structure and concentration of the pigments, sugar metabolites and metal ions

(Jackman and others 1987; Francis 1989; Fossen and others 1998). On the other hand, mechanisms have been discovered that effectively improve the color intensity and stability of common anthocyanin pigments. For example in red wine, anthocyanin monoglucosides like malvidin-3-*O*-glucosides form pyranoanthocyanin pigments through condensation reaction (**Figure 2**) with metabolites (e.g. pyruvic acid) during wine maturation (Alcalde-Eon and others 2006), and hence have enhanced stability (e.g. pH near 4.0 and the presence of SO_2 used as antioxidant and preservative) (Morata and others 2007). These derived pyranoanthocyanin pigments are more *resistant* to bleaching by bisulfite and oxidation than are their precursor anthocyanins (Bakker and Timberlake 1997; He and others 2006; Morata and others 2007). Many of these pyranoanthocyanins have been synthesized from anthocyanins and phenolic acids, acetaldehydes and acetone. They have all exhibited stable brighter colors (Fulcrand and others 1998).

In general, the synthesis of the anthocyanin-pyruvic acid adducts results from the cyclic addition of pyruvic acid onto carbon **4** and -OH group at the carbon **5** positions on the anthocyanin molecule (**Figure 2**). No information is available on the reaction of 3-deoxyanthocyanins with the phenolic acids or pyruvic acid, and how such reactions may affect their stability.

Degradation of common anthocyanin pigments during extraction, food processing and storage present a disadvantage for their application as food colorants. Therefore, increased stability to pH changes, SO_2, temperature, ascorbic acid and light of 3-deoxyanthocyanin pigments compared to commercial anthocyanin pigments, may be important to the food industry and enhance the potential utilization of these beneficial compounds. However, information on how glycosylation, acylation and methoxylation patterns affect the stability of sorghum 3-deoxyanthocyanins pigments is unavailable.

2.2. Factors contributing to anthocyanin instability

Several reactions occurring in foodstuff during processing and storage degrade the color of anthocyanins and limit their application as commercial colorants (Fossen and others 1998).

2.2.1. pH

In general, anthocyanin molecules occur in four species (**Figure 3**) existing in equilibrium: the quinoidal anhydrobase, A (blue), the flavylium cation, AH^+ (red), the pseudobase or carbinol, PB (colorless), and the chalcone, C (colorless or light yellow) (Chen and Hrazdina 1982; Lewis and others 1995; Heredia and others 1998). However, they occur preferably in their more stable and colored form of flavylium cation in very acidic solutions (Gonnet 1998).

As the pH increases, the kinetic and thermodynamic competition occurring between the hydration reaction on position **2** of the flavylium cation and the proton transfer reactions related to its acidic hydroxyl groups increases (Torskangerpoll and Andersen 2005). Thus, this favors the quinoidal (blue) forms (Heredia and others 1998) which are very susceptible to degradation due to light (Janna and others 2006), heat (Baranac and others 1996) and availability of oxygen.

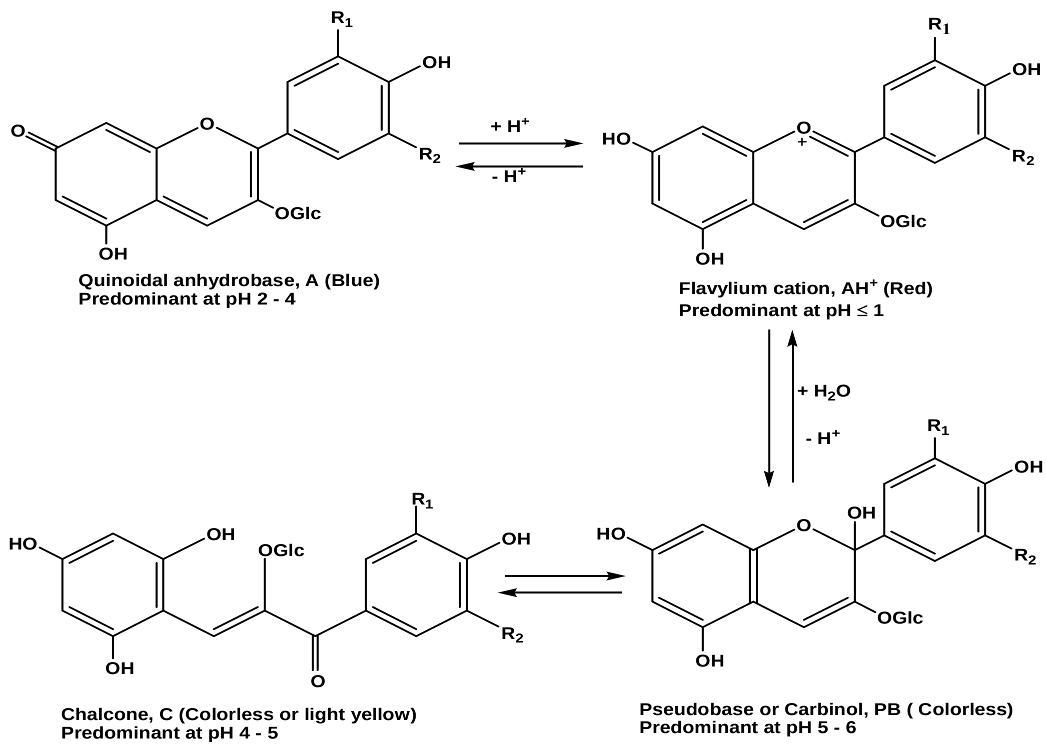


Figure 3: Structural transformations of anthocyanins. R_1 and R_2 are usually H, OH, or OCH_3.

Total anthocyanin color is observed in strongly acidic solutions (pH less than 2.0), where they express negative deviation from Beer's law (color increase is less than linear with increase in pigment concentration) as reported by Bridle and Timberlake (1997). For instance, in very acidic media (pH < 0.5) the red cation AH^+ is the dominant structure. As the pH increases, its concentration decreases as hydration of the colorless carbinol pseudobase occurs (Mazza and Brouillard 1987). These authors also reported that proportions of the colorless chalcone C and the blue quinoidal base A increases with increasing pH at the expense of the red cationic form AH^+ to about pH 4.5. Between pH 4 and 6, they observed very little amounts of both colored forms AH^+ and A.

However, the cationic form AH^+ of 3-deoxyanthocyanins is much higher at pH 6 compared to that of common anthocyanins, and its quinoidal form A dominates at pH 4.5 – 5.0. Compared to common anthocyanins, the methoxyl, carboxyl and glycosyl groups play no role in determining its acid-base equilibrium constant. A much higher constant suggests that the flavylium salts lose their proton more easily at carbon **7** than at carbon **4'**. Additionally, Giusti and Wrolstad (2001) reported that monomeric anthocyanins exhibit almost no absorbance at pH 4 – 5. On the other hand, apigeninidin and luteolinidin showed significant absorbance at pH 4 – 5 (Awika and others 2004b). Thus, unlike common anthocyanins, these 3-deoxyanthocyanins may be of importance as natural colorants in foods and beverages at near neutral pH.

2.2.2. Light

Daylight (or short wavelengths) and incandescent lamp (or long wavelengths) affect the color parameters of the anthocyanins in different solutions (Francis 1989; Gonnet 1998; Janna and others 2007). Consequently, Bordignon-Luiz and others (2007) established that reporting on color without specification of reference to light conditions cannot be achieved correctly.

Jurd (1964b) suggested that the position of the equilibrium between flavylium salts and 2-hydroxychalcones in aqueous solutions is markedly affected by light (**Figure 4**). The NMR and UV spectral data indicated that the chalcones formed at equilibrium had the *trans*-configuration which photo-isomerized rapidly to *cis* 2-hydroxychalcones, which then cyclized in acid solutions to flavylium salts.

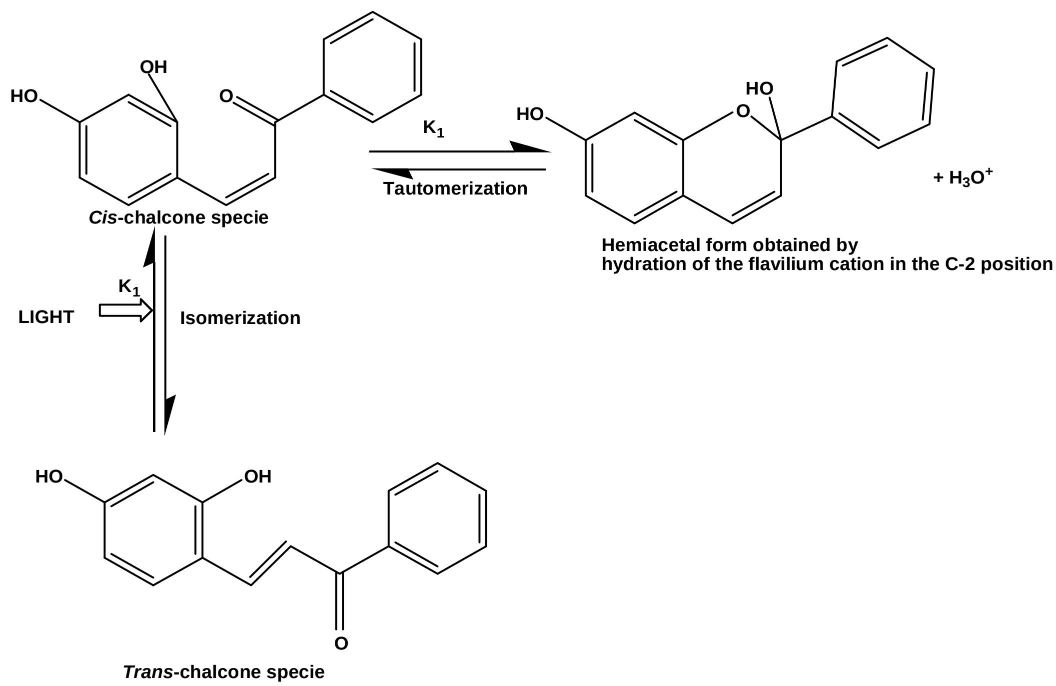


Figure 4: Structural re-arrangement of anthocyanins due to the effect of light.

UV-irradiation induces speedy anthocyanin degradation regardless of the pH of the solution (Abyari and others 2006). At a pH of 2.0 and 25°C, these authors evaluated the effect of the absence and the presence of light (400 Lux) for 90 days on the destruction of anthocyanins from four varieties of *Malus* spp. They found a significant discoloration of the anthocyanin pigments exposed to light compared to their respective samples stored in the dark. Palamidis and Markakis (1975) also exposed grape anthocyanin pigments to light for 135 days at 20°C and reported a 50% pigment discoloration, compared to 30% effect on those stored in the dark. Abyari and others (2006) found that co-pigmentation with some phenolics significantly prevented UV-irradiation degradation over a period of time, especially with tannic acid as the co-pigment. Parisa and others (2007) reported that the presence of co-pigments in the anthocyanin solution prevented the degradation effect of UV-irradiation over a period of time on anthocyanin pigments significantly. Similar results were also found by Bakowska and others (2003) and Kucharska and others (1998). Timberlake (1989) suggested an increase in flavylium cation restructuring from the influence of light, which explains why Abyari and others (2006) reported higher amounts of chalcone in the *Malus* spp extracts than the flavylium cation in the anthocyanin samples kept in the dark. However, no information on the effect of light on 3-deoxyanthocyanin pigments is available, especially in comparison to the common anthocyanins under similar conditions.

2.2.3. Temperature

Pigment stability is also affected by temperature (Baranac and others 1996; Janna and others 2007). High temperature leads to the degradation of the anthocyanins during storage through destabilization of the anthocyanin molecular structure (Bakhshayeshi and others 2006; Ochoa and others 2001; Shaked-Sachray and others 2002; Bolivar and Cisveros-Zevallos 2004).

Also, the co-pigment complexes are exothermic and very heat labile (Dangles and Brouillard 1992). Brouillard and Dangles (1994) suggested that increase in temperature caused a significant disruption of the organized lattice liquid-water structure, permitting a reduction in the degree of water-hydrogen bonding, leading to a reduction in co-pigmentation effectiveness. In this way, the flavylium ions are released and hydrated to the colorless hemiacetal form. However, increasing the temperature of the anthocyanin-containing solution with no co-pigments had no effect on the absorbance spectrum in the visible range (Dangles and Brouillard 1992). Therefore, thermal energy is a significant factor that dictates the thermodynamic conditions of the co-pigmentation process (Abyari and others 2006).

Rubinskiene and others (2005) also evaluated the effect of temperature on the stability of black currant berries anthocyanins, verifying increased degradation of the anthocyanin pigments subjected to high temperatures (85 and 95°C). Bakhshayeshi and others (2006) and Giusti and Wrolstad (2001) suggested this was due to the thermal hydrolization of the 3-glycoside structure, leading to the instability of the anthocyanin molecule. Increase in storage temperature accelerated anthocyanin destruction significantly in soft drinks, thus producing the chalcone responsible for browning in anthocyanin-containing foods (Palamidis and Markakis 1975; Spayd and others 2002). These undesirable brown colored compounds during thermal treatment and storage (Maccarone and others 1985; Fiore and others 2005) explain the observed allure red colorant in sterilized commercial juices. Anthocyanin thermal degradation is dependent on the time and temperature of the treatment and the subsequent storage conditions, which increases with increasing storage temperature (Fallico and others 1996).

However, no data are available on effects of severe thermal processing, e.g. sterilization treatment (i.e. autoclaving or pasteurization) on the stability of 3-deoxyanthocyanin pigments.

2.2.4. Oxygen

Oxygen occurs in the form of superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and singlet oxygen (O_2). Oxygen availability was reported as mutually destructive to anthocyanins in the presence of ascorbic acid (Starr and Francis 1968; Sondheimer and Kertesz 1953; King and others 1980), which confirmed that the color bleaching of the anthocyanins and anthocyanidins by ascorbic acid occurred via oxidative cleavage of the pyrilium ring (De Rosso and Mercadante 2007b; Walkowiak-Tomczak and Czapski 2007). De Rosso and Mercadante (2007a) reported that vitamin C degradation rates of acerola and açaí anthocyanin solutions in an inert (nitrogen) atmosphere were 1.3 – 1.4 times slower than in air, both in the presence and the absence of light. Deguchi and others (2000) also showed that the presence of oxygen from the air significantly degraded hordeumin (a protein-tannin-anthocyanin complex), given that the $t_{1/2}$ values of all the samples kept in the presence of atmospheric oxygen were lower when compared to the values of the samples kept under nitrogen flow, at pH 5.0, 6.0 and 7.0. This rate of anthocyanin destruction in oxygen was pH dependent and directly proportional to the amount of the pigment which exists in the form of the pseudo base.

Thus, ozonolysis of 3-methoxyflavylium salts in acetic acid solutions yield substituted *O*-benzoyloxyphenylacetic acids (Jurd 1964b), at a pH of about 5.8 as shown in **Figure 5** below. Similar results were also reported with hydrogen peroxide oxidation, formed by oxidation of natural anthocyanidin 3-glycosides in aqueous solutions. This leads to a pH-dependent oxidative discoloration of the anthocyanin pigment observed in many plant juices between pH 5 – 7 (Jurd 1964b).

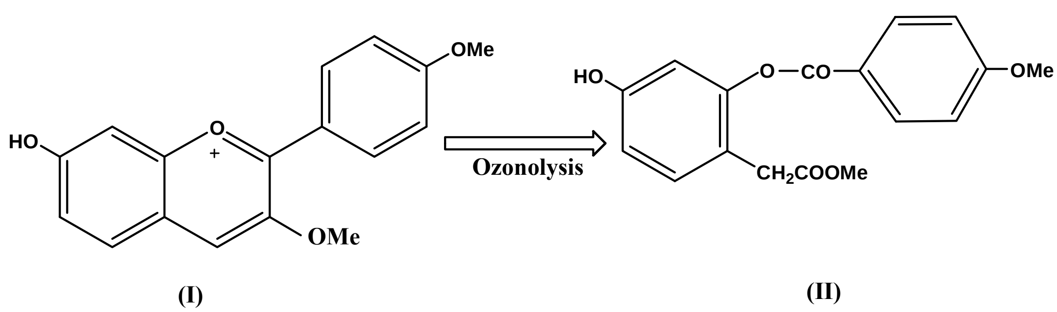


Figure 5: Transformation of a Flavylium salt, (I) into *O*- benzoyloxyphenylacetic acid, (II) during ozonolysis.

However, Hrazdina and Franzese (1974) confirmed that structure considerably affected this oxidative reaction. This resultant oxidative ring contraction (**Figure 5**) may not be exclusive to the common anthocyanins. The 3-deoxyanthocyanins may undergo similar oxidative reaction, involving initial formation of a hydroperoxide and its subsequent rearrangement as observed with common anthocyanins. Currently, there is no information on oxidative effect on 3-deoxyanthocyanin pigments in comparison to natural anthocyanins.

2.2.5. Enzymes

Enzymes are not only responsible for the synthesis of the anthocyanins; they also contribute to the observed discoloration of anthocyanin pigments.

2.2.5.1. Pectic enzyme

Several crude fungal enzyme preparations exerted a significant enzyme-catalyzed bleaching effect on extracts of berry fruit pigments within a pH range of 3.0 to 4.5 (pH range of most natural fruit juices), through the enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar, with a further transformation of the aglucone into colorless derivatives (Huang 1955). Thus, the decolorizing activity of the pectic enzyme is pH- and time dependent.

2.2.5.2. Polyphenol oxidase (PPO) enzyme

The browning reaction observed in anthocyanin-containing solutions is caused by the polyphenol oxidase (PPO) enzyme (Williams and others 1986) which masks the red color of the anthocyanins (Wesche-Ebeling and others 1996), via co-polymerization mechanism (Wesche-Ebeling and Montgomery 1990).

Generally, due to the sparing solubility of anthocyanidins in aqueous media, anthocyanase enzyme may be added to remove the pigment sediments (Wissemann and Lee 1980) from the solution in the form of aglucone sediments, in situations where the hydrolysis of the anthocyanidin is desirable. Thus, enzymes can be used to solubilize anthocyanidins, especially around the walls of wine bottles during storage. However, the influence of enzymes on the 3-deoxyanthocyanin pigments is largely unknown, but can be assumed to be similar to the co-polymerization of anthocyanin pigments during enzymatic browning reaction observed in plum juice extracts (Wesche-Ebeling and others 1996).

2.2.6. The presence of co-pigments including flavonoids, polyphenols, alkaloids, amino acids, metals and organic acids

Co-pigment effect, the phenomenon which makes the color of anthocyanins bluer, brighter and more stable is divided into intermolecular and intra-molecular co-pigmentation. UV-Vis and NMR spectroscopy (Dangles and El hajji 1994; Houbiers and others 1998; Yoshida and others 2000) and fluorescence spectroscopy (Wigand and others 1992; Alluis and others 2000) have been used to study co-pigmentation (Berké and de Freitas 2005).

The most studied group of co-pigments are polyphenols: chlorogenic acid (Mazza and Brouillard 1990), flavonoids i.e. rutin and quercetin 3-*β*-*D*-galactoside (Chen and Hrazdina 1981; Davies and Mazza 1993; Baranac and others 1997; Gonnet 1999), tannic acid (Cai and others 1990; Marquette and Trione 1998), ferulic acid (Eiro and Heinonen 2002), sinapic and rosmarinic acids (Rein and Heinonen 2004), caffeic acid (Wesche-Ebeling and others 2003), gallic acid, pentagalloylglucose, purines, pyrimidine (Berké and de Freitas 2005), and also some amino acids (Asen and others 1972; Chandra and others 1993). These have shown increased polymerization in red wines (Singleton and Trousdale 1992) hence helped in retaining their red color.

In general, an organic acid, an aromatic acyl group, or a flavonoid is covalently bonded to an anthocyanin chromophore (Brouillard 1981; Bloor and Falshaw 2000), or through loose intermolecular interactions in which flavonoids (colorless), other anthocyanins, or phenolic compounds, react with weak hydrophobic forces with the planar polarized nuclei of the anthocyanin-colored (quinoidal) forms (Mazza and Brouillard 1990; Eiro and Heinonen 2002) as shown in **Figure 6**.

Co-pigmentation is detected as both a hyperchromic effect and a bathochromic shift (Malien-Aubert and others 2001; Eiro and Heinonen 2002). It plays a big role in stabilizing the structural forms of the anthocyanin molecules, consequently enhancing their color intensity (Brouillard 1982; Mazzaracchio and others 2004). However, there is no information about co-pigmentation reactions on the rare 3-deoxyanthocyanin pigments. In food science, co-pigmentation is considered an important interaction, as color is one of the quality factors strongly affecting consumer choice of food.

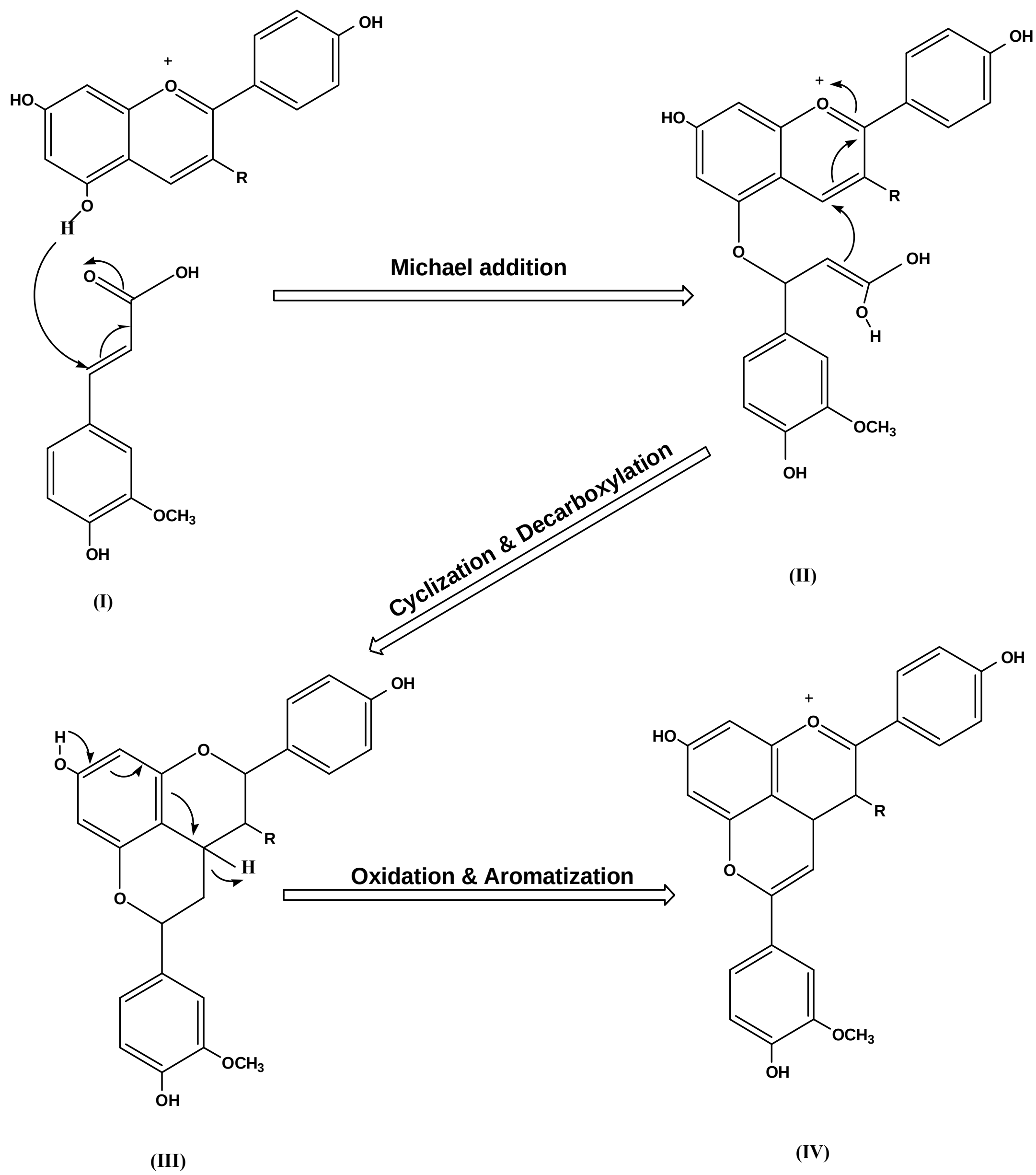


Figure 6: Proposed reaction mechanism for the formation of new pyranoanthocyanin adduct with pelargonidin 3-glucoside and ferulic acid (co-pigment). R = glucoside residue.

2.2.7. Solvent

The anthocyanins are highly water-soluble (Janna and others 2006). The anthocyanin nucleus is electron deficient and hence very reactive and unstable.

$$\mathbf{AH^+ + H_2O \Leftrightarrow A + H_3O^+}$$

Due to their positive charge, they are highly susceptible to nucleophilic attack (Garcia-Viguera and Bridle 1999), from reagents like peroxides and SO_2, principally at carbons **2** and **4**. Additionally, H^+, OH^- and H_2O species are highly reactive towards anthocyanins in water, and influence the spectral properties of anthocyanin structures in many aqueous solutions (Lewis and others 1995). Thus, to retain the intensity of the anthocyanin pigment color, it is necessary to protect the flavylium ring against water attack by reducing the extent of the hydration reaction through co-pigmentation (Lewis and others 1995).

2.2.8. Substitution on the chromane ring (A and C rings)

Hydroxylation, methoxylation, acylation and the type and number of sugars and co-pigments attached to the anthocyanins have a great effect on the stability of these pigments (Mazza and Miniati 1993; Lewis and others 1995). The the presence of di-methoxyl substitution contributes a higher stability to the anthocyanin molecule than does mono-methoxyl substitution implying that the natural or added methoxyl- groups lends relatively higher stability (Markakis 1982). Acylated analogues are more stable

(Saito and others 1995), depending on the position of the acyl group, position of the sugar moiety and the length of the *sugar spacer* on the anthocyanin structure.

Anthocyanin structure with sugar moieties acylated with acids are more stable to heat, light, pH and SO_2 (Asen and others 1972; Yoshida and others 1991; Giusti and Wrolstad 1996), depending on the type of chemical substitution (Garcia-Viguera and Bridle 1999; Brouillard and others 1982) and aromatic hydroxylation (Brouillard 1982). The stability is achieved by preventing condensation and hydration reactions that result in the loss of flavylium pigmentation. This also leads to improved color intensity by reducing their sensitivity to pH changes. Additionally, aromatic acyl groups may improve color stability via intra- and inter-molecular co-pigmentation and self association mechanisms (Brouillard 1988; Yoshida and others 2000; Nerdal and Andersen 1992).

Based on observations of some relatively simple anthocyanins and anthocyanidins *in vitro*, molecular optimizations have proved that aromatic acyl groups protect the aglycone against hydration in the carbon **2** and carbon **4** positions during intramolecular copigmentation (Torskangerpoll and Andersen 2005). This greatly influences the color of anthocyanins.

Iacobucci and Sweeny (1983) showed that for non-substituted anthocyanins at carbon **4**, adding hydroxyl substituents on the A or B rings improve the overall stability, while methoxylation of similar hydroxyls reduces their performance in solution. Further reduced stability is observed with an ethylene function between carbon **2** and the B ring due to extended conjugation. On the other hand, the **4-**

substituted (i.e. **4**-methyl- and **4**-phenyl- substituted) anthocyanins showed no difference in stability when compared to apigeninidin under optimized storage conditions. However, incorporating vitamin C and air had a higher discoloration effect on apigeninidin than on the **4**-substituted anthocyanins. Additionally, compared to apigeninidin, the anthocyanin pigments with methyl- groups at carbon **4** and hydroxyl- groups at carbon **5**, had the highest resistance to photo-oxidative discoloration. Similarly, the hydroxyl- group at carbon **5** (as in all natural anthocyanins) and substitution at carbon **4** significantly stabilized the colored (A and AH^+) forms by preventing hydration reactions that initiate formation of the colorless (B and C) forms (Brouillard and others 1982; Torskangerpoll and Andersen 2005).

Also, Iacobucci and Sweeny (1983) demonstrated that 3-deoxyanthocyanidins fade fast at pH 7. Generally, methylation at carbon **4** has a slight improvement on their overall stability. Similarly, the extent of methoxylation and hydroxylation influenced the color stability of 3-deoxyanthocyanidin pigments. Addition of a 4-carboxy group significantly increased their stability. Consequently, these authors suggested that 4-carboxy-3-deoxyanthocyanidin compounds could be of importance, in comparison to all natural anthocyanins, to color foods and beverages at neutral pH. However, there is no data on how substitution patterns affect color, solubility and stability of 3-deoxyanthocyanin pigments relative to natural anthocyanins.

2.3. The chromatic behavior of anthocyanins in the presence of SO_2 and ascorbic acid

2.3.1. Sulfur dioxide, SO_2

Sulfur dioxide is a biofunctional acid which is widely used in foods; for example, in winemaking as an antioxidant and bacteriostatic agent, it dissociates almost instantaneously into three fractions depending on the thermodynamic constant and the pH. These three fractions are molecular sulfur dioxide (SO_2), sulphite (SO_3^{2-}), and bisulphite (HSO_3^-).

$$\mathbf{H_2O + SO_2 \Longleftrightarrow H^+ + (HSO_3)^- \Longleftrightarrow 2H^+ + SO_3^{2-}}$$

Water	+	*Molecular sulphur dioxide*	*Hydrogen ion*	+	*Bisulphite*	*Hydrogen ion*	+	*Sulphite*

Early incorporation of SO_2 in the must during vinification protects phenolics that precipitate during the fermentation since SO_2 occupies the carbon **4** position of the flavylium cation (Timberlake and Bridle 1967a, b; Amerine and others 1967; Somers and Wescombe 1982), hence reducing the rate of color loss during wine aging (Picinelli and others 1994). The reversibility of this reaction may lead to polymerization and subsequent precipitation of the SO_2-bound phenolics in the wine during storage. Therefore, timing of this SO_2 addition during wine fermentation is extremely critical to prevent color degradation and microbial spoilage (Jurd 1964a; Hatfield and others 2003).

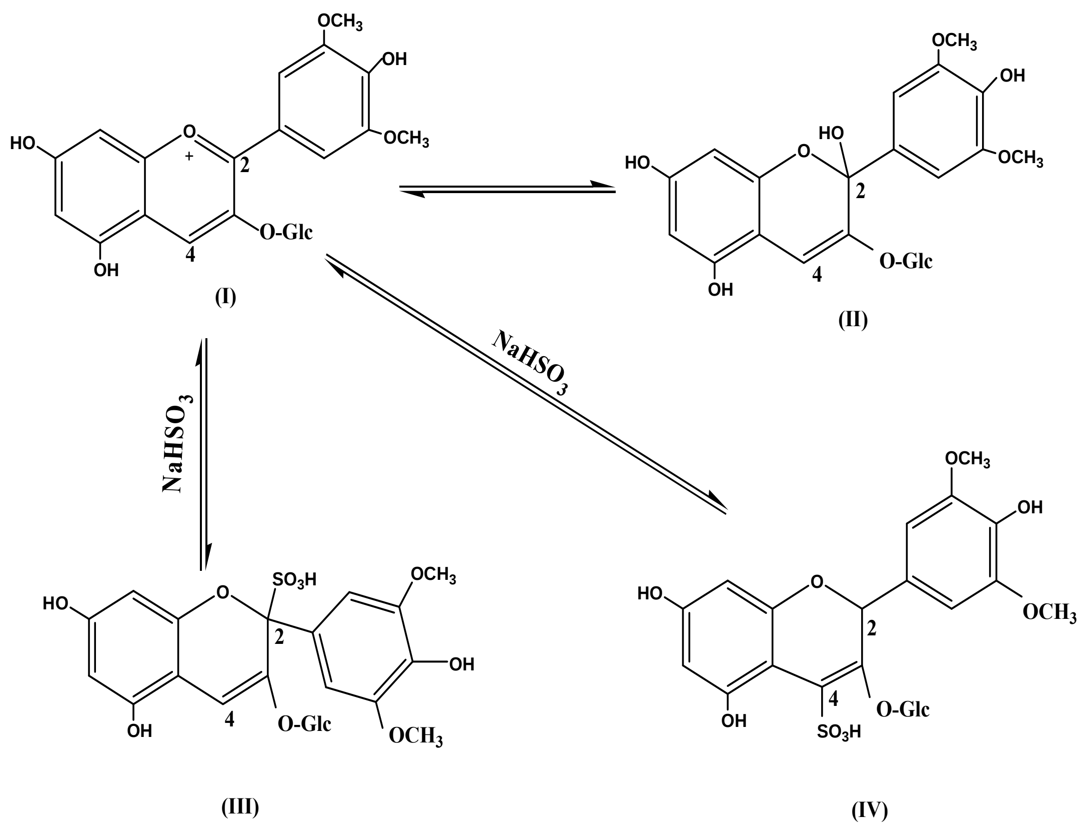


Figure 7: Bisulfite addition on carbon 2 or 4 on the anthocyanin molecule.

Moreover, dissolved SO_2 behaves as a powerful nucleophile. Sulfite bleaching results from the addition of hydrogen sulfite to the C ring of the flavylium cation, generating a colorless hydrogen sulfite adduct (Timberlake and Bridle 1967a, b; Berké and others 1998; Salas and others 2005). In anthocyanin solutions, the nucleophilic addition of bisulfite anions and water occurs at carbon **2** and **4** as shown in **Figure 7** forming colorless sulfonates **(III)** or **(IV)** (Berké and others 1998).

However due to preferential addition at carbon **4** position (more accessible due to its lower steric hindrance with respect to the carbon **2** position), sulfite bleaching of anthocyanins is highly prevented in a wide range of pH in the anthocyanin-pyruvic acid adducts as shown in **Figure 2**. This desirable observation is expected to have similar protective influence on 3-deoxyanthocyanin pigments.

2.3.2. Ascorbic acid (Vitamin C)

Ascorbic acid (**Figure 8**) is commonly added to fruit juices and other beverages to prevent browning and to provide an additional source of vitamin C. As a supplement, consumption of these dietary antioxidants offers protection against some pathological events (Martí and others 2001). The degradative effect of vitamin C on anthocyanin stability and its consequent discoloration have been studied in model solutions (Poei-Langstron and Wrolstad 1981; Iacobucci and Sweeny 1983b; Garcia-Viguera and Bridle 1999).

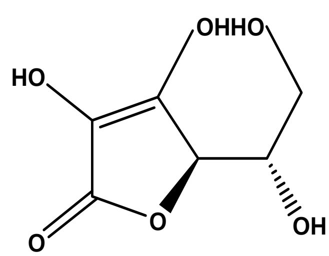


Figure 8: Ascorbic acid.

Instability of anthocyanins in the presence of ascorbic acid occurs due to the oxidative cleavage of the pyrulium ring by free radical mechanism, with the ascorbic acid acting as a pro-oxidant (Iacobucci and Sweeny 1983a). Poei-Langston and

Wrolstad (1981) and Jurd (1972) proposed that anthocyanin color decreased more quickly under nitrogen sparging than under oxygen sparging conditions, which suggested the decolorization effect was through a condensation reaction in the presence of ascorbic acid.

Other mechanisms earlier proposed include direct condensation of ascorbic acid on the carbon **4-**position of the anthocyanin molecule (Jurd 1972; Poei-Langston and Wrolstad 1981) contributing to the collective degradation and ultimate loss of the anthocyanins stability (Rodriguez-Saona and others 1999; Brenes and others 2005) similar to the bisulfite ion reaction. Therefore, the nature of the substituent at carbon **4** is a key factor influencing the resistance to discoloration by both sulfur dioxide and ascorbic acid.

Hydroxylation and methoxylation, together with type and numbers of sugar moieties attached to the anthocyanin chains also have an influence on the overall stability (Mazza and Miniati 1993). Greater stability against ascorbic acid degradation was observed when the sugar residues were acylated with acids (Asen and others 1972; García-Viguera and Bridle 1999).

Additionally, King and others (1980) confirmed that the rate of decolorization of the anthocyanins by vitamin C was dependent on the amount of oxygen available in the solution. Starr and Francis (1968) also reported a speedy degradation of cranberry juice when the highest amounts of vitamin C and oxygen were introduced into the system. These results proved that under oxygenated conditions, the introduction of transition metals accelerated vitamin C and anthocyanin degradation and break-down in their mutual the presence. Under these conditions, the copper-catalyzed disintegration of vitamin C is responsible for the observed pigment color loss (Timberlake 1960a, b). However, there is no data on the effect of ascorbic acid on 3-deoxyanthocyanin compounds.

2.3.3. Improving on the available knowledge in the study of 3-deoxyanthocyanin pigments

Since there is enough information on pyruvic acid reaction with common anthocyanins, it will be utilized as the co-pigment in this research. It is expected that the reaction mechanism between pyruvic acid and 3-deoxyanthocyanins will be similar to the condensation reaction between pyruvic acid and anthocyanins observed in red wines (**Figure 2**). Successful synthesis of novel 3-deoxypyranoanthocyanins (3-deoxyanthocyanin + pyruvic acid) adducts will significantly improve their stability compared to their precursors. Hopefully, the 3-deoxypyranoanthocyanins will express deeper colors than other similar pigments at the same pH level. Obtaining such more stable compounds will have several attractive advantages including their ease of storage over long periods of time and possible processing into shelf-stable colorful products. Sorghums have a very high concentration of 3-deoxyanthocyanins. In general, these compounds contribute the intense blue, purple and red colors in sorghum bran extract and are more prevalent in the pigmented sorghum varieties. Determining the color characteristics of the 3-deoxyanthocyanins in model food systems will significantly enhance their potential application as natural food colorants.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals, samples and reference compounds

3.1.1. Chemicals

HPLC grade hexanes, methanol, HCl, acetonitrile, water, L-ascorbic acid (99.7%), sodium meta-bisulfite and formic acid were purchased from Fisher Scientific (Hanover Park, IL). Pyruvic acid (*d* = 1.265, 98%) was obtained from Sigma-Aldrich (St. Louis, MO).

3.1.2. Sample and reference compounds

Apigeninidin, luteolinidin, 5-methoxyapigeninidin, 7-methoxyapigeninidin, 5,7-dimethoxyapigeninidin and 5,7-dimethoxylutelinidin standards were from ALSACHIM (Strasburg, France). For comparison, red cabbage powder was purchased from Voigt Global Distribution LLC (Kansas City, MO) and grape blue powder from Exberry (GNT group, Redwood City, CA). Black sorghum (TX 430) bran was obtained from Texas A&M University, College Station, TX.

3.1.3. Sorghum bran handling

Black sorghum (TX 430) bran was finely ground using a UDY mill (Model 3010-030, Fort Collins, CO), to pass through 0.1 mm mesh. Grinding was necessary to improve pigment extraction efficiency from the black sorghum brans. Black sorghum powder (2 grams) was defatted by extracting four times (20 mL each) in sequence with hexanes, shaken for 20 minutes in an Open-Air Reciprocating Shaker (Model SHKA 2506-1, Dubuque, IA), and then centrifuged at 3100 g-force for 30 minutes at 13ºC. The solid residue was dried overnight at room temperature and stored at -35°C until used.

3.1.4. The 3-deoxyanthocyanin pigment extraction

The crude pigment extraction was performed twice with 20 mL 1% HCl in methanol for 10 minutes with the aid of 500 watts, 20 kHz High Intensity Ultrasonic Liquid Processor (Model VC-505, Sonics and Materials, Inc., Newton, CT), with a 13 mm alloy probe, at 35% amplitude for 2 minutes. High Intensity Ultrasound is used in many food applications such as emulsification, sterilization, extraction, degassing, filtration, drying and enhancing oxidation (Leadley and Williams 2002; Wang and Wang 2004; Mason 1998). Morel and others (2000), Moulton and Wang (1982) and Wang (1975) confirmed that High Intensity Ultrasound enhanced protein extraction by increasing solubility but also decreased protein molecular weight (Morel and others 2000).

The samples were adjusted to 25 mL volume after sonication and before shaking for 45 minutes. The slurry were centrifuged at 3100 g-force for 25 minutes at 13ºC and then decanted. A further rinse with 20 mL of 1% HCl in methanol was done before being centrifuged a second time for additional pigment recovery from the bran matrix. This was a modification of the extraction procedure outlined by Wang and Wang (2004). These two aliquots were combined and the methanol and any residual hexanes were evaporated at room temperature using a rotary evaporator (Brinkmann, Model 0560-8), under vacuum (500mmHg vac). The concentrated crude sorghum 3-deoxyanthocyanin extract was kept in the dark at -35ºC until used.

3.1.5. Model solutions

Distilled water was adjusted to pH 2.0, 3.0, 3.2 and 5.0 using 0.1M HCl to make 50 mL model solutions in which the samples were separately dissolved (in tinted glass bottles). These were the controls. Similar model solutions but with the addition of 198.135 mg pyruvic acid (i.e. pH 2.0, 3.0, 3.2 and 5.0) were also prepared. The sample amounts listed in **section 3.1.6** below would separately give a final anthocyanin:pyruvic acid molar ratio of 1:50. The final desired solution pH (at 24°C) was further adjusted by using 0.1M NaOH and 0.1M HCl. The solutions with pyruvic acid were the treatments.

3.1.6. Synthesis of anthocyanidin-pyruvic acid adducts

Samples of apigeninidin (4.55 mg), luteolinidin (5.05 mg), 5-methoxyapigeninidin (5.25 mg), 7-methoxyapigeninidin (5.25 mg), 5,7-dimethoxyapigeninidin (4.95 mg), 5,7-dimethoxyluteolinidin (5.65 mg) standards and 148.15 mg of both red cabbage and grape blue powder were each incubated separately for 5 days at 37ºC, in model solutions adjusted to pH 2.0, 3.0, 3.2 and 5.0 using 0.1M HCl and 0.1M NaOH, to synthesize respective anthocyanin-pyruvic acid adducts. The incubations were in 50 mL of distilled water at the various pH levels, in tinted glass bottles (to protect the samples from UV-light), with and without pyruvic acid (pyruvic acid:anthocyanin molar ratio of 50:1) (Oliveira and others 2006). For the crude black sorghum extract, a 1 mL concentrated sample was pipeted and similarly incubated for 5 days. Samples without pyruvic acid were the controls. Pyruvic acid is known to increase the stability of anthocyanins in red wines (Morata and others 2007; Oliveira and others 2006).

3.2. Methods for the objectives

3.2.1. Research objective 1. Establish stability of sorghum 3-deoxyanthocyanins against SO_2 and ascorbic acid bleaching at different pH levels relative to commercial anthocyanin pigments

3.2.1.1. SO_2 assay

To make 3000 ppm SO_2 solution, 180 mg of sodium meta-bisulfite was dissolved in 30 mL of deionized water. Pipeting 200 µL of this solution into 9.8 mL solution (2 mL sample + 7.8 mL distilled water at each pH level tested) gave a final SO_2 concentration of 60 ppm. The stability of the six 3-deoxyanthocyanin standards, red cabbage powder, grape blue powder and the crude black sorghum pigment extract against SO_2 (0 and 60 ppm) bleaching at pH 2.0, 3.0, 3.2 and 5.0 was measured in the absence of pyruvic acid using the spectrophotometric analysis below, at 7-day interval for 21 days. After the addition of SO_2, the various pH of the solutions were adjusted using 0.1M HCl and 0.1M NaOH until the desired pH level was achieved.

3.2.1.2. Ascorbic acid assay

For vitamin C solutions, 938 mg of ascorbic acid were separately dissolved in 300 mL distilled water. The reaction mixtures for all the pH levels studied (sample:ascorbic acid 1:4 v/v) of each solution had a final vitamin C concentration of 500 ppm. After the addition of ascorbic acid, the solutions' pH were adjusted using 0.1M HCl and 0.1M NaOH until the desired pH level was obtained (i.e. pH 2.0, 3.0, 3.2 and 5.0). They were first vortexed and allowed to stand for 20 minutes. Spectroscopic absorbance readings in the absence of pyruvic acid were taken as explained below, on the first day, and after every 7 days storage period, for 21 days. The ascorbic acid concentrations were freshly prepared each day. All experiments were done in duplicates and their *means* reported.

3.2.1.3. UV-Vis absorption spectra

Absorption spectra were recorded over time using a Shimadzu UV-1650PC spectrophotometer (10mm path-length cell), from 250 to 720 nm. Duplicate solutions were prepared and the *mean* values of the spectrophotometric measurements reported. Solutions were properly mixed using a digital vortex mixer before each assay.

Research objective 2. Determine effectiveness of pyruvic acid addition on sorghum 3-deoxyanthocyanin stability against SO_2 and ascorbic acid bleaching

3.2.2.1. HPLC-DAD analysis

The synthesis of anthocyanin-pyruvic acid adducts were studied over time (7-day interval), at different pH levels using HPLC-MS analysis. All samples were filtered before being transferred into the injection vials. Qualitative analysis was achieved using an Agilent 1100 HPLC system, equipped with a diode array detector (DAD), quaternary pump and an automatic injector. All the samples were analyzed on a reversed phase 150 × 2.00 mm i.d., 5 micron (Phenomenex, Torrance, CA) C-18 column and thermostated at 35ºC. Sample injection volume was 5.00 μL; UV-Vis spectra were recorded from 200 – 720 nm and the monitoring wavelength was 480 nm. The mobile phase consisted of (A), 1% formic acid in water, and (B), 1% formic acid, 50% acetonitrile in water. The 43-min elution gradient was as follows: 0-2 min, 10% isocratic B; 2-30 min, 10-70% B; 30-32 min, 70-100% B; 32-36 min, 100% isocratic B; 36-37 min, 100-10% B; 37-43 min, 10% isocratic B; followed by 2 minutes of re-equilibration of the column before the next run at a flow rate of 0.250 mL/min.

3.2.2.2. LC-MS analysis

The MS analysis was performed using a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer equipped with an API2 source, Performance Pack (with wider orifice in the skimmer and an extra turbo pump on the source) and an Electrospray Ionization (ESI) interface (ThermoFinnigan, San Jose, CA). The mass spectrometer was connected to an integrated Thermo-Finnigan LC system consisting of a P4000 quaternary LC pump and SCM1000 vacuum degasser, an AS3000 autosampler, and a UV6000LP diode-array detector.

The electrospray needle voltage was 4.5 kV and the heated inlet capillary equilibrated at 250°C. All voltages were optimized to maximize ion transmission and minimize unwanted fragmentation. Spectra were recorded in a positive ion mode between *m/z* 150 and 1000.

3.2.2.3. UV-Vis absorption spectra

The pigments' color stability towards bleaching effects of SO_2 and ascorbic acid were also studied spectrophotometrically at pH levels 2.0, 3.0, 3.2 and 5.0 for 21 days as explained in **Objective 1**, to assess the influence of pyruvic acid addition.

3.2.2.4. Data analysis

For SO_2 analysis, the main effects *within* and *among* samples were estimated using a **2 × 2 × 4** factorial design {[two levels of pyruvic acid × two levels of SO_2 concentration × four pH levels]}. Similarly, the ascorbic acid statistical analysis was estimated with a **2 × 2 × 4** factorial design {[two levels of pyruvic acid × two levels of vitamin C concentration × four pH levels]}. The standard error of means (SEM) and standard deviations was used for post ANOVA analysis. Parallel comparisons were done across samples both under similar and varied pH levels.

3.2.3. Research objective 3. Determine effects of commercial sterilization conditions on 3-deoxyanthocyanins stability

3.2.3.1. Autoclaving process

Autoclaving was achieved by heating the anthocyanin samples (i.e. pH 3.2) at 15 p.s.i. to 121.1°C for 15 minutes using a Market Forge Autoclave (Market Forge Co., Everett, MA; Model STM-E 208) and then cooling immediately in an ice bath. Non-sterilized samples at every pH level were the controls. Data on the effect of the sterilization treatment on 3-deoxyanthocyanin pigments stability at these pH levels would be very significant in understanding how severe thermal processing conditions would affect utilization of these compounds in foods, and would further confirm the protective effect of the pyruvic acid addition to these beneficial pigments.

3.2.3.2. Sample analysis

Samples (both with and without pyruvic acid) were monitored by HPLC as explained in HPLC-DAD analysis in **Objective 2**.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Crude black sorghum pigments

Apigeninidin (39.9%), luteolinidin (16.0%), 5-methoxyapigeninidin (13.1%), 7-methoxyapigeninidin-5-glucoside (1.9%), 7-methoxyluteolinidin-5-glucoside (1.8%), luteolinidin-5-glucoside (5.0%) and apigeninidin-5-glucoside (5.1% of the total anthocyanin content in the crude sorghum pigments) were confirmed in sorghum by HPLC-DAD retention times and spectral characteristics, as well as mass spectra (**Figure 9**). The proportion of yellow, apigeninidin, and orange, luteolinidin, (55.9% of the total) was comparable to 36 – 50% reported by Awika and others (2004a, b) in black (Tx430) sorghum bran. This confirms that luteolinidin and apigeninidin are the dominant 3-deoxyanthocyanins components of black sorghum. The 3-deoxyanthocyanins showed absorption maxima lower than 490 nm, whereas common anthocyanins have absorption maxima above 500 nm in pH 1 buffer (Awika and others 2004b). Thus, these pigments could be useful natural substitutes for the yellow and orange artificial colorants.

Although some information is available on the stability of synthetic 3-deoxyanthocyanins in beverage systems, especially at pH below 4.0 (Jurd 1964a, b), the color, intensity and stability of these extracted crude natural 3-deoxyanthocyanin sorghum pigments have not been evaluated in model solutions. Hence, performance of these sorghum 3-deoxyanthocyanin pigments as food colorants in comparison to 3-deoxyanthocyanin standards was monitored spectrophotometrically at their respective maximum absorption wavelength (λ_{max} (nm)) at pH 2.0, 3.0, 3.2 and 5.0. The resistance to discoloration was a measure of their performance in these solutions.

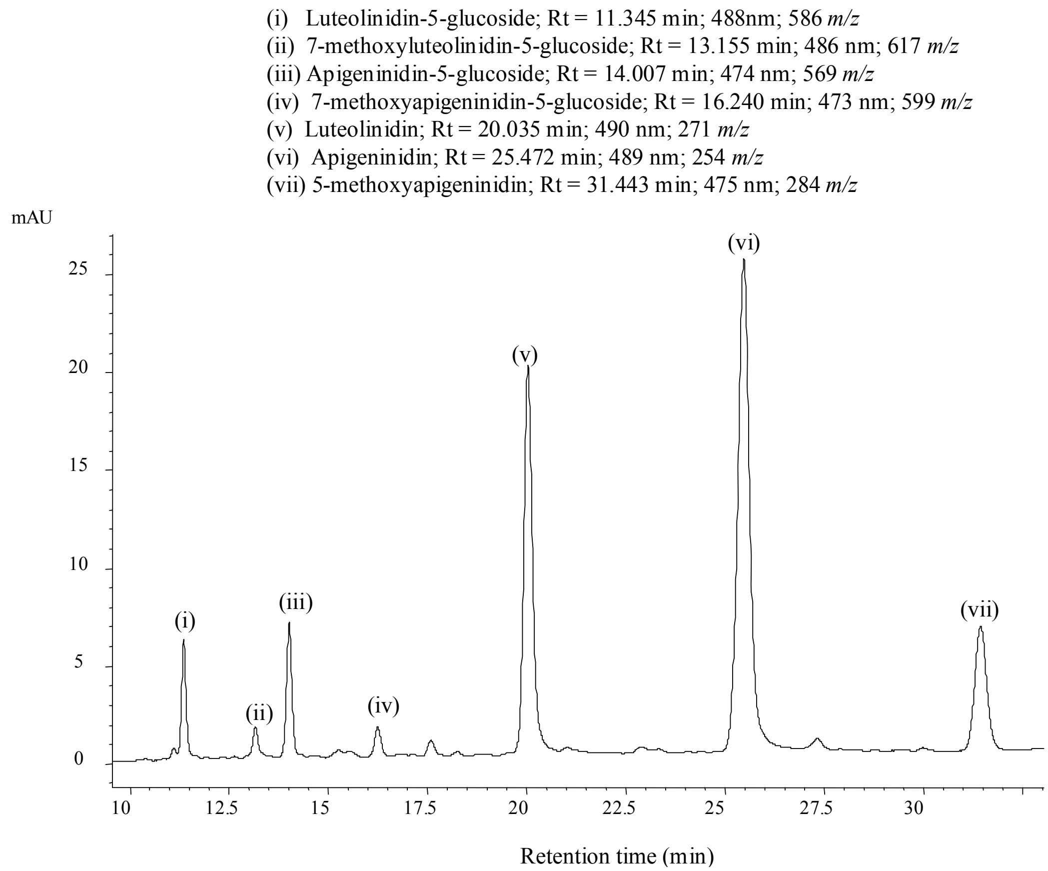


Figure 9: HPLC chromatogram of crude sorghum pigment.

4.2. Effect of SO_2 and ascorbic acid on 3-deoxyanthocyanin pigments stability at different pH levels

The influence of pH on the rate of fading of 3-deoxyanthocyanin pigments was studied at 27°C, in the presence of oxygen and fluorescent light for 21 days.

4.2.1. Stability of 3-deoxyanthocyanins pigments

The spectroscopic measurements were taken at the λ_{max} (nm) of every sample studied, scanning between 250 – 700 nm. The absorbance at day 0 were normalized to 1.00; and relative absorbance after 21 days reported. The color intensity displayed by these 3-deoxyanthocyanin pigments changed with the changing pH of the solution (**Table 1**). The stability of crude sorghum and red cabbage pigments to light, oxygen and at 27°C was similar at 3.2. However, sorghum pigment extract and red cabbage pigment showed remarkably better stability at pH 2.0 than the 3-deoxyanthocyanin standards. Similarly, stability of sorghum pigment was much higher at pH 3.0 (98% color retention) than the 3-deoxyanthocyanin standards (35% - 68% color retention). Thus, the natural pigments could be good colorants at low pH values (i.e. pH 2.0 and 3.0) relative to the standards.

All pigments were relatively stable at pH 3.2. However, the standard pigments showed increased intensity at pH 5.0 except 5-methoxyapigeninidin and 5,7-dimethoxyluteolinidin. Comparatively, the natural pigments' intensities reduced at pH 5.0. The decrease in absorbance intensities may be due to the general instability of anthocyanins to degradative effects of light, oxygen and temperature at different pH levels. But, even though the pigments' intensities also reduced at pH 2.0 and 3.0 for all samples, sorghum and red cabbage retained more color compared to the standards. Thus, natural colorants were more stable at lower pH (the pH of most fruit juices and many beverages) than the 3-deoxyanthocyanin standards.

Control Sample/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	0.97 ± 0.11	0.98 ± 0.06	1.00 ± 0.02	0.85 ± 0.01	0.11
Red cabbage	0.92 ± 0.02	*	1.00 ± 0.01	0.83 ± 0.02	0.08
Grape blue powder	*	*	1.03 ± 0.01	*	0.04
Apigeninidin	0.77 ± 0.16	0.68 ± 0.02	1.00 ± 0.02	1.38 ± 0.04	0.25
Luteolinidin	0.72 ± 0.15	0.42 ± 0.03	1.00 ± 0.03	1.42 ± 0.01	0.30
5-methoxyapigeninidin	0.58 ± 0.05	0.35 ± 0.03	1.00 ± 0.10	0.81 ± 0.08	0.37
7-methoxyapigeninidin	*	0.48 ± 0.01	1.00 ± 0.11	*	0.16
5,7-dimethoxyapigeninidin	0.79 ± 0.08	0.55 ± 0.14	1.00 ± 0.13	1.08 ± 0.05	0.78
5,7-dimethoxyluteolinidin	0.81 ± 0.11	0.60 ± 0.04	1.00 ± 0.04	0.23 ± 0.01	0.87

Table 1: Absorbance at respective λ_{max} (nm) for control samples after 21 days at 27°C, in the presence of light and oxygen, at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at α = 0.05.

4.2.2. Effect of SO_2 on stability of the control samples

At pH 2.0, there was a significant hyperchromic shift (increase in λ_{max} (nm)) of these 3-deoxyanthocyanin pigments when incubated with 60 ppm SO_2 for 21 days (**Table 2**). This was likely due to a co-pigmentation effect. The magnitude of this unique co-pigmentation effect (darkening of all the anthocyanin solutions with increased visual saturation of pigment color) was dependent on the type of 3-deoxy-anthocyanin pigment. Crude sorghum pigment showed greater pigment color and stability than red cabbage at pH 2.0, 3.2 and 5.0. The highest color intensity was observed at pH 5.0. It also showed a higher increase in absorbance at pH 3.2 than grape blue powder. Generally, sulfite effect on these natural pigments was to increase their absorbance as pH increased.

However, apigeninidin, luteolinidin and 5-methoxyapigeninidin standards showed much higher increase in absorbance at pH 2.0 than at pH 3.0, 3.2 and 5.0. They showed 6 – 8 times increased color intensity at pH 2.0 after 21 days. At pH 3.0, the co-pigmentation effect was much weaker for these pigments (1.2 – 2.3 times increase in intensity). At pH above 3.0, the bleaching effect of SO_2 was apparent in these pigments, except for apigeninidin, 5-methoxyapigeninidin and 7-methoxyapigeninidin.

The dimethoxylated pigments behaved differently in the presence of SO_2. At pH 2.0, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin showed 12 and 23 times increase in color intensity, respectively, after 21 days. However, both were completely bleached at pH 3.0 and 3.2, whereas only 5,7-dimethoxyapigeninidin was totally bleached at pH 5.0. 5,7-dimethoxyluteolinidin retained 83% of its color at pH 5.0. Among all samples, 5,7-dimethoxyapigeninidin was the most unstable to changes in pH in the presence of 60 ppm SO_2 because it showed complete discoloration at pH 3.0, 3.2 and 5.0. Hence, the dimethoxylated pigments could only be effective colorants at very low pH levels (i.e. pH 2.0) in the presence of SO_2. The general effect of pH on SO_2 co-pigmentation with 3-deoxyanthocyanin standards was the loss of the co-pigmentation effect, leading to reduced pigment intensity as the pH increased.

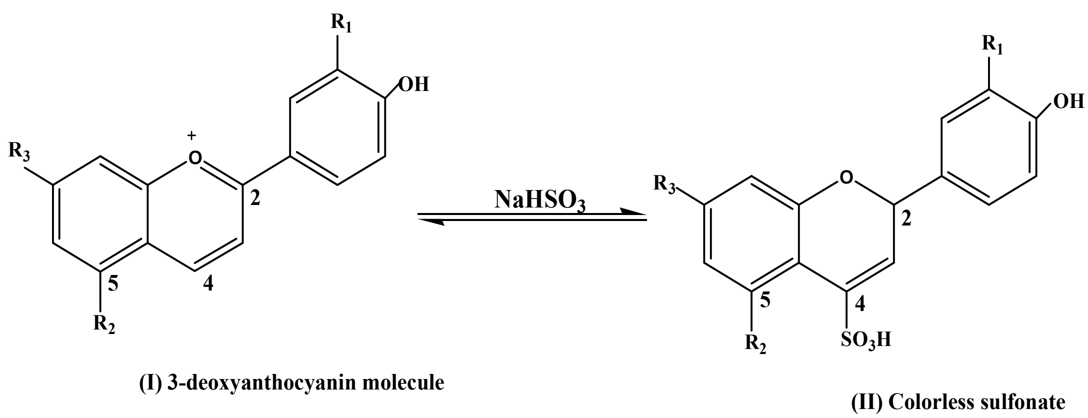
In the 3-deoxyanthocyanin molecules, the nucleophilic addition of anionic sulfite occurs preferentially at carbon **4** position rather than at carbon **2** due to steric hindrance at this position (**Figure 10**). This reaction causes bleaching of natural anthocyanin molecules in solution. However, it apparently leads to co-pigmentation of 3-deoxyanthocyanin pigments. For the standards, the lower the solution pH, the greater the co-pigmentation effect (**Table 2**).

Control Samples/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	1.56 ± 0.16	1.17 ± 0.03	1.65 ± 0.02	2.20 ± 0.17	0.11
Red cabbage	1.24 ± 0.02	*	1.20 ± 0.00	1.79 ± 0.01	0.08
Grape blue powder	*	*	1.14 ± 0.04	*	0.06
Apigeninidin	5.99 ± 0.12	2.29 ± 0.01	1.96 ± 0.02	1.08 ± 0.01	0.25
Luteolinidin	7.45 ± 0.09	1.59 ± 0.01	S^b	S^b	0.30
5-methoxyapigeninidin	7.96 ± 0.04	2.07 ± 0.01	1.66 ± 0.01	0.67 ± 0.09	0.37
7-methoxyapigeninidin	*	1.22 ± 0.01	1.05 ± 0.03	*	0.22
5,7-dimethoxyapigeninidin	12.10 ± 0.06	S^b	S^b	S^b	0.78
5,7-dimethoxyluteolinidin	23.78 ± 0.04	S^b	S^b	0.83 ± 0.01	0.87

Table 2: Relative absorbance at respective λ_{max} (nm) for control samples incubated with 60 ppm SO_2 after 21 days at 27°C, at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested and S^b means samples reported as totally bleached ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at $\alpha = 0.05$.

This phenomenon may be due to improved self association of these 3-deoxyanthocyanin pigments, causing monomeric anthocyanins to behave as co-pigments themselves. The precise mechanism is unclear and this unique phenomenon should be further investigated, in the presence and the absence of oxygen, light and heat. The observed hyperchromic shift can be explained by progressive formation of new intensely colored 3-deoxyanthocyanin compounds by intermolecular-sulfite addition.

However, it can also be suggested that a significant substitution for the hydroxyl with SO_2 may be responsible for this phenomenon, which improves the overall stability and color intensity, similar to the effect of hydroxylation at carbon **4** and **5** that prevent hydration reactions responsible for anthocyanin color degradation (Brouillard and others 1982; Iacobucci and Sweeny 1983a; Torskangerpoll and Andersen 2005). This low pH co-pigmentation phenomenon could possibly be utilized in coloring low acid foods and beverages like sodas.



Compounds:

Apigeninidin: R_1 = H; R_2 = R_3 = OH
Luteolinidin: R_1 = R_2 = R_3 = OH
5-methoxyapigeninidin: R_1 = H; R_2 = OCH_3; R_3 = OH
7-methoxyapigeninidin: R_1 = H; R_2 = OH; R_3 = OCH_3
5,7-dimethoxyapigeninidin: R_1 = H; R_2 = R_3 = OCH_3
5,7-dimethoxyluteolinidin:R_1 = OH; R_1 = R_2 = OCH_3

Figure 10: Sulfite addition reaction: 3-deoxyanthocyanin molecules, (I); and resultant colorless sulfonates, (II).

4.2.3. Effect of ascorbic acid on stability of the control samples

Crude sorghum pigment extract was more stable to ascorbic acid degradation than red cabbage pigment at all pH levels (**Table 3**). The stability of sorghum pigment was lowest at pH 2.0 (69% color retention) in the presence of 500 ppm ascorbic acid. However, this was much higher than red cabbage (15% color retention). At pH 3.2 and 5.0, crude sorghum pigment showed increased color intensity, suggesting co-pigmentation effect with 500 ppm ascorbic acid. Red cabbage did not show this effect at similar pH, even though it showed increase in stability to ascorbic acid degradation as pH increased from 3.2 to 5.0.

Control Samples/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	0.69 ± 0.03	1.01 ± 0.07	1.89 ± 0.10	1.26 ± 0.04	0.14
Red cabbage	0.15 ± 0.02	*	0.18 ± 0.04	0.70 ± 0.02	0.11
Grape blue powder	*	*	0.59 ± 0.05	*	0.05
Apigeninidin	0.09 ± 0.09	S^b	0.36 ± 0.02	0.44 ± 0.02	0.24
Luteolinidin	0.92 ± 0.02	0.38 ± 0.01	0.47 ± 0.07	1.30 ± 0.01	0.23
5-methoxyapigeninidin	S^b	S^b	0.18 ± 0.01	1.36 ± 0.01	0.21
7-methoxyapigeninidin	*	S^b	0.15 ± 0.02	*	0.15
5,7-dimethoxyapigeninidin	S^b	S^b	0.03 ± 0.01	0.83 ± 0.02	0.31
5,7-dimethoxyluteolinidin	S^b	S^b	0.03 ± 0.05	3.69 ± 0.01	0.30

Table 3: Absorbance at respective λ_{max} (nm) for control samples after 21 days at 27°C, incubated with 500 ppm ascorbic acid at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested and S^b means samples reported as totally bleached ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at α = 0.05.

The 3-deoxyanthocyanin standards were much less stable to ascorbate bleaching than crude sorghum pigments at pH 5.0. Only luteolinidin retained significant pigmentation at pH 2.0 and 3.0.

At pH 3.2, the non-methoxylated forms were the most stable to ascorbic acid bleaching among the standards. Apigeninidin and luteolinidin showed 36% and 47% color retention, respectively. Also, the monomethoxylated forms (15 – 18% color retention) were more stable than dimethoxylated forms (approx. 3% color retention). However, the mono- and dimethoxylated 3-deoxyanthocyanins standards were completely bleached at pH 2.0 and 3.0, suggesting poor stability at pH $\leq$ 3.0.

At pH 5.0, the 3-deoxyanthoxyanin standards showed increased color intensity in the presence of ascorbate except apigeninidin and its dimethoxylated form. Luteolinidin, 5-methoxyapigeninidin and 5,7-dimethoxyluteolinidin pigments recorded an increase in color intensity at pH 5.0, suggesting similar pigment co-pigmentation phenomenon observed in sorghum at pH 3.2 and 5.0. The 5,7-dimethoxyluteolinidin pigment showed the most increase in absorbance (3.7 times) at day 21 relative to day 0 in the presence of ascorbic acid.

The color enhancement of crude sorghum extract and the 3-deoxyanthocyanin standards at pH 5.0 suggests effectiveness of ascorbic acid in fortifying sorghum-pigment-containing foods, either for nutritive or preservation purposes, at high pH levels.

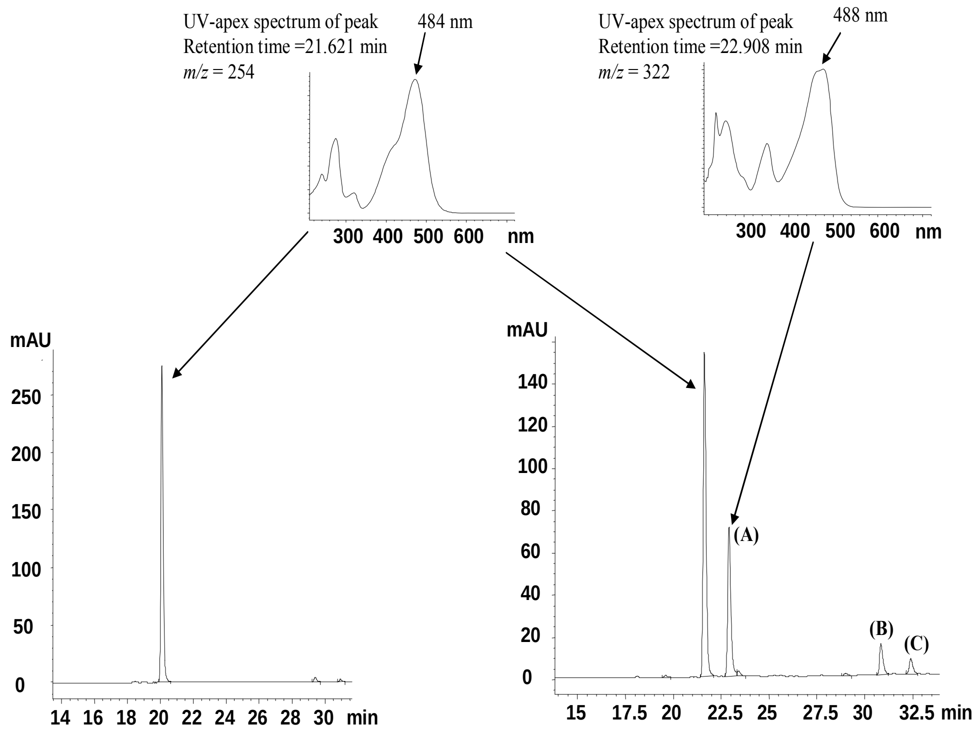
4.3. Synthesis of 3-deoxyanthocyanin-pyruvic acid adducts

4.3.1. Significance of pyruvic acid addition

The degradation of anthocyanins by food additives like SO_2 and ascorbic acid limits their use as *natural* food colorants. Oliveira and others (2006) reported that addition of pyruvic acid increased the stability of red wine anthocyanin pigments through the formation of more stable and brighter pyrano-anthocyanin compounds. Thus, the reaction of pyruvic acid and 3-deoxyanthocyanin pigments was anticipated to have similar effects.

4.3.2. HPLC-DAD/MS analysis

Synthesis of the 3-deoxypyranoanthocyanins (**Figure 11(a) – (d)**) was gradual. The yield of all the identified and characterized pyruvic acid adducts was approximately between 11 - 47% after 10 days at pH 3.2. These pigments were characterized by HPLC/DAD-MS. The progressive synthesis of these adducts over time was in agreement with Oliveira and others (2006) report on red wine pyrano-anthocyanin pigments at pH 2.0. Synthesis of 3-deoxy-pyranoanthocyanins was much greater with apigeninidin and luteolinidin than with 7-methoxyapigeninidin, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin at pH 3.2. Structural orientation of these methoxylated molecules in solution may be responsible for this phenomenon, inhibiting easy access of pyruvic acid onto the carbon **4** and **5** positions. However, pH influence on the synthesis of these pyruvic acid adducts was not investigated.



(I) Apigeninidin + pyruvate; day 0 (II) Apigeninidin-pyruvate adduct; day 10

Figure 11(a): HPLC profile (480 nm) of apigeninidin, before (I); and after reaction with pyruvic acid (A), (II), pH 3.2, after 10 days. UV-apex spectra and *m/z* shown confirmed the differences between pigments. (B) and (C) are unknown apigeninidin-pyruvic acid adducts.

During the formation of the pyrano-apigeninidin adduct, **(A)**, other pyruvic acid adducts (**(B)** and **(C)**) were also identified (**Figure 11(a)**). Whereas adducts **(A)** (λ_{max} = 488 nm; Rt = 22.908 min) and **(C)** (λ_{max} = 489 nm, Rt = 32.402 min) showed slight bathochromic shifts, adduct **(B)** showed a great hypsochromic shift (λ_{max} = 461 nm, Rt = 30.841 min), suggesting either a displacement of an –OH group on "B-ring" by pyruvic acid or a formation of an ester. Adducts **(A)**, **(B)** and **(C)** represented 27.12%, 6.16% and 3.37% of the total peak areas, respectively. However, the pigment characteristics, stability and structure of **(B)** and **(C)** were not investigated but their quantity in solution cannot be neglected and they may have a positive influence on the overall stability of the samples with pyruvic acid.

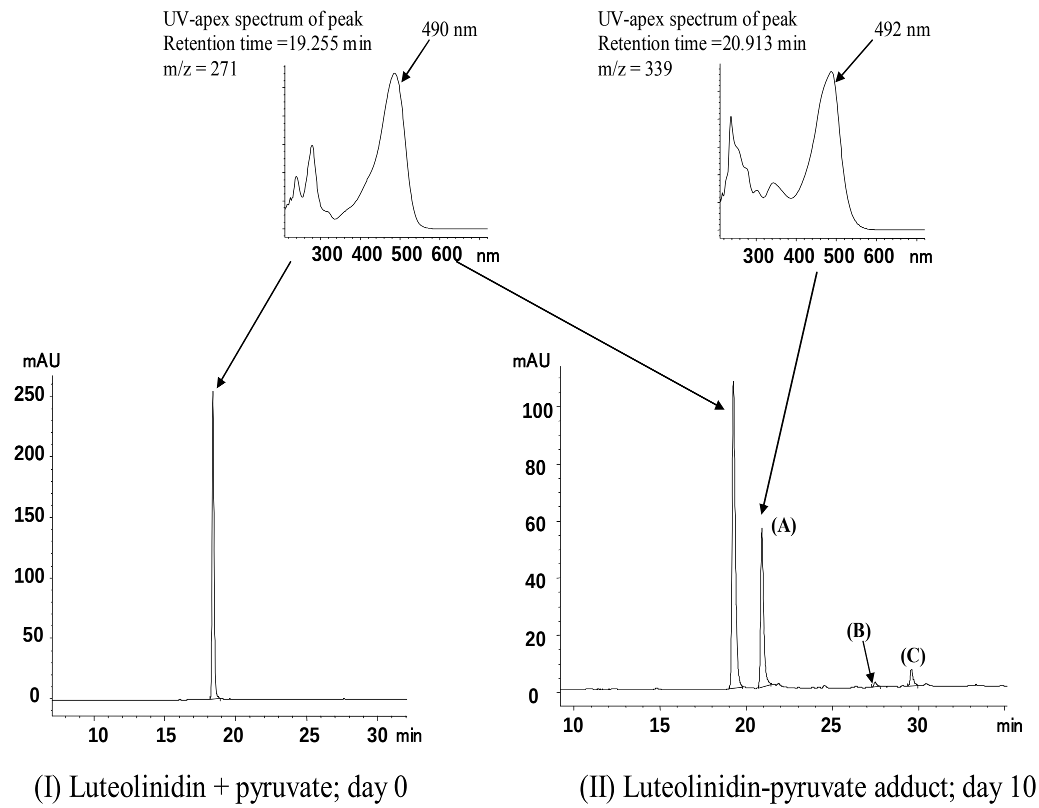
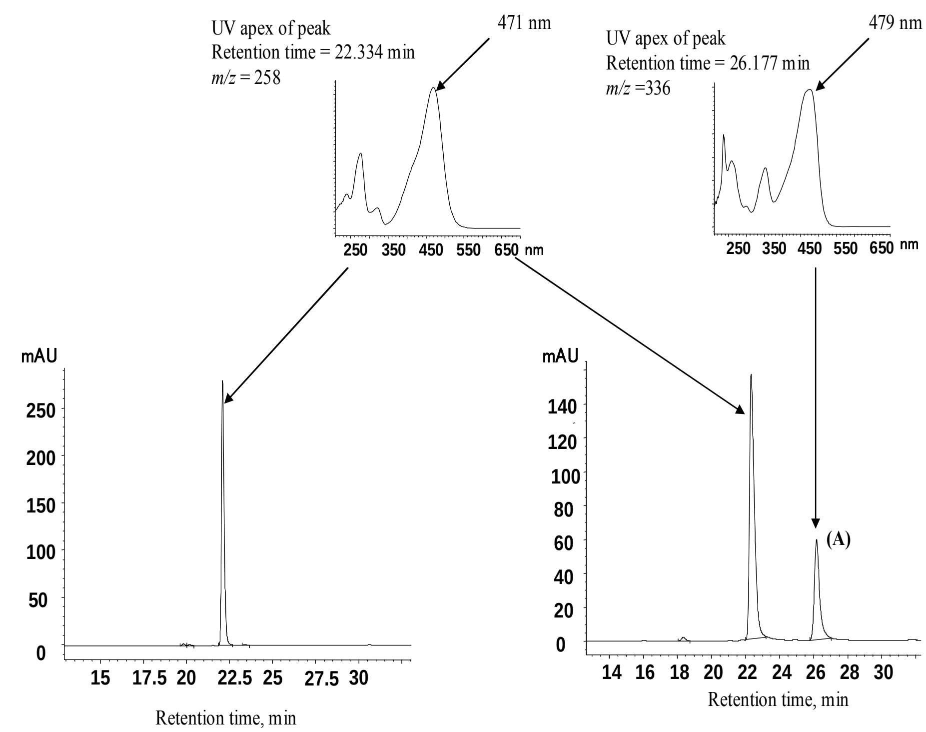


Figure 11(b): HPLC profile of luteolinidin, before (I); and after reaction with pyruvic acid (A), (II), pH 3.2, after 10 days at 480 nm. UV-apex spectra and *m/z* shown confirmed the differences between pigments. (B) and (C) are unknown luteolinidin-pyruvic acid adduct.

Similarly, pyrano-luteolinidin, **(A)**, showed a slight bathochromic shift (**Figure 11(b)**). This pyruvate adduct represented 28.31% of the total peak area. The HPLC profile indicated other synthesized luteolinidin-pyruvic acid adducts similar to the others observed for apigeninidin but at lower levels, **(B)** (λ_{max} = 478 nm, Rt = 27.463 min) and **(C)** (λ_{max} = 476 nm, Rt = 29.572 min). This hypsochromic shift indicates likely pyruvate addition on the “B-ring”, displacing an -OH group. Adducts **(B)** and **(C)** represented 0.97% and 3.19% of the total area, respectively, and should also be investigated independently for color, stability and structural characteristics.



(I) 7-methoxyapigeninidin + pyruvate; day 0 (II) 7-methoxyapigeninidin-pyruvate adduct; day 10

Figure 11(c): HPLC profile of 7-methoxyapigeninidin, before (I); and after reaction with pyruvic acid (A), (II), pH 3.2, after 10 days at 480 nm. UV-apex spectra and *m/z* shown confirmed the differences between pigments.

There was no observed peak for 5-methoxyapigeninidin-pyruvic acid adduct. This was attributed to the occupation of the carbon **5** position (by a methoxyl group) of the flavylium molecule which hindered the formation of the pyran ring. For 7-methoxyapigeninidin, *only* one 7-methoxyapigeninidin-pyruvic acid adduct, **(A),** was observed, representing 22.46% of the total area (**Figure 11(c)**). The inability of 7-methoxyapigeninidin to produce other significant adducts as observed with apigeninidin and luteolinidin could be due to structural orientation of the resultant adduct molecule in solution that may hinder addition of any other substituent groups.

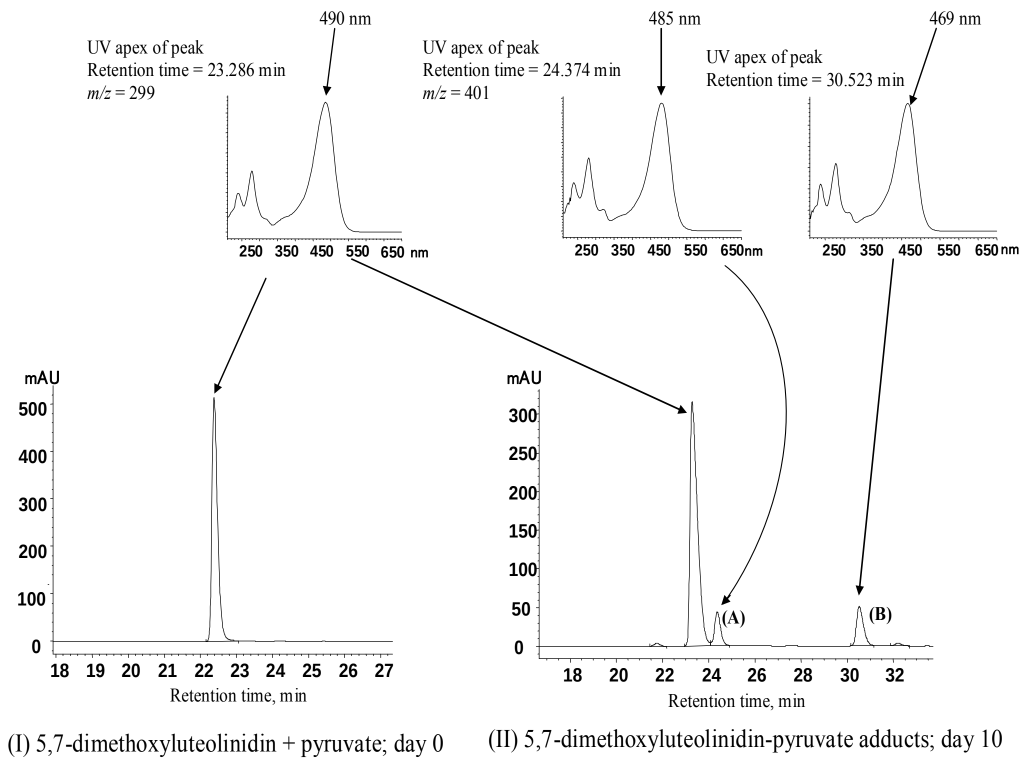


Figure 11(d): HPLC profile of 5,7-dimethoxyluteolinidin, before (I); and after reaction with pyruvic acid (A), (II), pH 3.2, after 10 days at 480 nm. UV-apex spectra and *m/z* shown confirmed the differences between pigments. (B) is an unknown 5,7-dimethoxyluteolinidin-pyruvic acid adduct.

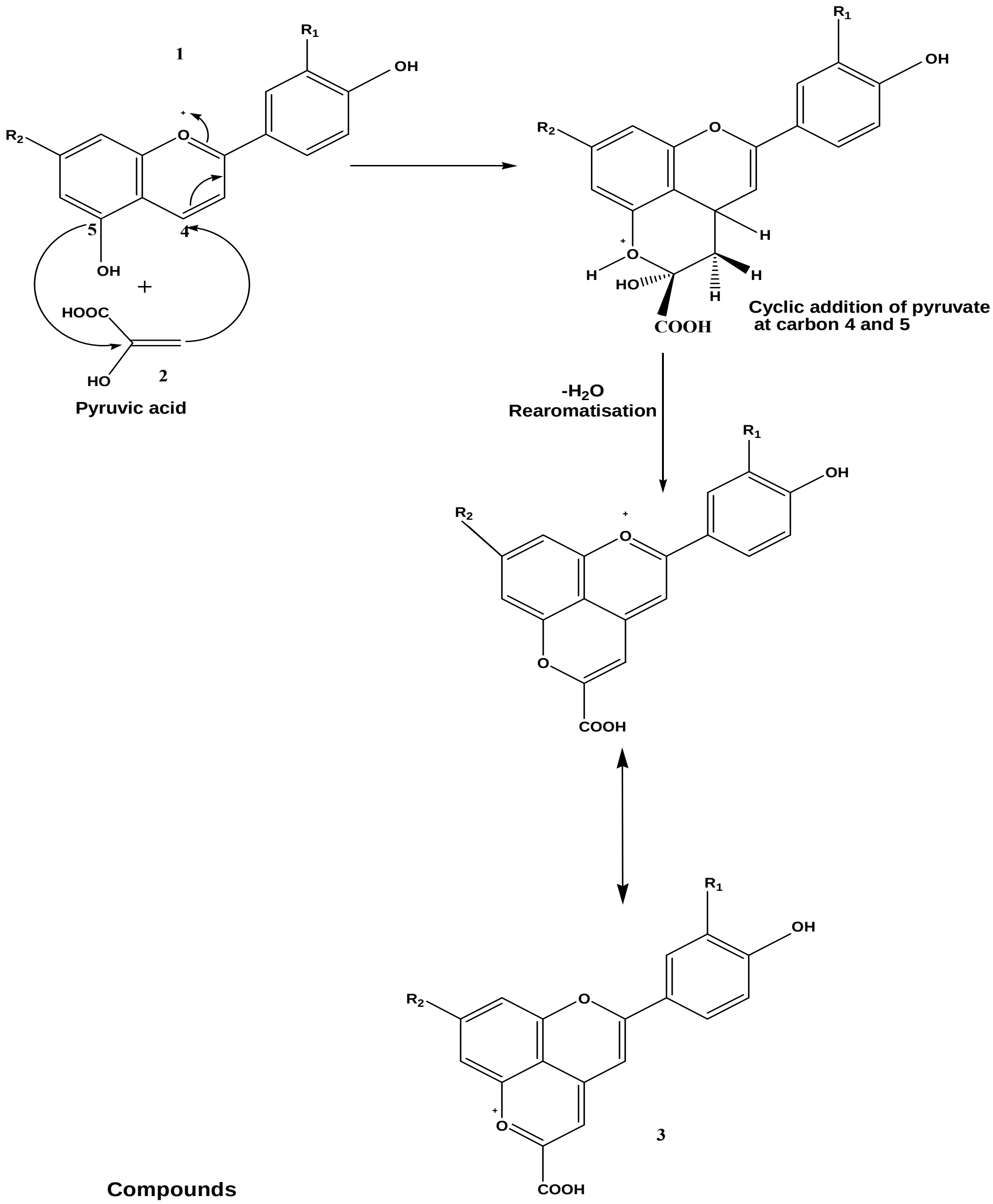
Both the dimethoxylated-pyruvic acid adducts showed hypsochromic shift (**Figure 11(d)**). The 5,7-dimethoxyluteolinidin-pyruvic acid adduct, **(A)** (λ_{max} = 485 nm; Rt = 24.374 min) and unidentified adduct, **(B)** (λ_{max} = 469 nm; Rt = 30.523 min) represented 8.38% and 11.18% of the total area, respectively.

On the other hand, 5,7-dimethoxyapigeninidin-pyruvic acid adduct (λ_{max} = 471 nm, Rt = 27.469 min; 385 *m/z*) had the least conversion rate of 4.17%, relative to its precursor pigment (λ_{max} = 479 nm; 283 *m/z*) (figure not shown).

4.3.3. Possible mechanisms of 3-deoxyanthocyanin-pyruvic acid reactions

4.3.3.1. Apigeninidin, luteolinidin and 7-methoxyapigeninidin

The higher stability observed with red wine anthocyanin-pyruvic acid adducts against sulfite degradation, is a result of cyclic addition of pyruvic acid onto the carbon **4** and **5** positions of the anthocyanins molecules. Similarly, for 3-deoxyanthocyanin pigments, this new fourth ring is formed if carbon **4** is unsubstituted and carbon **5** has an –OH group (**Figure 12**). This was verified for apigeninidin, luteolinidin and 7-methoxyapigeninidin pigments using their respective mass to charge (*m/z*) ratios that confirmed the addition of pyruvic acid and loss of two hydrogen atoms through condensation reaction. This phenomenon was expected to have the same protective role against ascorbic acid bleaching of the 3-deoxyanthocyanin pigments, as observed in red wine anthocyanin pigments by Oliveira and others (2006) against sulfite.

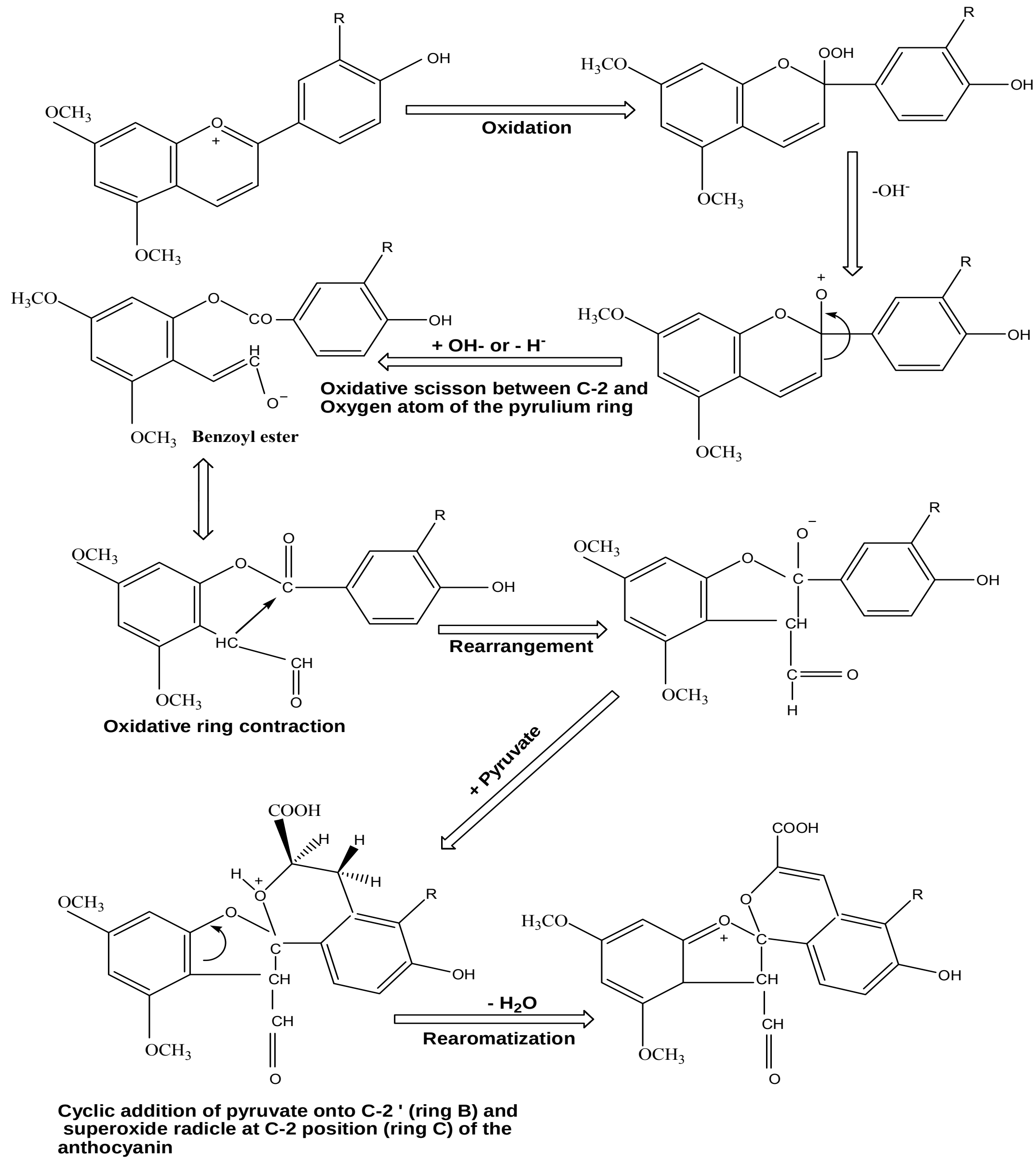


Compounds

a) Pyrano-apigeninidin: R_1 = H; R_2 = OH; 322 *m/z*
b) Pyrano-luteolinidin: R_1 = R_2 = OH; 339 *m/z*
c) 7-methoxyapigeninidin-pyruvic acid adduct: R_1 = H; R_2 = OCH_3; 336 *m/z*

Figure 12: Proposed scheme for the formation of 3-deoxyanthocyanin-pyruvic acid adducts: apigeninidin, luteolinidin or 7-methoxyapigeninidin molecule, 1; pyruvic acid, 2; and the 3-deoxyanthcyanin-pyruvic acid adducts, 3.

4.3.3.2. The dimethoxylated 3-deoxyanthocyanin pigments



Compounds:

a) 5,7-dimethoxyapigeninidin-pyruvic acid adduct; R = H; 385 *m/z*
b) 5,7-dimethoxyluteolinidin-pyruvic acid adduct; R = OH; 401 *m/z*

Figure 13: Proposed mechanism for the formation of dimethoxylated 3-deoxyanthocyanin-pyruvic acid adducts, following reaction mechanism proposed by Jurd (1964b).

The formation of 5,7-dimethoxyapigeninidin-pyruvic acid and 5,7-dimethoxyluteolinidin-pyruvic acid adducts involved oxidative scission of the flavylium molecule at carbon **2** and oxygen atom, followed by oxidative C-ring contraction and cyclic addition of pyruvic acid at carbon **2'** and the superoxide radical at carbon **2** positions (**Figure 13**). This unique phenomenon was exclusive for dimethoxylated forms, and is thought to be responsible for the hypsochromic shift observed compared to their parent anthocyanin precursors.

4.3.4. Effect of pyruvic acid on stability of 3-deoxyanthocyanin pigments

The addition of pyruvic acid generally had a hyperchromic effect on the color intensities of both sorghum and red cabbage pigments at pH 2.0, 3.0 and 5.0 (**Table 4**). At pH 5.0, red cabbage pigment expressed a higher increase in absorbance at day 21 than sorghum pigment extract, whereas sorghum recorded a higher absorbance increment than red cabbage at pH 2.0; but they were both stable at pH 3.2 after 21 days.

Also, in comparison to **Table 1**, pyruvic acid improved the color intensities of the 3-deoxyanthocyanin standards across different pH levels, except for 5,7-dimethoxyapigeninidin. Among the standards, the non- and the monomethoxylated analogs showed increase in absorbance at pH 5.0 after 21 days (**Table 4**). The increase was significantly higher than that observed in the absence of pyruvate (**Table 1**) and may be a result of structural transformations during the formation of the pyran ring at carbon **4** and **5**, resulting in the observed hyperchromic effect. In general, pyruvate improved overall stability of all pigments tested across different pH levels (**Tables 1 and 4**).

Sample With Pyruvic Acid/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	1.15 ± 0.12	1.19 ± 0.02	1.00 ± 0.03	1.04 ± 0.14	0.11
Red cabbage	1.06 ± 0.05	*	1.00 ± 0.02	1.28 ± 0.01	0.08
Grape blue powder	*	*	1.00 ± 0.03	*	0.04
Apigeninidin	1.04 ± 0.07	0.63 ± 0.01	1.00 ± 0.03	2.09 ± 0.18	0.25
Luteolinidin	1.05 ± 0.11	0.77 ± 0.01	1.00 ± 0.01	1.59 ± 0.03	0.30
5-methoxyapigeninidin	1.02 ± 0.02	0.88 ± 0.01	1.00 ± 0.08	1.41 ± 0.05	0.37
7-methoxyapigeninidin	*	0.69 ± 0.01	1.00 ± 0.07	*	0.16
5,7-dimethoxyapigeninidin	1.18 ± 0.15	0.72 ± 0.01	1.00 ± 0.02	0.68 ± 0.01	0.78
5,7-dimethoxyluteolinidin	0.95 ± 0.06	0.76 ± 0.01	1.00 ± 0.01	0.88 ± 0.01	0.87

Table 4: Absorbance at respective λ_{max} (nm) for samples incubated with pyruvic acid after 21 days at 27°C, at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at α = 0.05.

4.3.5. Effect of SO_2 on samples with pyruvic acid

At pH 2.0, SO_2 caused significant increase in absorbance with all the control samples, possibly due to co-pigmentation (**Figure 14(a); Table 2**).

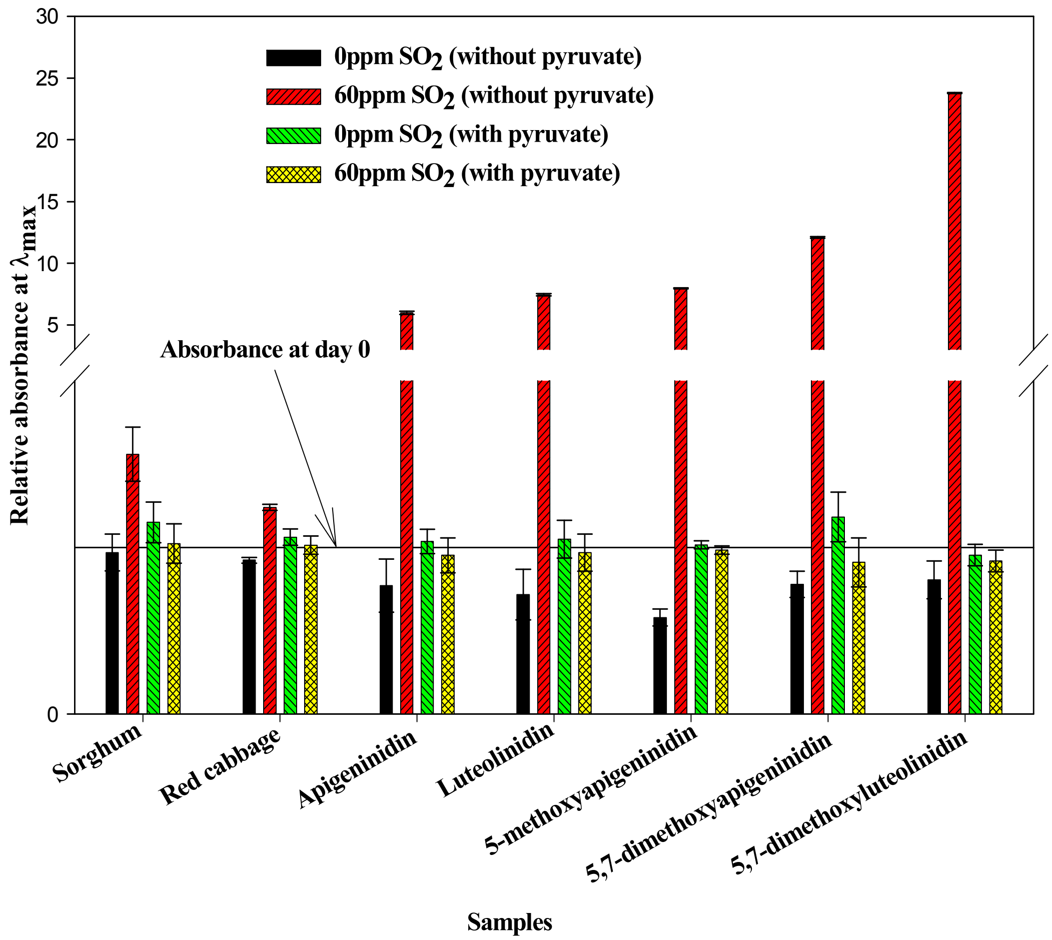


Figure 14(a): Effect of 60 ppm SO_2 and pyruvic acid on stability of sorghum, red cabbage and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 2.0. Absorbance readings normalized to 1.00 relative to day 0. Error bars represent standard deviations.

However, the presence of pyruvic acid seemed to prevent this co-pigmentation phenomenon, possibly through the addition of a pyran ring at carbons **4** and **5** positions. Between the natural colorants, the co-pigmentation effect was higher in sorghum than in red cabbage pigment. On the other hand, addition of sulfite to sorghum and red cabbage samples with pyruvic acid caused no significant bleaching.

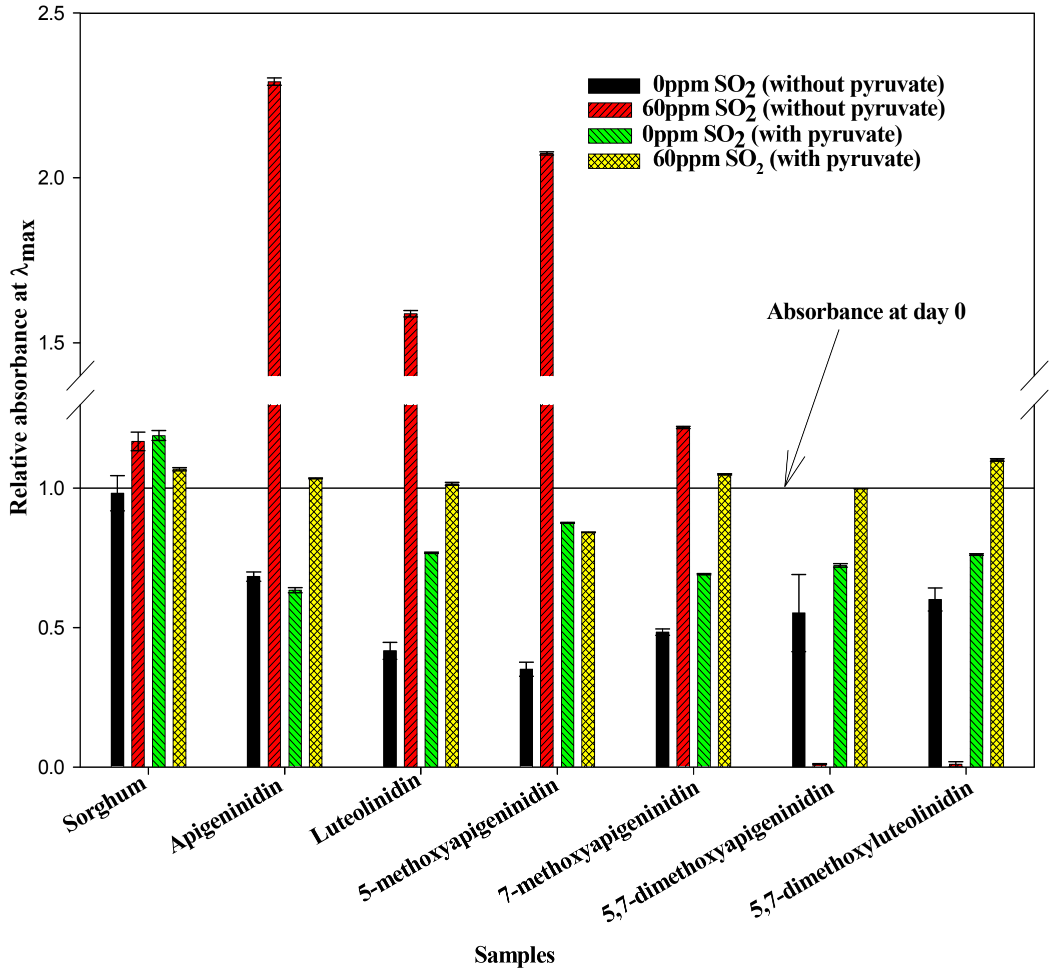


Figure 14(b): Effect of 60 ppm SO_2 and pyruvic acid on stability of sorghum and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 3.0. Absorbance readings normalized to 1.00 relative to day 0. Error bars represent standard deviations.

The increased color intensity of these 3-deoxyanthocyanin pigments at pH 2.0 in the presence of SO_2 is an important feature, especially in their putative application in coloring low acid foods like sodas. Thus, at day 21 and at pH 2.0, SO_2 seemed to behave as a co-pigment in the absence of pyruvic acid.

At pH 3.0, pyruvic acid caused a reduction in pigment intensity in crude sorghum extract in the presence of SO_2, indicating pyruvate may not improve stability of sorghum extract (**Figure 14(b); Tables 4 and 5**). Among 3-deoxyanthocyanin standards, samples with pyruvic acid were generally more stable in the presence of SO_2 at pH 3.0. The most exceptional protection of 3-deoxyanthocyanins against SO_2 bleaching at this pH was observed for dimethoxylated analogs, which were completely bleached in the presence of SO_2 without pyruvate, but retained 100% of their color in the presence of pyruvate.

At pH 3.2, sulfite caused greater increase in color intensity in sorghum pigment in the absence of pyruvic acid than in red cabbage and grape blue powder pigments (**Figure 14 (c); Tables 2 and 4**). Addition of pyruvic acid also caused increase in color intensity of sorghum and red cabbage pigments but not grape blue powder. In general, pyruvic acid addition was detrimental to color intensity of the natural extracts in the presence of SO_2 at this pH. Among the 3-deoxyanthocyanin standards, pyruvic acid significantly improved stability against SO_2 bleaching, except for 5,7-dimethoxyapigeninidin. For example, SO_2 completely bleached luteolinidin and its dimethoxylated form in the absence of pyruvic acid; but showed remarkable stability in the presence of pyruvic acid. However, pyruvic acid apparently did not protect 5,7-dimethoxyapigeninidin pigment from SO_2 degradation after 21 days at pH 3.2.

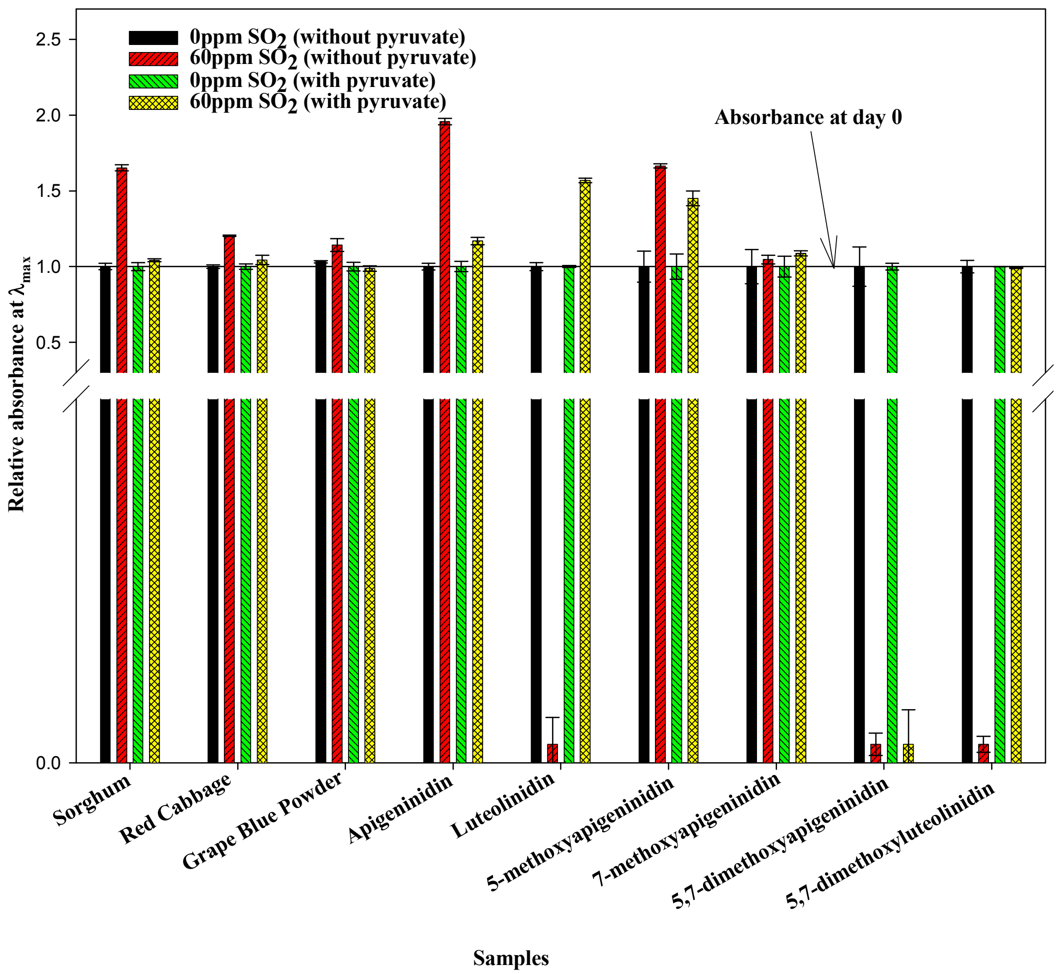


Figure 14(c): Effect of 60 ppm SO_2 and pyruvic acid on stability of sorghum, red cabbage, grape blue powder and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 3.2. Absorbance readings normalized to 1.00 relative to day 0. Error bars represent standard deviations.

At pH 5.0, there was no significant difference in the stability of sorghum and red cabbage pigments against 60 ppm SO_2, both in the presence and the absence of pyruvic acid (**Figure 14 (d); Tables 1 and 4).** Among the 3-deoxyanthocyanin standards, apigeninidin showed the highest increase in color intensity in the presence of pyruvic acid alone but was completely bleached in the presence of pyruvate and sulfite.

A similar pattern was observed for 5-methoxyapigeninidin pigment, suggesting that at pH 5.0, SO_2 bleaches these 3-deoxyanthocyanin pigments only when pyruvate is present. This could be that SO_2 is mutually detrimental to apigeninidin and 5-methoxyapigeninidin pigments in the presence of pyruvic acid by destroying the co-pigmentation effect via nucleophilic attack that also destroys the pigments. Only the dimethoxylated analogs were protected against SO_2 bleaching by pyruvic acid.

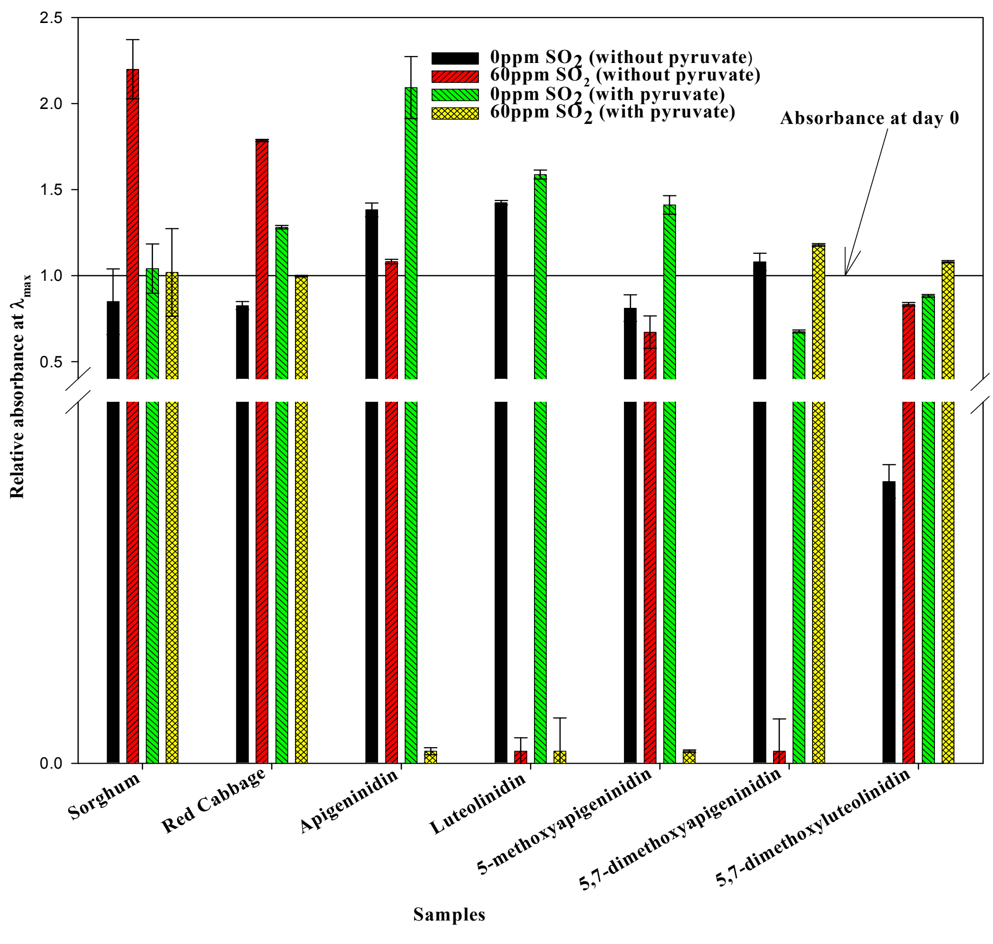


Figure 14(d): Effect of 60 ppm SO_2 and pyruvic acid on stability of sorghum, red cabbage and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 5.0. Absorbance readings normalized to 1.00 relative to day 0. Error bars represent standard deviations.

In summary, SO_2 led to increase in color intensity of all pigments at pH 2.0. A probable cause for this phenomenon may have been co-pigmentation. The dimethoxylated forms recorded the highest increase in pigment intensity effect (**Figure 14(a); Table 2**).

Samples With Pyruvic Acid/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	1.02 ± 0.12	1.07 ± 0.01	1.04 ± 0.01	1.02 ± 0.25	0.16
Red cabbage	1.01 ± 0.05	*	1.04 ± 0.03	1.00 ± 0.01	0.11
Grape blue powder	*	*	0.99 ± 0.02	*	0.06
Apigeninidin	0.95 ± 0.07	1.04 ± 0.01	1.17 ± 0.02	S^b	0.35
Luteolinidin	0.97 ± 0.11	1.02 ± 0.01	1.57 ± 0.02	S^b	0.42
5-methoxyapigeninidin	0.98 ± 0.03	0.84 ± 0.01	1.45 ± 0.05	S^b	0.53
7-methoxyapigeninidin	*	1.05 ± 0.01	1.09 ± 0.02	*	0.22
5,7-dimethoxyapigeninidin	0.91 ± 0.15	1.00 ± 0.01	S^b	1.18 ± 0.01	1.11
5,7-dimethoxyluteolinidin	0.92 ± 0.07	1.10 ± 0.01	0.99 ± 0.01	1.08 ± 0.01	1.22

Table 5: Relative absorbance at respective λ_{max} (nm) reported as percent color retention for samples incubated with 60 ppm SO_2 and pyruvic acid after 21 days at 27°C, at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested and S^b means samples reported as totally bleached ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at α = 0.05.

Addition of pyruvic acid apparently inhibited this co-pigmentation at this pH (**Figure 14(a)**). Also, addition of pyruvate both in the presence and the absence of SO_2 at pH 3.0 reduced the pigment intensity of sorghum extract pigment, suggesting pyruvate may not be effective in stabilizing crude sorghum extract pigments at this pH. But 3-deoxyanthocyanin standards with pyruvic acid recorded a general stability against SO_2 at this pH (**Figure 14(b); Table 5**). At pH 3.2, SO_2 and pyruvic acid independently caused increased color intensity to both crude sorghum and red cabbage pigments. However, in the presence of SO_2 at this pH, pyruvic acid was damaging to pigment intensity for these natural pigments (**Figure 14(c)**). At pH 5.0, pyruvic acid showed protection against SO_2 on only 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin pigments (**Figure 14(d); Table 5**).

4.3.6. Effect of ascorbic acid on samples with pyruvic acid

At pH 2.0, crude sorghum pigment stability against 500 ppm ascorbic acid bleaching in the absence of pyruvic acid (68.5% color retention) was higher than that of red cabbage pigment (15.1% color retention) (**Figure 15(a); Table 3**). Addition of pyruvic acid improved the color stability of both sorghum and red cabbage pigments to ascorbic acid degradation (71.5% color retention for sorghum and 28.3% color retention for red cabbage) (**Table 6**). However, these natural pigments showed higher resistance to ascorbate color degradation in the absence of pyruvic acid than the 3-deoxyanthocyanin standards. Pyruvic acid significantly improved the stability of 3-deoxyanthocyanin standards to ascorbic acid bleaching except for luteolinidin (α = 0.05); though the overall stability was still poor (10.6% - 40.2% color retention) (**Figure 15(a); Table 6**).

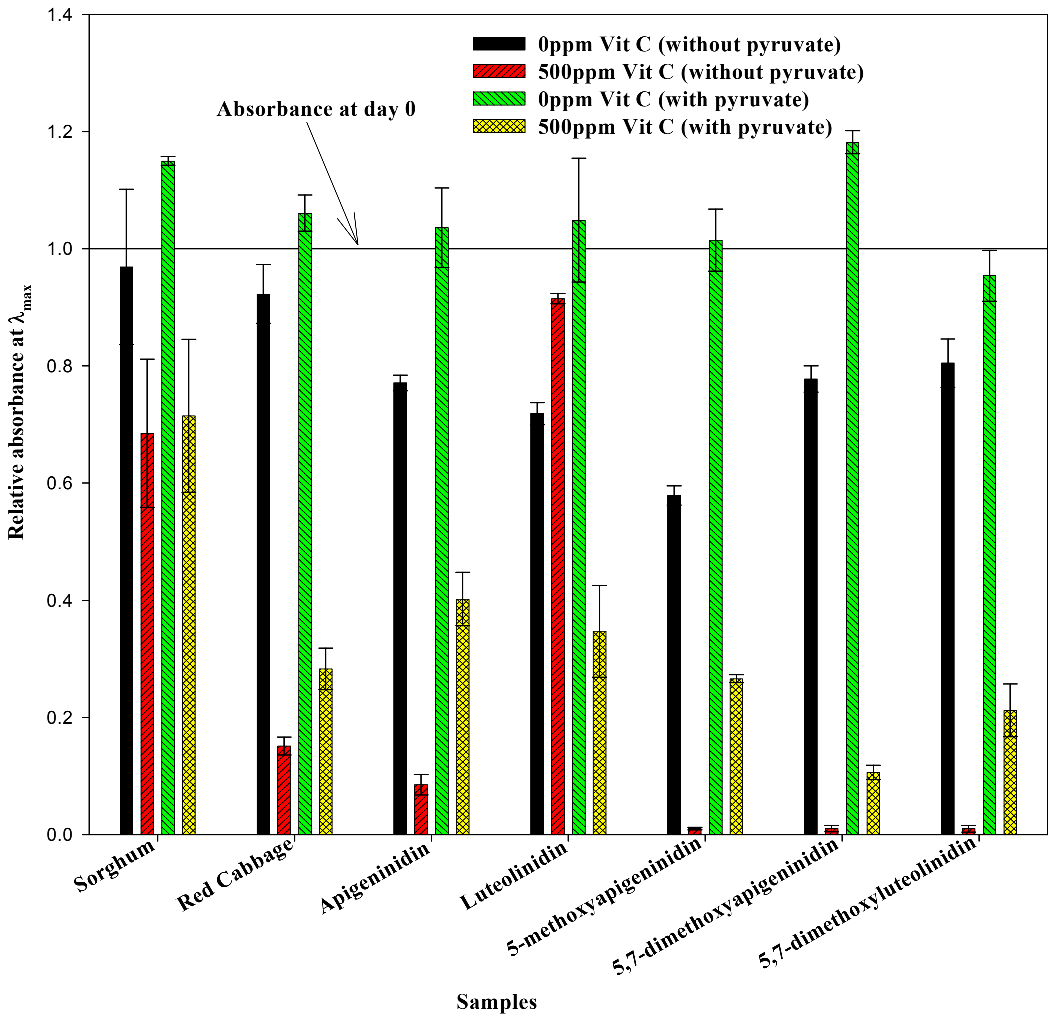


Figure 15(a): Effect of 500 ppm ascorbic acid and pyruvic acid on sorghum, red cabbage and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 2.0. Absorbance readings were normalized to 1.00 relative to day 0. Error bars represent standard deviations.

At pH 3.0, crude sorghum pigments showed significant color stability in the presence of ascorbic acid, without pyruvic acid addition (**Figure 15(b)**). Pyruvic acid apparently did not protect it from 500 ppm ascorbic acid degradation, but it still retained 91.1% of its color (**Figure 15(b); Table 6**). Among 3-deoxyanthocyanin standards, pyruvic acid significantly protected apigeninidin, 7-methoxyapigeninidin and

5,7-dimethoxyluteolinidin from bleaching by ascorbic acid after 21 days (**Figure 15(b); Table 4**).

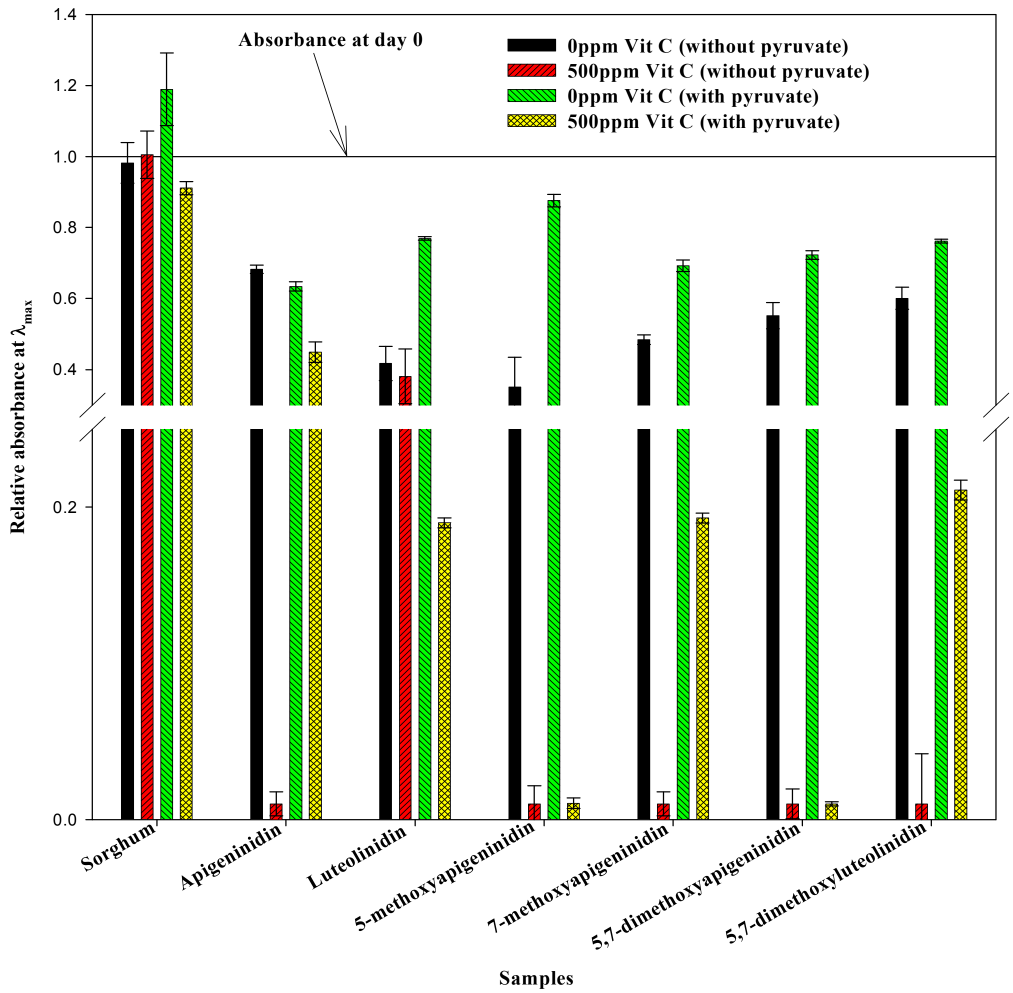


Figure 15(b): Effect of 500 ppm ascorbic acid and pyruvic acid on sorghum and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 3.0. Absorbance readings were normalized to 1.00 relative to day 0. Error bars represent standard deviations.

At pH 3.2, crude sorghum extract showed increase in color intensity in the presence of 500 ppm ascorbic acid, both with and without pyruvic acid addition (**Figure 15(c); Tables 3 and 6**). This indicates possible co-pigmentation reaction at this pH. Addition of pyruvic acid was somehow detrimental to the copigmentation effect (**Figure 15(c)**).

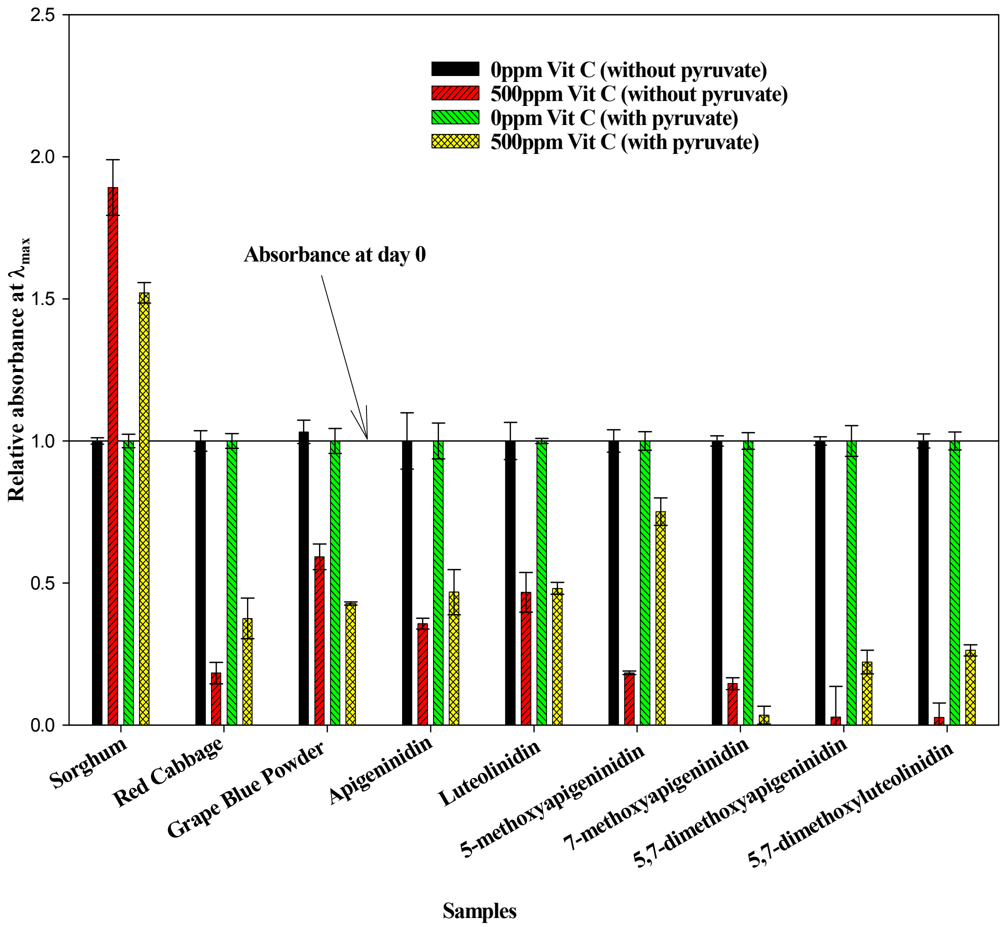


Figure 15(c): Effect of 500 ppm ascorbic acid and pyruvic acid on sorghum, red cabbage, grape blue powder and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 3.2. Absorbance readings were normalized to 1.00 relative to day 0. Error bars represent standard deviations.

The stability of the other natural colorants and 3-deoxyanthocyanin standards was generally very poor in the presence of ascorbic acid at this pH. Pyruvic acid addition marginally improved the stability of red cabbage extract and 3-deoxyanthocyanin standards against ascorbic acid bleaching at pH 3.2; however, pyruvic acid was detrimental to stability of grape blue powder and 7-

methoxyapigeninidin in the presence of ascorbic acid (**Figure 15(c)); Tables 3 and 6**). Thus, crude sorghum pigment was most suitable as natural colorant at this pH level in the presence of ascorbic acid.

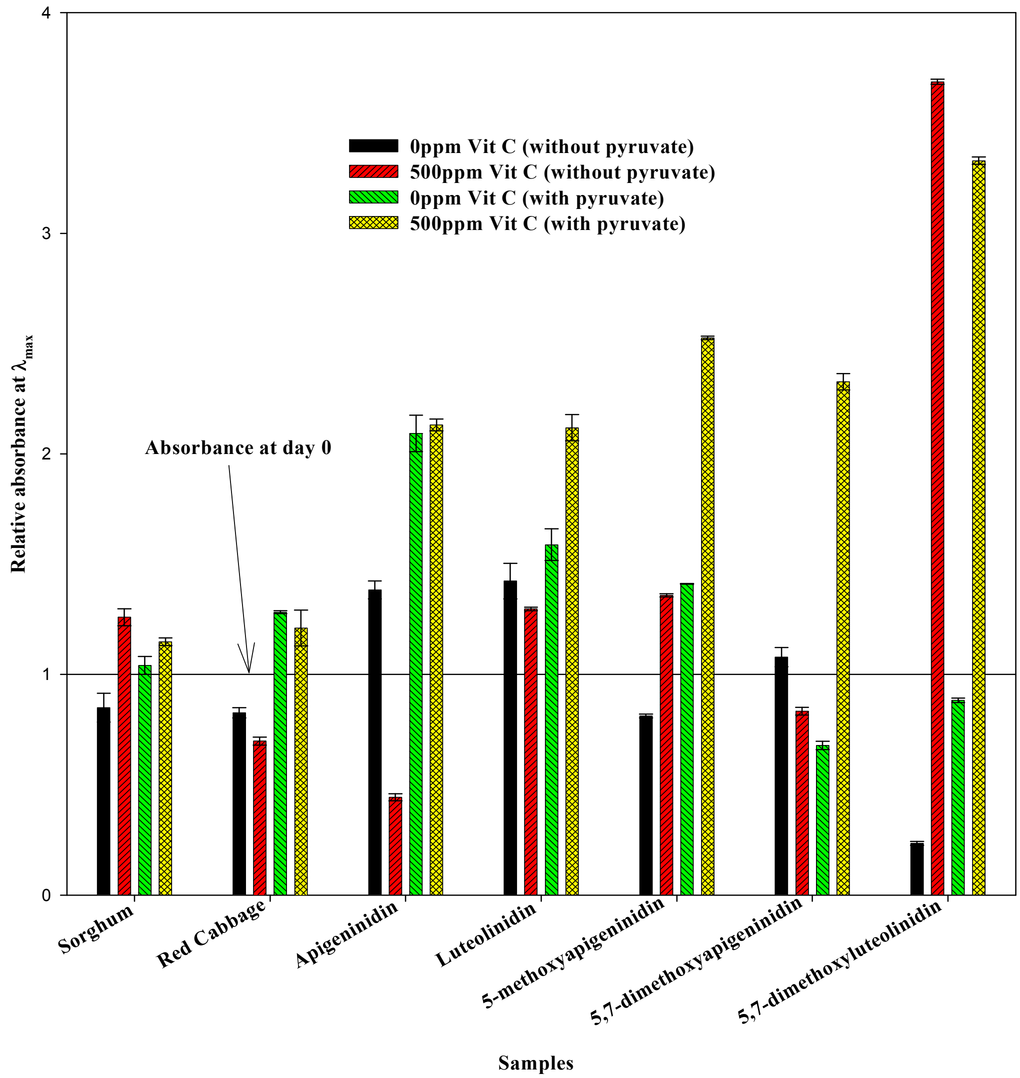


Figure 15(d): Effect of 500 ppm ascorbic acid and pyruvic acid on sorghum, red cabbage and 3-deoxyanthocyanin standards after 21 days (27°C) at pH 5.0. Absorbance readings were normalized to 1.00 relative to day 0. Error bars represent standard deviations.

At pH 5.0, pyruvic acid significantly protected sorghum extract and red cabbage pigments against 500 ppm ascorbic acid degradation (**Figure 15(d)**). However, it produced more dramatic increase in color intensity of red cabbage extract than sorghum pigment extract, both in the presence and the absence of ascorbic acid.

Among the 3-deoxyanthocyanin standards, ascorbic acid addition in the presence of pyruvic acid produced a dramatic increase in absorbance after 21 days at pH 5.0, relative to day 0 (**Figure 15(d); Tables 3 and 6**). This suggests intermolecular co-pigmentation interactions among the 3-deoxyanthocyanin-pyruvic acid compounds with ascorbic acid.

Samples With Pyruvic Acid/pH	2.0	3.0	3.2	5.0	Std Error of Means
Sorghum	0.72 ± 0.31	0.91 ± 0.02	1.52 ± 0.04	1.15 ± 0.02	0.20
Red cabbage	0.28 ± 0.04	*	0.38 ± 0.07	1.21 ± 0.08	0.16
Grape blue powder	*	*	0.43 ± 0.01	*	0.08
Apigeninidin	0.40 ± 0.05	0.45 ± 0.03	0.47 ± 0.08	2.13 ± 0.03	0.34
Luteolinidin	0.35 ± 0.08	0.19 ± 0.01	0.48 ± 0.02	2.12 ± 0.06	0.33
5-methoxyapigeninidin	0.27 ± 0.01	S^b	0.75 ± 0.05	2.53 ± 0.01	0.30
7-methoxyapigeninidin	*	0.19 ± 0.01	0.04 ± 0.03	*	0.22
5,7-dimethoxyapigeninidin	0.11 ± 0.12	S^b	0.22 ±0.04	2.33 ± 0.04	0.44
5,7-dimethoxyluteolinidin	0.21 ± 0.05	0.21 ± 0.01	0.26 ± 0.02	3.33 ± 0.02	0.42

Table 6: Absorbance at respective λ_{max} (nm) for samples incubated with 500 ppm ascorbic acid and pyruvic acid after 21 days at 27°C, at different pH levels. Absorbance values were normalized to 1.00, with respect to day 0. Missing values marked with (*) indicate samples not tested and S^b means samples reported as totally bleached ($\bar{x}$ ± SD, n = 3). Mean differences within a row that are ≥ Standard Error of Means are significantly different at α = 0.05.

In summary, at pH 2.0 the natural pigments had higher resistance to ascorbic acid bleaching than the 3-deoxyanthocyanin standards in the absence of pyruvic acid (**Figure 15(a)**). At pH 3.0, pyruvic acid did not protect sorghum pigments from ascorbic acid degradation. However, sorghum showed significant color stability at 500 ppm ascorbate and in the absence of pyruvic acid (**Figure 15(b); Table 6**). Also, pyruvate generally protected the 3-deoxyanthocyanin standards from ascorbic acid bleaching at this pH.

At pH 3.2, the presence and the absence of pyruvic acid both improved the color intensity of crude sorghum pigment extract with 500 ppm ascorbic acid (**Figure 15(c); Tables 3 and 6**), possibly through co-pigmentation reactions, but pyruvic acid addition was detrimental to the observed co-pigmentation effect. All samples had general poor stability in the presence of ascorbic acid at pH 3.2. Finally, at pH 5.0 ascorbic acid with pyruvic acid produced dramatic increase in color absorbance for all the 3-deoxyanthocyanin standards after 21 days (**Tables 3 and 6**). This may be attributable to copigmentation interactions among 3-deoxyanthocyanin-pyruvic-ascorbic acid molecules.

4.4. Effect of high temperature processing

4.4.1. Spectroscopic analysis of heat treated samples

Samples at pH 3.2 were autoclaved for 15 minutes at 121.1°C. The non- and monomethoxylated samples showed hyperchromic shift after thermal treatment in the absence of pyruvic acid (**Table 7**), suggesting occurrence of browning phenomenon at high temperatures. These browning reactions are undesirable in foods, especially in anthocyanin containing juices exposed to high sterilization temperatures reported by Maccarone and others (1985) and Fiore and others (2005). Palamidis and Markakis (1975) and Spayd and others (2002) suggested that this effect resulted from accelerated anthocyanin degradation which produced the chalcone responsible for browning observed in anthocyanin-containing soft drinks.

Samples	**Before heating**		**After heating**	
	λ_{max}	Abs	λ_{max}	Abs
Apigeninidin control	471.50	0.5330	472.50	0.5361
Apigeninidin + pyruvic acid	468.50	1.0216	463.00	0.5067
Luteolinidin control	482.00	0.5763	482.50	0.8800
Luteolinidin + pyruvic acid	482.00	0.9521	471.00	0.4215
7-methoxy-apigeninidin control	466.50	1.0774	467.50	1.0962
7-methoxy-apigeninidin + pyruvic acid	468.00	0.8118	463.50	0.4221
5,7-dimethoxy-luteolinidin control	481.50	2.3284	482.50	1.4025
5,7-dimethoxyluteolinidin + pyruvic acid	477.00	1.4226	465.00	0.5232
Sorghum control	478.00	1.0080	478.00	0.4934
Sorghum + pyruvic acid	477.50	0.8339	474.50	0.2176

Table 7: Sample absorbance values at λ_{max} (nm) before and after thermal treatment at pH 3.2 after 21 days.

Crude sorghum pigment and dimethoxylated 3-deoxyanthocyanin standards showed reduced color intensity after heating, both in the presence and the absence of pyruvic acid. Thus, pyruvic acid did not protect the 3-deoxyanthocyanin pigments against heat degradation.

4.4.2. HPLC analysis of heat treated samples

4.4.2.1. Crude sorghum pigment

The sorghum sample was the most stable, both in the absence and the presence of pyruvic acid, and showed no significant change in profile (**i**) through (**vii**) during thermal treatment (**Figure 16**). It was impossible to prove formation of any new pyruvic acid-adducts using the retention times and peak absorption wavelengths. HPLC-DAD analysis showed that pigments in (**2**), (**3**) and (**4**) were similar in both their retention times and λ_{max} (nm) values to pigments in **(1)**. During the incubation with pyruvic acid for 21 days, crude sorghum extract pigment reduced by approximately 15 - 30% of their original peak areas (**Figure 16(1) and (3)**).

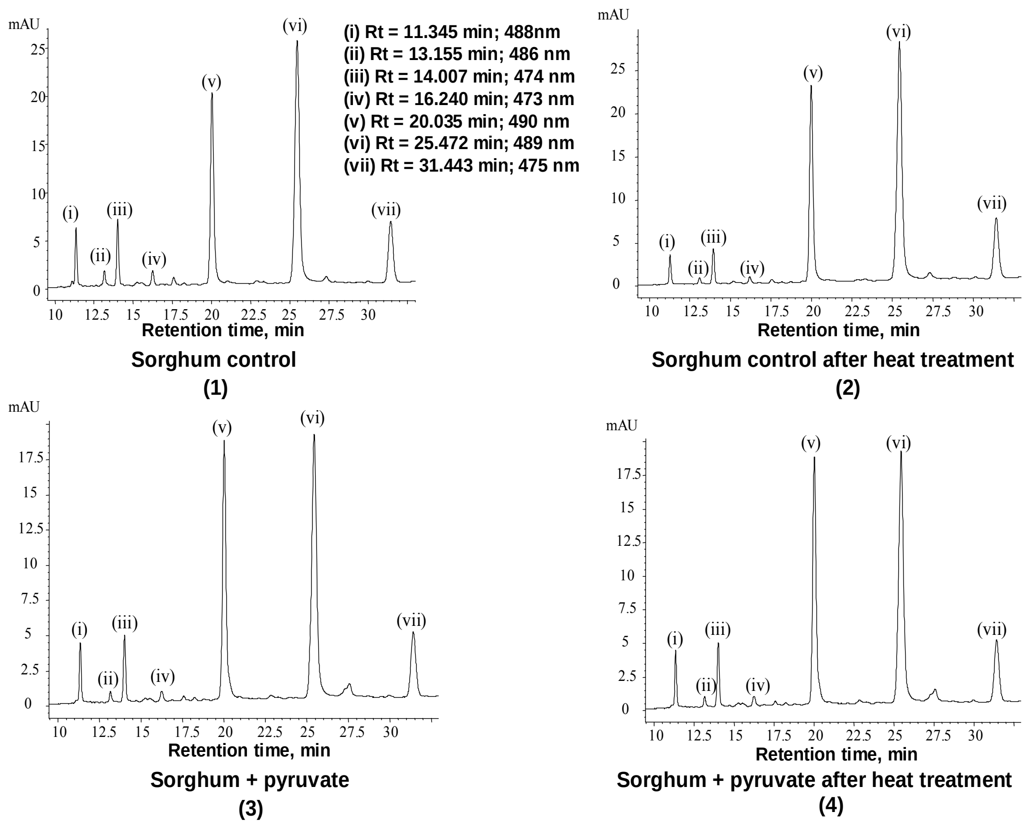


Figure 16: HPLC chromatograms of sorghum, (1); sorghum + pyruvic acid, (3) (before heating), and after heating, (2) and (4), at pH 3.2.

This result proved that for crude sorghum pigments: **(a)** there was no thermal influence on formation of any new adducts, both with and without pyruvic acid, and **(b)** pyruvic acid did not protect sorghum pigments against color degradation by heat.

4.4.2.2. Non- and monomethoxylated 3-deoxyanthocyanin standards

In the absence of pyruvic acid, HPLC-DAD analysis showed that these pigments lost 2.80 – 14.04% of their original peak areas after thermal processing (**Figure 17; (1) and (2)**). However, the increase in color absorbance reported in **Table**

7 may be due to browning of these pigments at high temperatures. These colored brown forms are complexes that may be very non-polar, hence were probably retained on the HPLC column, leading to the fewer pigment peaks and lower peak areas observed. Thus, their transformation into more colored brown forms caused the high hyperchromic shift reported in **Table 7**.

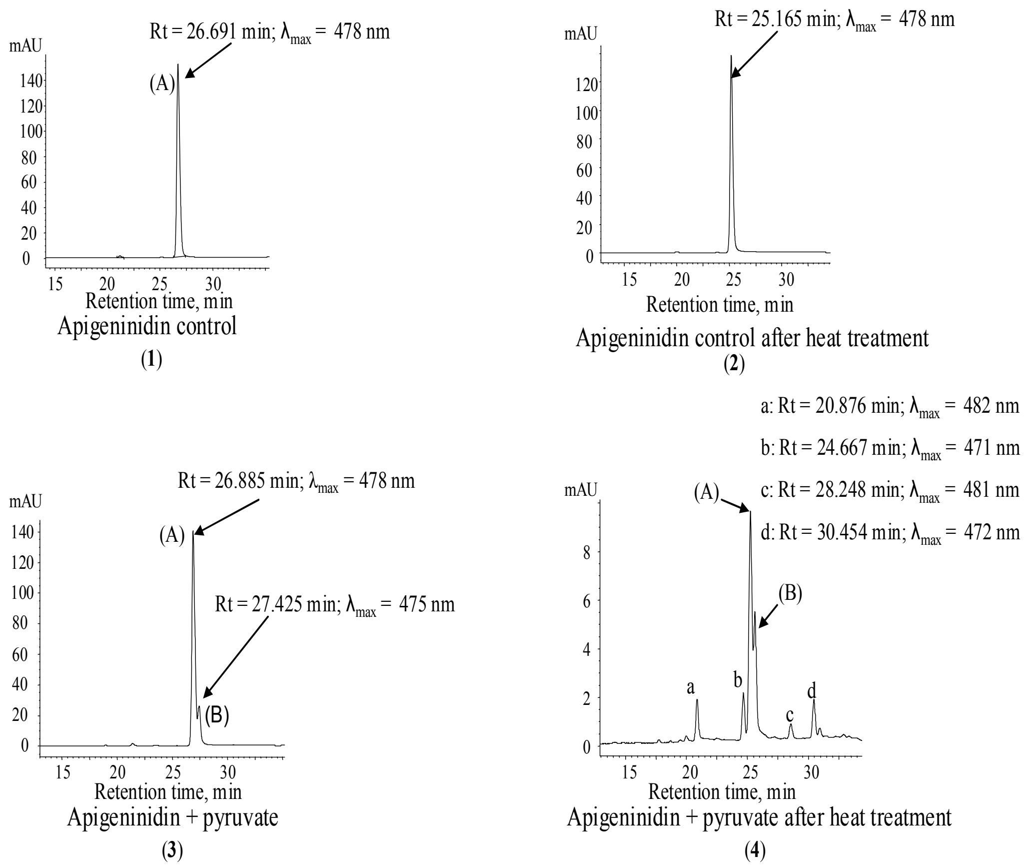


Figure 17: HPLC chromatograms of apigeninidin, A, (1); apigeninidin + pyruvic acid, (3) (before heating), and after heating, (2) and (4), at pH 3.2. Adduct (B) is pyrano-apigeninidin and 'a', 'b', 'c' and 'd' are unknown.

In the presence of pyruvic acid, formation of many different unique adducts (pigments **'a'** through **'d'**) were observed during thermal treatment of samples with UV absorption spectra between 470 – 485 nm (**Figure 17; (3) and (4)**).

Monomethoxylated analogs had fewer of these adducts than apigeninidin and luteolinidin (figures not shown). This phenomenon, which may be due to the effect of pyruvic acid on 3-deoxyanthocyanin molecules at higher temperatures, should be further investigated to elucidate the structural influence and stability of these adducts. The overall loss in initial pigment intensities (**Table 7**) may be due to the synthesis of these other adducts (that may be absorbing at lower wavelengths) from thermal degradation of pyruvic acid. However, the complete lack of secondary peaks in the absence of pyruvic acid (**Figure 17(2)**), and the presence of several other adducts in the presence of pyruvic acid, (**Figure 17(4)**), after heating, proved the significance of pyruvic acid in the synthesis of the secondary peaks observed in (**4**).

4.4.2.3. The dimethoxylated 3-deoxyanthocyanin pigments

In the absence of pyruvate, 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin showed 39.08% and 43.75% reduction in initial pigment peak areas, respectively (**Figure 18; (1) and (2)**). However, the seemingly high absorbance reported in **Table 7** may be due to transformation of these molecules towards the intensely colored brown forms through the effect of high temperature. The near spectral resemblance of the peak apex spectra (figure not shown) and the closeness of the λ_{max}

(nm) of these dimethoxy-adducts (**Figure 18**), suggested that there occurred thermal transformation on the molecular orientation of the parent 3-deoxyanthocyanin structure.

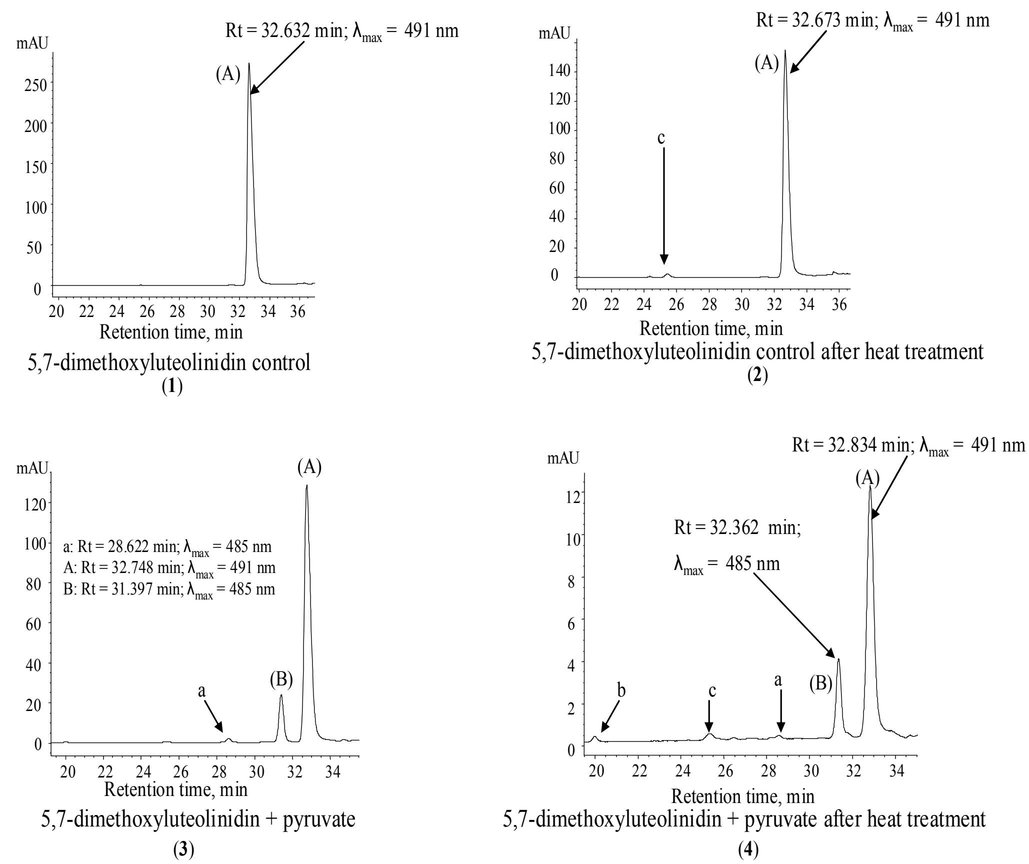


Figure 18: HPLC chromatograms of 5,7-dimethoxyluteolinidin (A), (1); 5,7-dimethoxyluteolinidin-pyruvic acid adduct (B), (3) (before heating), and after heating, (2) and (4), at pH 3.2. Adducts 'a', 'b' and 'c' are unknown.

Also, addition of pyruvic acid to these dimethoxylated analogs apparently offered no protection against heat. Approximately 91.3% of initial pigment peak area reduction occurred in the presence of pyruvic acid compared to 44% of initial pigment area reduction observed in the absence of pyruvate after heating (**Figure 18(2) and (4)**). **'a'** and **(B)** are pyruvic acid adducts since they are absent in **(1)** and **(2)** and only present in **(3)** and **(4)** (**Figure 18**)**.** However, adduct **'b'** is a heat induced compound (**Figure 18(2) and (4)**), since it was formed even in the absence of pyruvic acid only after heating. The dimethoxylated samples also showed secondary synthesis of adduct **'b'** (**Figure 18(4)**) during similar heat treatment in the presence of pyruvic acid. This adduct was absent when the samples were heated without pyruvic acid (**Figure 18; (2)**) suggesting pyruvate and heat were major factors in its synthesis. This unpredictable phenomenon may affect incorporation of 3-deoxyanthocyanin pigments in foods that undergo sterilization processing because the final color may be unknown, and should be further investigated.

CHAPTER 5

CONCLUSIONS, RECOMMENDATIONS AND SUMMARY

5.1. Conclusions

Crude sorghum pigment showed higher stability to sulfite and ascorbic acid bleaching at pH 2.0 and 3.0 than red cabbage pigment, both in the presence and the absence of pyruvic acid. However, at pH 5.0 there was no significant difference on the stability of sorghum and red cabbage pigments against SO_2, both in the presence and the absence of pyruvic acid.

In general, 60 ppm SO_2 led to increase in color intensity of all pigments at pH 2.0 possibly through co-pigmentation effect but pyruvic acid seemed to prevent this effect at this pH. Again, pyruvate may not effectively stabilize crude sorghum pigment extract at pH 3.0 and 3.2 since it reduces color intensity, in the presence of sulfite. At pH 5.0, pyruvic acid showed protection against SO_2 in only the dimethoxylated analogs.

On the other hand, natural pigments had higher resistance to 500 ppm ascorbic acid bleaching than the 3-deoxyanthocyanin standards in the absence of pyruvic acid. At pH 3.0, pyruvic acid did not protect sorghum pigments from ascorbic acid degradation, but it offered marginal protection to the 3-deoxyanthocyanin standards from ascorbic acid bleaching at this pH.

Possible co-pigmentation reaction between crude sorghum pigments and ascorbate occurred at pH 3.2, both in the absence and the presence of pyruvic acid; this may have improved its color intensity. Similar co-pigmentation effect was observed at pH 5.0 with the 3-deoxyanthocyanin standards in the presence of both ascorbic acid and pyruvate.

Thus, from the results obtained:

(a) Solution pH had the greatest effect on 3-deoxyanthocyanin pigments stability against SO_2 and ascorbic acid degradation ($\alpha = 0.05$).

(b) Increase in solution pH and addition of pyruvic acid both caused hyperchromic shift in 3-deoxyanthocyanin pigments (without SO_2 and ascorbic acid).

(c) SO_2 is an excellent co-pigment with 3-deoxyanthocyanin pigments at pH 2.0 in the absence of pyruvic acid.

(d) Ascorbic acid is a co-pigment with 3-deoxyanthocyanin pigments at pH 5.0.

(e) Pyruvic acid had marginal protection on the 3-deoxyanthocyanins against sulfite and ascorbic acid degradation, but did not protect against thermal degradation.

(f) Increase in methoxylation generally reduced the resistance of 3-deoxyanthocyanin pigments against heat.

(g) New pyrano-3-deoxyanthocyanin pigments were verified via HPLC-DAD and LC/MS analyses.

(h) Thermal processing triggers production of new 3-deoxyanthocyanin-pyruvic acid adducts.

5.2. Recommendations for further research

Based upon findings of the study, the following recommendations were made:

(a) More studies are needed to establish the influence of different 3-deoxy-anthocyanin substituents, substituent patterns, and the extent of substitution e.g. methylation on the effectiveness of pyruvic acid reaction during the formation of the pyrano-3-deoxyanthocyanin adducts.

(b) In this study, color expression of various 3-deoxyanthocyanin compounds was a result of both the individual pigment and its adduct in solution. Further studies are therefore required to determine the stability and color of the 3-deoxyanthocyanin-pyruvic acid adducts against SO_2 and ascorbic acid degradation independently, with and without light or oxygen, in comparison to their precursor pigments under the same pH and temperature conditions.

(c) Thermal treatment was shown to initiate further synthesis of new 3-deoxy-anthocyanin-pyruvic acid adducts. Their individual chemical structures, colors and stability must be studied carefully to elucidate their characteristics under various pH conditions.

(d) Apply the same method of synthesizing dimethoxylated 3-deoxyanthocyanin-pyruvic acid adducts (i.e. 5,7-dimethoxyapigeninidin and 5,7-dimethoxyluteolinidin), with and without oxygen purging, to prove the importance of oxygen and/or oxidative cleavage of the pyrilium ring during their synthesis.

(f) More studies are needed to find out whether different methods of crude sorghum pigment extraction can affect its color intensity and stability in various pH solutions, with or without light and pyruvic acid.

(g) With the proof that synthesis of 3-deoxyanthocyanin-pyruvic acid adducts are gradual, further studies are required to explain whether there is an optimum yield per mole of sample for every mole of pyruvic acid using the same experimental procedure utilized in this study, time taken to reach that optimum yield level, and also the optimum pH for attaining that maximum yield for every sample tested. This information will be helpful in the future large scale production of these seemingly stable adducts.

5.3. Summary

The purpose of the research was to: **(a)** establish the stability of sorghum 3-deoxyanthocyanins and other standards against SO_2 and ascorbic acid degradation at different pH levels, **(b)** determine the effectiveness of pyruvic acid addition on sorghum 3-deoxyanthocyanin stability against sulfite and ascorbic acid bleaching, and **(c)** establish the effects of thermal sterilization conditions on sorghum pigments stability relative to other standards. The experimental procedure used for synthesizing the 3-deoxyanthocyanin-pyruvic acid adducts simulated model solutions that would be found in common beverages.

The synthesis of 3-deoxyanthoxyanin-pyruvic acid adducts (pyruvic acid:anthocyanin = 50:1 molar ratio) after 5 days of incubation was progressively monitored by HPLC-DAD analysis, followed by mass spectrometry. Duplicate solutions were prepared and their color stability towards degradative effects of SO_2 and ascorbic acid were studied at pH levels 2.0, 3.0, 3.2 and 5.0. UV-Vis absorption spectra were also recorded over time using a Shimadzu UV-1650PC spectrophotometer (10 mm path-length cell) from 250 - 720 nm. Thermal processing was achieved by heating the samples at 15 p.s.i. to 121.1°C for 15 minutes using an autoclave and then immediately cooling in an ice bath.

Results strongly indicated that solution pH had the greatest effect on their stability ($\alpha = 0.05$), and SO_2 and ascorbic acid are co-pigments with 3-deoxyanthocyanin pigments in the absence of pyruvic acid at low pH (i.e. pH 2.0) and high pH (i.e. pH 5.0), respectively. Thus, in solutions with 3-deoxyanthocyanins, SO_2 (as a preservative) and ascorbic acid (for nutritive value) could be effective additives at low and high pH, respectively, by improving the pigment color during storage. Pyruvic acid had marginal protective influence on the stability on the 3-deoxyanthoxyanin pigments against sulfite and ascorbic acid degradation but not heat. Various recommendations were highlighted for further research.

APPENDIX

1. SAS DATA

1.1. ANOVA tables for samples with SO_2

(a) 5-methoxyapigeninidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	38.78754589	2.28162035	2.73	0.0078
Error	30	25.04002055	0.83466735		
Corrected Total	47	63.82756644			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.607693	81.14981	0.913601	1.125821

(b) 7-methoxyapigeninidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	1.58083718	0.17564858	1.19	0.3703
Error	14	2.06183076	0.14727363		
Corrected Total	23	3.64266794			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.433978	41.74652	0.383762	0.919268

(c) Apigeninidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	15.66478111	0.92145771	2.50	0.0136
Error	30	11.04616092	0.36820536		
Corrected Total	47	26.71094203			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.586456	56.40524	0.606799	1.075785

(d) 5,7-dimethoxyapigeninidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	106.9547979	6.2914587	1.71	0.0964
Error	30	110.2429740	3.6747658		
Corrected Total	47	217.1977719			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.492430	170.1802	1.916968	1.126434

(e) 5,7-dimethoxyluteolinidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	173.5539643	10.2090567	2.27	0.0240
Error	30	134.7625753	4.4920858		
Corrected Total	47	308.3165395			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.562908	170.6152	2.119454	1.242243

(f) Grape Blue Powder

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00561423	0.00112285	0.11	0.9851
Error	6	0.05994672	0.00999112		
Corrected Total	11	0.06556095			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.085634	9.369538	0.099956	1.066815

(g) Luteolinidin

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	16.75541291	0.98561252	1.87	0.0656
Error	30	15.83168606	0.52772287		
Corrected Total	47	32.58709897			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.514173	79.38298	0.726445	0.915115

(h) Red Cabbage

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.86906903	0.06685146	1.75	0.1184
Error	22	0.83802494	0.03809204		
Corrected Total	35	1.70709396			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.509093	16.65977	0.195172	1.171516

(i) Sorghum Extract

Dependent Variable: SO_2

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	2.63473095	0.15498417	1.96	0.0521
Error	30	2.37193996	0.07906467		
Corrected Total	47	5.00667091			

R-Square	Coeff Var	Root MSE	SO_2 Mean
0.526244	23.05579	0.281184	1.219583

1.2. ANOVA tables for samples with ascorbic acid

(a) 5-methoxyapigeninidin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	14.58209068	0.85777004	3.13	0.0031
Error	30	8.22183076	0.27406103		
Corrected Total	47	22.80392144			

R-Square	Coeff Var	Root MSE	VitC Mean
0.639455	60.33749	0.523508	0.867634

(b) 7-methoxyapigeninidin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	2.23454012	0.24828224	1.75	0.1684
Error	14	1.98891047	0.14206503		
Corrected Total	23	4.22345059			

R-Square	Coeff Var	Root MSE	VitC Mean
0.529079	55.83501	0.376915	0.675052

(c) Apigeninidin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	11.13611334	0.65506549	1.91	0.0590
Error	30	10.29007545	0.34300252		
Corrected Total	47	21.42618879			

R-Square	Coeff Var	Root MSE	VitC Mean
0.519743	72.73993	0.585664	0.805148

(d) 5,7-dimethoxyapigenindin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	10.19350174	0.59961775	1.03	0.4586
Error	30	17.49537397	0.58317913		
Corrected Total	47	27.68887572			

R-Square	Coeff Var	Root MSE	VitC Mean
0.368144	92.12039	0.763662	0.828982

(e) 5,7-dimethoxyluteolinidin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	18.82075926	1.10710349	2.08	0.0389
Error	30	15.98511478	0.53283716		
Corrected Total	47	34.80587404			

R-Square	Coeff Var	Root MSE	VitC Mean
0.540735	82.36842	0.729957	0.886210

(f) Grape Blue Powder

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.55962537	0.31192507	17.80	0.0015
Error	6	0.10515632	0.01752605		
Corrected Total	11	1.66478169			

R-Square	Coeff Var	Root MSE	VitC Mean
0.936835	13.17058	0.132386	1.005164

(g) Luteolinidin

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	13.63505917	0.80206230	2.49	0.0142
Error	30	9.67559096	0.32251970		
Corrected Total	47	23.31065013			

R-Square	Coeff Var	Root MSE	VitC Mean
0.584928	62.72732	0.567908	0.905360

(h) Red Cabbage

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	4.44107323	0.34162102	4.47	0.0010
Error	22	1.67948071	0.07634003		
Corrected Total	35	6.12055394			

R-Square	Coeff Var	Root MSE	VitC Mean
0.725600	31.27693	0.276297	0.883389

(i) Sorghum Extract

Dependent Variable: VitC

Source	df	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	1.81086435	0.10652143	0.85	0.6263
Error	30	3.74372477	0.12479083		
Corrected Total	47	5.55458912			

R-Square	Coeff Var	Root MSE	VitC Mean
0.326012	35.40587	0.353257	0.997737

REFERENCES

Abyari M, Heidari R, Jamei R. 2006. The effects of heating, UV irradiation and pH on stability of Siahe Sardasht grape anthocyanin-copigment complex. J Biol Sci 6(4):638-45.

Alcade-Eon C, Escribano-Bailon MT, Santos-Buelga C, Rivas-Gonzalo JC. 2006. Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study. Anal Chim Acta 563(1-2):238-54.

Alluis B, Perol N, Elhajji H, Dangles O. 2000. Water-soluble flavonol (=3-hydroxy-2-phenyl-4H-1-benzopyran-4-one) derivatives: Chemical synthesis, coloring, and antioxidant properties. Helv Chim Acta 83(2):428-43.

Amerine MA, Berg HW, Cruess WV. 1967. The Technology of Winemaking. Westport, CT: AVI Publishing Company Inc.

Andersen OM. 2001. Encyclopedia of life sciences. London: Nature Publishing Group. MacMillan Reference Ltd. Available: http://www.els.net

Asen S, Stewart RN, Norris KH. 1972. Co-pigmentation of anthocyanins in plant tissues and its effects on color. Phytochemistry 11(3):1139-44.

Awika JM, Rooney LW. 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry 65(9):1199-221.

Awika JM, Rooney LW, Waniska RD. 2004a. Anthocyanins from black sorghum and their antioxidant properties. Food Chem 90(1-2):293–301.

Awika JM, Rooney LW, Waniska RD. 2004b. Properties of 3-deoxyanthocyanins from sorghum. J Agric Food Chem 52(14):4388-94.

Awika JM, McDonough CM, Rooney LW. 2005. Decorticating sorghum to concentrate healthy phytochemicals. J Agric Food Chem 53(16):6230–4.

Bakhshayeshi MA, Khayami M, Heidari R, Jamei R. 2006. The effects of light, storage temperature, pH and variety on stability of anthocyanin pigments in four *Malus* varieties. Pak J Biol Sci 9(3):428-33.

Bakker J, Timberlake CF. 1997. Isolation, identification and characterization of new color-stable anthocyanins occurring in some red wines. J Agric Food Chem 45(1):35-43.

Bakowska A, Kucharska AZ, Oszmianski J. 2003. The effect of heating, UV-irradiation, and storage on stability of the anthocyanin-polyphenol co-pigment complex. Food Chem 81(3):349-55.

Baranac JM, Petranovic NA, Dimitric-Markovic JM. 1996. Spectrophotometric study of anthocyan copigmentation reactions. J Agric Food Chem 44(5):1333-6.

Baranac JM, Petranovic NA, Dimitric-Markovic JM. 1997. Spectrophotometric study of anthocyanin copigmentation reactions. 3. Malvin and the nonglycosidized flavone morin. J Agric Food Chem 45(5):1698-700.

Berké B, Chèze C, Vercauteren J, Deffieux G. 1998. Bisulfite addition to anthocyanins: Revisited structures of colorless adducts. Tetrahedron Lett 39(32):5771-74.

Berké B, de Freitas VAP. 2005. Influence of procyanidin structures on their ability to complex with eonin. Food Chem 90(3):453-60.

Bettini V, Fiori A, Martino R, Mayellaro R, Ton P. 1985. Study of the mechanism whereby anthocyanosides potentiate the effect of catecholamines on coronary vessels. Fitoterapia 54(2):67-72.

Bloor SJ, Falshaw R. 2000. Covalently linked anthocyanin-flavonol pigments from blue *Agapanthus* flowers. Phytochemistry 53(5):575-9.

Bolivar ACC, Cisveros-Zevallos L. 2004. Stability of anthocyanin-based aqueous extract of Andean purple corn and red-flushed sweet potato compared to synthetic and natural colorants. Food Chem 86(1):69-77.

Bordignon-Luiz MT, Gauche C, Gris EF, Falcao LD. 2007. Color stability of anthocyanins from Isabel grapes (*Vitis labrusca* L.) in model systems. LWT – Food Sci Technol 40(4):594-9.

Boyd W. 2000. Natural colors as functional ingredients in healthy foods. Cereal Foods World 45(5):221-2.

Brenes CH, Del Pozo-Insfran D, Talcott S. 2005. Stability of co-pigmented anthocyanins and ascorbic acid in a grape juice model system. J Agric Food Chem 53(1):49-56.

Bridle P, Timberlake CF. 1997. Anthocyanin as natural food colors – selected aspects. Food Chem 58(1-2):103-9.

Brouillard R. 1981. Origin of the exceptional color stability of the *Zebrina* anthocyanin. Phytochemistry 22(3):1311-23.

Brouillard R. 1982. Anthocyanins as Food Colors. Markakis P, editor. London: Academic Press. p 1- 40.

Brouillard R. 1988. Flavonoids and flower colors. In: Harbone JB, editor. The Flavonoids. Advance in research since 1980. London, UK: Chapman and Hall.

Brouillard R, Dangles O. 1994. Anthocyanin molecular interactions. The first step in formation of new pigments during wine aging. Food Chem 51(4):365-71.

Brouillard R. Iacobucci GA, Sweeny JG. 1982. Chemistry of anthocyanin pigments. 9.[1] UV-Visible spectrometric determination of the acidity constants of apigeninidin and three related 3-deoxyflavilium salts. J Am Chem Soc 104(26):7585-90.

Cai Y, Lilley TH, Haslam E. 1990. Polyphenolanthocyanin co-pigmentation. J Chem Soc Chewn Commun (5):380-3.

Chandler BV, Harper KA. 1962. A procedure for the absolute identification of anthocyanins: The pigments of blackcurrant fruit. Aust J Chem 15(4):114-20.

Chandra A, Nair MG, Iezzoni AF. 1993. Isolation and stabilization of anthocyanins from tart cherries (*Prunus cerasus* L.). J Agric Food Chem 41(7):1062-5.

Chen LJ, Hrazdina G. 1981. Structural aspects of anthocyanin-flavonoid complex formation and its role in plant color. Phytochemistry 20(2):297-303.

Chen LJ, Hradzina G. 1982. Structural transformation reactions of anthocyanins. Experientia 38(9):1030-2.

Chigurupati N, Saiki L, Gayser Jr C, Dash AK. 2002. Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. Int J Pharm 241(2):293-9.

Clifford MN. 2000. Anthocyanins – nature, occurrence and dietary burden. J Sci Food Agric 80(7):1063-72.

Dangles O, Brouillard R. 1992. A spectroscopic method on the anthocyanin copigmentation interaction and applied to the quantative study of molecular complexes. J Chem Soc Perkin Trans 2(2):247-57.

Dangles O, El hajji H. 1994. Synthesis of 3-methoxy- and 3-(β-$_{D}$-glucopyranosyloxy) flavylium ions. Influence of the flavylium substitution pattern on the reactivity of anthocyanins in aqueous solutions. Hel Chim Acta 77(6):1595-610.

Davies AJ, Mazza G. 1993. Co-pigmentation of simple and acylated anthocyanins with colorless phenolic compounds. J Agric Food Chem 41(5):716-20.

Deguchi T, Shohara S, Ohba R, Ueda S. 2000. Effects of pH and light on the storage stability of the purple pigment, hordeumin, from uncooked barley bran fermented broth. Biosci Biotech Biochem 64(10):2236-9.

De Rosso VV, Mercadante AZ. 2007a. The high ascorbic acid content is the main cause of the low stability of anthocyanin extracts from acerola. Food Chem 103(3):935-43.

De Rosso VV, Mercadante AZ. 2007b. Evaluation of color and stability of anthocyanins from tropical fruits in an isotonic soft drink system. Innov Food Sci Emerging Technol 8(3):347-52.

Dykes L, Rooney LW. 2006. Sorghum and millet phenols and antioxidants. J Cereal Sci 44(3):236-51.

Dykes L, Rooney LW, Waniska RD, Rooney WL. 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. J Agric Food Chem 53(17):6813-8.

Eiro MJ, Heinonen M. 2002. Anthocyanin color behavior and stability during storage: Effect of intermolecular co-pigmentation. J Agric Food Chem 50(25):7461-6.

Elliot JG. 1999. Application of antioxidant vitamins in foods and beverages. Food Technology 53(2):46-8.

Fallico B, Lanza MC, Maccarone E, Asmundo CN, Rapisarda P. 1996. Role of hydroxycinnamic acids and vinylphenols in the flavor alteration of blood orange juices. J Agric Food Chem 44(9):2654-7.

Fiore A, La Fauci L, Cervellati R, Guerra MC, Speroni E, Costa S, Galvano G, De Lorenzo A, Bacchelli V, Fogliano V, Galvano F. 2005. Antioxidant activity of pasteurized and sterilized commercial red orange juices. Mol Nutr Food Res 49(12):1129-35.

Fossen T, Cabrita L, Andersen ǾM. 1998. Color and stability of pure anthocyanins influenced by pH including the alkaline region. Food Chem 63(4):435-40.

Francis JF. 1989. Food colorants: Anthocyanins (review). Crit Rev Food Sci Nutr 28:273-314.

Fulcrand H, Benabdeljalil C, Rigaud J, Cheynier V, Moutounet MA. 1998. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. Phytochemistry 47(7):1401-7.

García-Viguera C, Bridle P. 1999. Influence of structure on color stability of anthocyanins and flavylium salts with ascorbic acid. Food Chem 64(1):21-6.

Giusti MM, Wrolstad RE. 1996. Radish anthocyanins extract as a natural red colorant for Maraschino cherries. J Food Sci 61(4):688-94.

Giusti MM, Wrolstad RE. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. Unit F1.2. In: Wrolstad RE, Schwartz SJ, editors. Current protocols in food analysis chemistry. New York: Wiley. F1.2.1-F1.2.13.

Gläßgen WE, Seitz HU, Metzger JW. 1992. High-performance liquid chromatography/electrospray mass spectrometry and tandem mass spectrometry of anthocyanins from plant tissues and cell cultures of *Daucus carota*. Biol Mass Spectrosc 21(6):271-7.

Gonnet JF. 1998. Color effects of co-pigmentation of anthocyanins revisited – 1. A colorimetric definition using CIELAB scale. Food Chem 63(3):409-15.

Gonnet JF. 1999. Color effects of co-pigmentation of anthocyanin revisited – 2. A colorimetric look at the solutions of cyanin co-pigmented by rutin using CIELAB scale. Food Chem 66(3):387-94.

Gous F. 1989. Tannins and phenols in black sorghum. Ph.D. Dissertation, Texas A&M University, College Station, TX.

Harborne JB. 1998. Phenolic compounds. In: Harbon JB, editor. Phytochemical methods: A guide to modern techniques of plant analysis. 3rd ed. New York: Chapman and Hall. p 66-74.

Harborne JB, Self R. 1989. Malonated cyanidin 3-glucosides in *Zea mays* and other grasses. Phytochemistry 26(8):2417-8.

Hatfield J, Morris J, Threlfall R. 2003. Minimizing color degradation in bluish wines. J Food Qual 26(5):367-80.

He J, Santos-Buelga C, Mateus N, de Freita V. 2006. Isolation and quantification of oligomeric pyranoanthocyanin-flavanol pigments from red wines by combination of column chromatographic techniques. J Chromatography 1134(1-2):215-25.

Heredia FJ, Francia-Aricha EM, Rivas-Gonzalo JC, Vicario IM, Santos-Buelga C. 1998. Chromatic characterization of anthocyanins from red grapes – I. pH effect. Food Chem 63(4):491-8.

Hipskind JD, Hanau R, Leite B, Nicholson RL. 1990. Phytoalexin accumulation in sorghum: identification of an apigeninidin acyl ester. Physiol Molec Plant Pathol 36(5):381-96.

Houbiers C, Lima JC, Macanita AL, Santos H. 1998. Color stabilization of malvidin 3-glucoside: Self-aggregation of the flavylium cation and co-pigmentation with the Z-chalcone form. J Phys Chem B 102(18):3578-85.

Hrazdina G, Franzese AJ. 1974. Oxidation products of acylated anthocyanins under acidic and neutral conditions. Phytochemistry 13(1):231-4.

Huang HT. 1955. Decolorization of anthocyanins by fungal enzymes. Agric Food Chem 3(2):141-6.

Iacobucci GA, Sweeny JG. 1983a. Effects of substitution on the stability of 3-deoxyanthocyanidins in aqueous solutions. J Agric Food Chem 31(3):531-3.

Iacobucci GA, Sweeny JG. 1983b. The chemistry of anthocyanins, anthocyanidins and related flavylium salts. Tetrahedron 39(19):3005-38.

Idaka E, Yamakita H, Ogawa T, Kondo T, Yamamoto M, Goto T. 1987. Structure of three diacylated anthocyanins isolated from red cabbage, *Brassica oleracea*. Chem Lett (6):1213-6.

Jackman RL, Yada RY, Tung MA, Speers RA. 1987. Anthocyanins as food colorants – a review. J Food Biochemistry 11(3):201-47.

Janna OA, Khairul A, Maziah M, Mohd Y. 2006. Flower pigment analysis of *Melastoma malabathricum*. Afri J Biotechnol 5(2):170-4.

Janna OA, Khairul AK, Maziah M. 2007. Anthocyanin stability studies in *Tibouchina semidecandra* L. Food Chem 101(4):1640-6.

Jurd L. 1964a. Reactions involved in sulfite bleaching of anthocyanins. J Food Sci 29(1):16-19.

Jurd L. 1964b. Anthocyanins and related compounds. III. Oxidation of substituted flavylium salts to 2-Phenylbenzofurans. Western Regional Research Laboratory. Agricultural Research Service, USDA. Albany, CA. 29:2602-05.

Jurd L. 1972. Some advances in the chemistry of anthocyanin-type plant pigments. In: Chichester CO, editor. The Chemistry of Plant Pigments. New York and London: Academic Press. p 1231-42.

Kalt W, Forney CF, Martin A, Prior RL. 1999. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. J Agric Food Chem 47(11):4638-44.

Kamei H, Kojima T, Hasegawa M, Koide T, Umeda T, Yukawa T, Terabe K. 1995. Suppression of tumor cell growth by anthocyanins in vitro. Cancer Invest 13(6):590-4.

Karaivanova M, Drenska D, Ovcharov R. 1990. A modification of the toxic effects of platinum complexes with anthocyanins. Eksperimetnalna Meditsna I Morfologiia 29(2):590-4.

King GA, Sweeny JG, Radford T, Iacobucci GA. 1980. The ascorbic acid-oxygen degradation of anthocyanidins. Bull Liaison-Groupe Polyphenols (9):121-8.

Kucharska AZ, Oszmianski J, Kopacz M, Lamer-Zarawska E. 1998. Application of flavonoids for anthocyanins stabilization. II. Conference: Flavonoids and their employments. Rzeszow. Poland.

Leadley C, Williams A. 2002. Power ultrasound - current and potential applications for food processing. Review No 32, Campden and Chorleywood Food Research Association.

Lewis CE, Walker JRL, Lancaster JE. 1995. Effect of polysaccharides on the color of anthocyanins. Food Chem 54(3):315-9.

Lietti A, Cristoni A, Picci M. 1976. Studies of *Vaccinium myrtillus* anthocyanosides. I. Vasoprotective and anti-inflammatory activity. Arzneimittel-Forschung 26(5):829-32.

Lo SC, Weiergang I, Bonham C, Hipskind J, Wood K, Nicholson RL. 1996. Phytoalexin accumulation in sorghum: Identification of methyl ether of luteolinidin. Physiol Molec Plant Pathol 49(1):21-31.

Maccarone E, Maccarone A, Rapisarda P. 1985. Stabilization of anthocyanins of blood orange fruit juice. J Food Sci 50(4):901-4.

Malien-Aubert C, Dangles O, Amiot MJ. 2001. Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. J Agric Food Chem 49(1):170-6.

Mares-Perlman JA. 1997. Contribution of epidemiology to understanding relations of diet to age-related cataract. Am J Clin Nutr 66(4):739-40.

Markakis P. 1982. Stability of anthocyanins in foods. In: Markakis P, editor. Anthocyanins as food colors. New York: Academic Press. p 163-80.

Marquette B, Trione D. 1998. The tannins: Wine titles. Proceedings, Australian Society of Viticulture and Oenology. Adelaide, Australia. p 12-17.

Martí N, Pérez-Vicente A, García-Viguera. 2001. Influence of storage temperature and ascorbic acid addition on pomegranate juice. J Sci Food Agric 82(2):217-21.

Mason TJ. 1998. Power ultrasound in food processing – the way forward. In: Povey, MJW, Mason TJ, editors. Ultrasound in food processing. London, UK: Thomson Science. p 105-26.

Mazza G, Brouillard R. 1987. Color stability and structural transformations of cyanidin 3,5-diglucoside and four 3-deoxyanthocyanins in aqueous solutions. J Agric Food Chem 35(3):422–6.

Mazza G, Brouillard R. 1990. The mechanism of co-pigmentation of anthocyanins in aqueous solutions. Phytochemistry 29(4):1097-102.

Mazza G, Cacace JE, Kay CD. 2004. Methods of analysis for anthocyanins in plants and biological fluids. J Ass Off Anal Chem Int 87(1):129-45.

Mazza G, Miniati E. 1993. Anthocyanins in fruits, vegetables and grains. Boca Raton, FL: CRC Press.149-99 p.

Mazzaracchio P, Pifferi P, Kindt M, Munyaneza A, Barbiroli G. 2004. Interactions between anthocyanins and organic food molecules in model systems. Int J Food Sci Technol 39(1):53-9.

Morata A, Calderón F, González MC, Gómez-Cordovés MC, Suárez JA. 2007. Formation of the highly stable pyranoanthocyanins (vitisins A and B) in red wines by addition of pyruvic acid and acetaldehyde. Food Chem 100(3):1144-52.

Morel M-H, Dehlon P, Autran JC, Leygue JP, Bar-L'Helgouac'H C. 2000. Effects of temperature, sonication time, and power settings on size distribution and extractability of total wheat flour proteins as determined by size-exclusion high-performance liquid chromatography. Cereal Chem 77(5):685-91.

Moulton KJ, Wang LC. 1982. A pilot-plant study of continuous ultrasonic extraction of soybean protein. J Food Sci 47(4):1127-9, 39.

Nakaishi H. 2000. Effects of black current anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alteration in healthy humans. Alt Med Rev 2000 Dec 5(6):553-62.

Nerdal W, Andersen ǾM. 1992. Intermolecular aromatic acid association of an anthocyanin (petanin) evidenced by two-dimensional nuclear Overhauser enhanced nuclear magnetic resonance experiments and distance geometry calculations. Phytochemical Analysis 3(4):182-9.

Nip WK, Burns EE. 1969. Pigment characterization in grain sorghum. I. Red varieties. Cereal Chem 46(5):490-5.

Nip WK, Burns EE. 1971. Pigment characterization in grain sorghum. II. White varieties. Cereal Chem 48(1):74-80.

Noda Y. 2000. Antioxidant activity of nasunin, an anthocyanin in eggplant peels. Toxicology. Aug 7. 148(2-3):119-23.

Ochoa MR, Kesseler AG, De Michelis A, Mugridge A, Chaves AR. 2001. Kinetics of color changes of raspberry, sweet (*Prunus avium*) and sour (*Prunus cerasus*) cherries preserve, packed in glass containers: light and room temperature effect. J Food Eng 49(1):55-62.

Oliveira J, Fernandes V, Miranda C, Santos-Buelga C, Silva A, de Freitas V, Mateus N. 2006. Color properties of four cyanidin-pyruvic acid adducts. J Agric Food Chem 54(18):6894-903.

Özkan M, Kirca A, Cameroglu B. 2004. Effects of hydrogen peroxide on the stability of ascorbic acid during storage in various fruit juices. Food Chem 88(4):591-7.

Palamidis N, Markakis T. 1975. Structure of anthocyanin. Food Sci 40(5):104-6.

Parisa S, Reza H, Elham G, Rashid J. 2007. Effect of heating, UV irradiation and pH on stability of the anthocyanin copigment complex. Pak J Biol Sci 10(2):267-72.

Pereira GK, Donate PM, Galembeck SE. 1997. Effects of substitution for hydroxyl in the B-ring of the flavylium cation. J Molec Structure 392:169-79.

Perera CO. 2005. Selected quality attributes of dried foods. Drying Technology 23(4):717-30.

Picinelli A, Bakker J, Bridle P. 1994. Model wine solutions: Effect of sulphur dioxide on color and composition during ageing. Vitis 33(1):31-35.

Poei-Langston MS, and Wrolstad RE. 1981. Color degradation in an ascorbic acid-anthocyanin-flavonol model system. J Food Sci 46:1218-22, 36.

Prior RL, Wu X. 2006. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. Free Radic Res 40(10):1014-28.

Rababah TM, Ereifej KI, Howard L. 2005. Effect of ascorbic acid and dehydration on concentrations of total phenolics, antioxidant capacity, anthocyanins, and color in fruits. J Agric Food Chem 53(11):4444-7.

Rein MJ, Heinonen M. 2004. Stability and enhancement of berry juice color. J Agric Food Chem 52(10):3106-14.

Rodríguez-Saona LE, Giusti MM, Wrolstad RE. 1999. Color and pigment stability of red radish and red-fleshed potato anthocyanins in juice model systems. J Food Sci 64(3):451-6.

Rubinskiene M, Viskelis P, Jasutiene I, Bobinas C. 2005. Impact of various factors on the composition and stability of black currant anthocyanins. Food Res Int 38(8-9):867-71.

Saija A. 1994. Pharmacological effects of anthocyanins from blood orange juice. Essenze-Deriv Agrum 64(2):229-33.

Saito N, Tatsuzawa F, Yoda K, Yokoi M, Kasahara K, Iida S, Shigihara A, Honda T. 1995. Acylated cyanidin glycosides in the violet-blue flowers of *Ipomoea purpurea.* Phytochemistry 40(4):1283-89.

Salas E, Dueńas M, Schwarz M, Winterhalter P, Cheynier V, Fulcrand H. 2005. Characterization of pigments from different high speed countercurrent chromatography wine fractions. J Agric Food Chem 53(11):3536-4546.

Schwarz M, Winterhalter P. 2003. A novel synthetic route to substituted pyranoanthocyanins with unique color properties. Tetrahedron Lett 44(41):7583-7.

Seitz LM. 2004. Effect of plant-type (purple vs. tan) and mold invasion on concentrations of 3-deoxyanthocyanidins in sorghum grain. AACC Annual Meeting Abstracts. URL: http://www.aaccnet.org/meetings/2004/abstract/a04ma384.htm Retrieved 06-13-07.

Shaked-Sachray L, Weiss D, Reuverin M, Nissim-Levi A, Oren-Shamir M. 2002. Increased anthocyanin accumulation in aster flowers at elevated temperature due to magnesium treatment. J Plant Phys 114(4):559-65.

Singleton VL, Trousdale EK. 1992. Anthocyanin-tannin interactions explaining differences in polymeric phenols between white and red wines. Am J Enol Vitic 43(1):63-70.

Somers TC, Wescomb LG. 1982. Red wine quality: The critical role of sulphur dioxide during vinification and conservation. Australian Grapegrower and Winemaker. Technical Issue No 220.

Sondheimer E, Kertesz ZI. 1953. Participation of ascorbic acid in the destruction of anthocyanin in strawberry juice and model systems. Food Res 18:475-9.

Spayd SE, Tahara JM, Mee DL, Feguson JC. 2002. Separation of sunlight and temperature effects on the composition of *Vitis vinifera.* J Food Sci 53(3):171-82.

Starr MS, Francis FJ. 1968. Oxygen and ascorbic acid effect on the relative stability of four anthocyanin pigments in cranberry juice. Food Technology 22(10):1293-5.

Striegl AW, Leitner E, Pfannhauser W. 1995. Qualitative und quantitative analyse der anthocyane in Schwarzen Apfelbeeren (*Aronia melanocarpa* Michx. Ell.) mittels TLC, HPLC und UV/VIS-Spectrometrie. *Z. Lebensm-Unters.Forsch* 201:266-8.

Sweeny JG, Iacobucci GA. 1981. Synthesis of anthocyanidins – III: Total synthesis of apigeninidin and luteolinidin chloride. Tetrahedron 37(8):1481-3.

Timberlake C. 1989. Plant pigments for coloring food. J B N F Nutr 14(2):113-7.

Timberlake CF. 1960a. Metallic components of fruit juices: III. Oxidation and stability of ascorbic acid in model systems resembling blackcurrant juice. J Sci Food Agric 11(5):258-68.

Timberlake CF. 1960b. Metallic components of fruit juices: IV. Oxidation and stability of ascorbic acid in model systems resembling blackcurrant juice. J Sci Food Agric 11(5):268-73.

Timberlake CP, Bridle P. 1967a. Flavylium salts, anthocyanidins and anthocyanins I.-Reactions with sulphur dioxide. J Sci Food Agric 18(10):473-8.

Timberlake CP, Bridle P. 1967b. Flavylium salts, anthocyanidins and anthocyanins II.-Reactions with sulphur dioxide. J Sci Food Agric 18(10):479-85.

Torskangerpoll K, Andersen OM. 2005. Color stability of anthocyanins in aqueous solutions at various pH values. Food Chem 89(3):427-40.

Vincieri FF, Romani A, Baldi A, Mulinacci N, Alberti MB. 1992. Analysis HPLC of anthocyanins present in fluid extracts from *Malva sylvestris* L. flowers and leaves. Bull Liaison-Groupe Polyphenols 16(1):339-42.

Walkowiak-Tomczak D, Czapski J. 2007. Color changes of a preparation form red cabbage during storage in a model system. Food Chem 104(2):709-14.

Wang LC. 1975. Ultrasonic extraction of proteins from autoclaved soybean flakes. J Food Sci 40(3):549-51.

Wang H, Cao G, Prior RL. 1997. Oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 45(2):304-9.

Wang L, Wang Y-J. 2004. Rice starch isolation by neutral protease and high-intensity ultrasound. J Cereal Sci 39(2):291-6.

Wesche-Ebeling P, Montgomery MW. 1990. Strawberry Polyphenoloxidase: its role in anthocyanin degradation. J Food Sci 55(3):731-4.

Wesche-Ebeling P, Argaíz-Jamet A, Hernández-Porras LG, López-Malo A. 1996. Preservation factors and processing effects on anthocyanin pigments in plums. Food Chem 57(3):399-403.

Wesche-Ebeling P, Argaiz-Jamet A, Patjane-Brito A. 2003. Stability of anthocyanins from strawberry in the presence of the co-pigment caffeic acid in a model carbonated beverage. IFT Annual Meeting – Chicago. 92A-13, Book of Abstracts p 221.

Wigand MC, Dangles O, Brouillard R. 1992. Complexation of a fluorescent anthocyanin with purines and polyphenols. Phytochemistry 31(12):4317-24.

Williams DC, Lim MH, Chen AO, Pangborn RM, Whitaker JR. 1986. Blanching of vegetables – which indicator enzyme to choose. Food Technol 40(6):130-40.

Wissemann KW, Lee CY. 1980. Polyphenoloxidase activity during grape maturation and wine production. Am J Enol Vitic 31(3):206-11.

Wu X, Prior RL. 2005. Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains. J Agric Food Chem 53(8):3101-13.

Wulf LW, Nagel CW. 1978. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera.* Am J Enol Vitic 29(1):42-9.

Yao LH, Jian YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, Chen SS. 2004. Flavonoids in food and their health benefits. Plant Foods Hum Nutr 59(3):113-22.

Yoshida K, Kondo T, Goto T. 1991. Unusually stable mono-acylated anthocyanin from purple yam *Dioscorea alata.* Tetrahedron Lett 32(40):5579-80.

Yoshida K, Toyama Y, Kameda K, Kondo T. 2000. Contribution of each caffeoyl residue of the pigment molecule of gentiodelphin to blue color development. Phytochemistry 54(1):85-92.