The Anthocyanins of Red Onions, *Allium cepa* L.: Extraction, Characterization, Complexation and Stabilization

A Thesis
Submitted to the Faculty of Graduate Studies
The University of Manitoba

By

Horst Donner

In Partial Fulfilment of the Requirements
for the Degree of
Master of Science

Department of Food Science
Faculty of Agricultural and Food Sciences

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Murphy's Law (or the optimist's creed)

Nothing is as easy as it looks.

Everything takes longer than you expect.

And if anything can go wrong – it will

At the worst possible moment.

ABSTRACT

The anthocyanins and colourless phenolics found in red onions, *Allium cepa* L., were extracted with a mixture of methanol, formic acid and water and then were fractionated using Amberlite CG-50 and a C_{18} solid phase extraction cartridge. Using high performance liquid chromatography (HPLC) with a reverse phase column and a photodiode array detector, the chromatographic profiles of the four red onion varieties 'Mambo', 'Red Bone', 'Red Granex' and 'Red Jumbo' were determined. A total of four major and four minor anthocyanins were present in red onions. The anthocyanins and colourless phenolics were identified using a rapid identification procedure based on spectrophotometric, and liquid and gas chromatographic techniques. The major anthocyanins were identified as cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-(6"-malonylglucoside), and cyanidin 3-(6"-malonyllaminaribioside). The four minor anthocyanins were identified as cyanidin 3-(3"-malonylglucoside), peonidin 3-glucoside, peonidin 3-malonylglucoside and cyanidin 3-dimalonyllaminaribioside. Using the same technique, the colourless phenolic compounds were identified as protocatechuic acid 4-glucoside, quercetin 7,4'-diglucoside, quercetin 3,4'-diglucoside, quercetin 3-glucoside, quercetin 4'-glucoside, and a 5,7-dihydroxy flavanone glucoside or a 5,7-dihydroxy dihydroflavanol glucoside. The presence of the major anthocyanins in red onions was confirmed, and four new minor anthocyanins were identified in the red onions from North America. Cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside were extracted, purified and used for the copigmentation study.

The copigmentation effect was studied using cyanidin 3-glucoside, cyanidin 3-malonylglucoside, cyanidin 3-malonyllaminaribioside, chlorogenic acid, protocatechuic acid and caffeine in aqueous buffers at pH 3.7, 4.7 and 5.7. By monitoring the changes in visible

absorbance using a spectrophotometer, it was observed that copigment structure and concentration, and pH have a dramatic influence on the copigmentation phenomenon. Pigment structure did have a slight effect but was minor relative to the effects of pH and copigment structure. The best copigment was chlorogenic acid, followed by caffeine, and protocatechuic acid which was the poorest copigment. The greatest increase in copigmentation was generally observed at pH 4.7 and pH 5.7, depending on the copigment used. However, the most intensely coloured solutions occurred at pH 3.7. The three copigments produced different colour hues and magnitudes of copigmentation when combined with the three anthocyanins. The equilibrium constants for the complexation reaction (K) and the stoichiometric constants (n) for the nine different anthocyanin, copigment and pH combinations were determined. When chlorogenic acid and caffeine, and chlorogenic acid, caffeine and protocatechuic acid were combined with cyanidin 3-glucoside in increasing amounts, there was an increased copigmentation effect and a significant increase in the range of colour produced.

Colour stability studies using cyanidin 3-glucoside solutions at pH 3.7, 4.7, and 5.7 without and with added chlorogenic acid, protocatechuic acid and caffeine in the presence and absence of light, demonstrated that intense exposure to fluorescent and incandescent light was detrimental to the photostability of copigmented cyanidin 3-glucoside solutions at any pH. The most photostable solution was cyanidin 3-glucoside with chlorogenic acid at pH 3.7. All other solutions discoloured more rapidly and some developed a precipitate. Samples stored in the dark under the same conditions were found to be more stable, especially at pH 3.7. However, at pH 4.7 and 5.7, the solutions were also discoloured, although not to the same extent as those solutions exposed to intense light. The lack of a stable red colour at pH 4.7 and 5.7 can be attributed to the formation of the colourless chalcone species from the red coloured flavylium cation. This study demonstrated that light and pH have detrimental effects on the colour retention

in anthocyanin solutions, while the addition of copigment can provide colour stability under suitable conditions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

1. INTRODUCTION

Consumers tend to judge the quality of food based on appearance which they associate with quality and certain odours, flavour, textures and other preconceptions (IFT, 1986). If the food does not have an appealing colour, the consumer may reject it, regardless of other quality attributes and sensory characteristics. The Food and Nutrition Board of the U.S.A. permits the use of food colours to restore the original appearance, ensure uniform colour intensity, intensify the naturally occurring colour, preserve the identity and character of the food, protect flavour and light sensitive vitamins during storage, lend an attractive appearance to unattractive foods, and to serve as a visual indicator of quality (FNB, 1971). Over the years, consumers have become more health conscious and have questioned the safety of food ingredients, especially the role of artificial food colourants. This concern arose after two Russian studies in 1970 dealing with FD&C Red No. 2, commonly known as amaranth, alleged that it was carcinogenic and embryotoxic (IFT, 1986). After conducting its own studies, the U.S. Food and Drug Administration (FDA) banned the use of amaranth; however, it is still permitted for use in Canada, Sweden, Denmark, Germany, Japan, and EEC countries. The other two red dyes currently used are FD&C Red No. 40, commonly known as allura red and FD&C Red No. 3, also known as erythrosine. These concerns have caused public controversy and a demand for alternatives to artificial food colourants. As a result, the food industry and research scientists have become interested in the use of natural plant pigments, like anthocyanins as alternatives to synthetic colourants.

Anthocyanins are widespread in plants and are responsible for most of the orange, red, and blue colours in the flowers, fruits, vegetables, leaves, roots and storage organs of higher plants (Francis, 1989a). Anthocyanins attract insects and birds to plants for pollination purposes

and act as a UV light screen in leaves (Harborne, 1967). Humans have used anthocyanins for their pharmacological properties and therapeutic purposes since they exhibit antiulcer activity, decrease the harmful effects of UV radiation, reduce capillary permeability and fragility, treat urinary tract infections and to improve night-time visual acuity, to improve adapting to low light conditions, and to decrease recovery time after exposure to glare (Cristoni and Magistretti, 1987; Kano and Miyakoshi, 1976; Boniface *et al.*, 1986; Ofek *et al.*, 1991; Morazzoni and Bombardelli, 1996). The other natural red pigments are betalains which are found in beets, lycopene in tomatoes, capsanthin in red peppers, and bixin in annatto (Francis, 1989b). The anthocyanins are the most diverse group and are found in many attractive, colourful foods, making them logical colour sources. They offer the advantage of no adverse health effects, water solubility and a bright red colour (Markakis, 1982). However, the widespread use of anthocyanins as food colourants has been limited due to their instability under processing and storage conditions. High temperature, high pH, oxygen, enzymes, light, metals and other compounds such as ascorbic acid, amino acids, sugars and phenolic compounds, will all cause a decrease in the colour of anthocyanin solution (Francis, 1989a and b). Other major considerations for use of anthocyanins as food colourants are their potential supply and cost, ease of purification, use, colouring efficiency, and aesthetic appeal (Francis, 1975). Fortunately, the number of complex acylated and more stable anthocyanins identified in recent years has increased which may lead to overcoming colour stability problems and eventually increase the industrial application of anthocyanins. Also, increased investigations of these complex anthocyanins in the presence of other phenolic compounds occurring naturally in plant products is leading to a better understanding of the copigmentation reactions between anthocyanins and copigments. This knowledge will hopefully result in an increased use of anthocyanins as natural food colourants in the future.

The objectives of the thesis research were as follows:

1. To identify the anthocyanins and colourless phenolics in red onions, *Allium cepa* L., using an identification procedure based upon high performance liquid chromatography (HPLC), gas chromatography (GC), acid and alkaline hydrolysis and spectral analysis.
2. To purify the major acylated anthocyanins found in red onions for study of their copigmentation properties.
3. To study the copigmentation phenomenon of cyanidin 3-glucoside, cyanidin 3-malonylglucoside, cyanidin 3-malonyllaminaribioside with chlorogenic acid, protocatechuic acid and caffeine at pH 3.7, 4.7 and 5.7.
4. To evaluate the stability of cyanidin 3-glucoside copigmented with chlorogenic acid, protocatechuic acid and caffeine at pH 3.7, 4.7 and 5.7 in the presence and absence of light.

2. LITERATURE REVIEW

2.1. Anthocyanins and their colour

The orange, red, violet and blue colours of fruits, vegetables and flower petals are due largely to anthocyanins. They are water-soluble phenolic compounds located in the cell vacuole and accumulate in the epidermal cells of fruits, flowers, roots and leaves and their main function in the plant is to attract insects, birds and other animals for pollination of flowers and seed dissemination (Mazza and Miniati, 1993a). Anthocyanins also function as light screens in leaves to protect against UV radiation and have been associated with resistance to pathogens in the *Brassica* species, sunflowers, pea seedlings and maize (Barber, 1965; Weisaeth, 1976; Burlov and Kostyuk, 1976; Kraft, 1977; Hammerschmidt and Nicholson, 1978). Humans and animals have consumed anthocyanins since the beginning of time without any ill effects. Anthocyanins have the been found to reduce capillary fragility and permeability, have antiulcer activity and protect against UV radiation, urinary tract infections and night blindness (Kano and Miyakoshi, 1976; Boniface *et al.*, 1986; Cristoni and Magistretti, 1987; Ofek *et al.*, 1991; Morazzoni and Bombardelli, 1996). Thus, they have been considered to be desirable substitutes for synthetic food colours which are under increased consumer scrutiny and must undergo extensive testing (Brouillard, 1982).

Anthocyanins belong to the family of chemicals known as the flavonoids, which are structurally characterized by a carbon skeleton made up of a $C_6C_3C_6$ unit. Anthocyanins strongly absorb visible light and can create an infinite variety of colours in plants. In plants, the pH of vacuole sap is always slightly acidic or neutral (Asen *et al.*, 1972). However, in experiments where environmental conditions such as pH and temperature were kept as close as possible to natural conditions, most anthocyanins were stable but in a colourless form. Therefore, it appears

mechanisms exist *in vivo*, which stabilize the coloured forms of anthocyanins. Substances which coexist with anthocyanins in cells include other phenolic compounds, proteins, amino acids, sugars, organic acids and minerals; these other compounds are known to prevent colour loss or enhance the colour through the phenomenon known as copigmentation. In order to understand copigmentation and its two types, inter- and intramolecular copigmentation, one must understand the structure of anthocyanins and the transformations that they undergo in water.

Naturally occurring anthocyanins are derivatives of the flavylium chromophore which is also known as 2-phenyl-1-benzopyrylium (Brouillard, 1981; Dangles and Elhajji, 1994). The basic flavylium chromophore, depicted below, consists of A, B and C rings (Figure 2.1), with the A and C rings forming 1-benzopyrylium ring and the B ring or phenyl group attached at the 2 position.

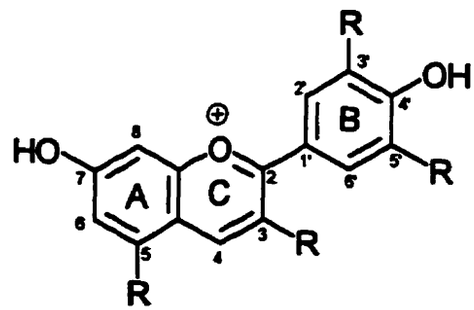


Figure 2.1: Flavylium chromophore.

Anthocyanins are usually isolated in the form of flavylium salts, the precise composition of which depends on the type of acid used in the isolation procedure. The flavylium cation represents the backbone of the anthocyanin to which hydroxyl (OH), methoxyl (OCH_3) and glycosyl groups are attached to give the tremendous variety and combinations of anthocyanins found in nature. The anthocyanidins are the anthocyanins without a sugar moiety. The six most common anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, malvidin and petunidin (Figure 2.2).

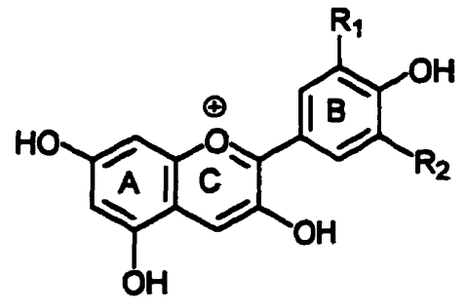


Figure 2.2: Common anthocyanidins and their absorbance maxima (λ_{max}).

Anthocyanidin	R_1	R_2	λ_{max} (nm)
Pelargonidin	H	H	520
Cyanidin	OH	H	535
Delphinidin	OH	OH	546
Peonidin	OCH_3	H	532
Petunidin	OH	OCH_3	543
Malvidin	OCH_3	OCH_3	542

(Timberlake, 1980)

Variation of anthocyanin colours is due to the differences in the degree of hydroxylation of the B ring, glycosylation of the C and/or B ring, acylation of the sugars, and pH and temperature of the milieu in which they are viewed (Mazza and Brouillard, 1987). At a low pH (pH<1), the colours for the six main anthocyanidins range from orange for pelargonidin, and orange-red for cyanidin, to red for peonidin, and to bluish-red for malvidin, petunidin and delphinidin (Robinson and Robinson, 1931; Francis, 1989a). Anthocyanidins are unstable and insoluble in water; however, glycosylation confers solubility and stability to the molecules. The most common glycosylating agents are monosaccharides such as glucose, galactose, rhamnose and arabinose as well as di- and trisaccharides (Timberlake and Bridle, 1975). In almost all anthocyanins, glycosylation occurs through the C-3 hydroxyl group of the C-ring. When more than one sugar moiety is present, the sugar may be attached to any of the hydroxyl groups present at the C-5, C-7 C-3', C-4' or C-5' positions on the rings. The sugars of the anthocyanin can be acylated with a variety of acids,

including acetic, malonic, succinic, benzoic, sinapic, ferulic, caffeic or cinnamic to form mono- or polyacylated anthocyanins (Harborne, 1967; Timberlake and Bridle, 1975; Brouillard, 1988; Mazza and Miniati, 1993). Acylation is important since the acyl groups interact with the pyrylium ring of the flavylium chromophore (Goto *et al.*, 1982) or they promote the association of the flavylium cation with a flavonoid molecule (Goto *et al.*, 1979). This interaction is the basis for copigmentation, which results in the tremendous variety of colours in foods containing anthocyanins.

An example of an acylated and glycosylated anthocyanin is platyconin which has a diacylated side chain (Figure 2.3). The structure was elucidated by Goto *et al.* (1983) using ^{1}H-NMR spectrometry. Of the six hydroxyl groups on delphinidin, four are unsubstituted. At C-3, a rutinosyl residue is present and at C-7, a linear chain of alternating glucosyl and caffeoyl groups is present. The variety of compounds possible, owing to the varying degrees of substitution and types of substituting agents accounts for the tremendous diversity of anthocyanins in fruits, vegetables, grains, leaves and flowers.

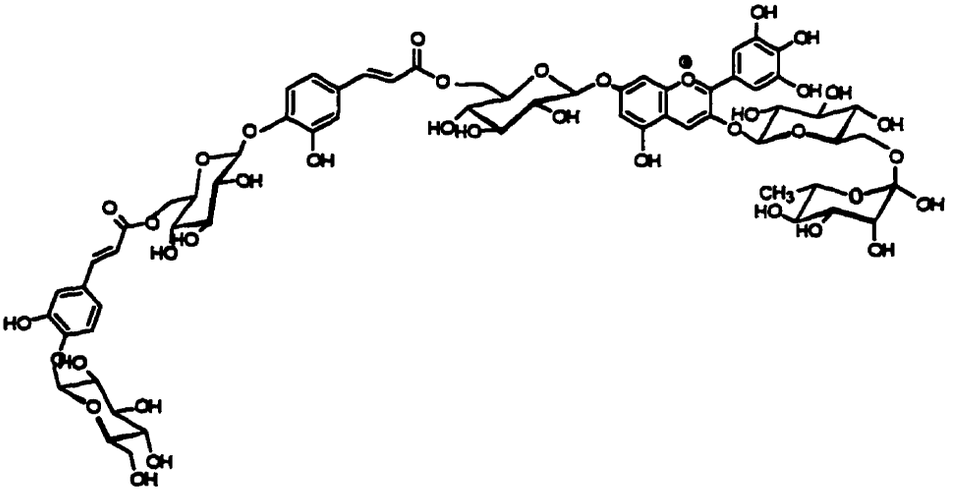


Figure 2.3: Structure of platyconin, the anthocyanin of *Platycodon grandiflorum* flowers. (Goto *et al.*, 1983).

2.2 Structural transformation of anthocyanins in aqueous solutions

The colour of a solution containing anthocyanins is dependent primarily on the structure of the anthocyanin present. Non-acylated and monoacylated anthocyanins behave like pH indicators, since they are red at a low pH, bluish at an intermediate pH and colourless at high pH (Mazza and Brouillard, 1987). The structural transformations observed when the pH of a solution is increased or decreased in an acidic or neutral anthocyanin solution were clarified by Brouillard and co-workers (1977, 1982, 1983). Through the use of kinetic, thermodynamic and spectroscopic techniques, it was demonstrated that in acidic aqueous solutions at 25°C, the four major anthocyanin species existing in equilibrium are the quinonoidal base (A), the flavylium cation (AH^+), the pseudobase or carbinol (B), and the *E*- and *Z*-chalcone, (C_E and C_Z).

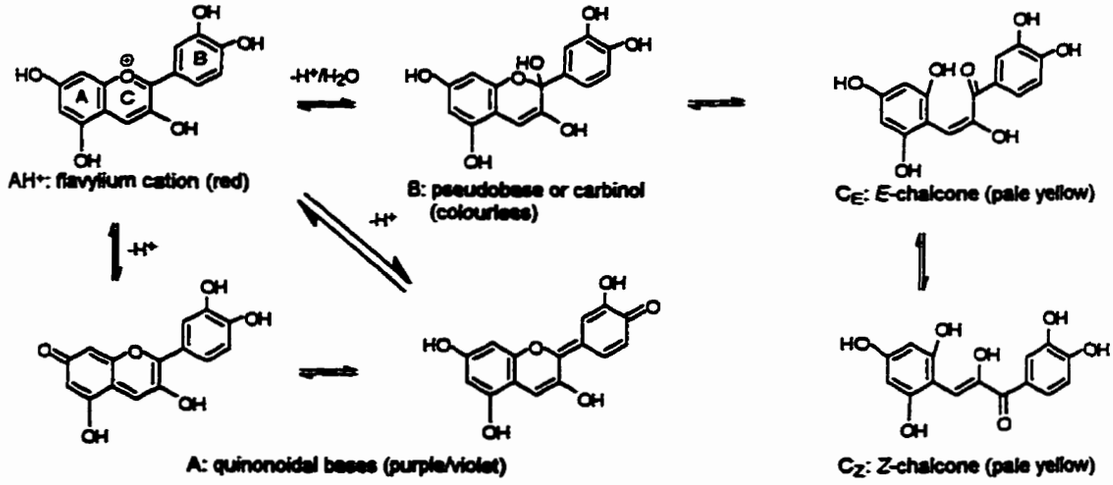


Figure 2.4: Anthocyanin forms in equilibrium.
(Dangles and Brouillard, 1992)

Of the four structures, the flavylium cation (AH^+) and the quinonoidal base (A) are the only coloured forms. At an acidic pH, the flavylium cation (AH^+) is red, the quinonoidal base or anhydrobase (A) is purple/violet, whereas the pseudobase or carbinol or hemiacetal (B) is colourless and the chalcone (C_E and C_Z) is pale yellow. Therefore, the degree of colouring of a solution at a given pH is dependent upon the relative amounts of the four species present

(Brouillard and Dubois, 1977; Mazza and Brouillard, 1987). At a low pH (approximately 0 to 1), the flavylium cation (AH^+) dominates with very little pseudobase (B) present to give a red solution. At pHs above 2.0, the flavylium cation (AH^+) loses protons (H^+) and as a result, blue/purple quinoinoidal bases (A) are formed. The AH^+ may also hydrate to colourless pseudobases or carbinols (B). These, in turn, equilibrate to the open chalcone form (C). Brouillard and co-workers (1977, 1982) demonstrated that the kinetic and thermodynamic competition between the hydration reaction of the pyrylium ring and the proton transfer was related to the acidic hydroxyl groups of the aglycone. They further demonstrated that no hydration of the quinonoidal bases occurs and confirmed the existence of an open chalcone structure.

The mechanisms for the interconversion between the quinonoidal base (A), the flavylium cation (AH^+), the pseudobase or carbinol (B) and the chalcone (C) occurred as is shown in Figure 2.4.

$$AH^+ \xleftrightarrow{K_a} A + H^+ \qquad (1)$$

$$AH^+ + H_2O \xleftrightarrow{K_h} B + H^+ \qquad (2)$$

$$B \xleftrightarrow{K_T} C \qquad (3)$$

Where: $K_a = \left(\frac{[A]}{[AH^+]}\right) a_{H^+}$ K_a = acid-base equilibrium constant

$K_h = \left(\frac{[B]}{[AH^+]}\right) a_{H^+}$ K_h = hydration equilibrium constant

$K_t = \frac{[B]}{[C]}$ Kt = equilibrium constant for ring-chain tautomeric equilibrium

a_H^+ = activity of the hydrogen ion (H^+) or the hydronium ion (H_3O^+)

$pH = -\log a_H^+$

In the acid-base equilibrium (Equation 1), the flavylium cation (AH^+) loses a proton at the C-5, C-7 and/or C-4' hydroxyl groups at a pH of 2-6 and results in the formation of quinonoidal bases in a fast acid-base equilibrium. In natural anthocyanins, most of the AH^+ (red) convert to A (blue) at the pK_a' value, which ranges from 3.50 to 4.85 (Brouillard, 1982; Mazza and Brouillard, 1987). The high acidity of the phenolic groups is due to their positive charge being delocalized over the entire flavylium ring system. If a second acidic hydroxyl group is present in the cation, an ionized quinonoidal base is formed by deprotonation to produce a resonance-stabilized quinonoidal anion in the pH range 6-8. The conjugated double bond system confers good resonance stabilization on the molecule.

In the hydration equilibrium (Equation 2), there is addition of a water molecule to the flavylium cation to yield a carbinol pseudobase and a hydronium ion. Brouillard (1982) determined that 3-O-glycosylated flavylium cations are readily and completely hydrated to the carbinol pseudobases at pH values ranging from 3 to 6. Nucleophilic attack by water at the C-2 position of the C ring is kinetically and thermodynamically favoured, although it may also occur at the C-4 position. When a glycosyl moiety is absent from position 3, the hydration process is less efficient and the carbinol pseudobase only forms at pH values of 4-5. The pK_h values of most anthocyanins are in the range of 2-3 (Brouillard, 1982; Mazza and Brouillard, 1987). The carbinol pseudobases produced are always colourless. Covalent hydration of the flavylium cation occurrs whenever poorly coloured anthocyanin solutions are observed due to the shift in the equilibrium favouring the colourless carbinol pseudobases.

The equilibrium (Equation 3) between the carbinol pseudobase (B) and the chalcone pseudobase (C) is called ring-chain tautomeric equilibrium and has been shown to occur at pH >2 and is favoured by a higher temperature (Brouillard and Delaporte, 1977). At room temperature

and in a slightly acidic aqueous solutions, tautomeric equilibrium is slow to establish because only small amounts of chalcone pseudobase (C) exist for natural anthocyanins under such conditions.

When flavylium salt is dissolved in a slightly acidic or alkaline solution, the neutral and/or ionized quinonoidal bases are immediately formed. This is followed by a slow conversion to the much more stable carbinol pseudobase or its ionized form. As a result, slightly acidic or slightly alkaline solutions of common anthocyanins have weak colour properties when equilibrium is obtained, since the hydration constant (K_h) is 10-100 times larger than the acid-base constant (K_a), which favours the hydration reaction (Brouillard, 1983). Therefore, in plants, one would expect anthocyanins to be poorly coloured. Consequently, to increase their colour and stability, the efficiency of the hydration reaction must be reduced. Consequently, to achieve an intensely coloured anthocyanin solution, good protection of the pyrylium ring against nucleophilic attack by water must be provided. This could be afforded by a reduction of K_h from 10^{-2} - 10^{-3} to 10^{-5} M. It is ironic that anthocyanins, whose natural environment is aqueous, must be protected from hydration of the flavylium cation in order to maximize their colouring capacity (Brouillard, 1988).

2.3 Copigmentation

Copigmentation can be defined as the phenomenon, which allows for the expression of a wide range of brilliant colours by anthocyanins in plants due to the molecular interaction between anthocyanin pigments and copigments. A copigment is a molecule that usually has no colour by itself, but when it is added to an anthocyanin solution, it greatly enhances the colour of the solution (Asen *et al.*, 1972). The copigmentation phenomenon was first documented by Robinson and Robinson (1931), and has since been found to be widespread in plants. The copigmentation phenomenon offers a logical explanation for the vast array of colours *in vivo*, where the

conditions of pH would normally prevent anthocyanins from producing colours (Asen *et al.*, 1970). The molecules found to act as copigments include flavonoids, polyphenolics, alkaloids, amino acids, organic acids and the anthocyanins themselves (Table 2.1) (Asen *et al.*, 1972). These compounds, especially flavonoids and polyphenols are frequently found in the vacuole sap along with the anthocyanins (McClure, 1979). However, only a few such as caffeic acid, chlorogenic acid and rutin (Figure 2.5) have been investigated in detail (Mazza and Brouillard, 1990; Davies and Mazza, 1991; Dangles *et al.*, 1993).

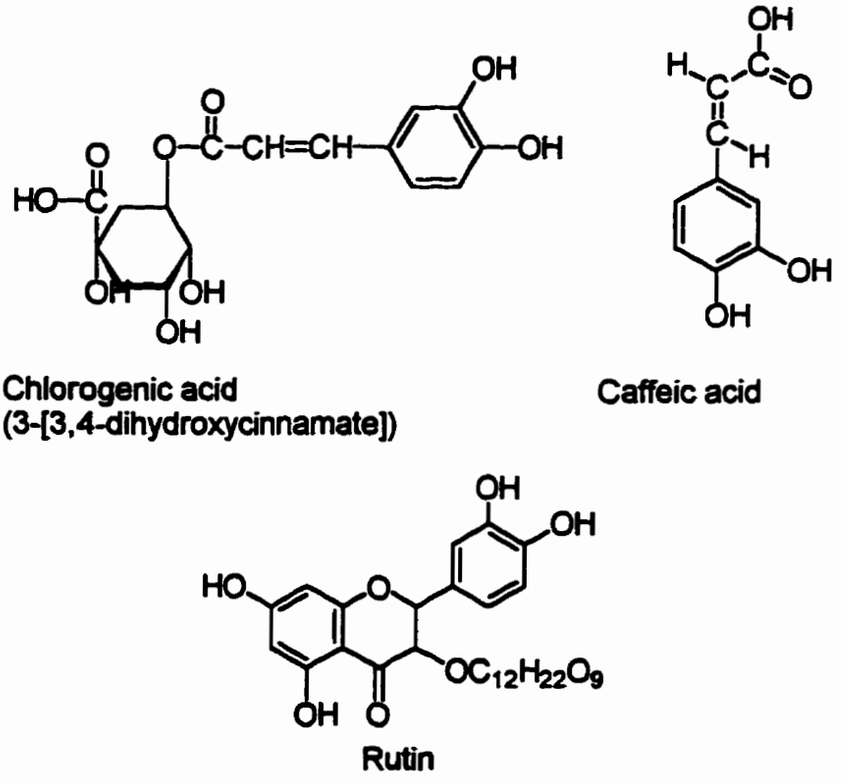


Figure 2.5: Structures of common copigments.

The copigmentation reaction occurs when a copigment is added to a sufficiently concentrated, slightly acidic aqueous solution of anthocyanins and produces an increase in the colour intensity and a change in the colour which is designated as the "bluing" effect (Figure 2.6) (Brouillard *et al.*, 1989). A copigment produces an increase in the absorbance in the visible range, which is known as a hyperchromic effect. The copigment also causes the wavelength of

maximum absorption to shift towards a longer wavelength, known as the bathochromic effect (Asen *et al.*, 1972). Two types of copigmentation are inter- and intramolecular copigmentation. Intermolecular copigmentation occurs when the copigment is non-covalently bound to the anthocyanin molecule, whereas in intramolecular copigmentation, the copigment is a constituent of the anthocyanin molecule itself (Brouillard, 1983).

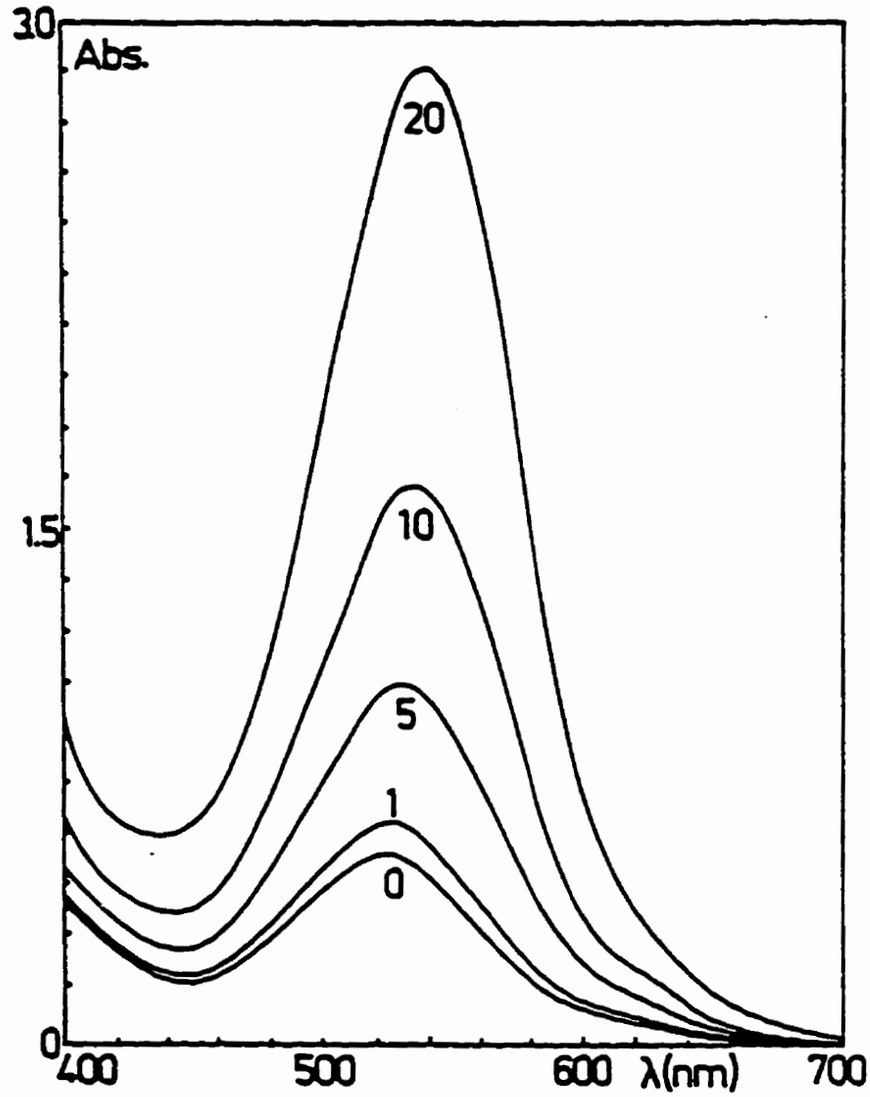


Figure 2.6: Visible spectra of malvin (7.73×10^{-4} M) and chlorogenic acid at 1:0, 1:1, 1:5, 1:10 and 1:20 copigment to pigment molar ratios: pH=3.65; T=20°C; l=1 cm; solvent, aqueous H_3PO_4-CH_3CO_2Na buffer; ionic strength=0.20 M.
(From Brouillard *et al.*, 1989)

Table 2.1: Copigmentation of cyanidin 3,5-diglucoside (2×10^{-3} M) at pH 3.32.

	Copigments (6×10^{-3} M)	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	A/mm at λ_{max}	%A increase at λ_{max}
	None	508	-	0.500	-
Aurone	Aureusidin*	540	32	2.135	327
Alkaloids	Caffeine	513	5	0.590	18
	Brucine	512	4	1.110	122
Amino acids	Alanine	508	0	0.525	5
	Arginine	508	0	0.600	20
	Aspartic acid	508	0	0.515	3
	Glutamic acid	508	0	0.530	6
	Glycine	508	0	0.545	9
	Histidine	508	0	0.595	19
	Proline	508	0	0.625	25
Benzoic acids	Benzoic acid	509	1	0.590	18
	o-Hydroxybenzoic acid	509	1	0.545	9
	p-Hydroxybenzoic acid	510	2	0.595	19
	Protocatechuic acid	510	2	0.615	23
Coumarin	Esculin	514	6	0.830	66
Cinnamic acids	*m*-Hydroxycinnamic acid	513	5	0.720	44
	p-Hydroxycinnamic acid	513	5	0.660	32
	Caffeic acid	515	7	0.780	56
	Ferulic acid	517	9	0.800	60
	Sinapic acid	519	11	1.085	117
	Chlorogenic acid	513	5	0.875	75
Dihydrochalcone	Phloridizin	517	9	1.005	101
Flavan-3-ols	(+)-Catechin	514	6	0.890	78
Flavone	Apigenin 7-glucoside*	517	9	0.840	68
C-glycosyl Flavone	8-*C*-Glucosylapigenin (vitexin)	517	9	1.690	238
	6-*C*-Glucosylapigenin (isovitexin)	537	29	1.705	241
	6-*C*-Glucosylgenkwanin (swertisin)	541	33	2.835	467
Flavonones	Hesperidin	521	13	1.095	119
	Naringin	518	10	0.985	97
Flavonols	Kaempferol 3-glucoside	530	22	1.693	239
	Kaempferol 3-robinobioside-7-rhamnoside (robinin)	524	16	1.423	185
	Quercetin 3-glucoside	527	19	1.440	188
	Quercetin 3-rhamnoside (quercitrin)	527	19	1.588	217
	Quercetin 3-galactoside (hyperin)	531	23	1.910	282
	Quercetin 3-rutinoside (rutin)	528	20	1.643	228
	Quercetin 7-glucoside (quercimeritrin)	518	10	1.363	173
	7-*O*-Methylquercetin-3-rhamnoside (xanthorhamnin)	530	22	1.576	215

*Formed a slight precipitate.

From Asen *et al.* (1972)

2.3.1 Intermolecular copigmentation

2.3.1.1 The mechanism

Brouillard *et al* (1989) and Mazza and Brouillard (1990) determined the chemical mechanism for the copigmentation reaction between anthocyanins and copigments. The mechanism was described as a two stage colour stabilizing and decolouration mechanism and was illustrated as follows:

$$AH^+ \xleftrightarrow{K_h} (B + C_E) + H^+ \qquad (4)$$

$$AH^+ + nCP \xleftrightarrow{K} AH(CP)^+ n \qquad (5)$$

Where: AH^+ = flavylium cation

B = carbinol or hemiacetal

C_E = *cis*-chalcone

CP = copigment

K_h = hydration reaction equilibrium constant

K = complexation equilibrium constant

n = number of copigment molecules linked to the flavylium cation

This general model for the copigmentation reaction was determined using malvidin 3,5-diglucoside (commonly known as malvin) and chlorogenic acid. It was determined that when a copigment such as chlorogenic acid is present with an anthocyanin such as malvin, the copigmentation effect is greatest at a pH of close to 3.6. The flavylium cation of malvin in the total absence of any copigment at this pH exists in the colourless carbinol or hemiacetal B and the *cis*-chalcone (C_E) forms in a fast equilibrium as depicted in equation (4) above. This first step was proven a prerequisite for full expression of plant pigmentation due to flavonoids. The first step resulted in a complete loss of anthocyanin colour due to the nucleophilic attack of water at

the C-2 position and the formation of mainly colourless carbinol structures. Small amounts of the flavylium cation AH^+, as well as a tiny amount of the fast equilibrating quinonoidal bases (A) and the trans-chalcone (C_Z) are present. As seen in Figure 2.5 (Brouillard, 1989), the absorbance of this solution was low due to the low concentration of flavylium cations. It was determined that malvin exists in a fast base-catalysed equilibrium between the carbinol or hemiacetal (B) and the *cis*-chalcone (C_E) (Brouillard *et al.*, 1990). Thus, equation (2) is valid. The K_h is indicative of the ease with which the flavylium cation equilibrates to give hemiacetal (B) and *cis*-chalcone (C_E).

When the copigment chlorogenic acid is added to the malvin solution at equilibrium at pH 3.6, there is a large increase in the absorbance of the flavylium cation, AH^+ (i.e., a hyperchromic shift). The presence of the copigment allows the second step of the mechanism to occur, with the return of colour. This led to the derivation of equation (5), which describes the mechanism of copigmentation. K, the equilibrium constant for the complexation reaction of the anthocyanin and the copigment, is defined as:

$$K = \frac{\left[AH(CP)_n^+\right]}{\left(\left[AH^+\right]_0 \left[CP\right]_0^n\right)}$$

K indicates the strength of the association between the copigment and the anthocyanin's flavylium cation (Brouillard *et al.*, 1989). According to Brouillard *et al.* (1989) the copigment suppresses the hydration reaction of the flavylium cation (AH^+) when in a complex with a copigment and this suppresses decolouration of the anthocyanin. Thus, the copigment protects the flavylium nucleus against nucleophilic attack by water at the C-2 and C-4 positions on the C ring. The loss of colour in an aqueous solution is prevented by the presence of a copigment, which prevents hydration and stabilizes the colour (Brouillard *et al.*, 1990; Mazza and Brouillard, 1990).

There is still some disagreement about whether a copigment stabilizes the flavylium cation or the quinonoidal bases (Scheffeldt and Hrazdina, 1978; Brouillard *et al.*, 1989). Brouillard *et al.* (1989) suggested that at low pH, the flavylium cation is stabilized, whereas at more neutral pH values, the quinonoidal bases are stabilized, but in a different way. These researchers suggested that quinonoidal bases are stabilized in part because of their increased concentration due to the acidities of the C-7 and C-4' hydroxyl groups of the flavylium cation not being affected by the complexation between the flavylium cation and the copigment. Furthermore, quinonoidal bases form weak complexes with the chlorogenic anion at pH values above five (Mazza and Brouillard, 1990). The flavylium cation on the other hand would preferentially be hydrated and therefore stabilized by the copigment such that hydration is prevented.

2.3.2 Factors affecting copigmentation

Factors influencing copigmentation include pH, the chemical structure and concentration of the copigment and pigment, the type of solvent and the temperature of the solution (Mazza and Brouillard, 1989). Mazza and Brouillard (1989) showed that the copigmentation phenomenon is very sensitive to pH. The pH is an important factor of colour expression in plants since anthocyanins exist as stable colourless forms under the normal physiological conditions. The copigmentation reaction takes place over the entire acidic pH range and even extends slightly into the alkaline range (Asen *et al.*, 1970). Anthocyanins act as pH indicators and at a particular pH, the anthocyanin solution contains a mixture of structural species in fast equilibrium. The effect of pH on solution colour is dependent upon the type and concentration of the copigment present. At low pH (i.e., 0.65) in the presence of excess copigment, the absorbance will remain roughly the same with only a slight decrease in the wavelength of maximum absorbance below 530 nm and a slight increase above 530 nm (Brouillard *et al.*, 1991). When chlorogenic acid is added to an

anthocyanin solution at pH 0.65, it causes a slight decrease in absorbance; however, the addition of other copigments such as caffeic acid and protocatechuic acid may result in no change in absorbance, or may actually cause the absorbance to increase. However, at a pH of 4, the absorption increases at all wavelengths in the visible region in a concentration-dependent manner (Brouillard *et al.*, 1991). If a copigment such as chlorogenic acid is added to a malvin system in excessive amounts at pH 3-6, the final absorbance could increase by 20-30 times from its initial value. Solutions of pelargonidin 3-glucoside and monardaein (a diacylated 3,5-diglucoside) have been shown to exhibit a maximum degree of copigmentation over pH ranges of 3.2-3.5 and 3.7-4.7 respectively, when chlorogenic acid was used as the copigment (Davies and Mazza, 1993).

The structure of the anthocyanin will also affect the magnitude of the copigmentation effect. Increasing degrees of the anthocyanin methoxylation on the B ring, as well as glycosylation increases the copigmentation effect (Mazza and Brouillard, 1990). The presence of a methyl substituent on the B ring was found to have a greater effect than diacyl substitution (Davies and Mazza, 1993). The difference in the substitution pattern on the B ring of the pigments malvin, monardaein and pelargonidin 3-glucoside was suggested to be responsible for the difference in the copigmentation effect between the three pigments in the presence of chlorogenic acid (Davies and Mazza, 1993). The copigmentation effect observed with pelargonidin 3-glucoside was less than the effects observed with malvin and monardaein. This phenomenon was attributed to the lower hydration constant of the later two pigments. Consequently, colour recovery was less when compared to a diglucoside (Davies and Mazza, 1993).

The structure of the copigment also determines its effectiveness as a copigment. Chen and Hrazdina (1981) studied 25 flavonoids and determined that a saturated C-2:C-3 bond on the C ring of the copigment decreases the effectiveness of its ability to function as a copigment. The

intensity of the coloured complex formation was dependent upon the number of free hydroxyl groups present on the flavonoid molecule, especially the 7-OH group. Methylation of the 3' and 5'-OH groups of the B ring decreases the ability of the flavonoids to form a complex (Chen and Hrazdina, 1981). Davies and Mazza (1993) determined using the three different phenolic compounds (caffeic acid, chlorogenic acid and rutin) that each of the copigments interacted differently with the pigments studied and affected the magnitude of copigmentation. The lack of a quinic acid moiety in caffeic acid when compared to chlorogenic acid (5-caffeylquinic acid) may increase the effectiveness of caffeic acid as a copigment (Davies and Mazza, 1993). Thus, it is the structure of the copigment and its affinity for the predominant pigment structure, which governs the magnitude of the copigmentation effect (Mazza and Brouillard, 1990).

The colour intensity of the anthocyanin-copigment solution is also determined by temperature (Mazza and Brouillard, 1990). As temperature is increased to 90°C, the copigmentation effect declines (i.e., more weakly coloured solutions result). If the anthocyanin-copigment solutions are subsequently cooled to 20°C, the absorbance and wavelength of maximum absorbance (λ_{max}) are restored. This indicates that neither the anthocyanin nor the copigment is destroyed. The reason for the colour restoration has been attributed to the unique solvent properties of water (Mazza and Brouillard, 1990). The extent of copigmentation is also affected by the nature of the solvent used (Brouillard *et al.*, 1989; Mazza and Brouillard, 1990). Water is the most common solvent for anthocyanins and it has unique properties differing from other polar solvents (Franks, 1972). The structure of water is a random, three-dimensional network of hydrogen-bonded water molecules which associate tetrahedrally (Stillinger, 1980). This lattice structure forces the anthocyanin and copigment into close contact by hydrophobic interactions to form a planar sandwich (Brouillard *et al.*, 1989; Goto *et al.*, 1986). When the temperature increases, the lattice structure is weakened, resulting in the weakening of the

association between the flavylium cation and the copigment (Brouillard *et al.*, 1989; Mazza and Brouillard, 1990). Therefore, the effect of temperature on copigmentation is indirect since it breaks the tetrahedral network of water at higher temperatures. As the temperature is decreased, the tetrahedral molecular network of water is restored. The absorbance of the solutions therefore increases due to the increase in the hydrophobic interactions between copigment and anthocyanin (Brouillard *et al.*, 1989).

The phenomenon of colour decrease and restoration also occurs when solvent and ionic strength are altered. When the ionic strength of the solvent is increased by adding sodium chloride, the effect of the chlorogenic acid copigment is slightly reduced (Mazza and Brouillard, 1990). This is because sodium chloride reduces the polarity of the water, creating disorder. Addition of solvents such as methanol or ethanol to copigmented anthocyanin solutions also reduces the copigmentation effect due to the reduced polarity of the solution. The lattice structure of water is disrupted, which weakens the hydrophobic interactions between copigment and anthocyanin (Brouillard *et al.*, 1989; Mazza and Brouillard, 1990). Therefore, copigmentation is characteristic of a solvent-solute interaction in aqueous solutions in which it occurs most strongly.

2.4 Measurement of the copigmentation effect

The magnitude of the copigmentation reaction can be assessed spectrophotometrically and is described by the relationship

$$\ln\left(\frac{(A-A_0)}{(A_0)}\right) = \ln(Kr_1) + n\ln[CP]_0 \qquad r_1 = \frac{A}{A_0}$$

Where: A_0 = absorbance of anthocyanin solutions at constant wavelength at or near the λ_{max} of the anthocyanin without copigment

A = absorbance of anthocyanin solutions at constant wavelength with copigment

$[CP]_0$ = analytical concentration of copigment

n = stoichiometric constant and the slope of the line

K = equilibrium constant for the complexation reaction and gives the strength of association between copigment and anthocyanin

r_1 = ratio of absorbance of the flavylium cation (at 525 nm) in 0.2 M HCl without copigment (A) over the absorbance (at 525 nm) of the flavylium cation with excessive amounts of copigment added to the same solution (A_c) (Brouillard *et al.*, 1989; Mazza and Brouillard, 1990).

The value of n denotes the stoichiometric constant between copigment and anthocyanin. For example, Mazza and Brouillard (1990) determined that the stoichiometric constant for cyanidin- and malvidin-diglucoside with the copigment chlorogenic acid was approximately 1.0 which means that one molecule of anthocyanin is stabilized by one copigment molecule. When the n value for an anthocyanin : copigment pair is about 0.5, then the association between the two is believed to be in a 1:2 molar ratio.

2.5 Intramolecular copigmentation

Intramolecular copigmentation also serves to protect the flavylium cation from nucleophilic attack by water. Intramolecular copigmentation is much like intermolecular copigmentation except that the copigment is covalently bound to the anthocyanin (Brouillard, 1981, 1982, 1983; Goto *et al.*, 1986; Iaccobucci and Sweeney, 1983). Intramolecular copigmentation is due to the acyl moieties which are attached to the glycosyl moieties which are usually located at positions C-3, 5 and 7 on the A and C rings. The acid groups are usually acetic, caffeic, *p*-coumaric, ferulic, *p*-hydroxybenzoic, malic, malonic, oxalic, sinapic or succinic acid (Mazza and Miniati, 1993b). The acyl moieties and the anthocyanin form a sandwich complex brought about by the three dimensional, tetrahedral water structure, which results in the

dprotection of the flavylium ring from nucleophilic attack by water. Therefore, polyacylated anthocyanins are more stable than nonacylated anthocyanins over the entire pH range.

The first polyacylated anthocyanin to be isolated was platyconin (or dicaffeoyldelphinidin-3-rutinoside 5-glucoside) a blue-violet coloured anthocyanin from *Platycodon grandoflorum* (Saito *et al.*, 1971). The largest anthocyanin ever isolated was the main pigment of *Ipomoea tricolour* Cav. cv Heavenly Blue which was peonidin 3-(dicaffeoylsophoroside)-5-glucoside, commonly known as heavenly blue anthocyanin (HBA). HBA, like most other polyacylated anthocyanins has a highly stable colour in a neutral solution and copigments such as quercetin have no effect on its colour. Using ^{1}H-NMR, the structure and conformation of HBA was determined (Goto *et al.*, 1982). By careful nuclear overhauser effect (NOE) measurements, the folded conformation of HBA was determined. Based on these results, one could make a model of HBA in the form of intramolecular sandwich-type stacking. In the model, the aromatic residues of acyl groups are stacked above and below the pyrylium ring of the flavylium cation. This greatly reduces the nucleophilic attack by water at the C-2 and the C-4 positions as the pH of the solvent increases. Consequently, water is prevented from hydrating the flavylium cation, which would otherwise form the colourless pseudobase. The flavylium ion, as well as the anhydrobase, are thus stabilized (Brouillard, 1981) resulting in the stabilization of the colour usually exhibited at a lower pH.

Another acylated anthocyanin which has been identified is gentiodelphin (Goto *et al.*, 1984). Gentiodelphin has a bluish-violet colour and is stable in weakly acidic or neutral aqueous solutions. Although the caffeic acid moieties on the glucosyl anthocyanidin moiety are different, it is hypothesized that sandwich type conformations occur, similar to those seen with HBA. Therefore, the anthocyanins were probably stabilized intramoleculary by forming the sandwich

type of stacking between the anthocyanidin nucleus and the two aromatic acyl moieties, with the hydrophilic sugar moieties covering them.

Monoacylated anthocyanins are not as stable in their neutral and ionized quinonoidal forms since one side of the pyrylium ring is protected while one side is exposed and prone to nucleophilic attack by water. Intermolecular copigmentation is more efficient with monoacylated anthocyanins than with polyacylated anthocyanins because a copigment must be present to protect the one exposed side. With intramolecular copigmentation, the acyl groups act as the copigment and thus no additional copigment should be theoretically required. But intermolecular copigmentation is more efficient with monoacylated anthocyanins than polyacylated anthocyanins. Intermolecular copigmentation is also more efficient with monoacylated anthocyanins than non-acylated anthocyanins (Hoshino *et al.*, 1980). Nonetheless, intramolecular copigmentation is more efficient than intermolecular copigmentation because it is not necessary to bring the copigment and anthocyanin together. When intramolecular copigmentation occurs, little or no intermolecular co-pigmentation was observed (Saito *et al.*, 1985; Asen *et al.*, 1977).

The structure of the acyl group, its position of attachment on the sugar, as well as the sugar structure and its location on the anthocyanin are also important in intramolecular copigmentation (Saito *et al.*, 1985). The stability of acylated anthocyanins increases with increasing content of organic acids (cinnamic and malonic acids) and also increased substitution of the aglycone (i.e., delphinidin is more stable than cyanidin or peonidin derivatives). The addition of organic acids has a greater stabilizing effect than aglycone structure (Saito *et al.*, 1985). It is unclear why an aliphatic acid such as malonic acid, which does not contain a C_6 ring like in caffeic acid, adds to the stability of the anthocyanin.

Davies and Mazza (1993) evaluated the copigmentation behaviour of monardaein (a diacylated form of pelargonidin 3,5-diglucoside) when it was complexed with the copigments chlorogenic acid and caffeic acid. Unlike all previous studies on copigmentation of polyacylated anthocyanins, they determined that the presence of a copigment such as chlorogenic acid did cause the copigmentation phenomenon to occur although monardaein was a diacylated anthocyanin. This indicates that even a diacylated anthocyanin can undergo copigmentation. As was suggested in the work done by Brouillard and co-workers (1989) and Mazza and Brouillard (1990), a stoichiometric constant close to unity characterized a 1:1 association between the pigment and the copigment. Only chlorogenic acid-based complexes were described as being 1:1. It was suggested by Davies and Mazza (1993) that a lack of a quinic acid moiety in the caffeic acid may have benefited the association of monardaein with the caffeic acid. Monardaein was found to exhibit a maximum degree of copigmentation over a pH range of 3.7-4.7 when complexed with chlorogenic acid. This pH range was much lower than the pH range required for the polyacylated anthocyanins previously studied. Davies and Mazza (1993) concluded that the number of oxygen substituents had a greater influence on the magnitude of copigmentation than the presence of diacylation. The copigmentation complexes formed between monardaein had a two-fold greater magnitude in copigmentation than monoglucosides pelargonidin 3-glucoside over a pH range of 2.7 to 4.7 (Davies and Mazza, 1993).

2.6 Recent advances in intra- and intermolecular copigmentation

Recently, many synthesized 3-glycosyloxylated flavylium ions and their derivatives as well as complex acylated anthocyanins have been studied by themselves and with copigments to determine how they are stabilized in aqueous solutions (Dangles *et al.*, 1993; Dangles and El Hajji, 1994; El Hajji *et al.*, 1997; Figueiredo *et al.*, 1996a and b, Mistry *et al.*, 1991). The earliest of these works studied the effects of caffeine, theophylline, vegetable tannins, ATP, DNA and RNA on the stabilization of the quinonoidal anhydrobase (A) (blue/violet colour) form of

malvidin 3,5-diglucoside (Mistry *et al.*, 1991). Proton NMR (^{1}H-NMR) was used to study the intermolecular copigmentation reaction. Goto (1986) originally proposed that 'Heavenly blue anthocyanin' (HBA) is stabilized via intra- or intermolecular copigmentation by the vertical 'hydrophobic stacking' of aromatic nuclei of both the anthocyanin and copigment. Brouillard *et al.* (1989) agreed and proposed that copigmentation was unique to an aqueous environment. Brouillard *et al.* (1989) and Brouillard and Mazza (1990) suggested that the copigment stabilizes the flavylium cation since their interaction results in a flavylium cation-copigment complex which does not hydrate. Consequently, more flavylium ions than other forms would be present at a given pH. At an intermediate pH of 3.7, copigmentation is the result of the flavylium cation-polyphenol complex and the shift of the equilibrium towards the flavylium cation (Mazza and Brouillard, 1990; Brouillard *et al.*, 1986). Using the slope from plots of $\ln((A-A_0)/A_0)$ versus $\ln[CP]_0$, malvidin 3,5-diglucoside was found to complex with each of quercetin 3-β-D-galactoside, β-1,2,3,4,6-pentagalloyl-D-glucose and aesculin in a 1:1 anthocyanin : copigment ratio (Mistry *et al.*, 1991). The effectiveness of the copigment is related to its planar structure, the potential surface area available for hydrophobic or 'π-π' overlap, and its electron-donating capacity (Mistry *et al.*, 1991). The N-methylated xanthines caffeine and theophylline were observed to have distinctive copigmentation effects (Mistry *et al.*, 1991). For example, caffeine and theophylline resulted in a shoulder at 620 nm when copigmented with malvidin 3,5-diglucoside, and the solution was red-violet due to the stabilization of the quinonoidal anhydrobase at pH 3.42. Copigmentation at pH 4.99, 6.20 and 6.85 led to violet and blue forms due to stabilization of the quinonoidal anhydrobase and the carbinol pseudobase. Turquoise-green colours were observed at pH values greater than 7.0. At pH 4.99, caffeine copigmentation showed a hyperchromic effect, but no bathochromic shift. At low pH values, galloyl esters and quercetin 3-β-D-galactoside were more effective copigments; however at higher pH values, caffeine and theophylline were better copigments than chlorogenic and p-coumarylquinic acid

(hydroxycinnamyl esters), galloyl esters, RNA, DNA and ATP (Mistry *et al.*, 1991). This lead Mistry *et al.* (1991) to conclude that since phenolic esters are electron rich systems, they associate strongly with the electron-deficient flavylium cation. Hydrophobic effects initially drive the association in an aqueous system.

Using four pelargonidin derivatives found in *Pharbitis nil* (morning glory) which have varying degrees of cinnamic acid acylation, competitive intra- and intermolecular copigmentation experiments illustrated that the former is a more efficient stabilization reaction (Dangles *et al.*, 1993). The varying degrees of cinnamic acid acylation and glucosylation on pelargonidin resulted in different colours and colour stability, with the wavelength of maximum absorbance ranging from 496 to 510 nm. Such a large bathochromic shift is usually attributed to intermolecular copigmentation; however, Dangles *et al.* (1993) showed that this large shift is due to intramolecular copigmentation. The glycosyl moieties act as spacers which act as covalent links between anthocyanidin and copigment. The glycoside must not cause stearic hindrance. Thus the strength of intramolecular copigmentation is determined by the position of cinnamic acid residue on the glycoside, the position of the spacer on the anthocyanidin, and the length of the glycoside spacer (mono-, di- or triglycoside) (Dangles *et al.*, 1993). A large spacer such as a β-diglucoside in alatanin C (Yoshida *et al.*, 1990) with a sinapic acid residue can promote colour stability comparable to that observed for a polyacylated anthocyanin, which is stabilized though a sandwich-type complex. From spectral data and the hydration equilibrium rate constant, two of the monoacylated β-D-glucosyl-β-galactoside pelargonidin pigments were more resistant to hydration than the non-acylated pigment. However, when compared to alatanin C, the diglucosyl spacers are shorter, resulting in less chromophore protection against nucleophilic attack at the C-2 position. The polyacylated pelargonidin derivative has a intermolecular copigmentation binding constant (K), 3 times smaller than the nonacylated pigment. The two caffeic acid residues present

in the molecule protect both sides of the molecule via a sandwich-type complex. Dangles *et al.* (1993) found the intermolecular copigmentation binding constant, K, for the non-acylated pelargonidin 3-sophorosyl 5-diglucoside to be small when compared to that of malvidin 3,5-diglucoside (Dangles and Brouillard, 1992). This is probably due to the bulkier sophorosyl group at the 3-position of pelargonidin 3-sophorosyl 5-glucoside when compared to a glucosyl group with malvidin 3,5-diglucoside. The single caffeic acid in the monoacylated pelargonidin 3-6-*trans*-caffeyl-sophoroside 5-glucoside does not exert a well defined intermolecular copigmentation binding constant (Dangles *et al.*, 1993). When the polyacylated pelargonidin 3-sophorosyl 5-glucoside was copigmented with chlorogenic acid, the stacked caffeic acid residues on either side of the molecule provide enough stearic hindrance that even the excessive addition of chlorogenic acid does not produce a hyperchromic shift. Thus, by binding one or two well placed copigment molecules on glycosyl residues, stable coloured anthocyanins at high pH can be achieved without excessive amounts of copigment in the vacuolar sap (Dangles *et al.*, 1993). Their work illustrated that deep stable colours can be better achieved through intramolecular versus intermolecular copigmentation where large quantities would be required to achieve the same colour intensity.

Dangles and Elhajji (1994) found that glycosyloxy group was less effective than a methoxyl group at C-3 at stabilizing the positively charged flavylium chromophore by enhancing the electrophilic character at C-2. The powerful electron-withdrawing effect of the pyranose ring on the '*exo*'-anomeric O-atom was illustrated using malvidin 3-glucoside and malvidin 3,5-diglucoside. When the C-5 hydroxyl group is glycosylated, the stabilizing effect of the 3-glycosyl group is lost due to the strong electron-withdrawing effect of the glycosyloxy moiety on the '*exo*'-anomeric O-atom. This is reflected in the pK_a values for 5-deoxy callistephin, delphinidin 3-glucoside, malvidin 3-glucoside and malvidin 3,5-diglucoside. When chlorogenic acid (alternatively known as 5-O-caffeoylquinic acid) is copigmented with a simple synthesized

model anthocyanin, 3-methoxy flavylium, it is found to form 2:1 copigment : pigment complexes. However, when chlorogenic acid was copigmented with glycosylated anthocyanins such as delphinidin 3-glucoside, malvidin 3-glucoside and malvidin 3,5-diglucoside, the glycosyl groups caused stearic hindrance on one side of the chromophore and prevented the approach of a second copigment. Through molecular modelling calculations of delphinidin 3-glucoside and malvidin 3,5-diglucoside, it was determined that the glycosyl groups lay outside of the benzopyrylium plane. Between these monoglycoside and the diglucoside, it was found that the delphinidin and malvidin 3,5-diglucoside's adopted a *cis*-arrangement with respect to the benzopyrylium plane and had a similar K value (binding constant). The 5-glucosyl group of malvidin 3,5-diglucoside did not oppose the binding of chlorogenic acid when compared to malvin 3-glucoside. The larger colour gains observed between 3-monoglycosides and 3,5-diglycosides at the same pH is due to thermodynamics of the hydration reaction. No colourless chalcones of the anthocyanin were found to interact with chlorogenic acid.

When non-glycosylated 3-methoxyflavylium was copigmented with caffeine at a weakly acidic pH of 3.7, it was observed that colour was increased up to a pigment : copigment ratio of 1:250; however, at a pigment : copigment of 1:700, the colour of the solution was reduced to a value less than that for the original solution containing no copigment. The loss of colour was attributed to hemiacetal and chalcone forms forming 1:1 and 1:2 complexes. It was found that the concentration of the *Z*-chalcone increased with addition of caffeine when monitored at 370 nm. When caffeine was copigmented with glycosylated pigments, colourless forms would also arise. However, their effect was not large enough to cause colour loss at high caffeine concentrations. The anthocyanin : caffeine association appeared to be 1:1 for anthocyanins, no matter the degree of glycosylation; however, the stoichiometric relationship between caffeine and the colourless forms was dependent upon the substitution pattern of the anthocyanin. With malvidin 3-glucoside, there was strong 1:2 complexation and with the 3, 5-diglucoside, there was strong 1:1

complexation. A possible explanation might be that the weak, non-covalent self-aggregates (dimers) of caffeine in aqueous solutions, interact with the hemiacetals and chalcones (Gill *et al.*, 1967).

Figueiredo *et al.* (1996b), using two series of structurally related anthocyanins from *Evolvulus pilosus* cv. Blue Daze and *Eichhornia crassipes*, which were acylated and glycosylated to varying degrees, determined that intramolecular hydrophobic interactions exist between the flavylium ion and the acid moiety. The sugar moieties were determined to affect the pigment's degree of hydrophobicity. Since all pigments had the same chromophore, the varying degrees of glycosyloxy substitution at positions C-3 and/or C-5 resulted in colour changes. At the same pH, larger bathochromic shifts and molar extinction coefficients (ε) occurred with greater amounts of acid substitution. A folded conformation can be achieved if the substituent group is long enough to fold over the pyrylium ring, thereby protecting the active C-2 and C-4 sites from nucleophilic attack by water. From the calculated hydration constants for each pigment, it was found that low hydration rate constant (k) values were synonymous with greater protection against nucleophilic attack due to increased acylation. The pK_h which denotes the rate constant for the equilibrium between the flavylium cation and the colourless hemiacetal and chalcones, increased in the series as the glycosyl chains became longer and more linear. This is indicative of intramolecular complexation between the planar rings of the aromatic acid and the planar pyrylium ring through π-π hydrophobic interactions. The rate of thermal degradation for the pigments with a folded conformation is inversely related to the increased degree of substitution. Delphinidin 3-gentiobioside, a linear disaccharide, had a large decrease in the molar absorption/extinction coefficient which also has been found in other 3-biosidic residues. In the hemiacetal form of delphinidin 3-gentiobioside, a hydrogen bond can form between the C-2 OH group and the free C-6 OH in the second sugar. This study on these anthocyanins strengthens the theory that 3-

monoglucosides are more resistant to nucleophilic attack than 3,5-diglucosides. It has been attributed to the '*exo*'-anomeric oxygen's electron-density being delocalized toward the pyranose ring, thereby reducing its capability to donate electrons towards the pyrylium ring, when a hydroxyl group is replaced by a β-D-glucopyranosyloxy group. The electrophilic character at the C-2 and/or C-4 positions is increased by this sugar substitution. Figueiredo *et al.* (1996b) concluded that the chalcone and hemiacetal formation is blocked by the intramolecular copigmentation between the planar pyrylium ring and the aromatic acids. With the presence of a disaccharide at C-3, the colourless hemiacetal form could be stabilized.

Figueiredo *et al.* (1996a) reported on a group of five natural cyanidin anthocyanins extracted from violet *Matthiola incana* flowers, four of which were acylated. These acylated anthocyanins deviated from the usual intramolecular, noncovalent interactions, which stabilize quinonoidal bases and flavylium cations. The five pigments were derivatives bearing sambubioside (2-O-(β-D-xylopyranosyl)-β-D-glucopyranosyl) at position C-3 and β-D-glucopyranosyl at the C-5 position. Acylation with malonic acid took place at C-6 of the 5-glucoside, with two cinnamic acid derivatives at the 6-gluco and 2-xylo positions of the sambubioside. The different acylation and glycosylation patterns caused a bathochromic shift and a small decrease in the ε as the structure became more substituted. In mildly acidic solution, there was an overall colour gain with the more acylated and glycosylated pigments. At mildly acidic pH values of 2.1 to 3.5, a shoulder characteristic of the quinonoidal base form appeared in the 550-650 nm region of the spectrum. As the pH increased, the shoulder increased and the flavylium cation absorption decreased gradually. When the absorbance of the flavylium cation was subtracted from the spectrum, the quinonoidal base was shown to contribute significantly to the spectra at a very low pH. This was unusual, since the quinonoidal base is usually observed

close to a neutral pH. To account for the observed behaviour of these four pigments at low pH, the following mechanism was proposed:

$$AH^+ \xleftrightarrow{K_t} CP \qquad (6) \qquad K_t = \frac{[CP]}{[AH^+]}$$

$$CP + H_2O \xleftrightarrow{K_{h\sigma}} B + H^+ \qquad (7) \qquad K_{h\sigma} = \frac{[B][H^+]}{[CP]}$$

$$CP \xleftrightarrow{K_{a\sigma}} A + H^+ \qquad (8) \qquad K_{a\sigma} = \frac{[A][H^+]}{[CP]}$$

(Figueiredo, *et al.*, 1996a)

Equation (6) describes the equilibrium between the flavylium cation (AH^+) at a very acidic pH <1, and the intramoleculary copigmented form (CP) at a pH of 1-2. The intramolecular complex occurs only between the chromophore and the acyl residue and it will subsequently undergo the hydration (equation 7) and deprotonation (equation 8) reactions. The hydroxyl groups located at C-4' and C-7 are deprotonated to form the quinonoidal bases. The proximity of the malonic acid to the C-7 hydroxyl group can result in a hydrogen bond, aiding deprotonation and decreasing the pK_a. Therefore, the hydroxyl groups of the aglycone are free so that the proton transfer can result and form a quinonoidal base. In the four acylated *Matthiola* anthocyanins, the difference in hydration protection is determined by the relative position of the aromatic acid ring relative to the B ring of the aglycone to form a sandwich structure. Increased proximity of the acid ring to the B ring of the aglycone decreases the possibility of nucleophilic attack by water. The colour stabilization and variation in *Matthiola incana* flowers arises due to formation of the quinonoidal base at low pH values. The malonyl groups lower the pK_a and protect against colour loss, enabling the flavylium cation and quinonoidal base to exist over a very large acid pH range.

El Hajji *et al.* (1997) proposed an alternative mechanism for the structural and spectral changes taking place with the malonylated *Matthiola* anthocyanins. The mechanism, which depends upon the acid-base and hydrogen bonding properties of the malonyl group, is illustrated below in Figure 2.7.

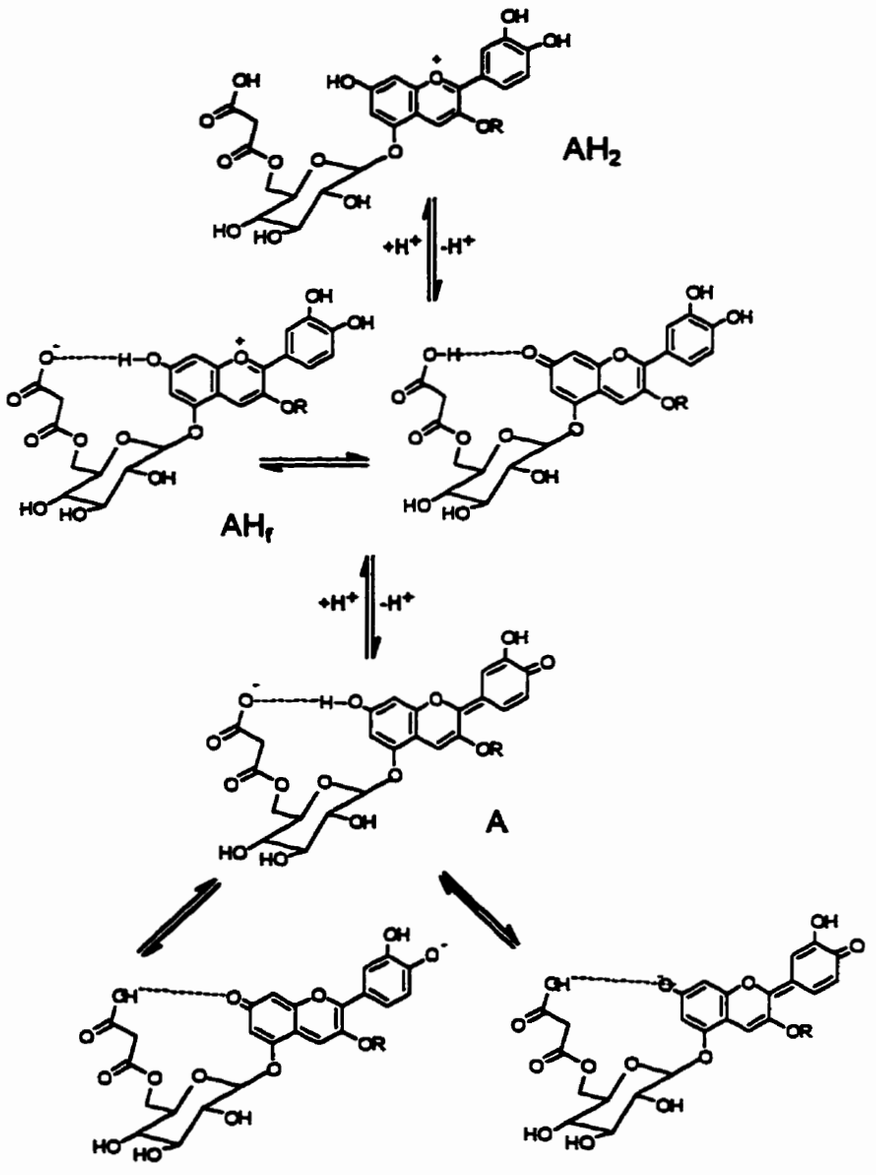


Figure 2.7: Postulated acid-base forms of malonated anthocyanins in strong to mildly acidic aqueous solutions.
(From El Hajji *et al.*, 1997)

In this mechanism, a proton transfer reaction initially occurs between the cationic AH_2 form, where the malonyl group is in a neutral protonated state, and the zwitterionic AH form where the malonyl group is in an anionic (deprotonated) state. This allows the zwitterionic AH form in an 'open' conformation to exist in equilibrium with the AH in a 'folded' conformation (AH_f) where the malonyl group and flavylium cation are hydrogen bonded. The folded AH_f conformation consists of two tautomers, the zwitterionic malonate-flavylium structure and the neutral malonic quinonoidal structure. Each of the AH_f conformations is able to form hemiacetal B by water addition. As the pH is further increased ($-H^+$), a proton transfer reaction occurs between AH_f and A, the anionic form. This anionic form A has two tautomers, one with a neutral quinonoid chromophore and anionic malonic group and the other with an anionic quinonoid chromophore and a neutral malonic group. It is assumed that the forms where the aromatic acyl residues is stacked on the chromophore and stabilized via intramolecular copigmentation dominate in the solution. At a pH of less than 1, the absorbance is due to the predominance of the AH_2 form. As pH increases to greater than 1.5, the AH, AH_f, A and B forms predominate.

Using this postulated mechanism, the observed spectral changes in the three malonylated pigments from *Matthiola incana* (stock) described by Figueiredo *et al.* (1996a) were reinterpreted by El Hajji *et al.* (1997). According to these authours, a pH of 0.7, the anthocyanins are in the cationic AH_2 form. As the pH is increased to 1.5, the malonyl carboxyl group is deprotonated, resulting in the zwitterion AH in equilibrium with AH_f. The zwitterionic AH form has a hydrogen bond between the carboxylate group of malonic acid (acceptor) and the acidic phenolic hydroxyl group of the flavylium donor at the 7-OH group. In the neutral AH form, the hydrogen bond occurs between the protonated malonyl group and the 7-keto group of the quinonoidal base. It was determined that the structure of AH_f is more accurately depicted by the neutral tautomeric form of AH_f than the zwitterionic form due to the quinonoidal base's shoulder. The lower pK_a of the malonyl carboxyl group is attributed to electrostatic interactions and hydrophobic stacking.

The decrease in absorbance intensity is attributed to the quinonoidal structure and the tightly packed acyl residues around the chromophore, resulting in lower molar extinction coefficients. The decrease in pK_a enables the quinonoidal bases to take part in the colour expression. El Hajji *et al.* (1997) using molecular-modelling calculations on glycosylated synthesized flavylium ions copigmented with chlorogenic acid, found that 1:1 flavylium-copigment complexes occurred. The simple synthesized flavylium pigment capable of copigmenting on both sides underwent 1:1 binding. When caffeine is copigmented with the synthetic pigments, it observes 1:1 vertical stacking interaction, selective of the colourless chalcones and results in colour loss. It has been shown that malvidin 3,5-diglucoside and caffeine copigmentation is stronger with neutral quinonoidal bases than with the flavylium ion (Dangles and Brouillard, 1992). The positively charged ion interacts weakly with the electron poor purine nucleus of caffeine but the neutral planar anthocyanin forms are attracted to caffeine.

2.7 Storage stability of copigmented anthocyanins

Over the years, a number of researchers have conducted stability studies on anthocyanins under different conditions in order to determine their potential as alternative food colourants. Green and Mazza (1988) studied the effect of acetaldehyde and catechin on a cyanidin 3-glucoside (1×10^{-4} M) in pH 3.5 sodium acetate – phosphoric acid buffer stored at 23°C in the dark. The UV-visible spectrum was monitored along with the HPLC profiles of the various pigment and copigment solution combinations over a 20 day storage study. It was determined that the addition of both catechin and acetaldehyde caused a 40% increase in absorbance and a 13 nm shift in the λ_{max}. The colour increase was attributed to the formation of Baeyer-type condensation of highly coloured intermediates composed of anthocyanins and catechin linked by CH_3CH bridges. The HPLC profiles indicated the presence of six new peaks while the peak areas of catechin and cyanidin 3-glucoside decreased, indicating that these new compounds have

components consisting of catechin and cyanidin 3-glucoside. When an aqueous saskatoon berry extract without acetaldehyde and catechin was monitored, the spectra exhibited a hypsochromic shift and a 32% decrease in absorbance. The absorbance decrease was attributed to the oxidative degradation and/or polymerization of the cyanidin 3-monoglycosides to colourless compounds. The presence and increased concentration of acetaldehyde in the saskatoon berry extract caused colour intensification. A violet precipitate was observed in the solution containing acetaldehyde after 10 days of storage. It was believed to be composed of acetaldehyde-anthocyanin polymers. The pigments did not form in the methanolic saskatoon extract since they were believed to be more soluble in water.

Liao *et al.* (1992) studied copigment complexes which formed with malvidin 3,5-diglucoside in a model red wine system using (-)-epicatechin, (+)-catechin and (+)-catechin-3-O-gallate as copigments. When malvin with and without the three types of catechol solutions were exposed to light for 44 months at 22°C, the absorbance at the wavelength of maximum absorbance decreased by 25%. Changes in colour and spectra were also noted over time in solutions containing copigment with an active phloroglucinol nucleus. While the absorption maximum at 525-540 nm decreased, there was a progressive increase in absorbance from 430-445 nm as the colour of the solution changed from red to yellow-orange. This colour change was due to a yellow-orange soluble copigment-pigment complex with an electron-deficient aromatic π system, which also copigmented with the residual copigment.

Miniati *et al.* (1992) determined that copigmented anthocyanin solutions stored at 5°C were more stable than samples stored at 20°C. They also found that during storage of a copigmented anthocyanin solution, all solutions lost colour. The most stable solutions were copigmented with quercetin, and gallic acid, with catechin showing poor colour stability. A pH

of 2.5 had the best colour retention, with pH 3.5 being slightly less and pH 4.5 having the poorest colour stability.

Baublis *et al.* (1994) compared the anthocyanin extract stability of Concord grapes, red cabbage, tradescantia and ajuga stored over 15 days in pH 3.5 sodium citrate buffer at 30°C and exposed to intense light (250 μmole sec^{-1} m^{-2}) from fluorescent lamps using HPLC and spectrophotometric methods of analysis. The tradescantia extracts had the greatest stability compared to the other extracts. The major anthocyanin in the tradescantia extract was hypothesized to be cyanidin 3,5,3'-triglucoside with three molecules of ferulic acid, one molecule of caffeic acid and one terminal glucose molecule (Shi *et al.*, 1993). Further analysis indicated that rutin, chlorogenic acid and caffeic acid were present in 1:3, 1:42, and 1:3 pigment : copigment ratios, respectively. Baublis *et al.* (1994) reported that the low concentration of the copigments did not aid the stability of the tradescantia extract, which degraded in an almost linear fashion when compared to exponential degradation of the other extracts. No brown discolouration was reported in the extracts after 15 days. Baublis *et al.* (1994) reported that the major anthocyanin in the tradescantia extract degraded 57% over 15 days.

Shi *et al.* (1992) conducted a study comparing the stability of anthocyanins in *Tradescantia pallida* extract with blackberry extract containing greater than 95% cyanidin 3-glucoside and enocyanin colourant over a year. The extracts were incorporated into model beverage systems containing 15% sucrose at pH 3.5, 4.5 and 5.5 in a citric acid/dibasic sodium phosphate buffer. The effect of added tannic acid and ascorbic acid was also determined. Tannic acid is reported to be the second most effective copigment (Robinson and Robinson, 1931). Maccarone *et al.* (1987) postulated that tannin formed molecular complexes with the anthocyanins that made them more resistant to chemical, photochemical and enzymatic

degradation. Shi *et al.* (1992) found that tannic acid as a copigment was detrimental to the stability of the anthocyanin solutions after a 4 month period, contrary to reports that it would stabilize the colour. It was postulated that the tannins favoured formation of intermolecular complexes creating less stable compounds. They also determined that the pigment content of the pH 5.5 cyanidin 3-glucoside beverage samples decreased since the pseudobase form is less stable at this pH, unlike the *T. pallida* samples. At pH 4.5, the cyanidin 3-glucoside samples were slightly less stable and at pH 3.5, the samples were more stable but still showed colour loss over increased storage time, relative to the *T. pallida* anthocyanins. It was found that at 16 weeks of storage, the cyanidin 3-glucoside samples had precipitation of pigment at pH 4.5 and 5.5, increasing the Hunter L value. The Theta values also increased with time.

Madhavi *et al.* (1996) compared the stability of the *in vitro* and *in vivo* extracts of major anthocyanins found in *Ajuga pyramidalis* metallica crisp cell cultures to extracts from *Tradescantia pallida* and cranberry, all containing cyanidin derivatives. The extracts were in a pH 3.5 sodium citrate buffer and were exposed to light from fluorescent lamps (total irradiance of 140 $\mu mol\ m^{-2}\ s^{-1}$) at 27°C for 32 days. The *in vitro* extract from the cell culture was more stable since it had a greater percentage of absorbance over the same time frame when compared to the nonacylated cranberry anthocyanins. It was postulated that this might be due to the presence of copigments found in the cell cultures along with the acylation on the major anthocyanin molecule. The formation of a precipitate under their experimental conditions was not reported (Madhavi *et al.*, 1996).

Cormier *et al.* (1997) compared the colour, thermal and pH stability of anthocyanins from grape cell suspension culture to other anthocyanin based food colorants using Hunter Lab and spectrophotometric analysis, and found that brown oxidized phenolics were present in grape skin and elderberry extracts. These samples were stored in a pH 3 citrate buffer system at 30°C over a

35 day period. Due to the presence of phenolics in the extract and the similar conditions used in the study, the brown precipitate was probably due to the formation of a complex between the anthocyanins and the phenolics. The precipitate and discolouration was probably aided by the exposure to intense light.

Inami *et al.* (1996) studied the heat and light stability of acylated cyanidin derivatives found in elderberries (*Sambucus canadensis* and *S. nigra*). It was found that acylation improved the heat and light stability but glycosylation only stabilized the anthocyanins in the presence of light. Cyanidin 3-*p*-coumaroyl-sambubioside 5-glucoside changed to three more complex cyanidin derivatives when exposed to light. Palamidis and Markakis (1975) observed that when grape anthocyanins used to make a carbonated beverage were stored under light and dark conditions, the sample stored in the presence of light always had a lower percent of anthocyanin retention. Van Buren *et al.* (1968) studied the effects of heat and light on wine anthocyanins. They found that when exposed to normal laboratory light, the wines containing monoglucosides tended to increase in the theta value towards yellow, unlike wines containing acylated anthocyanins which had no change or lower theta values.

2.8 Anthocyanins and other phenolics in red onions

Onions (*Allium cepa* L.) are members of the *Allium* family within the genus *Amaryllidaceae*. Other members of the *Allium* family include garlic (*A. sativum* L.), chive (*A. schoenprasum* L.), and leek (*A. ampeloprasum* L.) (Bailey and Bailey, 1976).

Onion cultivars can be coloured silvery white, yellow, brownish and red (Lee, 1951). The onion is a biennial plant with a large single underground bulb. The bulb is usually a well rounded, flattened sphere when mature. It consists of multiple leaves/scales on the inside, with a dry, membranous outer bulb coat. Although the dried outer scales are inedible, they are usually

the most highly pigmented part of the onion. The inner multiple scales/leaves are more fleshy and edible but become progressively less pigmented as they get closer to the core of the bulb. Pigmentation usually decreases from the top to the bottom of the bulb. In the edible scales/leaves, the pigmentation is localized in the outer membrane of each leaf. The extent and degree of coloration on each leaf and of the bulb varies within the same cultivar and within a sample of the onions harvested at a given time.

Onions can be consumed raw, such as in a salad, as a cooked table vegetable, but their most important use is as a flavouring agent or garnish in sauces and other foods (Lee, 1951). Approximately 91% of the fresh weight of an onion is edible. On average, 100g of edible onion contains 89 g water, 1.2 g protein, 0.2 g fat and 7.9 g carbohydrate (including oligosaccharides) (Holland *et al.*, 1991). Many studies have been conducted on onions and an excellent, extensive review has been published on many different aspects of onions (Fenwick and Hanely, 1985).

2.8.1 Anthocyanins

The first anthocyanin identified in the skin of red onions was cyanidin 3-pentoseglycoside using acid hydrolysis, colour reactions and spectrophotometric analysis (Robinson and Robinson, 1931). Using paper chromatography, Fouassin (1956) identified three cyanidin derivatives, including a monoside and a diglucoside and a unidentified cyanidin derivative. Brandwein (1965) identified only peonidin 3-arabinoside as the major anthocyanin constituent in the 'Southport Red Globe' cultivar of red onions. In two separate studies, Fuleki (1969, 1971) identified the major anthocyanins in the red onion cultivars 'Ruby', 'Southport Red Globe' and another Texas grown cultivar. Seven cyanidin derivatives predominated but the presence of a peonidin monoside was also confirmed. The major cyanidin derivatives existed as cyanidin 3-glucoside and cyanidin 3-diglucoside. The 3-diglucoside did not have the same chromatographic properties as cyanidin 3-sophoroside (β, 1-2 glucose-glucose linkage) and

gentiobioside (β, 1-6 glucose-glucose linkage). Peonidin 3-glucoside was present in small amounts along with very small quantities of a cyanidin monoside and two cyanidin diglycosides. Later work by Du *et al.* (1974) showed the unique cyanidin 3-diglucoside was cyanidin 3-laminaribioside (β, 1-3 glucose-glucose linkage) using UV-visible spectroscopy, paper chromatography and hydrogen peroxide hydrolysis with a series of standard sugars.

In the anthocyanin identification studies conducted prior to 1982, the predominant acidulant used in the pigment extraction was a mineral acid such as hydrochloric acid. Moore *et al.* (1982a and b) illustrated the importance of using a milder acidulant like formic acid since it would prevent deacylation of potentially acylated anthocyanins in red onions. They suggested that many red onion anthocyanins may be acylated with cinnamic acid but one could not detect the acylation unless one used UV/visible spectral analysis, NMR, and gas liquid chromatography (GLC) methods. Terahara and Yamaguchi (1985) used thin layer chromatography (TLC) and UV/visible spectral measurements to verify that there were four anthocyanins present, all of which had cyanidin as the aglycone. The major anthocyanin was identified as cyanidin 3-glucoside acylated with malonic acid. Terahara *et al.* (1994) re-investigated the anthocyanin profile in the Japanese cultivar 'Kurenai' using H^1-NMR and fast atom bombardment - mass spectrometry (FAB-MS) to establish the chemical structures after mild extraction and chromatography of the pigments. The four cyanidin-based anthocyanins were identified as cyanidin 3-glucoside, 3-malonylglucoside, 3-laminaribioside, and 3-malonyllaminaribioside. The following year, Andersen and Fossen (1995) reported the identification of two novel anthocyanins, which were identified in a related species of *Allium, Allium victorialis*. Using spectroscopy, chemical degradation and homo- and hetero-nuclear two-dimensional NMR techniques, the anthocyanins were identified as cyanidin 3-O-(3", 6"-O-dimalonyl- β-glucopyranoside) and cyanidin 3-O-(3"-O-malonyl-β-glucopyranoside). Ferreres *et al.* (1996)

assessed the changes taking place among the anthocyanin and flavonoids in stored shredded red onions. Using the edible portion of the Spanish red onion cultivar 'Morada de Amposta', they reported the anthocyanins to be cyanidin 3-glucoside, 3-arabinoside, 3-malonylglucoside and 3-malonylarabinoside along with other flavonoids such as quercetin 3,4'-diglucoside, 7,4'-diglucoside, 3-glucoside, dihydroquercetin 3-glucoside and isorhamnetin 4'-glucoside. The anthocyanin and flavonoid contents were 233 mg kg^{-1} and 933 mg kg^{-1} fresh weight, respectively. The most recent study on red onion and other *Allium* species anthocyanins was conducted by Fossen *et al.* (1996). Using modern techniques such as H^1-NMR, MS and HPLC, they were able to describe qualitatively and quantitatively the anthocyanin contents of red onions (cultivars 'Red Baron', 'Comred', and 'Tropea'), a top onion *A. cepa* var. *viviparum*, chive (*A. schoenoprasum*) and *A. altaicum*. They identified the following six cyanidin derivatives: cyanidin 3-glucoside, 3-(3"-glucosylglucoside), 3-(3"-malonylglucoside), 3-(6"-malonylglucoside), 3-(6"-malonyl-3"-glucosylglucoside) and 3-(3",6"-dimalonylglucoside) along with trace amounts of four other anthocyanins, cyanidin 3,5-diglucoside, peonidin 3,5-diglucoside, and two pelargonidin 3-glycosides.

2.8.2 Colourless phenolics

The colourless phenolics in red onions have been as extensively studied as the anthocyanins (Fenwick and Hanley, 1985). The flavonoids which have been identified include quercetin, quercetin 3-glucoside, quercetin 4'-glucoside (spiraeoside), quercetin 7-glucoside, quercetin 3,4'-diglucoside, quercetin 3,7-diglucoside, quercetin 7,4'-diglucoside, kaempferol 4'-glucoside, kaempferol 7,4'-diglucoside in onion cultivars such as 'Carmen Hybrid', 'Sweet Spanish Utah', 'Sweet Spanish Hybrid', 'Early Yellow Globe', 'Yellow Globe Hybrid', 'Red Hamburger', 'Walla Walla', 'Evergreen', 'Long White Bunching', 'Hyper', 'Hygro', 'Topaz', 'Superba', 'Southport Red', 'White' and 'Yellow Globe' (Fenwick and Hanley, 1985). The flavonols especially quercetin are present in concentrations of 2.5 to 6.5 g/100 g dry weight in

coloured onions where as only 1 mg/100 g is present in white onions (Mazza and Miniati, 1993c). The edible portions of red onions also contained twice as much flavonols as yellow cultivars (Mazza and Miniati, 1993). The outer skins contain phenolics such as protocatechuic acid and its methyl esters, phloroglucinol, phloroglucinol carboxylic acid, pyrocatechol, ferulic acid, hydroxybenzoic acid, caffeic acid and vanillic acid in large amounts (Fenwick and Hanley, 1985).

2.9 Analysis of anthocyanins and other phenolics

The analysis of anthocyanins involves their extraction, purification and identification. Protocols for the extraction, purification and identification of anthocyanins are well established (Harborne, 1967; Mabry *et al.*, 1970a; Markham, 1982b). More sophisticated techniques such as proton^{1}- and carbon^{13} - nuclear magnetic resonance (H^{1}- and C^{13}-NMR), and fast atom bombardment - mass spectrometry (FAB-MS) are being used more often to obtain detailed structural information, such as the oxygenation pattern, the number and position of methoxyl groups, the number and linkage between sugars, the molecular weight, and the determination of the nature and site of sugar attachment (Markham, 1982b). These techniques have been used recently to identify the anthocyanins in red onions and related plants (Andersen and Fossen, 1995; Fossen *et al.*, 1996; Terahara *et al.*, 1994).

2.9.1 Extraction and purification procedures

Anthocyanins are located in vacuoles of pigmented cells. Numerous extraction procedures utilize solvents consisting of different proportions of acids, and polar solvents such as methanol, ethanol and water. The methanol or ethanol present in the solvent will denature the cell membranes and enzymes. The water soluble anthocyanins will then dissolve in the water alcohol based solvent. The acid acts as the acidifying agent since it is necessary to have a low pH for the red flavylium cation to remain stable. In the past hydrochloric acid was used as the acidifying agent and it was later determined that hydrochloric acid destroyed the acid linkages.

Acids such as acetic, formic and trifluoroacetic acids are now used because they do not hydrolyze the labile acyl groups found on some anthocyanins (Moore *et al.*, 1982a and b). Methanol and ethanol have low boiling points, which allow for easy concentration of the extract. To minimise degradation, the extract is concentrated to near dryness under a vacuum at a temperature close to 30°C (Francis, 1989a).

2.9.2 Spectrophotometric analysis

Ultraviolet-visible absorption spectroscopy is an important technique used to identify anthocyanin and flavonoid structures, the oxygenation pattern, the location of unsubstituted phenolic hydroxyl groups, and indirectly, the location of a sugar or methyl group attached to one of the phenolic hydroxyls (Markham, 1982c). Many reference spectra are available to aid in the spectra interpretation (Harborne, 1967; Jurd, 1962; Mabry *et al.*, 1970b). Shift reagents are added to a small amount of flavonoid (0.1 mg in methanol) or anthocyanin (0.1 mg in 0.4 M methanolic HCl) solutions in a stepwise manner. The compound can be characterized depending on how the spectrum changes after the shift reagent is added. The shift reagents used are sodium methoxide, sodium acetate, boric acid and aluminum chloride. Sodium methoxide will reveal the hydroxylation pattern and the acidic hydroxyl groups present. Sodium acetate will detect the presence of a free 7-hydroxyl group. When boric acid is added after the sodium acetate, it will detect the presence of an *ortho*-dihydroxy group. Aluminum chloride is used to detect the presence of *ortho*-dihydroxyl groups in anthocyanins since it forms an acid-stable complex between the hydroxyl and neighbouring ketones and an acid–labile complex with *ortho*-dihydroxyl groups. Interpretation details are available (Harborne, 1967; Mabry *et al.*, 1970a and b; Markham, 1982c).

2.9.3 Chromatographic analysis of anthocyanins

Chromatography is the only method of separation used to separate the individual anthocyanins in the crude extracts for identification purposes. A number of chromatographic methods have been developed over the years and are summarized in literature reviews and books by Lee and Hong (1992) and Markham (1982a and b). The basic chromatographic procedures traditionally used are column, paper and thin layer chromatography and paper electrophoresis. In the last number of years, solid phase extraction combined with high performance liquid chromatography (HPLC) has become the procedure of choice (Baldi *et al.*, 1995; Hong and Wrolstad, 1990a and b; Lee and Hong, 1992; Romani *et al.*, 1996). Paper and thin-layer chromatography were the chromatographic techniques used to conduct the pioneering work of anthocyanin identification. With these two methods, the samples are spotted onto the paper sheets or thin layer chromatography plates, and are resolved using a variety of solvents (Markham, 1982b). Paper chromatography can be used on a preparative scale to isolate compounds for identification. Thin layer chromatography offers the advantage of different stationary phases, and faster resolution time over paper chromatography (Lee and Hong, 1992). Both methods require the use of spray reagents to resolve the spots. Paper electrophoresis has been used on a few occasions to distinguish malonated anthocyanins (Davies, 1992; Harborne and Boardley, 1985). It offers little or no advantage over paper chromatography (Lee and Hong, 1992). Open column chromatography has been used for large scale preliminary purification or preparative scale separation of anthocyanins and flavonoids. A number of adsorbent materials have been used, ranging from cellulose, ion-exchange resins, polyamide, polyvinylpyrrolidone (PVP), silica and Sephadex G- and LH-20 series of gels (Markham, 1982b; Lee and Hong, 1992). The solvents used with column chromatography are similar to those used in thin layer chromatography. Column chromatography is very tedious but is a useful large scale separation process.

Over the last number of years, solid phase extraction cartridges have become increasingly popular for cleaning up crude anthocyanin extracts (Baldi *et al.*, 1995; Hong and Wrolstad, 1990a,b; Romani *et al.*, 1996). A solid phase extraction cartridge consists of a small, disposable, plastic cartridge or column packed with an adsorbent. The commercially available solid phase extraction cartridges are packed with adsorbents based on reverse phase, normal phase and ion-exchange separation mechanisms. Available reversed phase packing materials consist of C_{18}, C_8, C_2 and CH bonded phases. Octadecylsilane (C_{18}) sorbent is the most common hydrophobic sorbent used for anthocyanin method development by extracting compounds in aqueous samples (Waters (Canada), 1996). The solvents used are often the same ones used for high performance liquid chromatography *e.g.* water, acidified water, methanol, ethyl acetate, acetonitrile and others (Baldi *et al.*, 1995; Romani *et al.*, 1996). The sample is simply applied to the cartridge which is washed with a number of solvents to elute the components of interest in the various fractions. Solid phase extraction cartridges are rapid, efficient, reproducible, economical and safe when compared to column chromatography (Waters, 1996). The fractions of interest can then be injected into an HPLC for separation and identification.

High performance liquid chromatography is a form of column chromatography which utilizes very small packing material particles of regular shape and high pressure to achieve acceptable flow rates and separation (Markham, 1982b). The major benefits are that one is able to quantitatively analyse the flavonoid and anthocyanin mixture, it is very sensitive, requires very little sample, offers excellent separation, is very fast and can be used on a preparative scale (Markham, 1982b; Hong and Wrolstad, 1990a and b; Hong and Lee, 1992). Flavonoid and anthocyanin analysis is typically performed using reverse phase HPLC (Hong and Lee, 1992). A reverse phase column consists of a hydrocarbon chain of a certain length bonded onto the silica matrix. For C_{18} packing material, the hydrocarbon chain is 18 carbon units long and is referred to as octadecylsilyl (ODS). In reverse phase chromatography, the compounds of interest are eluted

in decreasing order of polarity. Elution is achieved by using a gradient consisting of acidified water, which is mixed with increasing amounts of methanol or acetonitrile to elute the less polar compounds. UV-visible or photodiode array detectors are used to provide preliminary characterization of the pigments. Computers, which are connected to the detector, are used to monitor retention times, spectral data, compare data to standards, and aid in quantitation. The spectral data and retention time provide information on the aglycone, the sugar and the presence of acylation. Dual wavelength detection at 280 nm and 500-550 nm is useful by providing preliminary identification of anthocyanin peaks since they absorb strongly in the visible region when in acidic solutions. A rapid method incorprating HPLC, GC and UV-vis spectrophotometry has been developed by Gao and Mazza (1994a) for the chemical charaterization of anthocyanins. This method has been used to identify the anthocyanins and phenolics in low- and highbush blueberries (Gao and Mazza, 1994b; Gao and Mazza, 1995a) and cherries (Gao and Mazza, 1995b).

2.9.4 Recent advances in identification techniques

More recently, HPLC with mass spectrometer detectors have been used to provide faster, more accurate identification of the peaks (Baldi *et al.*, 1995). Also, information from FAB-MS and NMR is now used to identify the structures of the compounds more accurately than the spectral methods used before. Methods using capillary zone electrophoresis with mass spectrometer detectors have been developed to identify anthocyanins (Bridle *et al.*, 1996; Schwenk *et al.*, 1995).

2.10 Conclusions

These new rapid isolation and identification techniques can be used to identify new, more complex anthocyanins found in nature. By studying the structure and their stability *in vivo* and *in vitro*, it can be determined how the vast array of orange, red and blue colours in nature are

stabilized. This information will also help determine whether any of these stable anthocyanins with or without a copigment can be used as another natural food colourant to complement those already in use today.

3. ANTHOCYANINS AND COLOURLESS PHENOLICS IN RED ONIONS

3.1 Introduction

Red onions, *Allium cepa* L. contain anthocyanins and colourless phenolics in the dried outer skins and in the epidermal layer of their fleshy scales. The colourless phenolics such as flavonols, quercetin and kaempferol glucosides were identified by Fenwick and Hanley (1985). Previously these compounds were characterized using paper and/or thin layer chromatography, UV-vis spectroscopy and chemical methods such as acid and alkaline hydrolysis (Fuleki, 1969, 1971; Starke and Herrmann, 1976). Studies on anthocyanins by Robinson and Robinson (1931), Brandwein (1965), Fuleki (1969, 1971), and Du *et al.* (1974) showed that the major pigment in red onions was cyanidin 3-glucoside, with lesser amounts of cyanidin 3-laminaribioside and other minor unidentified cyanidin, peonidin and pelargonidin glycosides. Moore *et al.* (1982a and b) reported the presence of several acylated cyanidin glycosides although identification of the acyl group was lacking. Terahara *et al.* (1994) determined that 'Kurenai' red onions from Japan contained the anthocyanins cyanidin 3-glucoside, cyanidin 3-laminaribioside, and their 6"-malonyl derivatives. While the present study was in progress, Andersen and Fossen (1995) reported cyanidin 3-(3",6"-dimalonyl glucopyranoside) and cyanidin 3-(3"-malonylglucopyranoside) in *A. victorialis* L., a close relative of *A. cepa* L. Recently, Fossen *et al.* (1996) reported four major and six minor anthocyanins in 'Red Baron', 'Tropea' and 'Comred' onions, including the 3-O-β-(3"-malonylglucoside), 3-O-β-(3", 6"-dimalonylglucoside), and 3,5-diglucoside derivatives of cyanidin, peonidin 3,5-diglucosides and two 3-glycosylated derivatives of pelargonidin.

The objective of this research was to identify the major anthocyanins and colourless phenolics present in four of the red onion cultivars grown in Canada and USA. Initially, the

objective was to verify the identity of the major anthocyanins and phenolics using a novel, rapid identification procedure. However, publication of the identity of the major onion anthocyanins by Terahara *et al.* (1994) and Fossen *et al.* (1996) while this study was in progress, resulted in a re-assessment of the study objectives. It was decided that the minor anthocyanins should also be identified using an improved separation procedure. Thus, the objectives of this study were several fold:

1) To isolate and identify the major colourless phenolics and anthocyanins in four red onion cultivars using chromatographic, spectral and chemical methods;
2) To quantify and compare the anthocyanin and colourless phenolic contents of several cultivars of red onions using HPLC;
3) To isolate and identify the minor anthocyanins using solid phase extraction and a novel HPLC-GC method for the identification of anthocyanin pigments; and
4) To collect the major anthocyanins for copigmentation experiments.

3.2 Materials and methods

Red onions (cv. 'Mambo' grown commercially in the Fraser Valley of British Columbia, Canada in 1994, cv. 'Red Jumbo' grown commercially in Washington State, USA in 1995, and cv. 'Red Bone' and 'Red Granex' grown commercially in California, USA in 1994) were used. Onions were harvested in early September when the tops were down, and stored at 1-2°C in a low humidity chamber for about four weeks before extraction of anthocyanin pigments. The 'Red Jumbo' cultivar samples were freeze-dried and used for the purification of certain anthocyanins.

3.2.1 Extraction and purification of major anthocyanins and colourless phenolics

The extraction was done using freeze dried onions and methanol : formic acid : water (MFW; 40:5:55) for 1 hour. The slurry was filtered through Whatman No. 4 filter paper and a 0.45 μm filter. An aliquot was injected into the HPLC analytical column for analysis.

The crude extract portion soluble in 5% formic acid (HFo) in water was applied to a small packed column of weakly acidic carboxylic type, cation exchange resin Amberlite CG-50 (Mallinckrodt Chemical Works). The dimensions of the column were as follows: length, 109 cm; diameter, 17 mm. The packing was washed with several column volumes of 5% HFo before the sample was applied. The sample was carefully applied onto the column all at once with a pipette and allowed to pass into the packing material before the eluent of 5% HFo was added. Most of the anthocyanins eluted in the first few fractions collected from the column. All collected fractions were concentrated to dryness on the rotary evaporator and then resolubilized in 5% HFo, filtered and run on the HPLC in order to determine which fractions contained the most anthocyanins (fraction #2). The eluent was changed to ethanol : formic acid : water (EFW, 50:1:49) once it appeared that most of the pigments (red band) had eluted from the column. The EFW improved the elution of a yellowish-brown layer, which contained mostly the flavonoids. The column was washed clean with acidified ethanol and then washed with EFW and then 5% HFo, each a few column volumes. The fraction containing the most anthocyanins and phenolics was then injected into the HPLC to identify the major anthocyanins and phenolics. All peaks which absorb strongly at 280 nm (colourless phenolics) and 525 nm (anthocyanins) were collected and freeze dried.

3.2.2 Extraction and isolation of major and minor anthocyanins using solid phase extraction

The above method did not allow for efficient separation of the minor and major anthocyanins for identification and collection purposes. Therefore, another method was developed and was a modification of that used by Baldi *et al.* (1995) and Romani *et al.* (1996). Extraction of minor anthocyanin pigments was carried out primarily using the dry outer scales of 'Red Jumbo' onions since this variety was more readily available. The dry outer scales were

milled in a coffee grinder. Anthocyanins were extracted from the milled material (30 g) with 3 x 300 mL of methanol : formic acid : water (MFW; 50:5:45; v:v:v) (Figure 3.1) and filtered through a Buchner funnel and Whatman No. 4 filter paper.

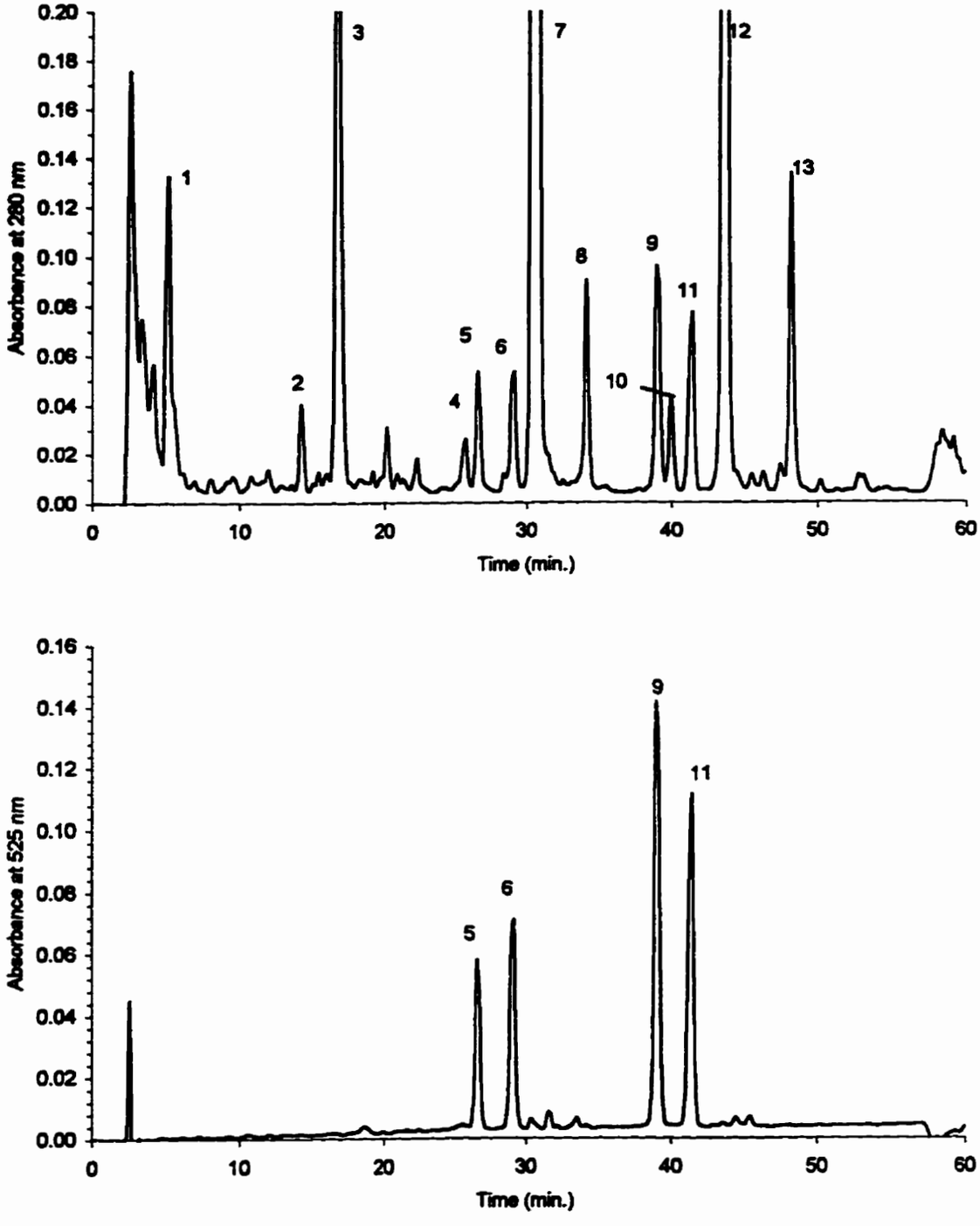


Figure 3.1: HPLC separation of colourless phenolics and anthocyanins in a methanol:formic acid:water extract of cv. 'Red Granex' red onions, monitored at 280 nm (top) for colourless phenolics and at 525 nm (bottom) for anthocyanins.

Extracts were combined and concentrated to near dryness on a Büchi rotary evaporator (Brinkmann Instruments Canada Ltd., Mississauga, ON) at 30°C to yield a crude extract. Aliquots of crude extract (100 μL) were loaded on a C_{18} Maxi-Clean (600 mg) solid phase extraction cartridge (Alltech Assoc. Inc., Deerfield, IL) which had been activated with 10 mL methanol, followed by 10 mL of 5% formic acid (HFo) in water. The loaded cartridge was sequentially washed with 10 mL of MilliQ water, 5% formic acid in water, ethyl acetate, methanol, and MFW (50:5:45, v:v:v). The methanol fraction contained the anthocyanins and was evaporated to dryness on a rotary evaporator. The anthocyanins were resolubilized in 5% formic acid and filtered through a polyvinylidene fluoride (PVDF) filter (Acrodisc LC13 0.45 μm, Gelman Sciences, Ann Arbor, MI) prior to high performance liquid chromatography (HPLC).

3.2.3 High performance liquid chromatography

HPLC analysis was carried out on a Waters liquid chromatography system (Millipore, Milford, MA) using a 250 x 4.6 mm i.d. Zorbax SB-C_{18} Stablebond analytical (5 μm) column and a 5 μm, 250 x 9.4 mm i.d. semipreparative column (Rockland Technologies Inc., Chadds Ford, PA). For the semipreparative column, a 500 μL loop was used and for the analytical column, a 50 μL loop was used. Column temperature was maintained at 30°C for the first anthocyanin and phenolic identification and then increased to 35°C for identification of the minor anthocyanins to obtain best resolution and retention time stability using a Waters temperature control module. A Waters 990+ photodiode array detector connected to a personal computer and plotter was used for peak detection and data analysis. Compounds were separated by gradient elution using formic acid : water (5:95, v/v) (solvent A) and HPLC grade methanol (solvent B) according to the following profile: 0-20 min, 10-24% B; 20-32 min, 24-32% B; 32-47 min, 32-45% B; 47-53 min, 45-50% B; 53-54 min, 50-80% B; 54-58 min, 80% B; 58-60 min, 80-10% B. The solvent flow rate was 1.0 mL/min for the analytical column and 4.2 mL/min for the semipreparative

column. All peaks that absorbed at 280 nm (flavonoids and phenolic acids) and 525 nm (anthocyanins) were collected using a fraction collector. Anthocyanin fractions were concentrated to dryness on a rotary evaporator, redissolved in 5% aqueous formic acid and re-chromatographed on a gradient consisting of formic acid : water (5:95 v/v) (solvent A) and HPLC grade acetonitrile (solvent B). This elution profile was: 0-55 min, 8-20% B; 55-56 min, 20-80% B; 56-59 min, 80% B; 59-60 min, 80-8% B. All peaks were collected for characterization by chemical, chromatographic and spectral methods as described below.

3.2.4 Identification of anthocyanins

Isolated anthocyanins were characterized using previously described methodology (Gao and Mazza, 1994a). Anthocyanins were hydrolysed in methanol - 2 N HCl (0.2 mg dry anthocyanin in 0.4 mL MeOH; then 0.2 mL of anthocyanin solution with 0.2 mL 2 N HCl) to cleave the pigments into aglycones, sugars, and acyl groups. Anthocyanidins and phenolic acids were identified by reverse-phase HPLC in a single analytical run using retention times and UV-visible absorption spectra. Aliphatic acylating acids and sugars were characterized by capillary gas-liquid chromatography (GLC) analysis of the trimethylsilyl derivatives of sugars and aliphatic acids and by the methyl esters of the aliphatic acids.

Retention times of anthocyanin standards were obtained from a crude extract of 'Fundy' blueberries, whose anthocyanin composition has been identified (Gao and Mazza, 1994b). Anthocyanidin retention times were obtained by acid hydrolysis of the anthocyanins from a 'Fundy' blueberry sample. A purity check of the purified anthocyanin allowed for determination of retention time. Purified pigment was recollected for alkaline and acid hydrolysis and spectral analysis.

3.2.5 Alkaline hydrolysis (deacylation of anthocyanin)

Each HPLC purified anthocyanin (less than 0.2 mg) was dissolved in 1 drop of 5% formic acid (HFo) and then flushed with nitrogen. While this solution was still under nitrogen, 2-3 drops of 2 N NaOH were added turning the solution from red to blue, then to green and finally to yellow, at which time 2-3 drops of concentrated 90% HFo were added to stop the alkaline hydrolysis. The hydrolysate was left to stand for two hours (at room temperature and in the presence of dim light) to allow reversion of the chalcone to the flavylium cation, and was injected into the HPLC system to determine the retention time of the deacylated anthocyanin.

3.2.6 Spectral analysis

In order to determine the amount of anthocyanin to use for sugar and acid analysis, shift reagent analysis was first performed (Markham, 1982c). Purified anthocyanins were dissolved in 0.01% (v/v) HCl in methanol and all phenolics were dissolved in 2 mL methanol for determination of UV and visible spectra (200-700 nm) using a Beckman DU-640 spectrophotometer (Beckman Instruments Canada Inc., Mississauga, ON). The sodium methoxide (NaOMe), sodium acetate (NaOAc) and sodium acetate + boric acid (NaOAc + H_3BO_3) shifts were recorded for colourless phenolics over the same wavelength range and interpreted as recommended by Harborne (1958), Mabry *et al.* (1970a and b) and Markham (1982c). For anthocyanins, the aluminum chloride and hydrochloric acid shifts were recorded and interpreted as described by Harborne (1958), Mabry *et al.* (1970a) and Markham (1982c). The position of the sugar on the anthocyanin molecule was determined from the spectral data along with the retention times of the known anthocyanins in 'Fundy' blueberries.

3.2.7 Acid hydrolysis

Anthocyanin samples (200 μg) were subjected to acid hydrolysis. After redissolving the anthocyanin in a mixture of 200 μL of 2 N HCl and 200 μL of MeOH, the resulting solution was

heated under N_2 atmosphere for 1 hour in boiling water, cooled in ice, and assayed for aglycone, sugars and methylated aliphatic acids. An aliquot (5 μL) of the hydrolysate was injected into the HPLC system to allow retention time and spectral characteristics of the anthocyanidin to be determined.

3.2.8 Aliphatic acid analysis

The acid hydrolysate was extracted with chloroform (100 μL) to identify any methylated aliphatic carboxylic acids present. After phase separation, 30 μL of the chloroform extract was pipetted into an amber vial containing 30 mg of sodium sulphate and shaken. An aliquot (5 μL) of the extract was injected onto a Hewlett-Packard 5890A gas chromatograph (GC) system (Hewlett-Packard, Avondale, PA) equipped with a flame ionisation detector (FID) and a 1 μm x 30 m J&W DB-1701 capillary column (J&W Scientific, Folsom, CA). A temperature gradient was used in the separation, wherein column temperature was held at 45°C for two minutes, then increased at a rate of 20°C/min to 250°C and held at 250°C for 15 min. Helium was the carrier gas (1 mL/min), with nitrogen as the makeup gas (30 mL/min), and the split ratio was 25. Standard carboxylic acids (oxalic, malonic and succinic acids) (Sigma Chemical Co., St. Louis, MO) were subjected to the same methylation procedure and analyzed under the same conditions outlined above.

3.2.9 Sugar analysis

The acid hydrolysate after chloroform extraction was used for sugar analysis by adding 3 x 200 μL aliquots of 1-pentanol (also known as amyl alcohol), shaking, and allowing the phases to separate. The upper (1-pentanol) layer containing anthocyanidin was removed and the remaining aqueous phase containing sugar(s) and acid(s) was dried under nitrogen. Dry sugar(s) and acid(s) were dissolved in 50 μL pyridine and derivatized using 25 μL each of

hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS). The mixture was shaken for 30 seconds and left to stand for five minutes prior to injection into the gas chromatograph system equipped with a FID detector and capillary column as described above. A thermal gradient was used for separation. The initial column temperature was 120°C, and was increased immediately at a linear rate of 20°C/min, followed by a 5°C/min increase after 3 min to a final column temperature of 230°C. All other conditions were as described above for methyl ester analysis. Sugar and dicarboxylic acid standards (rhamnose, glucose, and galactose; oxalic, malonic and succinic acids) were subjected to the same derivatization procedures, as were the samples.

3.2.10 Determination of anthocyanin content

Total anthocyanin content was determined by HPLC under the same conditions used for identification. Results were expressed as cyanidin 3-glucoside. Analysis was performed on the freeze-dried whole onion. Approximately 1 g of freeze-dried onion was combined with 10 mL MFW and allowed to mix for 20 minutes. MFW extract was separated from the solid material by filtration with a Buchner funnel. An aliquot of the crude extract was filtered through a 0.45 μm PVDF filter and 50 μL was injected into the HPLC for analysis. For each variety, one extract was made on a single onion bulb and three different bulbs were analyzed for each variety.

3.2.11 Collection and precipitation of anthocyanins for copigmentation experiment

The anthocyanin peaks of interest, identified as cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside, were collected using the semipreparative column and the same gradient and other conditions as stated previously. Each peak was collected in a separate test tube and then pooled with the previously collected fractions in a 250 mL round bottom flask. The collected pigment peaks were dried on a daily basis using a Büchi rotary evaporator (Brinkmann Instruments Canada Ltd., Mississauga, ON) with the water bath temperature set at

30°C and then stored at -40°C. The pigments were re-chromatographed to check their purity using 5% formic acid in water (solvent A) and 100% acetonitrile (solvent B), using the corresponding gradient mentioned earlier. The purified peaks were re-collected, and flushed with nitrogen to evaporate the solvents. The resulting pure anthocyanins were dried onto the walls of the vial. In this form, they could not be easily and accurately weighed for use in the copigmentation experiment.

To overcome this difficulty, a precipitation procedure was used that resulted in a powdered, easily weighed anthocyanin which did not deacylate the anthocyanin (Terahara *et al.*, 1994). The pooled sample of each anthocyanin was dissolved in less than 100 μL concentrated HFo acid; this was followed by addition of 1 mL of MeOH. The dissolved anthocyanin was pipetted into a 1.5 mL Eppendorf plastic disposable micro centrifuge tube (VWR Canlab Ltd., Mississauga, ON). The solution was dried by gently flushing with nitrogen gas. Once dry, approximately 20 μL of concentrated trifluoroacetic acid (100 %) (Fischer Scientific Ltd., Nepean, ON) was added in a fume hood causing the anthocyanin to dissolve and form a dark orange solution. To precipitate the anthocyanin, 200 μL of concentrated diethyl ether (100 % ethyl ether) (Fischer Scientific Ltd., Nepean, ON) was added. Upon addition, one could immediately see the anthocyanin come out of solution and form a purple precipitate. The Eppendorf tube was closed and the mixture was mixed on a vortex for 30 seconds. To aid in the settling of the precipitate, the solution was centrifuged at 10,000 rpm for 5 minutes using an Eppendorf micro centrifuge (model 5415C) (VWR Canlab Ltd., Mississauga, ON). After centrifugation, the diethyl ether layer was gently pipetted off and another 200 μL of diethyl ether was added, vortexed and then centrifuged. This step was repeated twice. Then the diethyl ether was decanted and the Eppendorf tube containing the anthocyanin pellet was placed in a glass desiccator filled with drierite and placed under vacuum for 3 days. After the 3 day drying period,

the anthocyanin was dry and ready for the copigmentation experiment. The pigment was checked for degradation and purity by re-chromatographing it using the same conditions used for its purification.

3.3 Results

3.3.1 Characterization of major anthocyanins and colourless phenolics

3.3.1.1 Characterization of major anthocyanins

From the data in Tables 3.1 and 3.2, all four anthocyanin peaks have cyanidin as their aglycone. Peaks 9 and 11 were acylated with malonic acid. Alkaline hydrolysis of peaks 9 and 11 indicated that peak 5 and 6 were the corresponding de-acylated anthocyanins. Gas chromatography of the derivatized sugars showed that all four pigments contained glucose as the glycoside. Based on the chemical, spectral and chromatographic results, the identities of the four anthocyanins are as follows: peak 5 is cyanidin 3-glucoside, peak 6 is cyanidin 3-laminaribioside, peak 9 is cyanidin 3-malonylglucoside and peak 11 is cyanidin 3-malonyllaminaribioside. These results were in agreement with those of Terahara *et al.* (1994) who reported the occurrence of the same anthocyanins in Japanese red onions.

Table 3.1: Spectral characteristics of anthocyanins in red onions.

Peak No.	λ_{max} (nm)	% peak area at 525 nm	$E_{440nm}/E_{vis\ max}$ (%)	$E_{uv\ max}/E_{vis\ max}$ (%)	$AlCl_3$ λ_{max} (nm)	$AlCl_3$ shift (nm)
5	528, 281	12.3	23	77	561	+41
6	528, 282	8.1	25	100	561	+41
9	528, 281	52.7	23	62	574	+46
11	527, 281	17.4	24	76	574	+47

Peak numbers refer to anthocyanins in Fig. 3.1

Table 3.2: HPLC and GC retention times (t_r) of anthocyanins and their corresponding sugars and acids after hydrolysis.

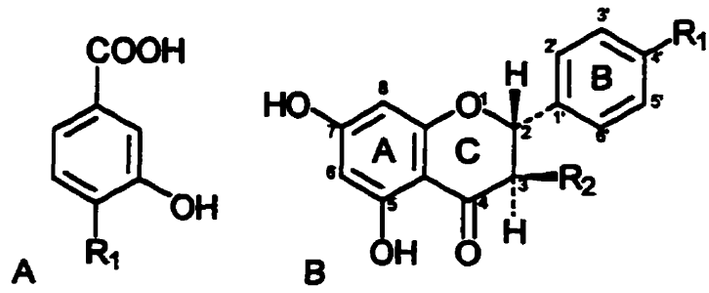
Peak No., t_r (min)	Aglycones, t_r (min)	Sugars, TMS-derivatives, t_r (min)	Dicarboxylic acids, TMS-derivatives, t_r (min)	Acid, methyl ester, t_r (min)	Identity
5, 26.63	35.70	18.25, 21.21	-	-	Cyanidin 3-glucoside
6, 29.09	35.82	18.22, 21.19	-	-	Cyanidin 3-laminaribioside
9, 39.02	36.36	18.16, 21.12	6.65	9.33	Cyanidin 3-malonylglucoside
11, 41.48	35.50	18.16, 21.09	6.63	9.25	Cyanidin 3-malonyllaminaribioside
Standards					
Delphinidin	26.24	-	-	-	
Cyanidin	36.04	-	-	-	
Petunidin	41.30	-	-	-	
Pelargonidin	45.43	-	-	-	
Peonidin	47.63	-	-	-	
Malvidin	48.76	-	-	-	
Glucose	-	18.27, 21.23	-	-	
Galactose	-	17.19, 19.07	-	-	
Arabinose	-	11.93, 12.73	-	-	
Methyl acetate	-	-	5.79	3.55	
Oxalic acid	-	-	8.20	8.39	
Malonic acid	-	-	6.68	9.35	
Succinic acid	-	-	10.66	10.42	

Peak numbers refer to anthocyanins in Fig. 3.1

3.3.1.2 Characterization of colourless phenolics

The spectral data for peaks 1, 2, and 3 indicated that they were either flavanones or dihydroflavonols due to the strong absorption at 275–295 nm (Markham, 1982c). Peaks 1 and 3 had very similar spectral properties except that a shoulder appeared for peak 3 at 322 nm. Peak 2 had a different methanol spectrum since it had peaks at 258 nm and 294 nm with a shoulder at 220 nm. By using the shift reagents, it was not possible to establish the identity of peaks 1 and 3. By comparing the HPLC retention times of these peaks with those of standards, it appears that peak 2 is probably protocatechuic acid. Acid hydrolysis indicates that all three peaks are glycosylated and GC analysis of the TMS derivatives indicated that glucose was present. Acid hydrolysis also showed that peak 1 is a product of the breakdown of peak 3. Therefore, peak 1 is

identified as protocatechuic acid 4-glucoside, peak 2 is protocatechuic acid and peak 3 is a glucosylated 5,7-dihydroxy dihydroflavonol or a glucosylated 5,7-dihydroxy flavanone. The structures are depicted below in Figure 3.2.



Peak No.	Structure	Name	R_1	R_2
1	A	Protocatechuic acid 4-glucoside	Glucose	H
2	A	Protocatechuic acid	H	H
3	B	5,7-dihydroxy dihydroflavanol glucoside	Glucose	OH
	B	5,7-dihydroxy flavanone glucoside	Glucose	H

Peak numbers refer to anthocyanins in Fig. 3.1

Figure 3.2: Structures of the identified colourless phenolics.

Peaks 4, 7, 8, and 12 were tentatively identified as flavonols because their spectral data indicated that they absorbed strongly at 346-365 nm and 251-266 nm. After analyzing the effects of the shift reagents on the spectra and comparing the spectra to those published by Mabry *et al.* (1970b), one can conclude that all four flavones were derivatives of quercetin. This was expected since previous researchers have identified quercetin and its glycosides as being present in onions (Brandwein, 1965; Starke and Hermann, 1976; Bilyk *et al.*, 1984, Kiviranta *et al.*, 1986; and Price and Rhodes, 1997).

Peak 12 was identified as quercetin 4'-glucoside (commonly know as spiraeoside). Its spectral properties and shifts after addition of the shift reagents are very similar to those of quercetin 4'-glucoside as published by Brandwein (1965). The sodium methoxide spectrum shift indicates that a 4'-hydroxy is present and the $AlCl_3$/HCl spectrum indicates a 5-OH is also present. This leaves the 4'-OH on the Bring as the only other place where the molecule can be

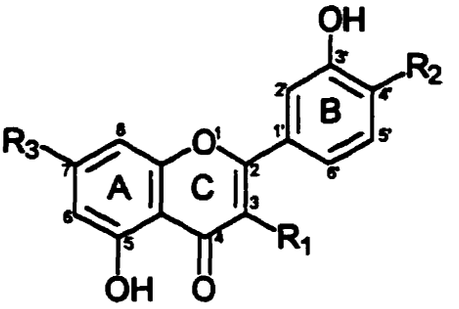
glycosylated. The GC analysis verifies that glucose is attached to the flavone. The identity of the flavone is further verified by acid hydrolysis. The hydrolysis product had the same retention time as the quercetin standard and their spectra matched. This lead one to conclude that peak 4 is quercetin 4'-glucoside or spiraeoside.

Peak 7 was identified as quercetin 3,4'-diglucoside. As with peak 4, its spectra and the shifts induced by the shift reagents were similar to the values for quercetin 3,4'-diglucoside by Brandwein (1965). The spectral shift data indicates that there is no free 4'-OH group and that therefore glucoside must be present at that position. There is a free 7-OH group and no B ring *ortho*-dihydroxy system. The acid hydrolysis product has the same retention time and spectra as the quercetin standard. The GC analysis indicates that the sugar is glucose. Therefore, based on this data, it was concluded that the identitiy of peak 7 is quercetin 3,4'-diglucoside.

Peak 8 was tentatively identified as quercetin 3-glucoside which has not been reported in onions before. Its methanol spectrum show one large peak in the region of 250-270 nm but this peak had two smaller apexes at the top. When the rest of the spectral data was compared to the results by Mabry *et al.* (1970b), the rest of the shifts seemed to be fairly close. The GC analysis indicated that this peak is also glycosylated with glucose and no acids were detected. This led one to conclude that it is quercetin 3-glucoside

Peak 4 was also identified as a quercetin derivative. From the spectral data and the data produced using the shift reagents, one can conclude that the 4'-hydroxy group is present along with perhaps a 3-hydroxy and/or 5-hydroxy. The acid hydrolysis yielded quercetin and the spectra and retention time matched with the standard. The GC analysis indicated that glucose is the glycosylating sugar. The identity of this peak can only be speculated upon. Its spectral properties seem to be close to those of quercetin 3',4',5,7-tetramethyl. However, if this were the

derivative, its acid hydrolysis product should have a different retention time and spectra than that of quercetin. Perhaps it is quercetin with laminaribioside as the sugar. However, it is most likely quercetin 7,4'-diglucoside, since this is a quercetin derivative which has been identified in large amounts by others (Bilyk *et al.*, 1984; Kiviranta *et al.*, 1986; Starke and Hermann, 1976). The data indicates that a 4'-OH, 7-OH, no B ring *ortho* dihydroxy groups and a 3-OH are present. The structures of the four quercetin derivatives are illustrated below in Figure 3.3.



Peak #	Name	R_1	R_2	R_3
4	Quercetin 7,4'-diglucoside	H	Glucose	Glucose
7	Quercetin 3,4'-diglucoside	Glucose	Glucose	H
8	Quercetin 3-glucoside	Glucose	H	H
12	Quercetin 4'-glucoside	H	Glucose	H

Peak numbers refer to anthocyanins in Fig. 3.1

Figure 3.3: Structures of the identified quercetin derivatives.

Table 3.3: HPLC and GC retention times (t_r) of colourless phenolics and flavones and their corresponding sugars and acids after hydrolysis.

Peak No.	Retention time, t_r (min)	Aglycones, t_r (min)	Sugars, TMS-derivatives, t_r (min)	Dicarboxylic acids, TMS-derivatives, t_r (min)	Acid, Methyl ester, t_r (min)	Identity
1	5.85	5.90, 17.90	18.25, 21.21	-	-	Protocatechuic acid 4-glucoside
3	14.80	5.11, 5.84, 11.11, 17.90	18.22, 21.18	-	-	5,7-dihydroxy flavanone or 5,7-dihydroxy dihydroflavonol
4	25.77	48.89	18.25, 21.21	-	-	Quercetin 7,4'-diglucoside
7	30.56	49.14	18.23, 21.18	-	-	Quercetin 3,4'-diglucoside
8	34.16	52.67	18.22, 21.18	-	-	Quercetin 3-glucoside
Standards						
Protocatechuic acid		5.83	-	-	-	
Quercetin		48.80	-	-	-	
Naringenin		49.50	-	-	-	
Glucose		-	18.27, 21.23	-	-	
Galactose		-	17.19, 19.07	-	-	
Arabinose		-	11.93, 12.73	-	-	

Peak numbers refer to anthocyanins in Fig. 3.1

Table 3.4: Spectral characteristics of colourless phenolics in red onions.

	MeOH, λ_{max} (nm)		NaOMe		NaOAc		NaOAc/H_3BO_3		$AlCl_3$		$AlCl_3$/HCl	
Peak #	Band II	Band I	Band II	Band I	Band II	Band I	Band II	Band I	Band II	Band I	Band II	Band I
1	258, 220 sh	293	274	300	291, 252sh		266	298	239, 285	320	221, 260	297, 360
3	291, 322sh		316, 346sh		322, 364sh		294, 331sh		314	410sh, 342sh	224, 292	
4	253, 268sh	365	268, 240sh	415	416, 260sh		262sh	366	264	421	263	422
7	266	348	272	379	273	376	266	350	272	355, 394	275, 256sh, 298sh	348, 392
8	251, 266	346	274	373	275	370	267	347	272, 296sh	355, 394	275, 258sh, 295sh	347, 393
12	252, 267sh	363	277	414, 319sh	275	386	296, 266sh, 250sh	366	263	302, 357, 421	260, 265	355, 421

sh = shoulder

Peak numbers refer to anthocyanins in Fig. 3.1

3.3.2 Quantification and distribution of anthocyanins and colourless phenolics

Table 3.5 is a quantitative survey of the anthocyanins and colourless phenolics present in the red onion cultivars 'Mambo' (grown in British Columbia), 'Red Bone' and 'Red Granex' (grown in California). The chromatogram of the crude extracts for 'Red Bone' onions at 280 and 525 nm is presented in Figure 3.4. The malonated anthocyanins were predominant. However, in both 'Red Bone' and 'Red Granex' cultivars, there were larger amounts of cyanidin 3-glucoside and 3-laminaribioside. Quercetin 3,4'-diglucoside and quercetin 4'-glucoside (peak 7 and 12) constituted the majority of the phenolics. 'Red Granex' contained approximately twice as much quercetin 3,4'-diglucoside as the other two cultivars.

Table 3.5: Contents[a] (milligrams per 100 g dry weight) of colourless phenolic, anthocyanins and quercetin glucosides in three onion cultivars.

			Cultivar		
Peak No.			Mambo	Red Bone	Red Granex
1	Phenolic acid	Protocatechuic acid	39.2 ± 4.8	50.9 ± 2.8	40.4 ± 3.1
3	Flavones	5,7-dihydroxy flavanone / dihydroxy dihydroflavanol glucoside	91.4 ± 7.4	124.4 ± 5.5	113.1 ± 15.3
5	Anthocyanins	Cyanidin 3-glucoside	14.9 ± 9.8	17.7 ± 6.2	28.2 ± 9.0
6		Cyanidin 3-laminaribioside	6.9 ± 4.4	44.9 ± 8.4	28.8 ± 2.6
9		Cyanidin 3-malonylglucoside	68.1 ± 11.7	98.1 ± 12.2	64.7 ± 11.6
11		Cyanidin 3-malonyllaminaribioside	19.1 ± 9.2	58.4 ± 7.8	40.5 ± 3.1
4	Quercetin glucosides	Quercetin 7,4'-diglucoside	6.7 ± 3.8	5.4 ± 0.6	8.9 ± 1.5
7		Quercetin 3,4'-diglucoside	299.1 ± 20.2	290.6 ± 14.4	566.5 ± 35.2
8		Quercetin 3-glucoside	5.8 ± 0.2	13.3 ± 0.8	22.5 ± 2.1
12		Quercetin 4'-glucoside	237.4 ± 11.6	166.7 ± 11.4	281.5 ± 16.4

[a] Mean and standard deviation of 3 replicates.

Peak numbers refer to anthocyanins in Fig. 3.1

3.3.3 Conclusions on the major anthocyanins and phenolics in red onions

A total of four anthocyanins and nine colourless phenolic compounds were found in red onions. The four anthocyanins were cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside. The other phenolic compounds were

protocatechuic acid 4-glucoside, quercetin 7,4'-diglucoside, quercetin 3,4'-diglucoside, quercetin 3-glucoside, quercetin 4'-glucoside, and a 5,7-dihydroxy flavanone glucoside or a 5,7-dihydroxy dihydroflavanol glucoside.

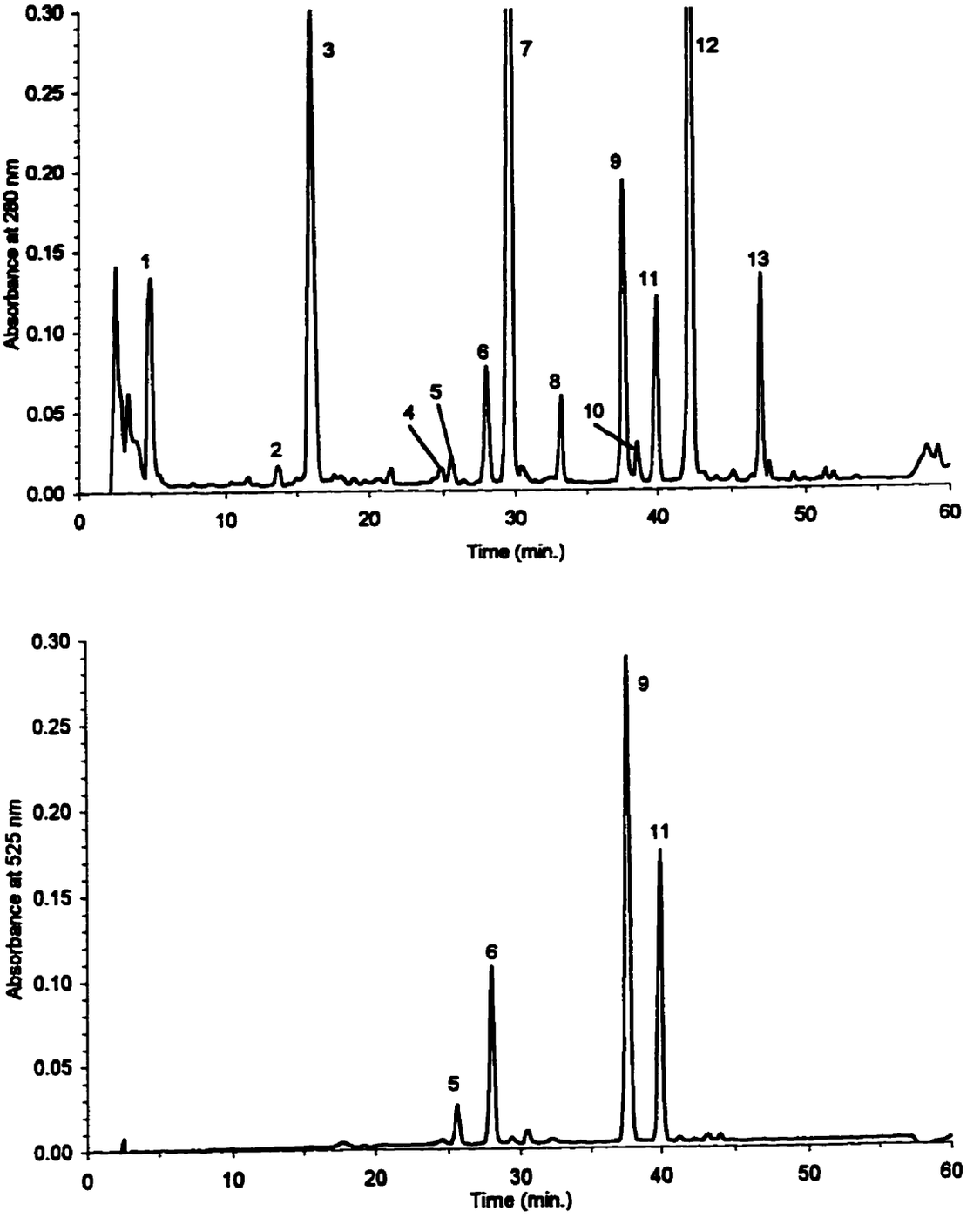


Figure 3.4: HPLC chromatogram of cv. 'Red Bone' crude extract in a methanol:formic acid:water, monitored at 280 nm (top) for colourless phenolics and at 525 nm (bottom) for anthocyanins.

3.3.4 Identification of major and minor anthocyanins

A typical HPLC chromatogram of the anthocyanins in the methanol fraction of red onion, cv. 'Red Jumbo', is shown in Figure 3.5. All onion cultivars showed qualitatively identical chromatographic profiles for anthocyanins (monitored at 525 nm) but with varying quantities of anthocyanins. For 'Red Jumbo' at 525 nm, eight anthocyanin peaks were present, four of which were major (peaks 1, 2, 5, and 6) and four were minor peaks (peaks 3, 4, 7, and 8) (Table 3.6).

Table 3.6 HPLC characterization of anthocyanins in MeOH-HFo extract of red onions, cv. 'Red Jumbo'.

Peak No.	Anthocyanin t_R (min)	% Peak area $\lambda = 525$ nm	Aglycone t_R (min)[1]	Deacylated Anthocyanin t_R (min)[2]
1	24.8	25.4	40.5	24.7
2	27.2	8.0	40.5	27.3
3*	29.7	1.2	40.4	24.9
4*	31.7	0.8	48.4	31.8
5	36.9	51.2	40.5	24.7
6	39.3	11.8	40.6	27.3
7*	42.6	1.1	48.5	31.6
8*	43.4	0.5	40.5	27.2
Standards				
Delphinidin			33.6	
Cyanidin			40.4	
Petunidin			43.4	
Pelargonidin			-	
Peonidin			48.5	
Malvidin			49.6	
Cyanidin 3-glucoside	25.0			
Peonidin 3-glucoside	31.8			

* newly identified anthocyanins, [1] determined after acid hydrolysis, [2] determined after alkaline hydrolysis

Peak numbers refer to anthocyanins in Fig. 3.5.

Table 3.7: GC characterization of aliphatic and silylated sugars of anthocyanins in red onions.

Peak #	Aliphatic acids (as methyl esters) t_R (min)	Silylated Sugars t_R (min)
1	-	18.35 and 21.40
2	-	18.33 and 21.38
3*	9.26	18.35 and 21.40
4*	-	18.33 and 21.38
5	9.26	18.36 and 21.42
6	9.26	18.36 and 21.42
7*	9.26	18.33 and 21.38
8*	9.26	18.36 and 21.41
Standards		
oxalic acid	8.31	
Malonic acid	9.26	
Succinic acid	10.34	
Rhamnose		15.05 and 15.63
Glucose		18.23 and 21.38
Galactose		17.24 and 19.14

* newly identified anthocyanins

Peak numbers refer to anthocyanins in Fig. 3.5.

Table 3.8: Spectral data of red onion anthocyanin peaks in 0.01% methanolic HCl.

Peak No.	λ_{max} (nm)	$E_{440\,nm}/E_{vis\,max}$ (%)	$E_{UV\,max}/E_{vis\,max}$ (%)	$AlCl_3$ λ_{max} (nm)	$AlCl_3$ shift (nm)
1	528, 281	23	77	561	+41
2	528, 282	25	100	561	+41
3*	528, 281	21	62	573	+45
4*	527, 282	27	75	537	+10
5	528, 281	23	62	574	+46
6	528, 281	24	76	574	+47
7*	527, 281	30	69	535	+ 8
8*	529, 282	27	73	573	+44

* newly identified anthocyanins

Peak numbers refer to anthocyanins in Fig. 3.5.

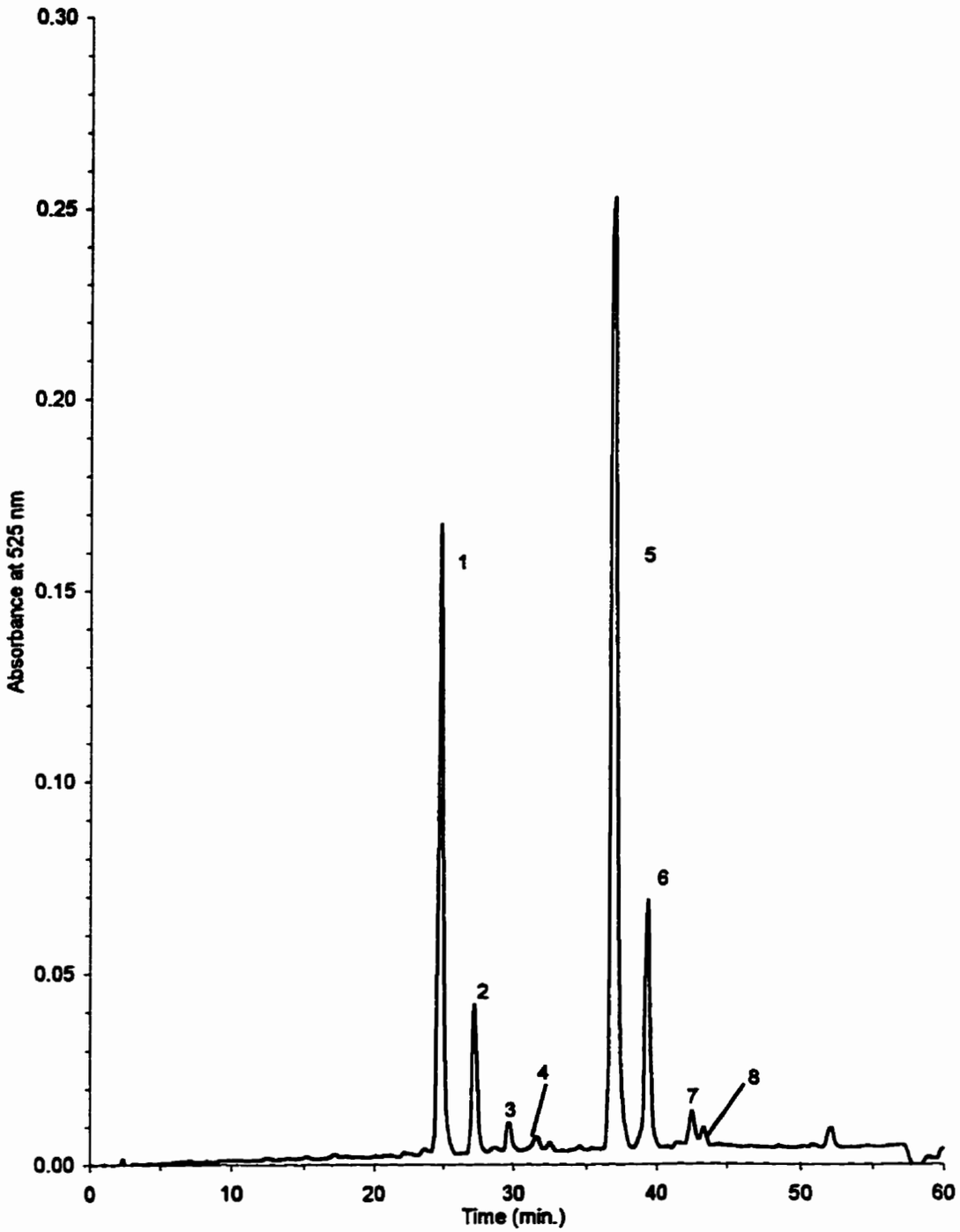


Figure 3.5: HPLC profile of anthocyanins recovered from cv. 'Red Jumbo' after purification using a C_{18} cartridge, monitored at 525 nm.

The four major peaks were easily separated from other components. At 280 nm, the chromatogram showed a complex mixture of phenolic compounds with minor anthocyanin peaks eluting or co-eluting with flavonols and other phenolics or other compounds. In order to adequately separate and identify these peaks, pre-fractionation of the raw extract was necessary. The complete procedure, which used a solid phase extraction cartridge, is shown in Figure 3.6.

Red onion scales

⇓

Extraction with MFW (40:5:55)

⇓

Crude extract ⇒ HPLC analysis

⇓

Concentration & resolubilization in aqueous HFo

⇓

Application of extract onto C_{18} cartridge

⇓

Wash with water followed by 5% HFo (10 mL)

⇓

Wash with ethyl acetate ⇒ other flavonoids (discarded)

⇓

Wash with methanol (10 mL)

⇓

Anthocyanins

⇓

HPLC separation and identification (Fig. 3.5)

Figure 3.6: Extraction, fractionation and concentration procedure for anthocyanins from red onions.

The methanol eluate obtained by this procedure contained all of the anthocyanins, which were easily resolved by HPLC for purification and identification (Figure 3.5). Most of the other

flavonoids eluted in the ethyl acetate eluent fraction and were discarded.

Peak 1 comprised 25.4 % of the anthocyanins in 'Red Jumbo' (Table 3.10). Total acid hydrolysis of this peak produced cyanidin and glucose, while alkaline hydrolysis did not produce a retention time shift indicating that peak 1 was non-acylated. Sugar analysis established that only glucose was present (Table 3.8). Spectral shift data and the comparison of the retention time of peak 1 to standards indicated that the aglycone was cyanidin; thus, the complete anthocyanin was cyanidin 3-glucoside (Table 3.7). Thus, in agreement with the findings of Terahara *et al.* (1994) and Fossen *et al.* (1996), peak 1 is cyanidin 3-glucoside.

Peak 5 was the major anthocyanin (51.2 %) in 'Red Jumbo' and total acid hydrolysis produced cyanidin and glucose, as for peak 1 (Table 3.10). Cyanidin 3-glucoside (peak 1) was yielded upon alkaline hydrolysis, indicating acylation. The acylating acid was identified as malonic acid using gas chromatographic analysis of the acid hydrolysate (Table 3.8). Using these results, peak 5 was identified as cyanidin 3-(6"-malonylglucoside), which concurs with results of Terahara *et al.* (1994) and Fossen *et al.* (1996).

Peaks 2 and 6 represented 8 % and 11.8 % of the total anthocyanins, respectively in 'Red Jumbo' onions. Acid hydrolysis of peak 2 and its spectral data led to its identification as cyanidin with a sugar moiety at the 3-position. The sugar moiety was confirmed to be glucose by sugar analysis, while alkaline hydrolysis results indicated the compound was not acylated (Tables 3.7 and 3.8, respectively). It was concluded that peak 2 was the cyanidin 3-laminaribioside as reported by Terahara *et al.* (1994) since it eluted later than peak 1 even though its chemical constituents were identical to peak 1. Glycosylation by laminaribiose probably renders the compound more hydrophobic, increasing its retention time on a C_{18} column. For peak 6, the total

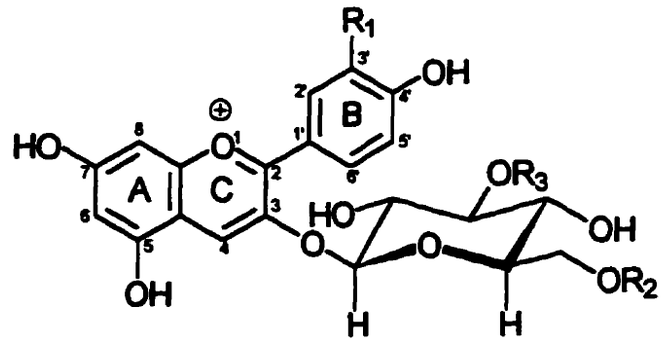
acid hydrolysis and sugar analysis produced the same result as peak 2. Alkaline hydrolysis of peak 6 yielded cyanidin 3-laminaribioside (peak 3); the acylating acid was malonic acid. Peaks 2 and 6 were therefore identified as cyanidin 3-laminaribioside and cyanidin 3-(6" malonyllaminaribioside), respectively. The four major anthocyanins of onions (peaks 1, 2, 5, 6) were confirmed to be cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside, respectively, as reported by Terahara *et al.* (1994) and Fossen *et al.* (1996).

In 'Red Jumbo', peak 3 was the most abundant of the minor peaks, and accounted for 1.2% of the total anthocyanins in this cultivar (Table 3.10). The aglycone was identified as cyanidin through acid hydrolysis, and spectral shift analysis indicated glycosylation at the 3-position (Table 3.7 and 3.9). The formation of cyanidin 3-glucoside after alkaline hydrolysis, the identification of malonic acid as the acylating acid, the shorter retention time of this peak than that of peak 5, indicated that this peak is an isomer of peak 5 with malonic acid attached at a different position on the glucose residue. It was concluded that peak 3 is cyanidin 3-(3" malonylglucoside).

Peak 8 represented 0.5 % of the total anthocyanin content in 'Red Jumbo' (Table 3.10). Similar to peak 6, acid hydrolysis, spectral data and sugar analysis indicated that this compound was cyanidin glycosylated at the 3-position. Results of alkaline hydrolysis and aliphatic acid identification suggested the compound was malonated cyanidin 3-laminaribioside. Although peaks 6 and 8 were identical in their chemical structures, the compound represented by peak 8 was more hydrophobic than peak 6, as indicated by the increased HPLC retention time of the former. It was postulated that peak 8 represents a dimalonated compound in which both glucose moieties of the laminaribiose are substituted with malonic acid.

Peaks 4 and 7, the other two minor anthocyanins, comprised 0.8 % and 1.1 % of the total anthocyanin content respectively, in 'Red Jumbo' onions (Table 3.10). Spectral analysis and acid hydrolysis results showed that the aglycone was peonidin glycosylated at the 3-position (Table 3.9). Alkaline hydrolysis of peak 7 produced peak 4 while aliphatic acid analysis identified the acylating acid as malonic acid and sugar analysis revealed the presence of glucose. Comparison of results with standards led to the tentative identification of peaks 4 and 7 as peonidin 3-glucoside and peonidin 3-malonylglucoside, respectively. This is the first report of peonidin 3-malonylglucoside in red onions.

The structures for all anthocyanins identified in red onions are illustrated in Figure 3.7.



Peak #	Name	R_1	R_2	R_3
1	Cyanidin 3-glucoside	OH	H	H
2	Cyanidin 3-laminaribioside	OH	H	glucose
3	Cyanidin 3-(3"-malonylglucoside)	OH	H	malonic acid
4	Peonidin 3-glucoside	OCH_3	H	H
5	Cyanidin 3-(6"-malonylglucoside)	OH	malonic acid	H
6	Cyanidin 3-(6"-malonyl-laminaribioside)	OH	malonic acid	glucose
7	Peonidin 3-malonylglucoside	OCH_3	malonic acid	H
8	Cyanidin 3-dimalonyllaminaribioside	OH	H	glucose & malonic acid

Peak numbers refer to anthocyanins in Fig. 3.5.

Figure 3.7: Structures of the anthocyanins identified in red onions.

3.3.5 Anthocyanin profiles of various cultivars

In all four North American grown red onion cultivars investigated, there were significant differences in the relative quantity of the anthocyanins although the anthocyanin profiles of the four cultivars were qualitatively similar (Table 3.9). In all four cultivars, cyanidin 3-malonylglucoside (peak 5) constituted between 30 and 51 % of the total anthocyanin content. In general, the second most abundant peak was cyanidin 3-malonyllaminaribioside (peak 6), followed by cyanidin 3-glucoside (peak 1) and cyanidin 3-laminaribioside (peak 2). The newly identified minor anthocyanins (peaks 3, 4, 7 and 8) were present in much lesser amounts in all four cultivars relative to peaks 1, 2, 5, and 6. Contrary to the results by Fossen *et al.* (1996), no pelargonidin derivatives were found in the four North American red onion cultivars tested. The presence of peonidin 3-glucoside (peak 4), previously reported in 'Ruby' and 'Southport Red Globe' red onions by Fuleki (1971), was confirmed. The total anthocyanin content ranged from a high of 219 ±34 mg/100g dry weight in 'Red Bone' to a low of 109 ±28 mg/100 g in 'Mambo' red onions.

Table 3.9: Relative amounts[@] of anthocyanins in red onions.

Peak #	Anthocyanin[a]	Cultivar			
		'Mambo'	'Red Bone'	'Red Granex'	'Red Jumbo'
1	Cyn 3-glc	9.4	6.8	12.5	25.4
2	Cyn 3-lam	4.4	17.8	13.6	8.0
3*	Cyn 3-mal 3"glc*	7.3	3.6	5.6	1.2
4*	Pn 3-glc*	1.1	4.7	6.4	0.8
5	Cyn 3-malglc	51.4	39.4	30.0	51.2
6	Cyn 3-mallam	10.7	23.5	19.3	11.8
7*	Pn 3-malglc*	3.4	1.7	2.7	1.1
8*	Cyn 3-dimallam*	12.2	2.4	9.8	0.5
Total anthocyanin content, (mg/100 g DW)		109 ± 28	219 ± 34	162 ± 26	-

[@] expressed as a percentage of total anthocyanin content.
Peak numbers refer to anthocyanins in Fig. 3.5.
* newly identified anthocyanin
[a] Cyn = cyanidin; Pn = peonidin; glc = glucoside; lam = laminaribioside; mal = malonyl.

3.3.6 Conclusions on the major and minor anthocyanins in red onions

Four major and four minor anthocyanins were identified in 'Red Jumbo', 'Red Granex', 'Red Bone', and 'Mambo' red onions. Major anthocyanins were identified as cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-(6"-malonylglucoside) and cyanidin 3-(6"-malonyllaminaribioside), while the minor anthocyanins were shown to be cyanidin 3-(3"-malonylglucoside), peonidin 3-glucoside, peonidin 3-malonylglucoside and cyanidin 3-dimalonyllaminaribioside. The presence of the major anthocyanins in red onions was confirmed, and four new minor anthocyanins were identified in the red onions from North America.

4. COPIGMENTATION OF RED ONION ANTHOCYANINS

4.1 Introduction

A number of factors affect the colour of anthocyanin solutions. The most important are structure of the anthocyanin and the pH of the solution (Brouillard and Dubois, 1977; Timberlake, 1980; Mazza and Miniati, 1993b). The model of Brouillard and Delaporte (1977) established that anthocyanins exist as a mixture of the flavylium cation, pseudobase or hemiacetal, chalcone and quinonoidal base forms at different pH values. Earlier, Asen *et al.* (1972) had determined that anthocyanins accumulate in the plant cell vacuole where the pH ranges from 3.5 to 5.5. Since most anthocyanins would be colourless in this pH range, mechanisms must be present which permit the vast array of colours that exist *in vivo*.

Copigmentation has been proposed as the major mechanism responsible for the orange and red to blue and violet colour diversity in most plants. Copigmentation is a molecular interaction that takes place between anthocyanin and copigment. A copigment is a colourless molecule on its own in solution, but when it is added to an anthocyanin solution, it will greatly enhance the colour (Asen *et al.*, 1972). The copigmentation effect is characterized by an increase in colour intensity (a hyperchromic effect), and a shift in the wavelength of maximum absorbance to a longer bluer wavelength (a bathochromic shift) (Asen *et al.*, 1972). The numerous sources of copigments range from flavonols, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals to anthocyanins themselves (Asen *et al.*, 1972). The copigmentation phenomenon is influenced by the structures of the anthocyanin and copigment, the pH of the solution, the solvent and the temperature of the solution (Mazza and Brouillard, 1987, 1990; Brouillard *et al.*, 1989).

Previous studies have focused on the copigmentation of commercially available diglucosides such as cyanidin 3,5-diglucoside (cyanin) and malvidin 3,5-diglucoside (malvin) with a good copigment such as chlorogenic acid (Mazza and Brouillard, 1990; Brouillard *et al.*, 1989). More recently, Davies and Mazza (1993) studied copigmentation of simple and acylated anthocyanins with different copigments such as chlorogenic acid, caffeic acid, and rutin. In addition, the copigmentation behaviour of copigments such as purines such as caffeine have recently been studied (Dangles and Elhajji, 1994; Dangles and Brouillard, 1992). A number of recent reports (Terahara *et al.*, 1994; Andersen and Fossen, 1995; Fossen *et al.*, 1996) confirmed the presence of a unique series of cyanidin 3-glycosides in red onions. Nonetheless, the effects of copigmentation with caffeine and protocatechuic acid on simple 3-glycosides or those acylated with malonic acid have not been investigated.

The copigments chlorogenic acid, protocatechuic acid and caffeine used in this study were chosen because of their solubility in an aqueous system, availability, and ability to act as a copigment (Dangles and Elhajji, 1994). The copigments used also represented different structural classes of molecules. Commercially obtained cyanidin 3-glucoside was more cost-effective, owing to the expense associated with purifying large amounts of this compound.

The purpose of this study was to determine the copigmentation effect and stability of the purified cyanidin 3-glucoside and the structurally unique malonylated cyanidin 3-glycoside derivatives from red onions with other phenolics.

The specific objectives were:

1) To determine the copigmentation characteristics of cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside with chlorogenic acid, protocatechuic acid, and caffeine.
2) To determine the effect of the type of glycosylation and/or the presence of malonic acid on copigmentation.
3) To determine the effect of pH on the copigmentation of the three anthocyanins with three colourless copigments.
4) To determine whether the copigments chlorogenic acid, protocatechuic acid and caffeine interact with cyanidin 3-glucoside, cyanidin 3-malonylglucoside, and cyanidin 3-malonyllaminaribioside via inter- or intramolecular copigmentation.
5) To determine the effects of multiple copigments on anthocyanin copigmentation.
6) To assess the photostability of cyanidin 3-glucoside at pH 3.7, 4.7, and 5.7 without and with chlorogenic acid, protocatechuic acid and caffeine in the presence and absence of light over a 35 day period.

4.2 Materials

The *ortho*-phosphoric acid was purchased from BDH Inc. (Toronto, ON) and the sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) from Mallinckrodt (St. Louis, MO). Details of the buffer preparation are outlined in Appendix 5. Water purified filtered using a MilliQ system (MilliPore Canada Ltd., Nepean, ON) was used to prepare the phosphoric acid and sodium acetate buffer. Cyanidin 3-glucoside (kuromanin chloride) was purchased from Extrasynthèse (Genay, France). Cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside were purified from red onion, *Allium cepa* L. as described in the previous section. Chlorogenic acid (3-caffeoylquinic acid) and protocatechuic acid (3,4-dihydroxybenzoic acid) were purchased from Sigma Chemical

Co. (St. Louis, MO). Caffeine (1,3,7-trimethylxanthine) was purchased from the Fluka Chemie AG (Buchs, Switzerland). All pigments and copigments were assayed for purity on the HPLC system described in the previous section. All chemicals were of reagent grade or better. The structures of the anthocyanins and copigments used are shown in Figure 4.1 below.

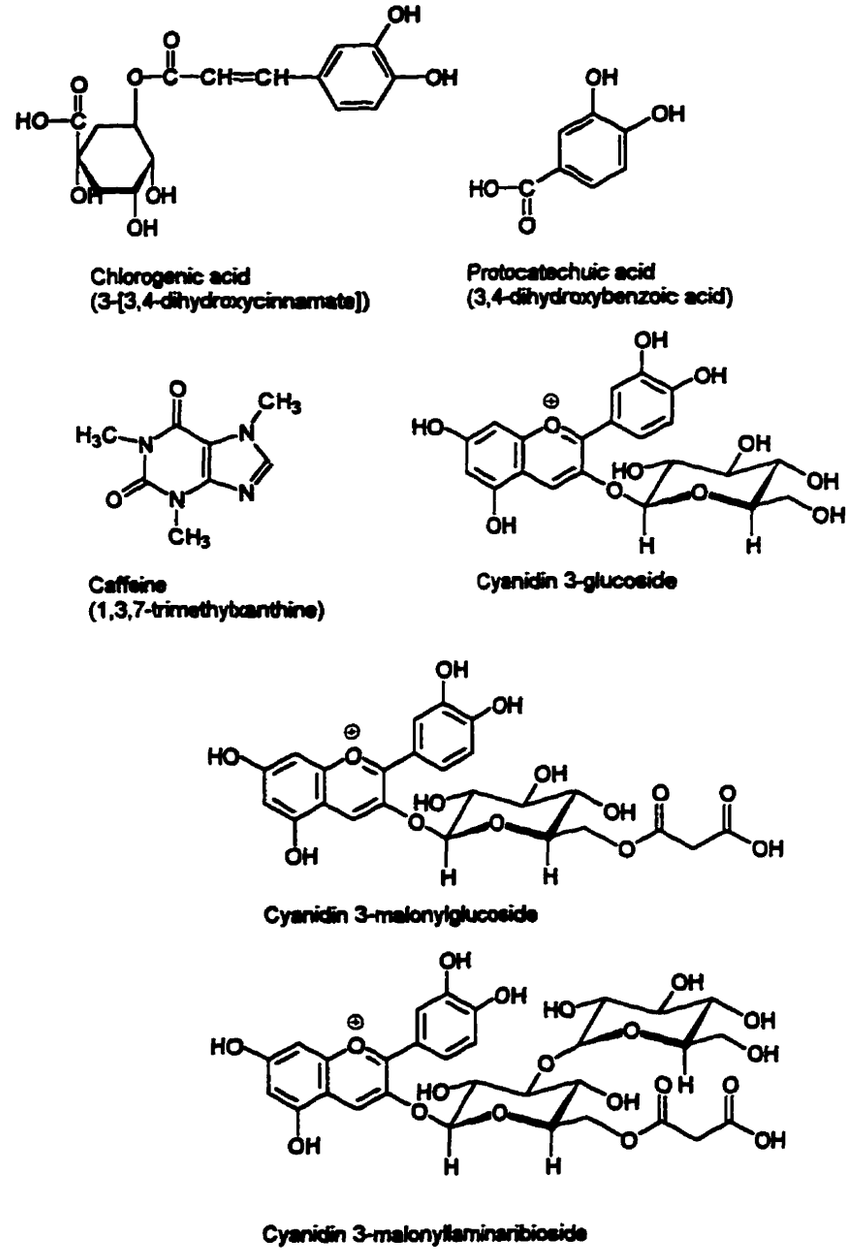


Figure 4.1: Structures of compounds used in copigmentation experiment.

4.2.1 Methods

4.2.1.1 Apparatus

Absorption spectra of the buffered anthocyanin solutions, with (A) and without (A_0) copigment were recorded from 400 to 700 nm using a Beckman DU640 spectrophotometer (Beckman Instruments (Canada) Inc., Mississauga, ON) using the wavelength scan software. The spectrophotometer was also fitted with Tristimulus colorimetry software from Beckman (Colour Determination and Matching (CIE, E308)). The cuvette was a standard 1 cm^2 quartz cuvette with a glass jacket on its opaque sides that allowed for temperature control of the solution. Cuvette temperature was controlled at 20°C±0.01°C by a recirculating water bath (Model 1166, VWR Scientific Canada Ltd., London, ON). A cuvette stir bar was used for mixing. A custom fitted cuvette holder containing a magnetic stirring device (The Chemical Co., Cleveland, OH) driven by compressed air was used to spin the stir bar. The pH was measured using a Fisher Accumet pH meter 25 and a Fisher Accu.pHast microprobe combination glass electrode (Fisher Scientific, Nepean, ON). A standard non-jacketed quartz cuvette was used to hold the blank solution.

The concentration of the buffers was 0.06 M *o*-phosphoric acid and 0.2 M sodium acetate. Stock solutions of anthocyanins (cyanidin 3-glucoside, cyanidin 3-malonylglucoside, and cyanidin 3-malonyllaminaribioside) were prepared by dissolving all of the pigment necessary for a series of experiments (1 anthocyanin x 3 copigments x 3 pH values) in the minimum amount of phosphoric acid buffer to be used. At least 0.2 mL of phosphoric acid was needed for each individual experiment; therefore, this defined the minimum amount used (0.2 mL x 9 solutions and at least 0.2 mL of excess in case an experiment had to be repeated). The ratios of phosphoric acid and sodium buffer required to obtain the pH values of 3.7, 4.7, and 5.7 are detailed in Appendix 2. A final anthocyanin concentration of 1.29×10^{-4} M, at a pH value close to the desired pH was obtained once the two buffers and stock were mixed in the appropriate amounts.

The solution was allowed to mix in the dark in the spectrophotometer at 20°C for 30-45 minutes prior to the start of experimentation.

To determine the copigmentation effect, the absorbance values without (A_o) and with (A) copigment were recorded at 525 nm. After allowing the anthocyanin solution to mix for 30-45 minutes, the pH of the solution was adjusted by pipetting a few µL of 5 M HCl or 5 M NaOH into the cuvette to obtain the desired pH. The spectrophotometer was then zeroed with the non-jacketed standard quartz cuvette filled with the same buffer solution used in the experiment minus the pigment. The cuvette containing the anthocyanin solution was replaced in the holder and the temperature was allowed to stabilize before the initial Tristimulus measurements (CIE L*a*b* 76) and initial absorbance (A_0) values were read. After recording the spectrum of the anthocyanin solution, a preweighed amount of copigment (chlorogenic acid, protocatechuic acid or caffeine) was added. After 15 minutes of stirring, the pH of the solution was measured, adjusted accordingly and then the spectrum of the anthocyanin with the copigment was recorded to determine its absorbance with the added copigment at 525 nm (A). Pigment to copigment ratios (P:CP) of 1:5 (CP concentration = 6.45×10^{-4} M), 1:10 (1.29×10^{-3} M), 1:15 (1.94×10^{-3} M), 1:20 (2.58×10^{-3} M), 1:50 (6.45×10^{-3} M), 1:100 (1.29×10^{-2} M), 1:150 (1.94×10^{-2} M), 1:200 (2.58×10^{-2} M), 1:250 (3.23×10^{-2} M), 1:300 (3.87×10^{-2} M), 1:350 (4.52×10^{-2} M)and 1:400 (CP concentration = 5.16×10^{-2} M) were used in the experiment. Spectra were obtained for all these concentrations; however, the Tristimulus measurements were taken at 1:0, 1:200 and 1:400 pigment : copigment molar ratios. The Tristimulus measurements were only read at concentrations of 1:0, 1:200, and 1:400 since these were the initial solution colour, at half the final pigment : copigment concentration, and final solution colour. The L*, a*, b* values would provide another way of describing the colour changes with increasing amount of added copigment.

4.2.1.2 Equilibrium constant determination

The equilibrium constant (K) was determined using the procedure used by Brouillard *et al.* (1989). In order to determine K, the r_1 value was first determined. The r_1 value is a ratio of the absorbance at 525 nm without copigment (A_0) over the absorbance at 525 nm for the solution with an excess amount of copigment (A). $r_1 = \frac{A}{A_0}$

First, a 50 μL aliquot of the original stock solution of anthocyanin was diluted in 0.2 M HCl. A known concentration of anthocyanin was dissolved in 0.2 M HCl to obtain a final concentration of 6.45×10^{-6} M and the initial absorbance at 525 nm (A_o) was read. Once the copigment was added, the absorbance at 525 nm was read (A) and then the next amount of copigment was added. The amount of copigment was considered excessive when additional amounts would no longer dissolve, causing turbidity. The r_1 was calculated for each concentration of added copigment, which was used to calculate K. The K values were averaged for each pigment : copigment combination and the main K was determined from the average K values at the three different pHs.

4.2.1.3 Multiple copigmentation experiments

For the multiple copigmentation experiments, the same apparatus and methodology were used as with the previous copigmentation experiments. For these experiments, the cyanidin 3-glucoside solution (1.29×10^{-4} M) was prepared from the stock solution at a pH of 4.7. A pH of 4.7 was used since it gave the greatest increase in absorbance upon addition of copigment. Two experiments were conducted. The first experiment involved the addition of chlorogenic acid and caffeine successively at pigment : copigment molar ratios of 1:5:0, 1:5:5, 1:10:5, 1:10:10, 1:25:10, 1:25:25, 1:50:50, 1:100:50, 1:100:100. The second experiment involved the addition of chlorogenic acid, caffeine and protocatechuic acid successively at pigment : copigment ratios of

1:10:0:0, 1:10:10:0, 1:10:10:10, 1:25:10:10, 1:25:25:10, 1:25:25:25, 1:50:25:25, 1:50:50:25, 1:50:50:50, 1:100:50:50, 1:100:100:50, 1:100:100:100, 1:200:100:100, 1:200:200:100, 1:200:200:200. The data was analyzed using the copigmentation model as in the previous experiments (Mazza and Brouillard, 1987).

4.2.1.4 Colour stability of cyanidin 3-glucoside:copigment complexes at 20°C in the presence and absence of light

An adequate amount of cyanidin 3-glucoside stock solution was prepared for a set of controls at pH 3.7, 4.7, and 5.7 and the three pH solutions with the three different copigments (chlorogenic acid, protocatechuic acid, and caffeine) for a total of twelve different solutions. This set of twelve solutions was doubled since the effect of light on the colour stability were to be studied over 35 days. A set of twelve solutions was stored in the presence of light and another twelve solutions were stored in the absence of light in a box lined with aluminium foil to prevent light from entering. The solutions were mixed and stored in 4 mL HPLC sample vials. All vials were stored in a ConViron chamber (Controlled Environments Ltd., Winnipeg, MB). The chamber temperature was maintained as close as possible to 20°C. All fluorescent and incandescent lights were on continuously for the 35 day period to mimic the intensity of natural light as much as possible. Samples exposed to light were placed on a table 1.2 m from the lights on a white sheet of paper set at 30° angle. The samples, which were kept in the dark, were placed in a plastic container and stored inside a small cardboard box beside the light exposed samples. Approximately every 5 days over a 35 day period, the pH, absorbance at 525 nm, spectra and CIE L*a*b* Tristimulus measurements were taken to determine how stable each anthocyanin : copigment solution was, based on colour and visual appearance. At the same time, the light intensity was measured using a LI-1600 steady state porometer with a LI-190S-1 quantum sensor (LiChor Inc., Lincoln, NE). The LI-COR quantum sensor measures photosynthetically active

radiation in the 400-700 nm waveband in micromoles per square meter per second ($\mu mol\ m^{-2}\ s^{-1}$, where 1 $\mu mol\ m^{-2}\ s^{-1}$ = 1 $\mu E\ m^{-2}\ s^{-1}$ = 6.023 x 10^{23} photons). Solutions were removed from the chamber to record spectra and CIE L*a*b* values. The samples were removed from the box prior to analysis and then replaced as soon as the analysis was done. Stability readings were determined as the ratio of absorbance at 525 nm at day X relative to the initial absorbance at 525 nm on day 0 and called relative absorbance.

$$\frac{A_{525}\ \text{day X}}{A_{525}\ \text{day 0}}$$

The change in CIE L*, a*, and b* values, absorbance at 525 nm, and wavelength of maximum absorbance (λ_{max}) were monitored for all samples over the 35 day period.

4.2.1.5 Data analysis

All data were tabulated, graphed and analyzed using Microsoft Excel 97 (Microsoft Inc., Redmond, WA). The CIE L*a*b* colour squares were created using Adobe Photoshop Version 4.0 (Adobe Systems Inc., Mountain View, CA).

4.3 Results

4.3.1 Influence of anthocyanin structure

When a copigment is added to an anthocyanin solution, it will change the colour of the solution and increase the colour intensity depending on the pH of the solution. The increase in colour intensity is known as a hyperchromic shift and the shift in wavelengths of maximum absorbance towards a longer wavelength is called a bathochromic shift. The spectra and spectral shifts that take place when increasing amounts of copigment are added to an anthocyanin solution are illustrated in Figures 4.2, 4.3 and 4.4. Figure 4.2 are the spectral scans of cyanidin 3-glucoside at pH 3.7, 4.7 and 5.7 without copigment and when copigmented with increasing

amounts of chlorogenic acid. Figures 4.3 and Figures 4.4 are the spectral scans of cyanidin 3-glucoside at pH 3.7, 4.7 and 5.7 copigmented with protocatechuic acid and caffeine, respectively. The spectra for cyanidin 3-glucoside with the different copigments illustrate a general increase in absorbance at pH 3.7, 4.7 and 5.7. The spectra of cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside exhibited spectra similar to those of cyanidin 3-glucoside. The degree of copigmentation was dependent upon the structure of the anthocyanin, and copigment and the pH. These three factors were analyzed separately by combining the data from the different sets of experiments and then comparing the change in absorbance at 525 nm with added copigment (A) to the original absorbance at 525 nm without copigment (A_0). When the change in absorbance ($A-A_0/A_0$) was plotted as a function of copigment concentration, the effect of increasing copigment concentration on colour was determined. In addition, the change in absorbance was then used to compare the effect of pigment and copigment structure and pH.

Figures 4.5 illustrate the absorbance change at 525 nm for cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside at pH 3.7, 4.7 and 5.7 respectively, with increasing concentration of chlorogenic acid. Figures 4.6 illustrates the absorbance change for three anthocyanins copigmented with protocatechuic acid and Figures 4.7 for the three anthocyanins copigmented with caffeine. In Figures 4.5, 4.6, and 4.7, cyanidin 3-glucoside generally had the largest absorbance change when one compares it to the two malonylated anthocyanins. However, the difference in absorbance change between the three pigments was never very large.

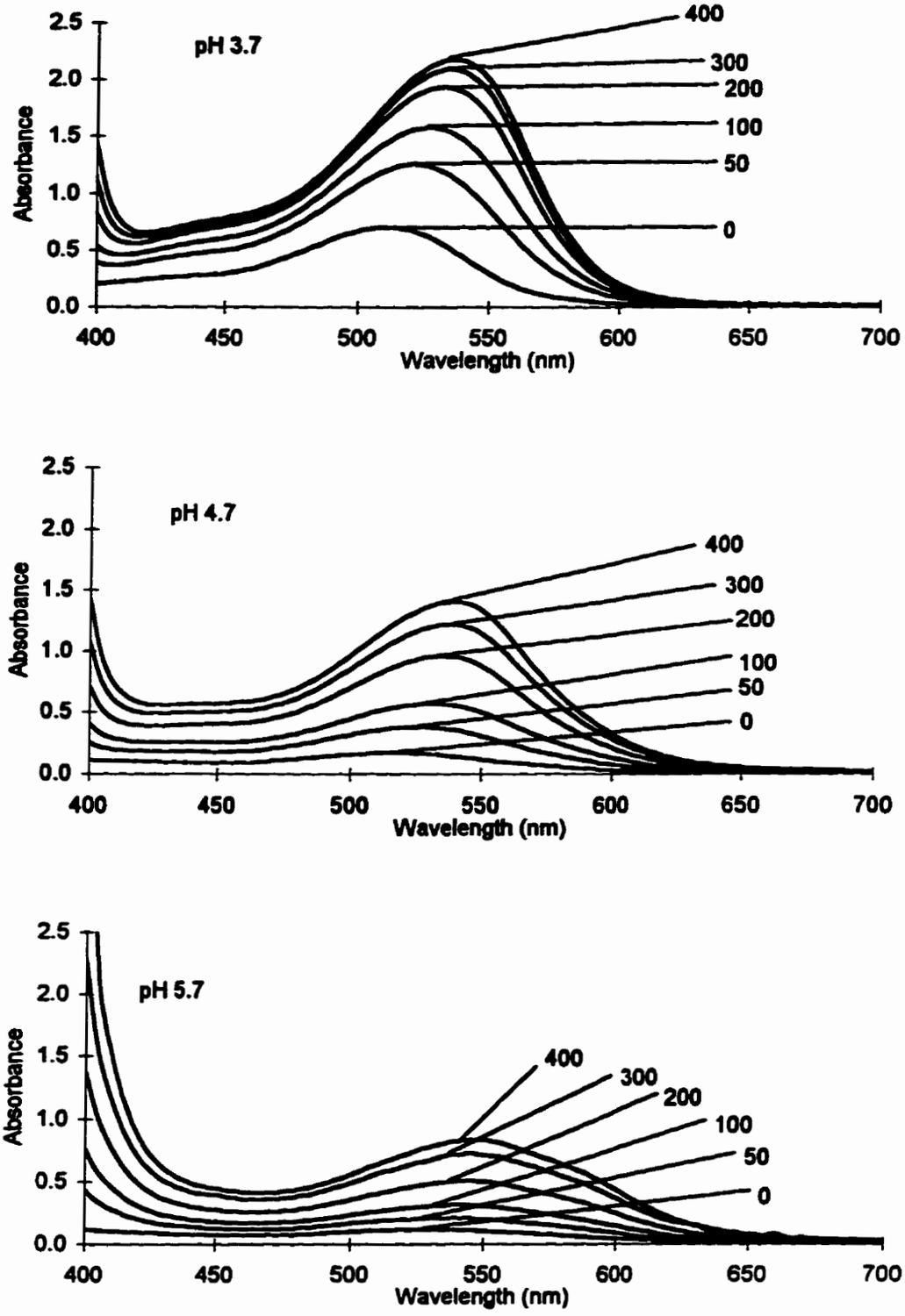


Figure 4.2: Spectral scans of cyanidin 3-glucoside (1.29×10^{-4} M) with increasing amounts of chlorogenic acid (P:CP = 1:0, 1:50, 1:100, 1:200, 1:300, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

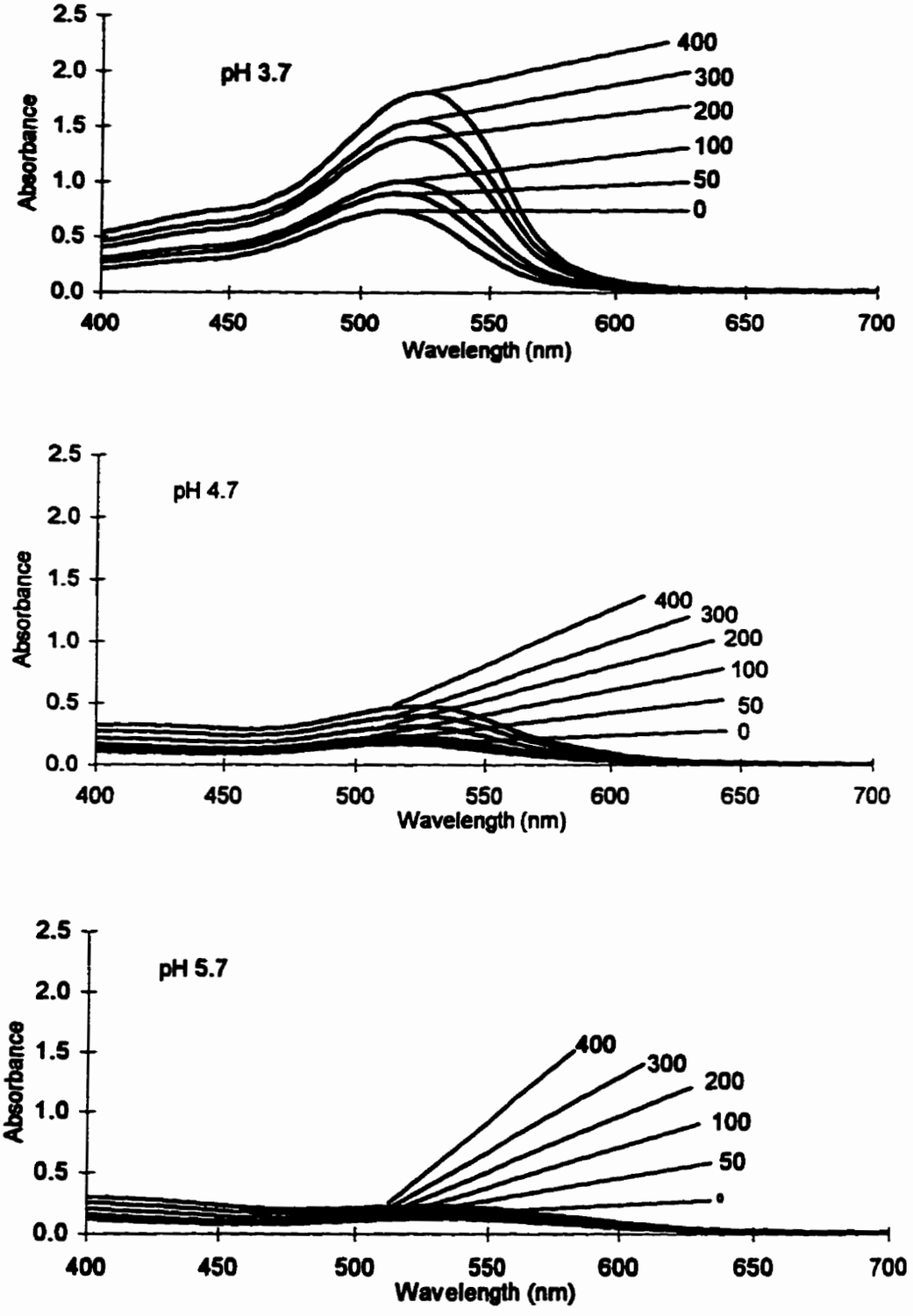


Figure 4.3: Spectral scans of cyanidin 3-glucoside (1.29×10^{-4} M) with increasing amounts of protocatechuic acid (P:CP = 1:0, 1:50, 1:100, 1:200, 1:300, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

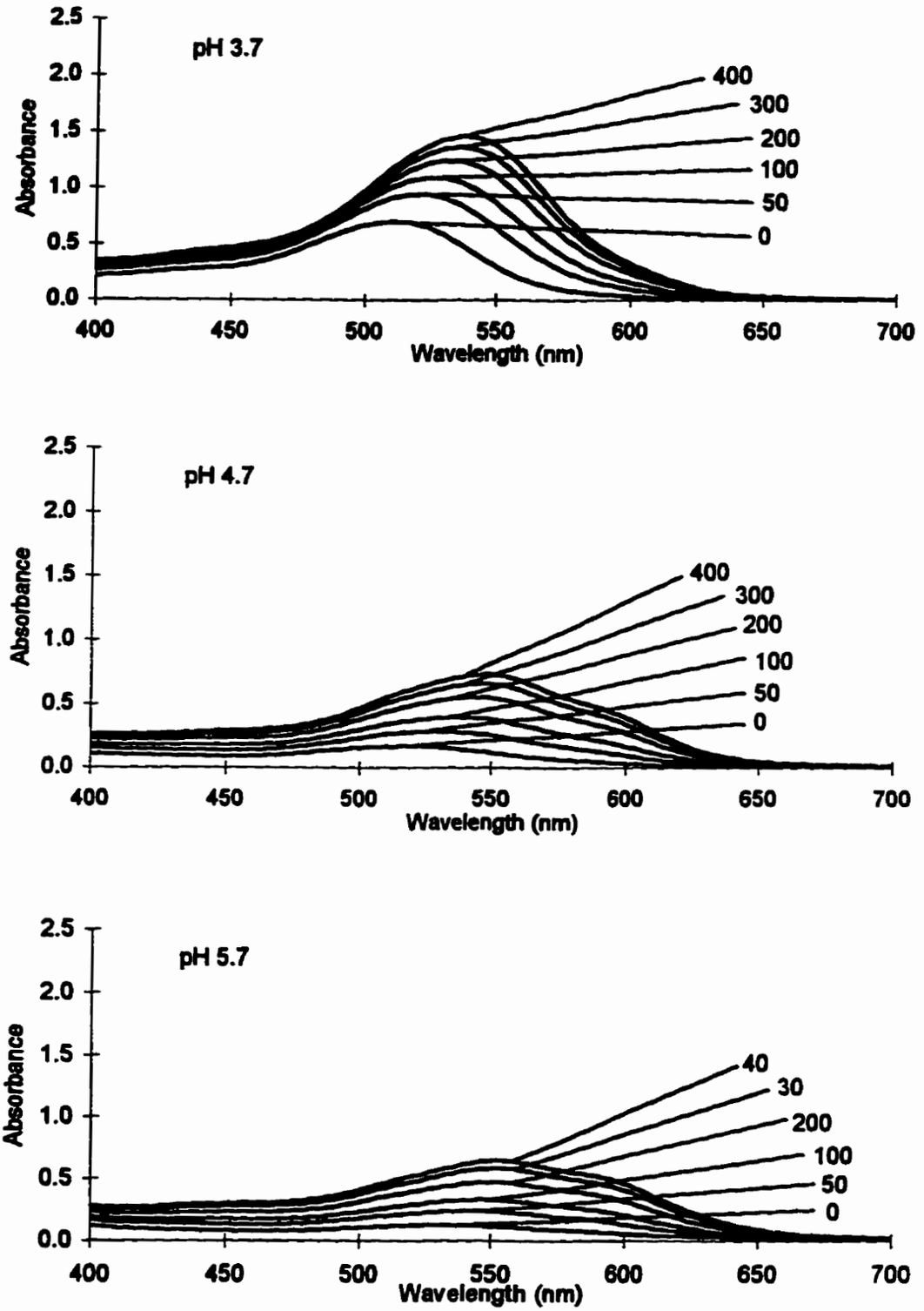


Figure 4.4: Spectral scans of cyanidin 3-glucoside (1.29×10^{-4} M) with increasing amounts of caffeine (P:CP = 1:0, 1:50, 1:100, 1:200, 1:300, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

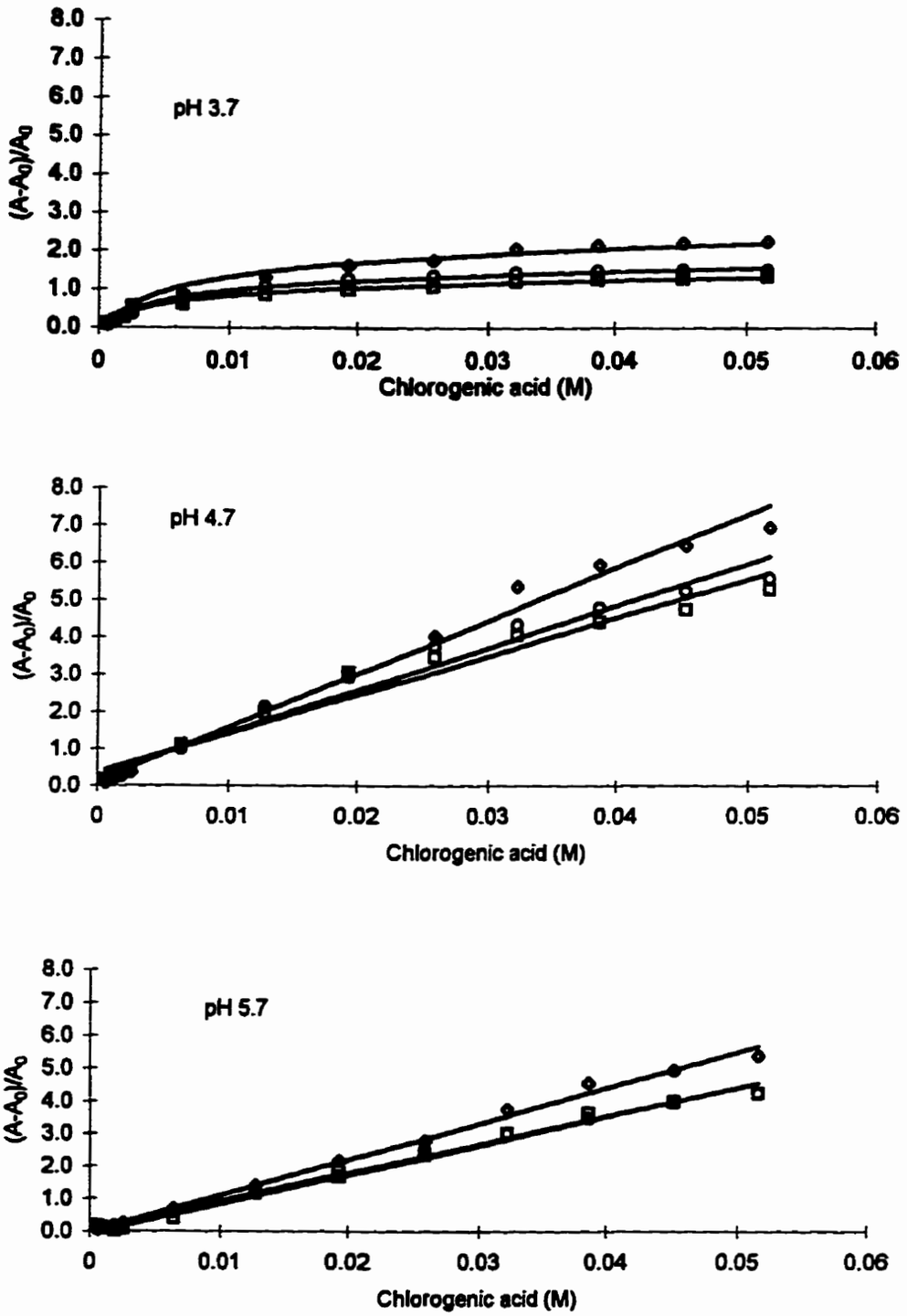


Figure 4.5: Absorbance change at 525 nm for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○) with increasing amounts of chlorogenic acid (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

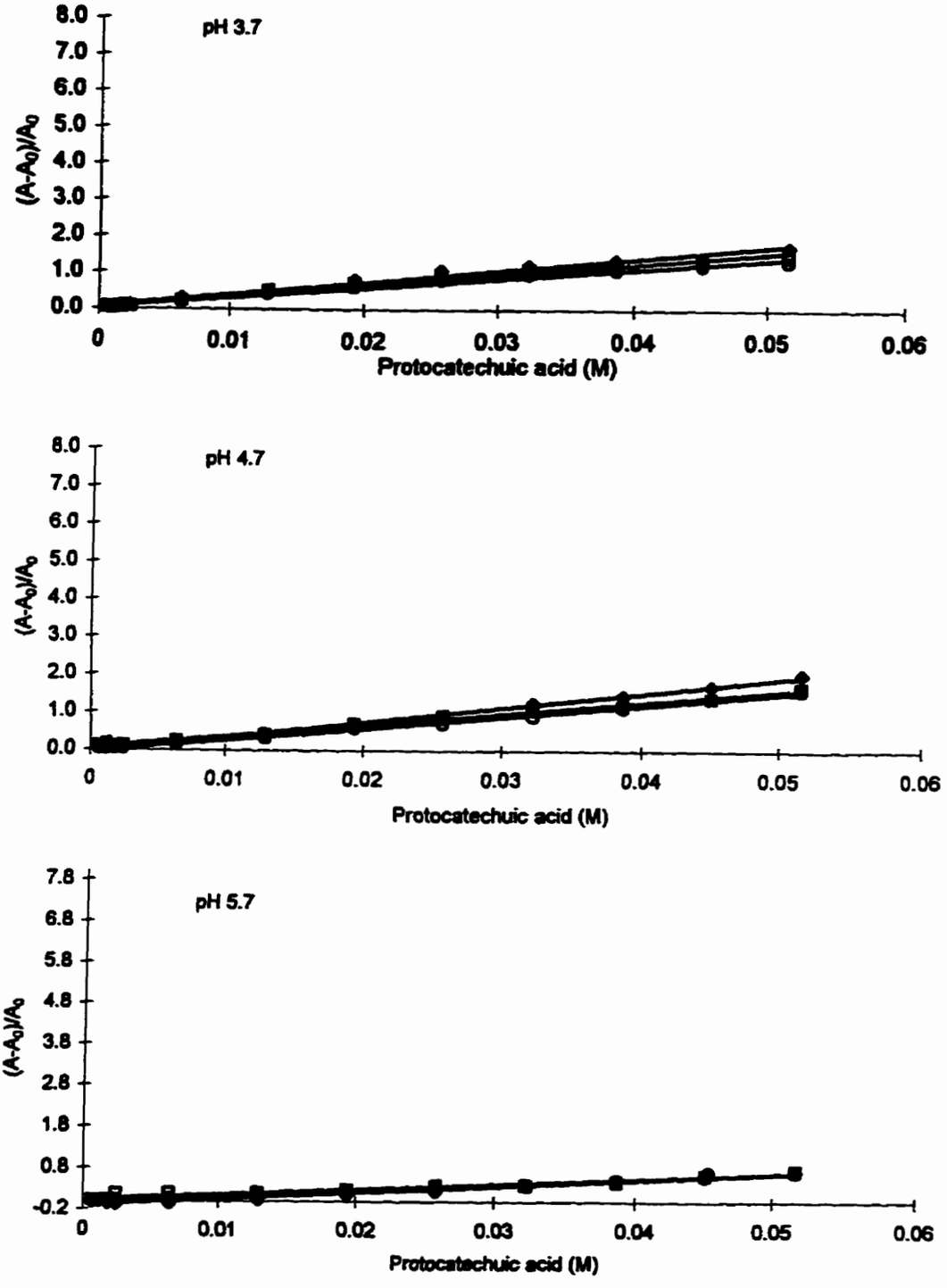


Figure 4.6: Absorbance change at 525 nm for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○) with increasing amounts of protocatechuic acid (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

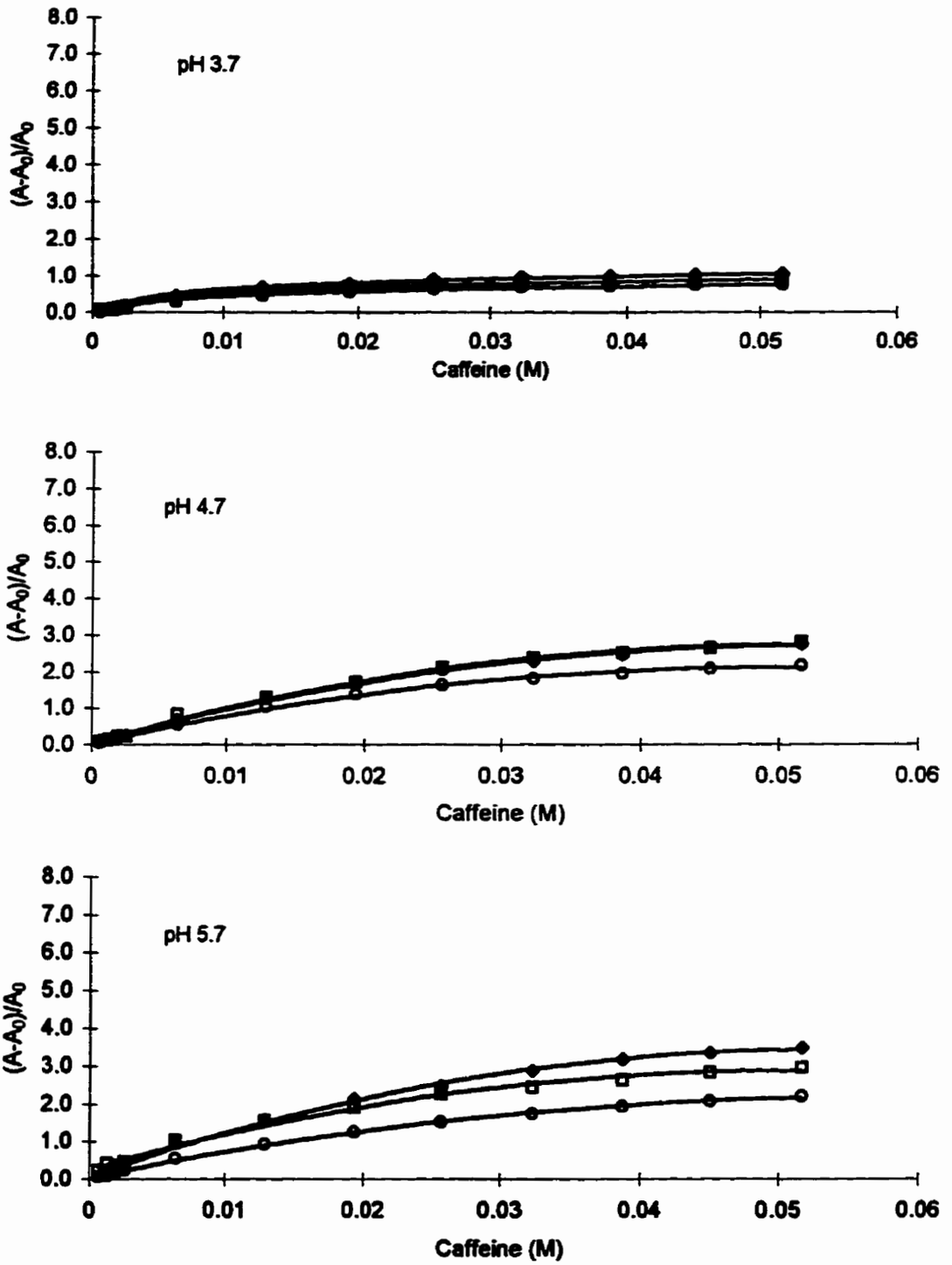


Figure 4.7: Absorbance change at 525 nm for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○) with increasing amounts of caffeine (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

Table 4.1: Influence of pigment and copigment structure on absorbance increase at the P:CP ratio of 1:400 and pH 3.7, 4.7 and 5.7.

Anthocyanin	Copigment	pH	$(A-A_0)/A_0$ at P:CP 1:400	Type of increase	Equation of line
Cyanidin 3-glucoside	chlorogenic acid	3.7	2.24	logarithmic	$y = 0.54 \ln x + 3.79$
		4.7	6.94	linear	$y = 143.37x + 0.13$
		5.7	5.38	linear	$y = 110.16x - 0.01$
	protocatechuic acid	3.7	1.70	linear	$y = 33.27x + 0.05$
		4.7	2.02	linear	$y = 38.67x - 0.04$
		5.7	0.74	linear	$y = 15.71x - 0.10$
	caffeine	3.7	1.05	logarithmic	$y = 0.25 \ln x + 1.79$
		4.7	2.75	quadratic	$y = -984.23x^2 + 102.57x + 0.03$
		5.7	3.47	quadratic	$y = -1215.80x^2 + 127.94x + 0.06$
Cyanidin 3-malonylglucoside	chlorogenic acid	3.7	1.32	logarithmic	$y = 0.29 \ln x + 2.17$
		4.7	5.28	linear	$y = 103.51x + 0.38$
		5.7	4.21	linear	$y = 89.43x - 0.67$
	protocatechuic acid	3.7	1.44	linear	$y = 29.77x + 0.04$
		4.7	1.66	linear	$y = 30.41x + 0.06$
		5.7	0.75	linear	$y = 12.77x + 0.04$
	caffeine	3.7	0.90	logaritmic	$y = 0.22 \ln x + 1.53$
		4.7	2.82	quadratic	$y = -1042.10x^2 + 105.89x + 0.042$
		5.7	2.96	quadratic	$y = -1039.00x^2 + 104.42x + 0.25$
Cyanidin 3-malonyllaminaribioside	chlorogenic acid	3.7	1.49	logarithmic	$y = 0.37 \ln x + 2.64$
		4.7	5.56	linear	$y = 113.54x + 0.30$
		5.7	4.23	linear	$y = 86.50x + 0.06$
	protocatechuic acid	3.7	1.32	linear	$y = 26.49x + 0.04$
		4.7	1.60	linear	$y = 31.04x - 0.05$
		5.7	0.71	linear	$y = 13.80x - 0.02$
	caffeine	3.7	0.75	logarithmic	$y = 0.19 \ln x + 1.31$
		4.7	2.17	plateau	$y = -838.77x^2 + 83.65x + 0.03$
		5.7	2.21	plateau	$y = -654.68x^2 + 75.05x + 0.03$

Tables 4.1 summarizes information in Figures 4.5, 4.6, and 4.7. When comparing the three pigments with three copigments at three different pH values, it is evident that there are similar increases in copigmentation amongst the three anthocyanins, cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside. Figures 4.5, 4.6, and 4.7 illustrate that when each anthocyanin is copigmented with a copigment at a certain pH, there is a similar pattern of absorbance change. All three anthocyanins behaved similarly when combined with the same copigment at the same pH. For example, cyanidin 3-glucoside, cyanidin 3-malonylglucoside, and cyanidin 3-malonyllaminaribioside all had linear absorbance increases up to a pigment : copigment ratio of 1:400 for pH 3.7, 4.7 and 5.7 (see Figure 4.6). When copigmented with caffeine up to a pigment : copigment ratio of 1:400 at the same three pH values, cyanidin 3-glucoside, cyanidin 3-malonylglucoside, and cyanidin 3-malonyllaminaribioside all exhibited a plateau in the absorbance increase (Figure 4.7). Figure 4.5 illustrates that at pH 3.7, the increase in absorbance of all three anthocyanins with the addition of increasing amounts of chlorogenic acid was initially rapid. However, at concentrations of chlorogenic acid above approximately 0.0025 M (1:20), absorbance values increased more slowly, to generate a curve with an absorbance plateau at 1:400 (Figure 4.5a). At pH 4.7 and 5.7, the addition of chlorogenic acid caused the absorbance of the solution to increase in a linear fashion at all copigment concentrations tested. Figures 4.5, 4.6, and 4.7 and Table 4.1 illustrate that each of the three copigments evaluated caused a greater increase in absorbance when added to cyanidin 3-glucoside than when the copigments were added to either cyanidin 3-malonylglucoside or cyanidin 3-malonyllaminaribioside. However, the absorbance increase with cyanidin 3-glucoside was only slightly higher than that observed with the other two anthocyanins tested. For example, cyanidin 3-glucoside, when copigmented with chlorogenic acid at pH 3.7, 4.7, and 5.7, always had a higher absorbance increase than cyanidin 3-malonylglucoside and

cyanidin 3-malonyllaminaribioside. The same trend held true for copigmentation with caffeine at pH 3.7 and 5.7. At pH 4.7 and with caffeine, the absorbance increase between cyanidin 3-glucoside and cyanidin 3-malonylglucoside was virtually identical (Figure 4.7b). The copigmentation with protocatechuic acid up to 1:400 at pH values 3.7, 4.7 and 5.7 resulted in minor differences between the three anthocyanins, relative to the other copigments used (Figure 4.6).

Generally, the largest absorbance increase took place with cyanidin 3-glucoside with all copigments. The absorbance of cyanidin 3-malonylglucoside solutions increased less and absorbance of cyanidin 3-malonyllaminaribioside solutions increased the least. Tristimulus colour measurements also revealed little difference in the colour between the three anthocyanins before and after copigment had been added to the solution at ratios of 1:200 (2.58×10^{-2} M) and 1:400 (5.16×10^{-2} M) (Figures 4.8, 4.9, 4.10). The original CIE L*a*b* values and hue angle are listed in Tables 4.2, 4.3, and 4.4.

Figure 4.8: CIE L*a*b* values for cyanidin 3-glucoside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

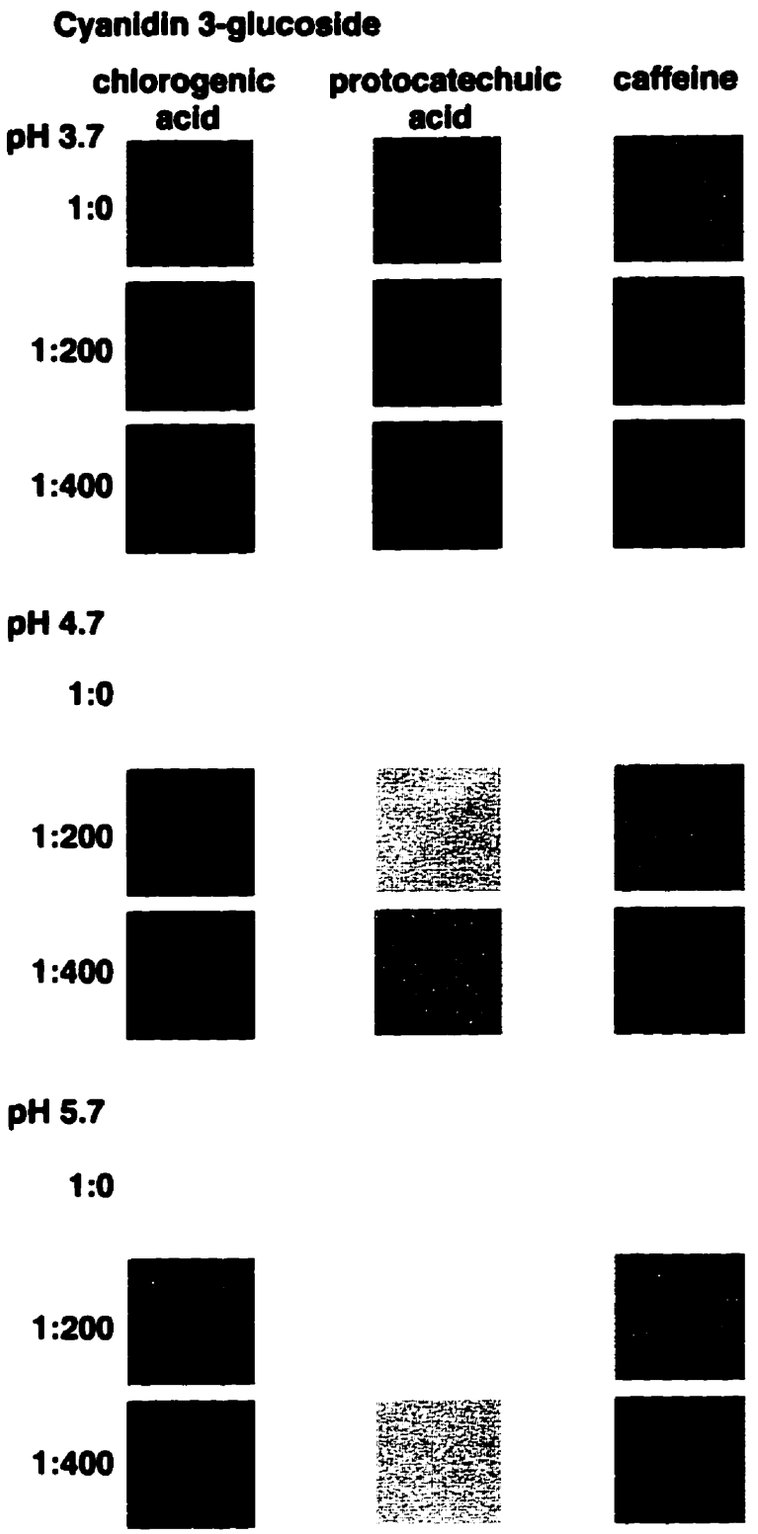

Cyanidin 3-glucoside
chlorogenic acid
protocatechuic acid
caffeine
pH 3.7
1:0
1:200
1:400
pH 4.7
1:0
1:200
1:400
pH 5.7
1:0
1:200
1:400

Table 4.2: CIE L*a*b* values for cyanidin 3-glucoside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

pH	Pigment : Copigment		Chlorogenic acid	Protocatechuic acid	Caffeine
3.7		L*	80.5	81.1	80.2
	1:0	a*	39.0	37.7	38.3
		b*	11.5	10.7	10.0
		Hue angle (θ)	16.4	15.8	14.6
		L*	54.6	70.7	60.0
	1:200	a*	70.4	52.8	59.6
		b*	10.7	13.1	-10.6
		Hue angle (θ)	8.6	13.9	-10.1
		L*	52.7	62.0	55.0
	1:400	a*	71.1	63.3	62.2
		b*	11.5	21.8	-16.0
		Hue angle (θ)	9.2	19.0	-14.4
4.7		L*	91.7	91.6	91.7
	1:0	a*	12.8	12.2	12.3
		b*	0.5	0.2	-0.2
		Hue angle (θ)	2.2	0.9	-0.9
		L*	64.4	85.6	71.2
	1:200	a*	48.9	19.9	29.9
		b*	-6.5	2.9	-15.1
		Hue angle (θ)	-7.6	8.3	-26.8
		L*	54.0	78.8	64.5
	1:400	a*	58.5	28.0	34.4
		b*	-5.2	5.6	-20.2
		Hue angle (θ)	-5.1	11.3	-30.4
5.7		L*	92.6	90.4	92.3
	1:0	a*	7.4	7.7	7.1
		b*	-0.3	-2.7	-1.1
		Hue angle (θ)	-2.3	-19.3	-8.8
		L*	72.0	89.0	71.7
	1:200	a*	23.3	8.6	19.9
		b*	-4.5	4.8	-14.4
		Hue angle (θ)	-10.9	29.2	-35.9
		L*	59.2	86.0	64.9
	1:400	a*	31.2	10.0	23.2
		b*	-5.7	8.8	-19.0
		Hue angle (θ)	-10.4	41.3	-39.3

L, 0 – 100 black to white; +a, red, -a, green; +b, yellow, -b, blue; Hue angle (θ), $\tan^{-1}$ (b*/a*).

Figure 4.9: CIE L*a*b* values for cyanidin 3-malonylglucoside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

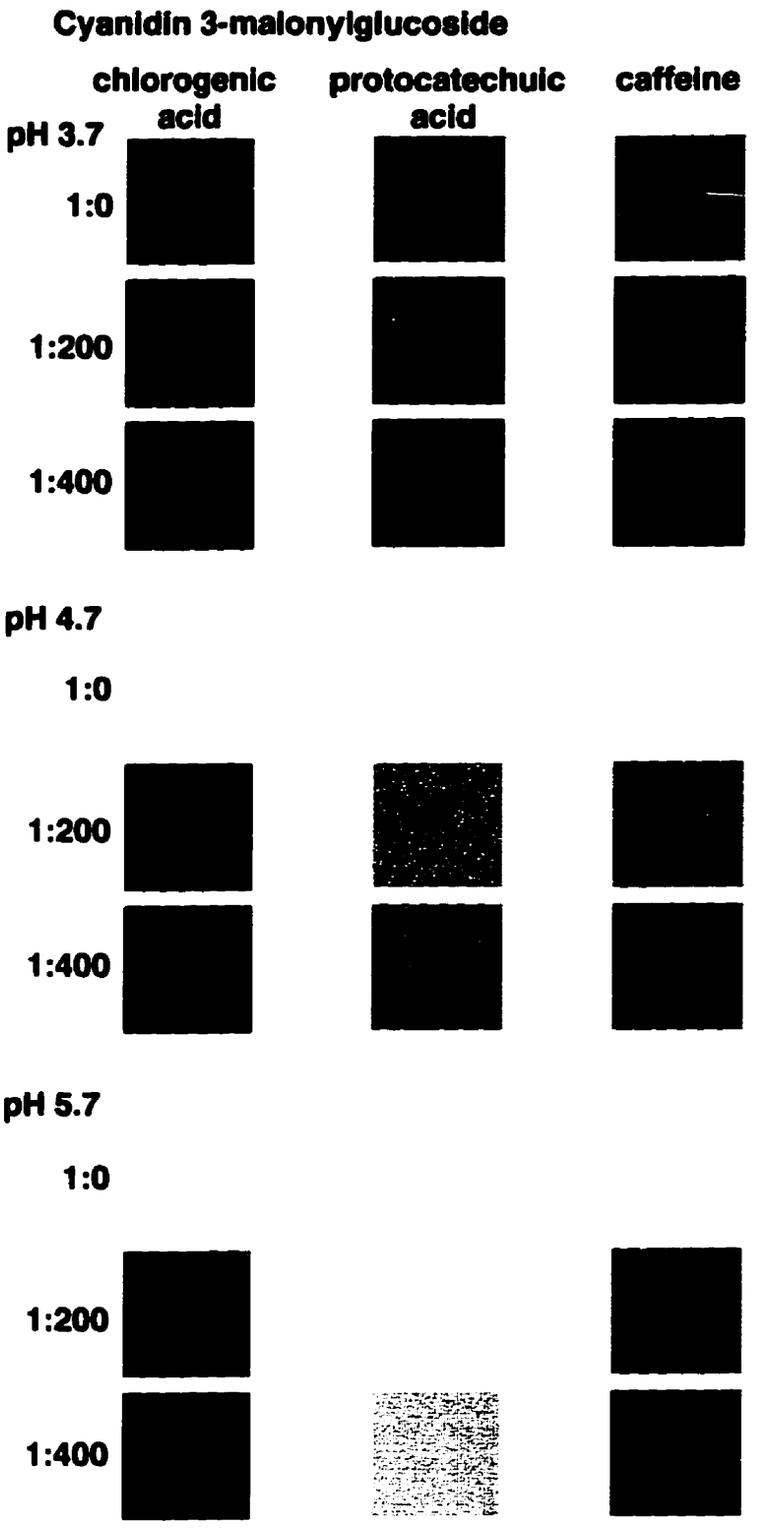

Cyanidin 3-malonylglucoside
chlorogenic acid
protocatechuic acid
caffeine
pH 3.7
1:0
1:200
1:400
pH 4.7
1:0
1:200
1:400
pH 5.7
1:0
1:200
1:400

Table 4.3: CIE L*a*b* values for cyanidin 3-malonylglucoside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

pH	Pigment : Copigment		Chlorogenic acid	Protocatechuic acid	Caffeine
3.7		L*	74.6	76.5	78.4
	1:0	a*	47.1	42.9	43.4
		b*	14.7	11.5	10.0
		Hue angle (θ)	17.3	15.0	13.0
		L*	54.5	65.5	57.9
	1:200	a*	69.6	58.7	62.2
		b*	9.2	16.6	-10.3
		Hue angle (θ)	7.5	15.8	-9.4
		L*	56.3	60.5	53.3
	1:400	a*	74.4	64.5	65.0
		b*	6.8	22.2	-16.1
		Hue angle (θ)	5.2	19.0	13.9
4.7		L*	90.6	90.3	91.7
	1:0	a*	14.2	13.0	14.0
		b*	-0.4	-0.2	0.1
		Hue angle (θ)	-1.6	-0.9	0.4
		L*	60.5	83.7	71.1
	1:200	a*	52.9	22.0	33.1
		b*	-8.6	3.0	-16.4
		Hue angle (θ)	-9.2	7.8	-26.4
		L*	54.5	77.3	64.8
	1:400	a*	59.8	30.4	37.8
		b*	-5.1	5.1	-20.8
		Hue angle (θ)	-4.9	9.5	-28.8
5.7		L*	90.7	91.2	91.3
	1:0	a*	7.6	7.5	8.2
		b*	-2.2	-2.3	-2.0
		Hue angle (θ)	-16.1	-17.0	-13.7
		L*	71.3	88.9	71.5
	1:200	a*	23.4	8.1	21.4
		b*	-6.4	4.0	-16.5
		Hue angle (θ)	-15.3	26.3	-37.6
		L*	60.1	87.2	65.1
	1:400	a*	31.5	9.5	25.4
		b*	-8.1	10.3	-20.9
		Hue angle (θ)	-14.4	47.3	-39.4

L, 0 – 100 black to white; +a, red, -a, green; +b, yellow, -b, blue; Hue angle (θ), $\tan^{-1}$ (b*/a*).

Figure 4.10: CIE L*a*b* values for cyanidin 3-malonyllaminaribioside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

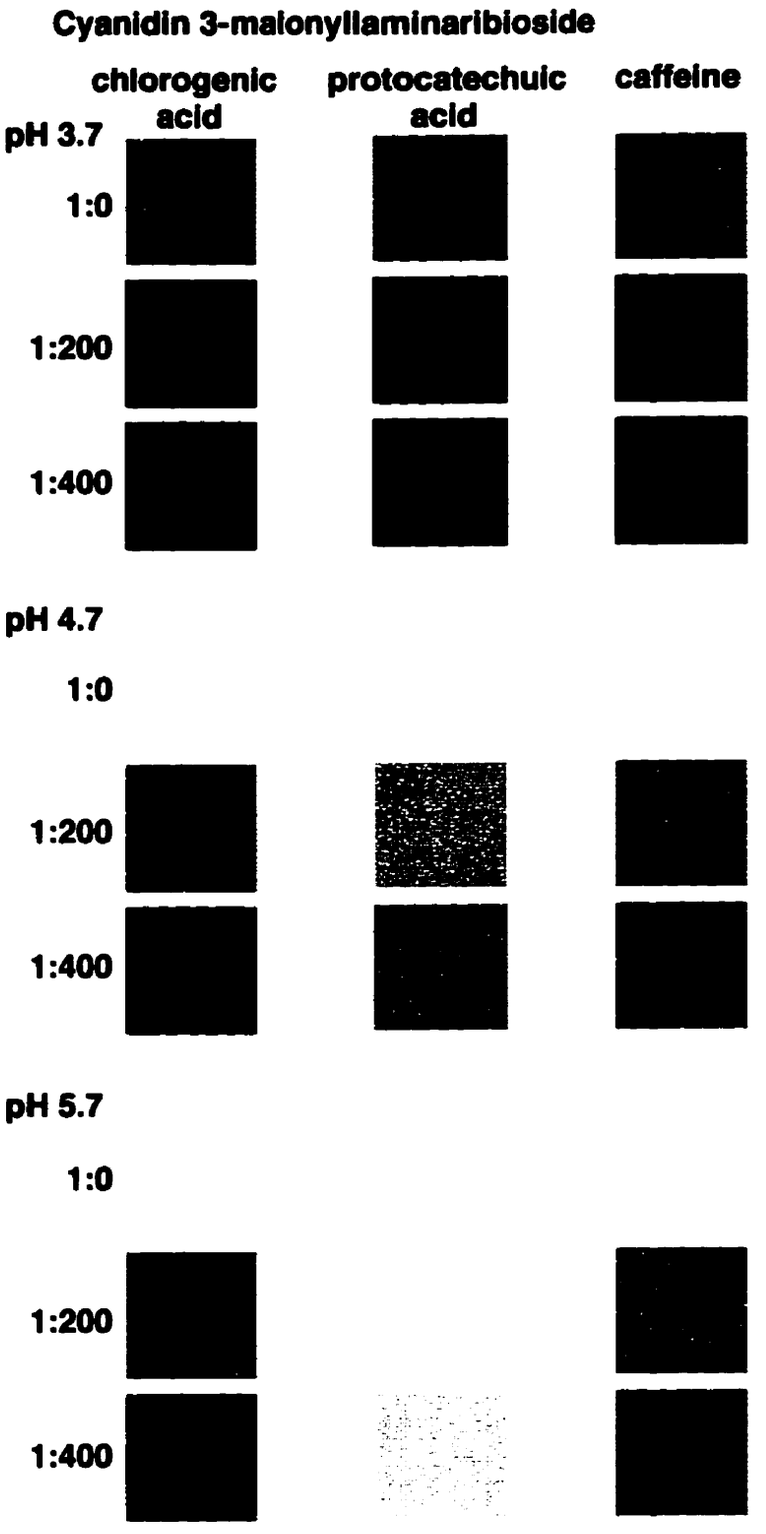

Cyanidin 3-malonylaminaribioside
chlorogenic acid
protocatechuic acid
caffeine
pH 3.7
1:0
1:200
1:400
pH 4.7
1:0
1:200
1:400
pH 5.7
1:0
1:200
1:400

Table 4.4: CIE L*a*b* values for cyanidin 3-malonyllaminaribioside at pH 3.7, 4.7, and 5.7 with no copigment (1:0) and increasing amounts of copigment (1:200, 1:400) chlorogenic acid, protocatechuic acid and caffeine.

pH	Pigment : Copigment		Chlorogenic acid	Protocatechuic acid	Caffeine
3.7		L*	78.1	78.5	78.0
	1:0	a*	40.0	39.5	40.0
		b*	13.2	13.1	13.5
		Hue angle (θ)	18.3	18.3	18.6
		L*	57.5	68.3	61.7
	1:200	a*	67.4	55.8	57.9
		b*	6.5	16.4	-7.7
		Hue angle (θ)	5.5	16.4	-7.6
		L*	55.3	63.6	57.8
	1:400	a*	68.6	61.9	60.1
		b*	7.2	21.0	-13.4
		Hue angle (θ)	6.0	18.7	-12.6
4.7		L*	90.8	91.1	91.0
	1:0	a*	13.4	13.0	13.0
		b*	4.0	4.0	4.0
		Hue angle (θ)	16.6	17.1	17.1
		L*	64.9	85.6	73.9
	1:200	a*	48.2	19.9	28.6
		b*	-5.3	7.1	-10.7
		Hue angle (θ)	-6.3	19.6	-20.5
		L*	56.6	79.3	68.4
	1:400	a*	56.6	27.9	32.5
		b*	-4.5	9.5	-15.6
		Hue angle (θ)	-4.5	18.8	-25.6
5.7		L*	91.1	92.6	90.4
	1:0	a*	9.6	8.8	9.2
		b*	3.3	3.7	3.1
		Hue angle (θ)	19.0	22.8	18.6
		L*	71.2	90.2	74.0
	1:200	a*	24.6	9.3	19.9
		b*	-2.4	8.6	-10.3
		Hue angle (θ)	-5.6	42.8	-27.4
		L*	59.9	87.1	68.4
	1:400	a*	31.8	10.4	23.2
		b*	-4.2	13.3	-14.8
		Hue angle (θ)	-7.5	51.9	-32.5

L, 0 – 100 black to white; +a, red, -a, green; +b, yellow, -b, blue; Hue angle (θ), $\tan^{-1}$ (b*/a*).

4.3.2 Influence of copigment structure

A good copigment augments the colour of the anthocyanin solution and causes batho- and hyperchromic shifts in the spectrum at mildly acidic to neutral pH values. The copigmentation effect of chlorogenic acid, protocatechuic acid and caffeine at pH values of 3.7, 4.7 and 5.7 with cyanidin 3-glucoside is illustrated in Figures 4.2 through 4.4. The effect of copigmentation with the three copigments evaluated was similar for the other pigments cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside. The type of copigment had a significant effect on copigmentation with the anthocyanin at the three pH values used. From Figures 4.5 to 4.7 and Table 4.5, it is evident that generally, chlorogenic acid resulted in the largest absorbance increase with each of the anthocyanins evaluated. The copigmentation effect was less with caffeine, while protocatechuic acid was found to be a poor copigment. The relationship between absorbance change and concentration of copigment (*i.e.*, linear or plateau) for a given anthocyanin depended on the copigment and the pH of the solution. Protocatechuic acid caused absorbance values to increase in a linear manner at all pH values tested, while absorbance values plateaued when caffeine was added to anthocyanin solution, regardless of pH. In the case of chlorogenic acid, a linear increase in absorbance was observed at pH values of 4.7 and 5.7; however, the increase plateaued when the solutions were maintained at pH 3.7.

The effect of the copigment on the absorbance of anthocyanin solutions depends on the pH as well as the presence or absence of malonic acid substitution on the cyanidin molecule. For caffeine and chlorogenic acid, the largest increases in absorbance were observed at pH 4.7 and 5.7, with the least copigmentation effect at pH 3.7. Protocatechuic acid caused the absorbance to increase more at pH 4.7 followed by pH 3.7, and finally pH 5.7.

The observed colour of the solution was markedly affected by the type of copigment added. Figures 4.8 to 4.10 illustrate the CIE L*a*b* colours for the three anthocyanin, copigment and pH combinations based on the CIE L*a*b* values in Tables 4.2-4.4. Table 4.6 lists the observed colour with the different cyanidin 3-glucoside, copigment and pH combinations. The most striking colour changes were brought about by chlorogenic acid and caffeine while protocatechuic acid was not as noticeable. Initially, the anthocyanin solutions were reddish orange, pink and faint pink at pH 3.7, 4.7 and 5.7, respectively. When chlorogenic acid was added, the final solution (1:400 pigment : copigment) was magenta at pH 3.7 and pale red/magenta at pH 4.7. Addition of protocatechuic acid to pH 3.7 and 4.7 solutions resulted in red and tan yellow colours. Caffeine changed the colour of pH 3.7 and 4.7 solutions to fuschia and purplish, respectively. At pH 5.7, all solutions had faint purple colours (Figures 4.8 to 4.10, Table 4.10).

Table 4.5: Influence of copigment and pigment structure on the absorbance increase at the P:CP ratio of 1:400 and pH 3.7, 4.7 and 5.7.

Copigment	pH	Anthocyanin	$(A-A_0)/A_0$ at P:CP 1:400	Type of increase	Equation of line
Chlorogenic acid	3.7	Cyn 3-glc[a]	2.24	logarithmic	$y = 0.54 \ln x + 3.79$
		Cyn 3-malglc[b]	1.32	logarithmic	$y = 0.29 \ln x + 2.17$
		Cyn 3-mallam[c]	1.49	logarithmic	$y = 0.37 \ln x + 2.64$
	4.7	Cyn 3-glc	6.94	linear	$y = 143.37x + 0.13$
		Cyn 3-malglc	5.28	linear	$y = 103.51x + 0.38$
		Cyn 3-mallam	5.56	linear	$y = 113.54x + 0.30$
	5.7	Cyn 3-glc	5.38	linear	$y = 110.16x - 0.01$
		Cyn 3-malglc	4.21	linear	$y = 89.43x - 0.07$
		Cyn 3-mallam	4.23	linear	$y = 86.50x + 0.06$
Protocatechuic acid	3.7	Cyn 3-glc	1.70	linear	$y = 33.27x + 0.05$
		Cyn 3-malglc	1.44	linear	$y = 29.77x + 0.04$
		Cyn 3-mallam	1.32	linear	$y = 26.49x + 0.04$
	4.7	Cyn 3-glc	2.02	linear	$y = 38.67x - 0.04$
		Cyn 3-malglc	1.66	linear	$y = 30.41x + 0.06$
		Cyn 3-mallam	1.60	linear	$y = 31.04x - 0.05$
	5.7	Cyn 3-glc	0.74	linear	$y = 15.71x - 0.10$
		Cyn 3-malglc	0.75	linear	$y = 12.77x + 0.04$
		Cyn 3-mallam	0.71	linear	$y = 13.80x - 0.02$
Caffeine	3.7	Cyn 3-glc	1.05	logarithmic	$y = 0.25 \ln x + 1.79$
		Cyn 3-malglc	0.90	logarithmic	$y = 0.22 \ln x + 1.53$
		Cyn 3-mallam	0.75	logarithmic	$y = 0.19 \ln x + 1.31$
	4.7	Cyn 3-glc	2.75	quadratic	$y = -984.23x^2 + 102.57x + 0.03$
		Cyn 3-malglc	2.82	quadratic	$y = -1042.10x^2 + 105.89x + 0.04$
		Cyn 3-mallam	2.17	quadratic	$y = -838.77x^2 + 83.65x + 0.03$
	5.7	Cyn 3-glc	3.47	quadratic	$y = -1215.8x^2 + 127.94x + 0.06$
		Cyn 3-malglc	2.96	quadratic	$y = -1039x^2 + 104.42x + 0.25$
		Cyn 3-mallam	2.21	quadratic	$y = -654.68x^2 + 75.05x + 0.03$

Note: cyn3glc[a], cyanidin 3-glucoside; cyn3malglc[b], cyanidin 3-malonylglucoside; cyn3mallam[c], cyanidin 3-malonyllaminaribioside.

Table 4.6: Observed colour of cyanidin 3-glucoside solutions.

Copigment	Ratio (P:CP)	Solution colour		
		pH 3.7	pH 4.7	pH 5.7
Chlorogenic acid	1:0	reddish orange	pink	faint pink
	1:400	magenta	pale red/magenta	dull reddish yellow
Protocatechuic acid	1:0	reddish orange	pink	faint pink
	1:400	red	tan yellow	tan yellow
Caffeine	1:0	reddish orange	pink	faint pink
	1:400	fuschia	purplish	dull grey purple

4.3.3 Influence of pH

There was a difference in the wavelength of maximum absorbance (λ_{max}) between the three pigments at the different pH values used (Tables 4.7 – 4.9). At pH 3.7, there was no significant difference in the λ_{max} of the three pigments ($P = 0.32$). At pH 4.7 and 5.7, there was a significant difference in the λ_{max} of the three pigments ($P \leq 0.05$). Cyanidin 3-malonyllaminaribioside generally had a slightly lower λ_{max} than did cyanidin 3-glucoside and 3-malonylglucoside. The same trend was observed at pH 5.7 except that the trend was more evident colourwise and spectrophotometrically in the spectral scans (Figure 4.6, 4.11, 4.12, 4.13, 4.14). The λ_{max} values did not change much with the anthocyanin structure, but the average absorbance at λ_{max} differed for equimolar concentrations of anthocyanins. The solutions at pH 3.7 had the most intense colours, which faded as the pH was increased to 4.7 and 5.7. All pigments displayed bathochromic and hyperchromic shifts in the presence of copigment (Figure 4.2-4.4). The bathochromic shifts induced by the copigment concentrations and the pH are tabulated in Tables 4.7 - 4.9. In general, as P:CP increased, a hyperchromic shift took place. The absorbance increase initially at low copigment amounts was usually large. Whereas when larger copigment amounts were added, the absorbance increase was no longer proportional. This resulted in an absorbance plateau where addition of copigment no longer resulted in a significant absorbance increase. The corresponding fitted equations were either logarithmic or polynomial in nature (Table 4.5) and there was a plateau increase since the absorbance change leveled off after a while. This phenomenon took place with chlorogenic acid at pH 3.7, and caffeine at pH 3.7, 4.7, and 5.7 (Table 4.1, 4.7 - 4.9). This was indicative of a high initial absorbance at 525 nm due to pH sometimes. On the other hand, sometimes the addition of copigment at various pH's resulted in a slow but steady absorbance increase. The absorbance change was referred to as a linear increase since the change fitted a linear regression equation (Table 4.5). This happened with

chlorogenic acid at pH 4.7 and 5.7 and protocatechuic acid at all pH's. This indicates that these copigments and pH had a continuous hyperchromic shift taking place resulting in an intensification of the colour, appealing or otherwise not. These changes are due to the pH and copigment structure.

Table 4.7: Effect of copigment concentration and solution pH on absorbance maxima (λ_{max}) for cyanidin 3-glucoside.

Copigment, (CP)	Ratio (P:CP)*	λ_{max} at pH 3.7	Shift (nm)#	λ_{max} at pH 4.7	Shift (nm)#	λ_{max} at pH 5.7	Shift (nm)#
Chlorogenic acid	1:0	511.6	N/A	516.4	N/A	527.9	N/A
	1:20	516.0	4.4	522.6	6.2	531.9	4.0
	1:100	527.3	15.7	531.4	15.0	540.0	12.1
	1:200	532.0	20.4	535.3	18.9	543.9	16.0
	1:400	536.2	24.6	539.2	22.8	546.4	18.5
Protocatechuic acid	1:0	511.4	N/A	515.4	N/A	530.2	N/A
	1:20	513.3	1.9	517.4	2.0	527.9	-2.3
	1:100	515.9	4.5	518.8	3.4	530.2	0
	1:200	519.2	7.8	521.4	6.0	527.8	-2.4
	1:400	524.0	12.6	524.5	9.1	525.6	-4.6
Caffeine	1:0	511.3	N/A	516.7	N/A	530.6	N/A
	1:20	515.9	4.6	523.4	6.7	538.2	7.6
	1:100	525.5	14.2	538.4	21.7	547.8	17.2
	1:200	531.7	20.4	544.7	28.0	551.1	20.5
	1:400	538.6	27.3	548.6	31.9	552.2	21.6

* (P:CP), pigment to copigment ratio; N/A, not applicable; # shift calculated from λ_{max} of 1:0.

Table 4.8: Effect of copigment concentration and solution pH on absorbance maxima (λ_{max}) for cyanidin 3-malonylglucoside.

Copigment, (CP)	Ratio (P:CP)*	λ_{max} at pH 3.7	Shift (nm)#	λ_{max} at pH 4.7	Shift (nm)#	λ_{max} at pH 5.7	Shift (nm)#
Chlorogenic acid	1:0	510.3	N/A	518.6	N/A	532.5	N/A
	1:20	na	na	520.2	1.6	532.4	-0.1
	1:10	528.5	18.2	531.6	13.0	540.9	8.4
	1:20	532.7	22.4	535.8	17.2	543.7	11.2
	1:40	537.7	27.4	539.5	20.9	546.2	13.7
Protocatechuic acid	1:0	511.5	N/A	516.3	N/A	532.4	N/A
	1:20	512.4	0.9	518.8	2.5	528.9	-3.5
	1:10	516.4	4.9	517.7	1.4	529.2	-3.2
	1:20	519.5	8.0	521.3	5.0	527.7	-4.7
	1:40	523.2	11.7	524.2	7.9	526.4	-6.0
Caffeine	1:0	511.5	N/A	518.6	N/A	532.8	N/A
	1:20	514.7	3.2	520.8	2.2	536.0	3.2
	1:10	524.0	12.5	537.2	18.6	547.6	14.8
	1:200	531.7	20.2	543.2	24.6	551.9	19.1
	1:400	536.3	24.8	547.4	28.8	552.1	19.3

• (P:CP), pigment to copigment ratio; N/A, not applicable; na, not available; # shift calculated from λ_{max} of 1:0.

Table 4.9: Effect of copigment concentration and solution pH on absorbance maxima (λ_{max}) for cyanidin 3-malonyllaminaribioside.

Copigment, (CP)	Ratio (P:CP)*	λ_{max} at pH 3.7	Shift (nm)#	λ_{max} at pH 4.7	Shift (nm)#	λ_{max} at pH 5.7	Shift (nm)#
Chlorogenic acid	1:0	510.7	N/A	508.7	N/A	499.8	N/A
	1:20	516.3	5.6	516.7	8.0	502.0	2.2
	1:100	528.3	17.6	530.4	21.7	535.2	35.4
	1:200	534.0	23.3	536.4	27.7	542.0	42.2
	1:400	538.1	27.4	538.8	30.1	545.1	45.3
Protocatechuic acid	1:0	511.0	N/A	510.9	N/A	498.9	N/A
	1:20	513.3	2.3	510.6	-0.3	498.1	-0.8
	1:100	516.0	5.0	513.0	2.1	499.1	0.2
	1:200	519.1	8.1	516.7	5.8	400.4	-98.5
	1:400	523.0	12.0	519.9	9.0	406.3	-92.6
Caffeine	1:0	511.3	N/A	509.4	N/A	498.5	N/A
	1:20	514.2	2.9	517.3	7.9	506.0	7.5
	1:100	524.4	13.1	534.4	25.0	545.3	46.8
	1:200	531.0	19.7	541.1	31.7	550.1	51.6
	1:400	537.0	25.7	547.1	37.7	552.1	53.6

* (P:CP), pigment to copigment ratio; N/A, applicable; # shift calculated from λ_{max} of 1:0.

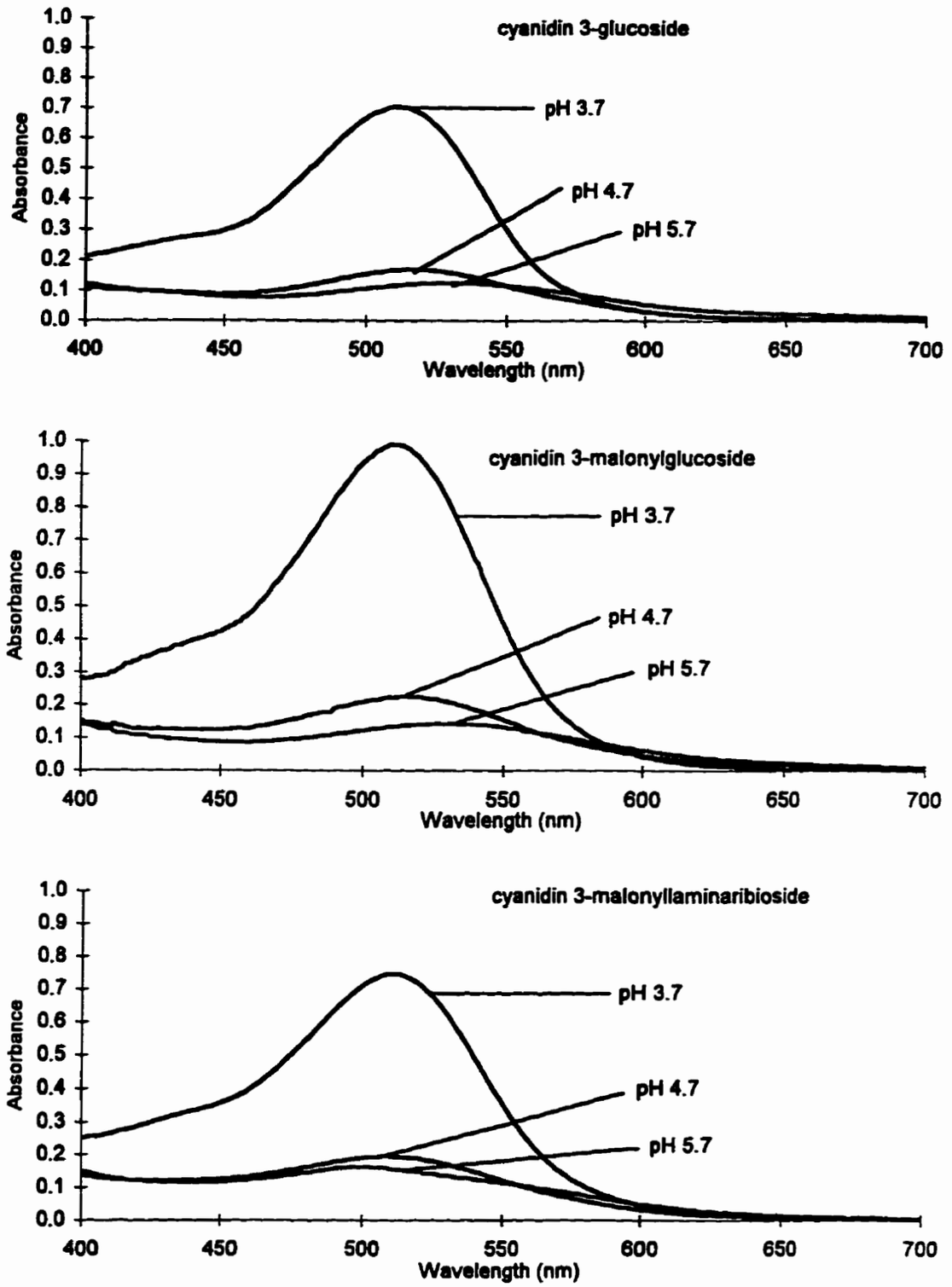


Figure 4.11: Spectral scans of cyanidin 3-glucoside (top), cyanidin 3-malonylglucoside (middle) and cyanidin 3-malonyllaminaribioside (bottom) (1.29×10^{-4} M each) at pH 3.7, 4.7 and 5.7 in NaOAc - H_3PO_4 buffer.

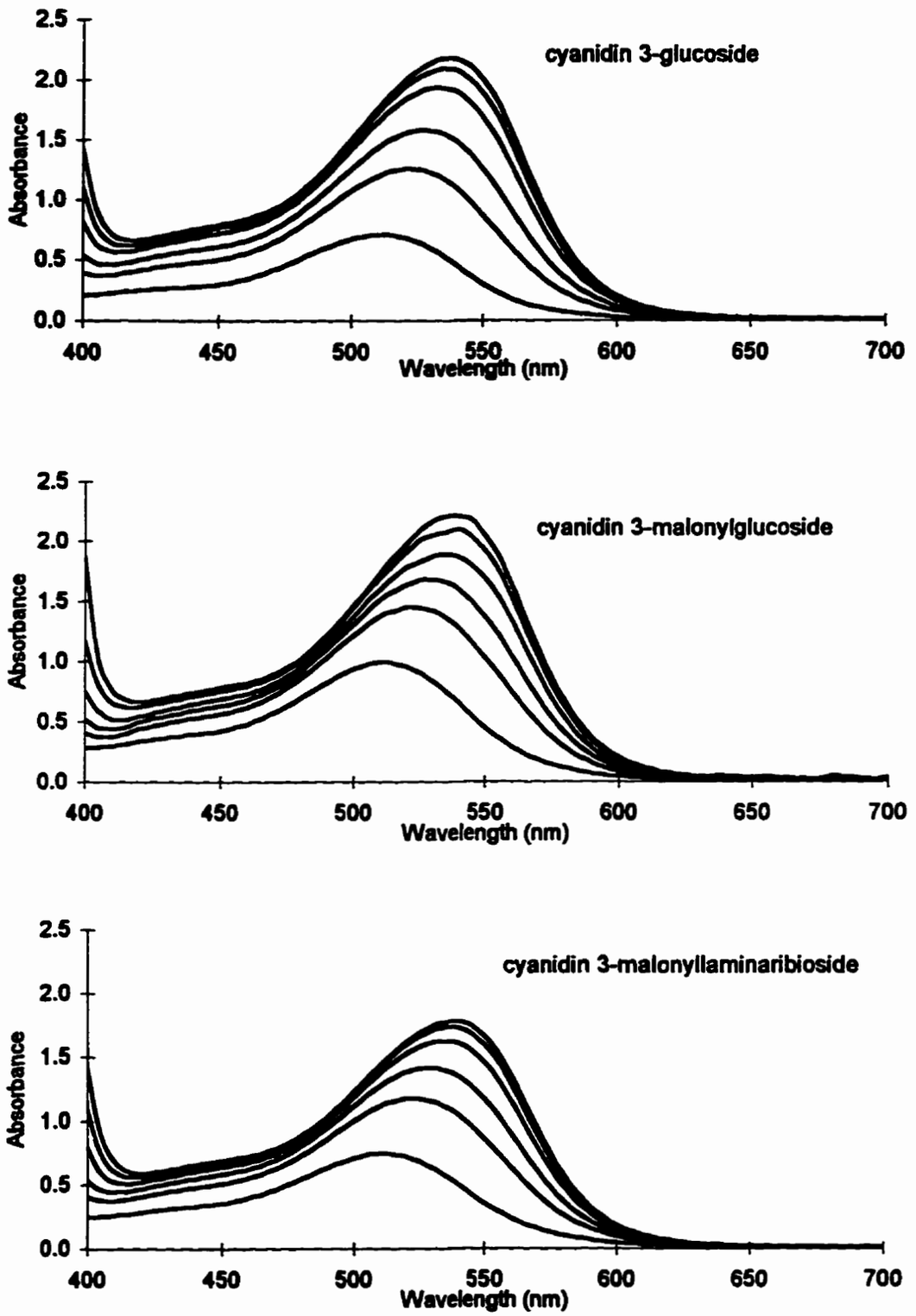


Figure 4.12: Copigmentation scans of cyanidin 3-glucoside (top), cyanidin 3-malonyl-glucoside (middle) and cyanidin 3-malonyllaminaribioside (bottom) with addition of chlorogenic acid at pH 3.7.

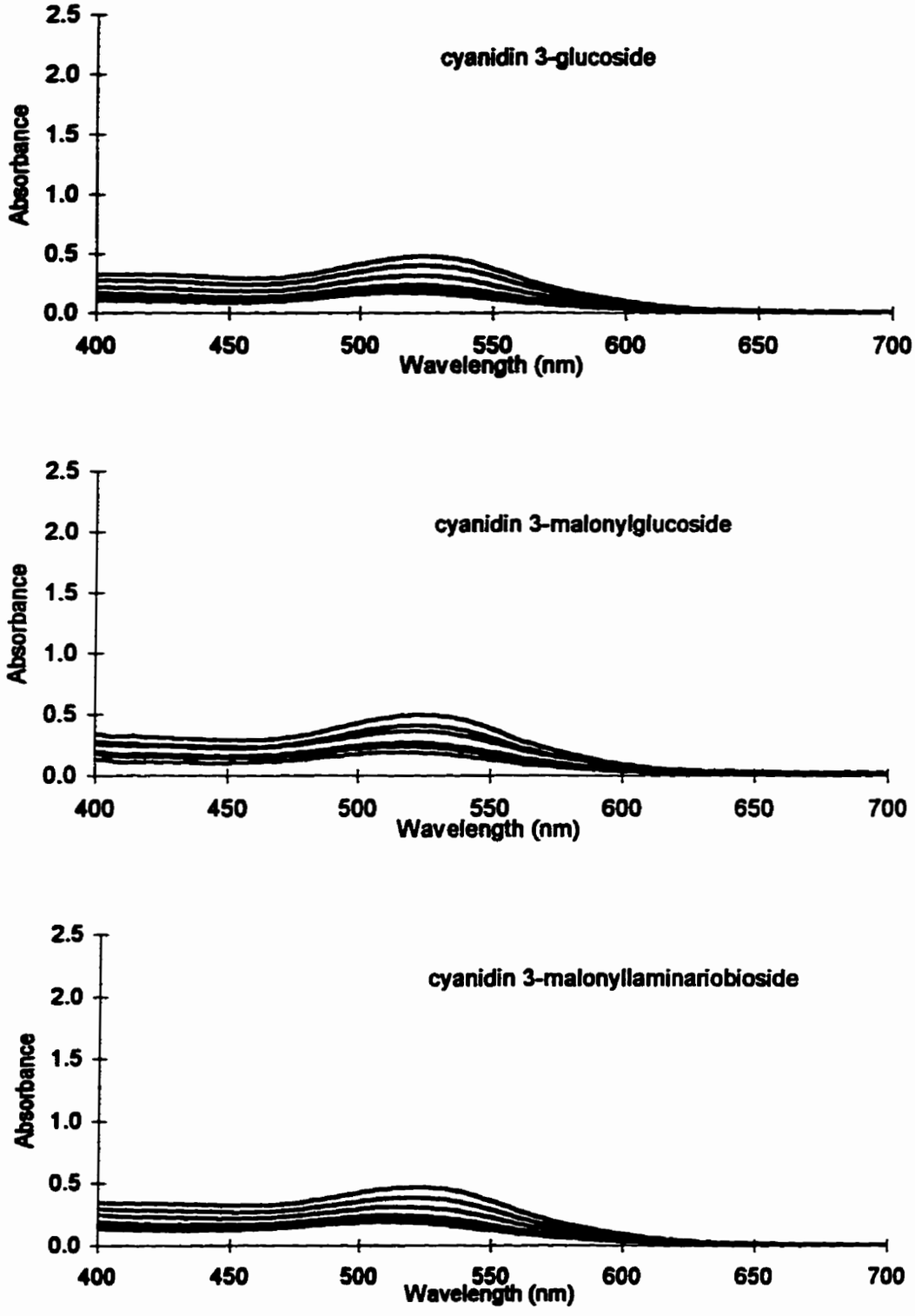


Figure 4.13: Copigmentation scans of cyanidin 3-glucoside (top), cyanidin 3-malonyl-glucoside (middle) and cyanidin 3-malonyllaminaribioside (bottom) with addition of protocatechuic acid at pH 4.7.

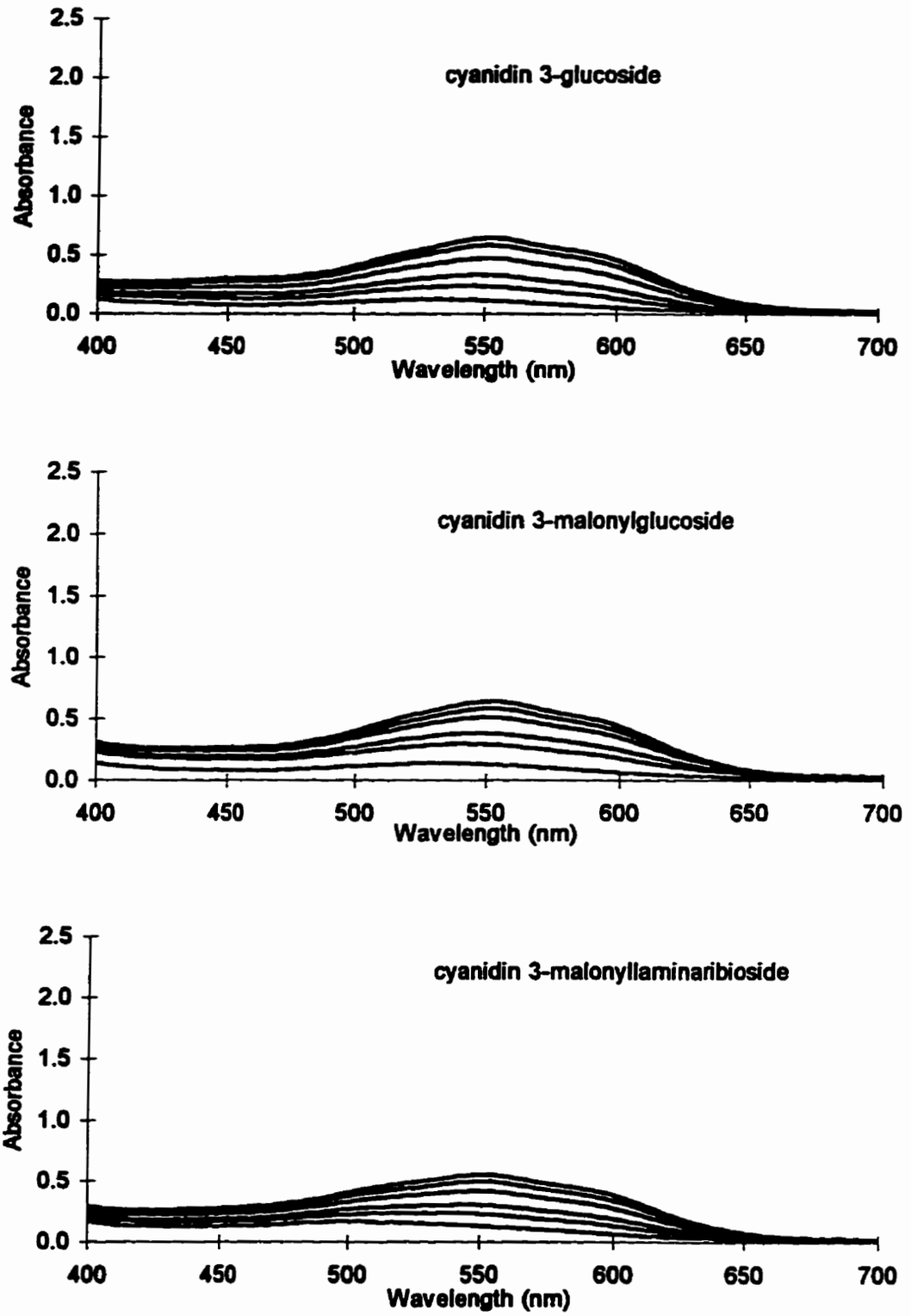


Figure 4.14: Copigmentation scans of cyanidin 3-glucoside (top), cyanidin 3-malonyl-glucoside (middle) and cyanidin 3-malonyllaminaribioside (bottom) with addition of caffeine at pH 5.7.

Table 4.10: Spectral characteristics of three anthocyanins at 1.29 x 10^{-4} M at pH 3.7, 4.7, and 5.7.

pH	Pigment	λ_{max} (nm), (n)*	Abs. @ λ_{max}, (n)*	Abs. @ 525, (n)*
3.7	Cyn 3-glc[a]	511.4 ± 0.5 (23)	0.722 ± 0.050 (23)	0.658 ± 0.045 (22)
	Cyn 3-malglc[b]	511.1 ± 0.6 (6)	0.885 ± 0.069 (6)	0.807 ± 0.060 (6)
	Cyn 3-mallam[c]	511.0 ± 0.7 (6)	0.740 ± 0.006 (6)	0.675 ± 0.007 (6)
4.7	Cyn 3-glc	516.3 ± 1.5 (23)	0.173 ± 0.008 (23)	0.169 ± 0.009 (18)
	Cyn 3-malglc	516.7 ± 1.5 (8)	0.202 ± 0.017 (8)	0.195 ± 0.017 (6)
	Cyn 3-mallam	509.9 ± 0.9 (7)	0.191 ± 0.002 (7)	0.179 ± 0.002 (6)
5.7	Cyn 3-glc	529.3 ± 1.9 (15)	0.127 ± 0.009 (15)	0.127 ± 0.009 (13)
	Cyn 3-malglc	532.6 ± 1.7 (7)	0.140 ± 0.004 (7)	0.138 ± 0.004 (6)
	Cyn 3-mallam	499.2 ± 0.7 (7)	0.162 ± 0.019 (7)	0.139 ± 0.011 (6)

Values shown indicate mean ± standard deviation; Cyn 3-glc[a], cyanidin 3-glucoside; Cyn 3-malglc[b], cyanidin 3-malonylglucoside; Cyn 3-mallam[c], cyanidin 3-malonyllaminaribioside; (n)*, sample size.

4.3.4 Stoichiometric constant, n (copigmentation mechanism)

The stoichiometric constant (n) was determined from the linear relationship between ln $[CP_0]$ and ln $((A-A_0)/A_0)$ for each anthocyanin, copigment and pH experiment. The n value which is the slope of the line obtained from linear regression analysis of the data, describes the degree of association between copigment and pigment under the particular experimental conditions. The n value also is an indicator of how many copigment molecules aid in stabilising an anthocyanin molecule. An n value of 1 or close to unity is indicative of a 1:1 association between pigment and copigment. The effect of the stoichiometric constant, n, on pigment structure, copigment structure and pH was examined.

4.3.4.1 Effect of pigment structure

Figures 4.15, 4.16, and 4.17 depict the linear relationships between ln $((A-A_0)/A_0)$ and ln $[CP]_0$ from which the n values were calculated. The n values from these linear equations are

tabulated in Table 4.11. Generally, all three anthocyanins had similar n values regardless of pH or copigment concentration. This indicated that the presence of malonic acid and the longer laminaribioside sugar molecule had no significant influence on the degree of association between anthocyanin and copigment. From the two sets of copigmentation experiments done with cyanidin 3-glucoside, one can note the range in n values under the different conditions. Chlorogenic acid resulted in a low n value of 0.68-0.69 at pH 3.7 and the n value increased to greater than 1 at pH 4.7 and 5.7. The n value for protocatechuic acid was close to 1 at pH 3.7 and 4.7 yet increased to 1.58 at pH 5.7. When caffeine was the copigment, a low n value of 0.68 was determined at pH 3.7 and at pH 4.7, it was approximately 0.8. One exception was cyanidin 3-glucoside with protocatechuic acid. The n value of 3.1 is high since not all data points (1:5 to 1:20) were used for the linear regression since these amounts of copigment decreased the absorbance causing a negative absorbance value change, which precluded conversion of these data points to natural log values. The n value was taken from the data which was plotted using the pigment : copigment ratios of 1:50 to 1:400. Thus, the equation that was obtained had a higher n value relative to the others. When the three anthocyanins were compared, the n values were usually close. The exception was cyanidin 3-malonylglucoside with chlorogenic acid at pH 3.7, 4.7 and 5.7. In this case, the n value was 10-15% lower than for cyanidin 3-glucoside and cyanidin 3-malonyllaminaribioside. At a pH of 3.7, the n values for the three anthocyanins ranged from 0.55 to 0.69 and at pH 4.7 and 5.7, the n values were from 0.85 to 1.05. At all pH values of 3.7, 4.7 and 5.7 for chlorogenic acid, the n values for cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside were lower by 0.1 when compared to those for cyanidin 3-glucoside and in the presence of chlorogenic acid.

Table 4.11: Influence of anthocyanin, copigment and pH on the stoichiometric constant (n) of copigmented solutions.

Copigment	pH	Cyanidin 3-glucoside	Cyanidin 3-malonylglucoside	Cyanidin 3-malonyllaminaribioside
Chlorogenic acid	3.7	0.69, 0.68[a]	0.55	0.66
	4.7	1.19, 0.97	0.85	0.92
	5.7	1.06, 1.03	0.96	1.05
Protocatechuic acid	3.7	0.90, 0.97, 1.33	1.04	0.99
	4.7	0.91, 1.10	0.73	1.31
	5.7	3.08, 1.58	1.05	1.05
Caffeine	3.7	0.68, 0.68	0.68	0.80
	4.7	0.82, 0.80	0.82	0.83
	5.7	0.85, 0.86	0.60	0.86

[a] n values calculated from the slope of line obtained when plotting ln $((A-A_0)/A_0)$ vs ln $[CP_0]$.

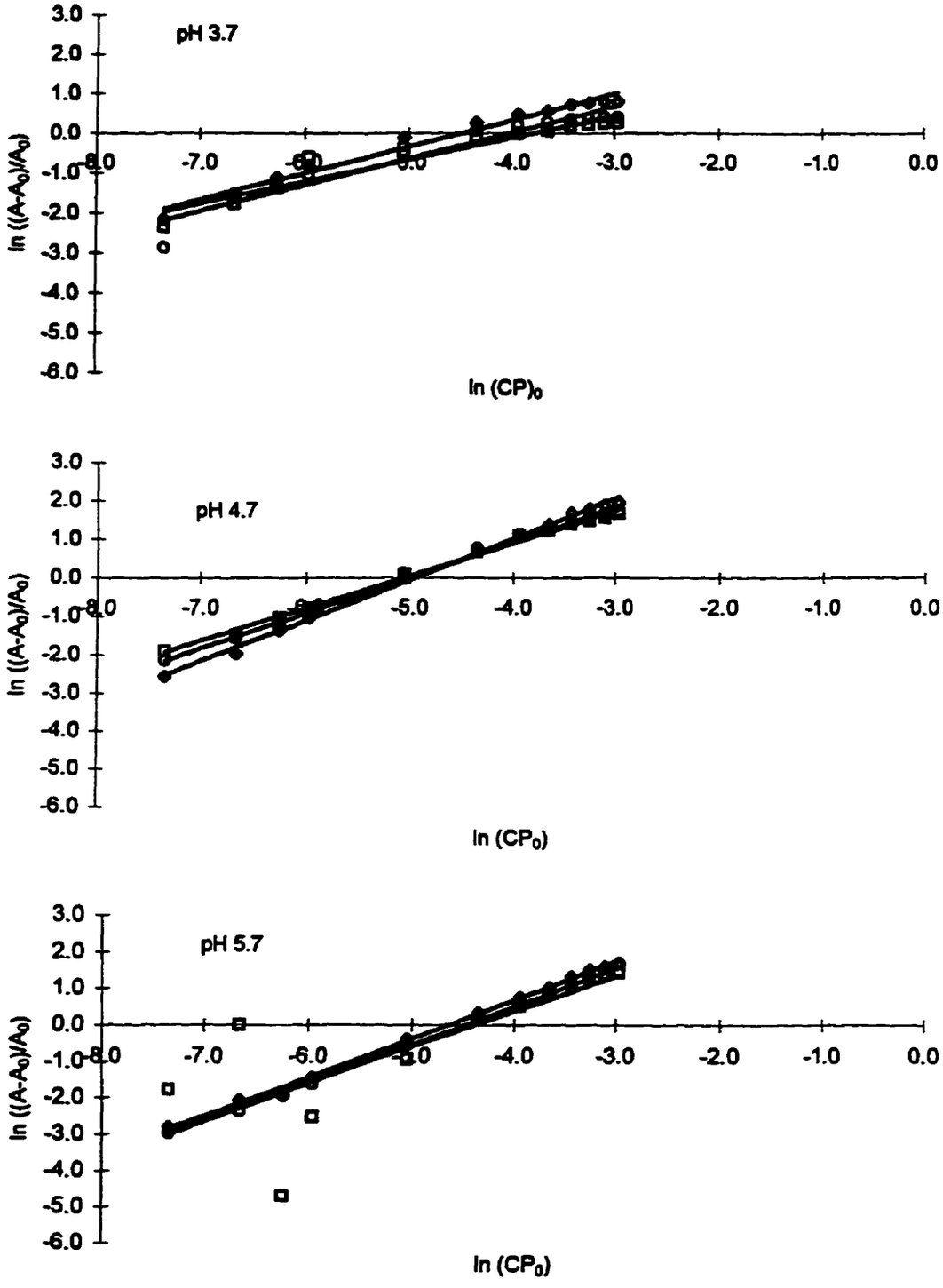


Figure 4.15: Plot of ln $((A-A_0)/A_0)$ versus ln$[CP_0]$ for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○)with increasing amounts of chlorogenic acid (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

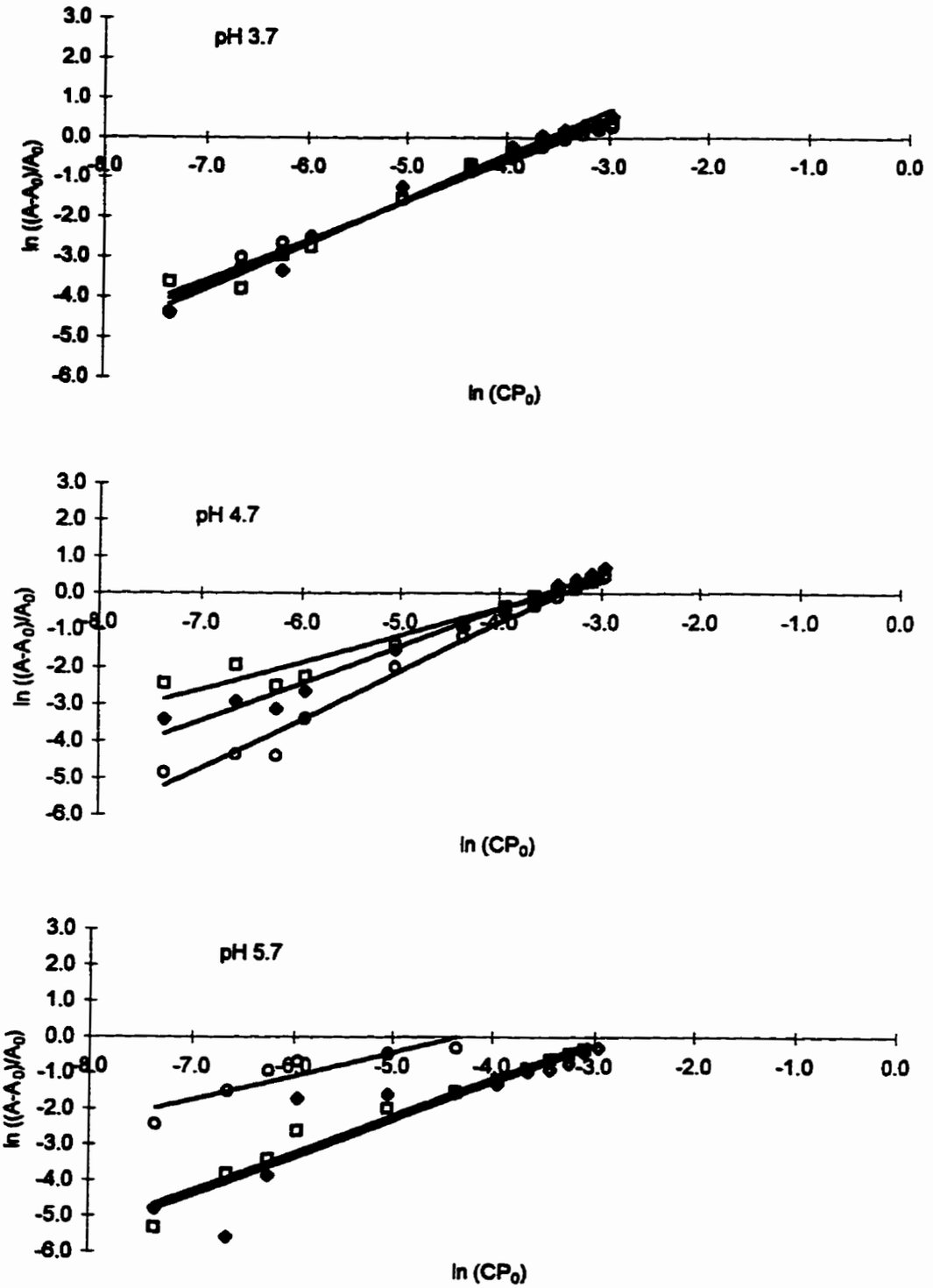


Figure 4.16: Plot of ln $((A-A_0)/A_0)$ versus ln$[CP_0]$ for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○) with increasing amounts of protocatechuic acid (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

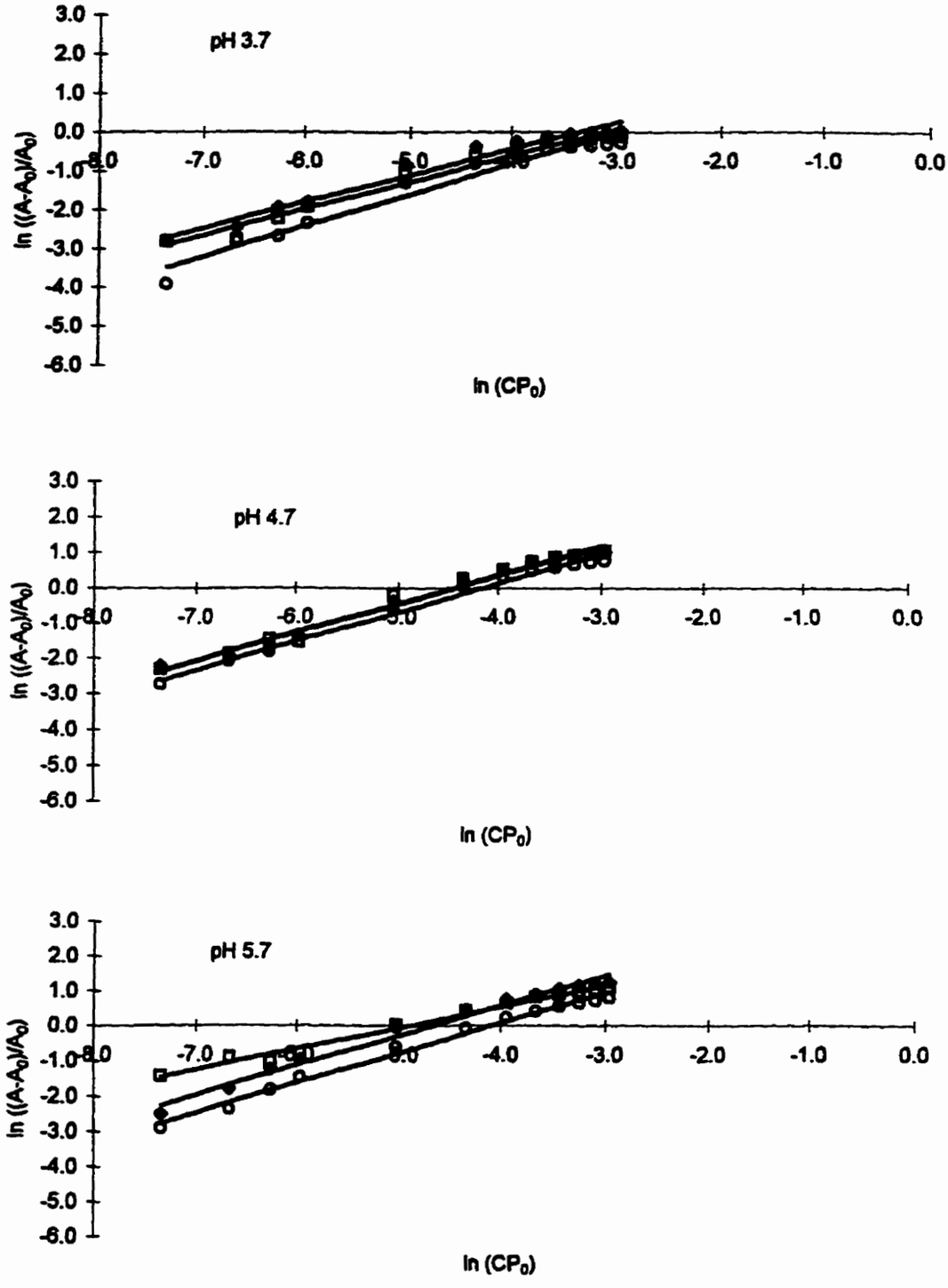


Figure 4.17: Plot of ln $((A-A_0)/A_0)$ versus ln$[CP_0]$ for cyanidin 3-glucoside (1.29×10^{-4} M) (◇), cyanidin 3-malonylglucoside (□), and cyanidin 3-malonyllaminaribioside (○) with increasing amounts of caffeine (P:CP = 1:0, 1:5, 1:10, 1:15, 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400) at pH 3.7 (top), 4.7 (middle) and 5.7 (bottom).

4.3.4.2 Effect of copigment structure

When the data in Table 4.11 are compared on the basis of copigment differences in the n value are more pronounced. Caffeine always had n values of less than unity (range = 0.68 - 0.86), no matter what the pH or pigment used was. This indicates that caffeine does not appear to form a 1:1 association with an anthocyanin, and thus does not fit the model proposed by Mazza and Brouillard (1990). On the other hand, protocatechuic acid resulted in n values close to 1.0 at pH 3.7 and 4.7, and values greater than 1.0 at pH 5.7 with cyanidin 3-glucoside. At a pH of 3.7 for all three pigments, the n value was 0.9 to 1.33, which indicates 1:1 association and in agreement with the currently accepted copigmentation mechanism. The same holds true at pH 4.7 with cyanidin 3-glucoside and at pH 5.7 with cyanidin 3-malonylglucoside and 3-malonylaminaribioside. The n values of 0.73 at pH 4.7 with cyanidin 3-malonylglucoside, 1.31 at pH 4.7 with cyanidin 3-malonyllaminaribioside and 1.6+ at pH 5.7 with cyanidin 3-glucoside indicates no adherence to the 1:1 anthocyanin : copigment model. The n values ranged from 0.99 to 1.12 with the exceptions being cyanidin 3-malonylglucoside at pH 4.7 (n = 0.73) and cyanidin 3-malonyllaminaribioside at pH 4.7 (n = 1.31). In general, protocatechuic acid had a 1:1 association with the three pigments at pH 3.7 and 4.7 only. The association of chlorogenic acid with cyanidin anthocyanins varied. At pH 4.7 and 5.7, the degree of association ranged from 0.85 to 1.05, indicating 1:1 association. However, at a pH of 3.7, the n value was from 0.55 to 0.68. This indicates that the chlorogenic acid may not associate on a 1:1 basis at a lower pH.

4.3.4.3 Influence of pH

All data gathered in the copigmentation experiments indicated that pH has an effect on the n value. At pH 4.7 and 5.7, in general, the n value ranged from 0.73 to 1.05, which indicated 1:1 association between anthocyanin and copigment. There were some extreme n values at pH

5.7 with cyanidin 3-glucoside and protocatechuic acid (n = 1.58 and 3.08), cyanidin 3-malonylaminaribioside and protocatechuic acid (n = 1.31), and with caffeine and cyanidin 3-malonylglucoside (n = 0.60). At pH 3.7, the results varied and the degree of association between pigment and copigment was more dependent upon the copigment structure. With protocatechuic acid and caffeine, the n values were less than 1, indicating possible 2:1 copigment to pigment association.

4.3.5 Equilibrium constant (K) determination

The equilibrium constant (K) which describes the degree of association (affinity) between the copigment and anthocyanin was highest at the pH of maximum copigmentation (Table 4.12). The anthocyanins solutions containing chlorogenic acid had higher K, when compared to protocatechuic acid and caffeine containing solutions. However, the K values of those solutions were not dramatically different when compared to those which contain chlorogenic acid.

Table 4.12: Effect of anthocyanin, copigment and pH on the equilibrium constant, K of copigmented solutions.

Pigment	Copigment	r_1[@]	K (M^{-1})		
			pH 3.7	pH 4.7	pH 5.7
Cyn 3-glc[a]	Chloro[#]	0.96	20.8 ± 0.2	197.0 ± 1.9	140.8 ± 1.3
	Proto[#]	1.02	54.6 ± 1.3	34.5 ± 0.8	17.8 ± 0.4
	Caffeine	1.07	9.2 ± 0.2	33.7 ± 0.7	50.6 ± 1.0
Cyn 3-malglc[b]	Chloro[#]	0.98	8.3 ± 0.1	74.2 ± 0.3	68.3 ± 0.3
	Proto[#]	1.14	32.9 ± 0.4	10.6 ± 0.1	15.9 ± 0.2
	Caffeine	1.06	8.0 ± 0.1	34.3 ± 1.6	17.4 ± 0.8
Cyn 3-mallam[c]	Chloro[#]	1.00	14.2 ± 0.1	99.7 ± 0.4	107.2 ± 0.4
	Proto[#]	1.11	26.0 ± 1.1	75.4 ± 3.2	18.2 ± 0.8
	Caffeine	1.07	10.1 ± 0.2	28.7 ± 0.4	31.2 ± 0.5

[@] r_1, A/A_0 ratio; Cyn 3-glc[a], cyanidin 3-glucoside; Cyn 3-malglc[b], cyanidin 3-malonylglucoside; Cyn 3-mallam[c], cyanidin 3-malonyllaminaribioside; Chloro[#], chlorogenic acid; Proto[#], protocatechuic acid.

4.3.6 Multiple copigment experiments

The initial copigmentation experiment using chlorogenic acid and caffeine at pH 4.7 with cyanidin 3-glucoside (1.29×10^{-4} M) produced a final absorbance increase of 2.9. Figure 4.18 indicates that with addition of more chlorogenic acid and caffeine, the change in absorbance increased rapidly but started to plateau at higher copigment concentrations. It should be noted that the chlorogenic acid was always added before the caffeine. Figure 4.18 illustrates that the addition of chlorogenic acid always resulted in a larger absorbance increase. Caffeine caused less of an absorbance change especially at higher copigment concentrations resulting in a plateau. When the natural logarithm was calculated for the absorbance change versus copigment concentration, the n value obtained was 0.83.

The second multiple copigmentation experiment at pH 4.7 with cyanidin 3-glucoside (1.29×10^{-4} M) involved the addition of chlorogenic acid, caffeine and protocatechuic acid successively. Figure 4.20 illustrates that addition of small concentrations of copigment resulted in similar absorbance increases. However, addition of higher concentrations of chlorogenic acid, caffeine and protocatechuic acid at higher concentrations lead to a gradual plateau in the absorbance increase. The addition of two superior copigments, chlorogenic acid and caffeine, prior to protocatechuic acid produced a gradual drop in the absorbance increase within this series, except at the final addition of protocatechuic acid. The n value from the logarithm of this data was 0.66. Figure 4.22 illustrates the CIE L*a*b* values for the multiple copigmented anthocyanin solutions at the initial and final copigment concentration levels.

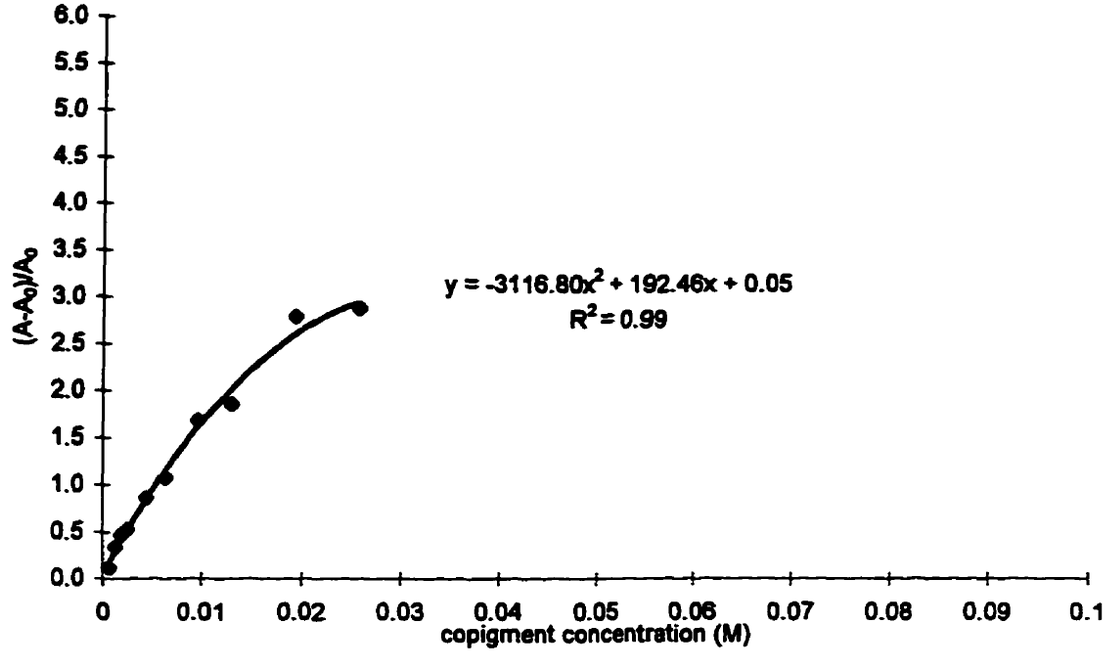


Figure 4.18: Absorbance increase of copigmented cyanidin 3-glucoside (1.29 x 10^{-4} M) with chlorogenic acid and caffeine (1:0:0, 1:5:0, 1:5:5, 1:10:5, 1:10:10, 1:25:10, 1:25:25, 1:50:25, 1:50:50, 1:100:50, 1:100:100) at pH 4.7 and 20°C

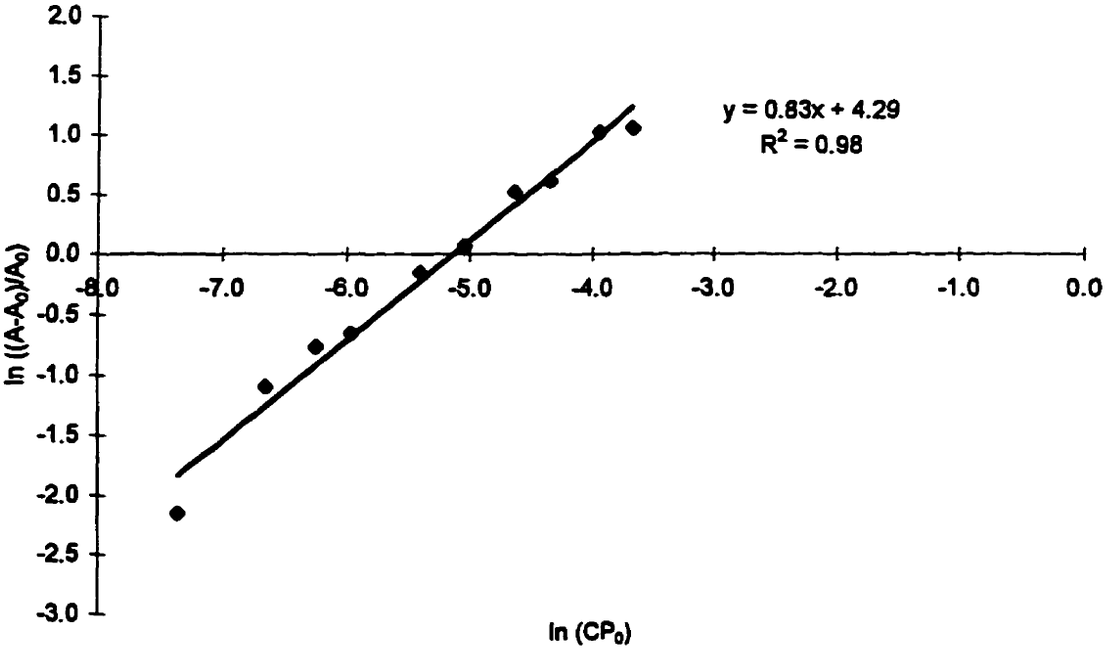


Figure 4.19: Copigmentation of cyanidin 3-glucoside (1.29 x 10^{-4} M) with chlorogenic acid and caffeine (1:0:0, 1:5:0, 1:5:5, 1:10:5, 1:10:10, 1:25:10, 1:25:25, 1:50:25, 1:50:50, 1:100:50, 1:100:100) at pH 4.7 and 20°C

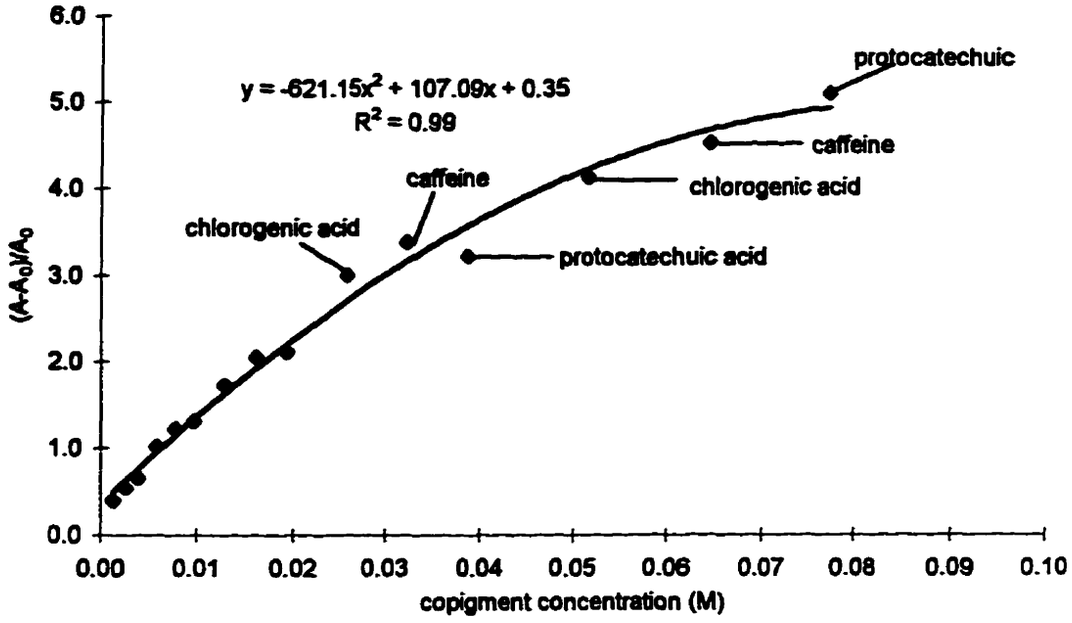


Figure 4.20: Absorbance increase with increasing amounts of cyanidin 3-glucoside solution (1.29×10^{-4} M) with chlorogenic, protocatechuic acid and caffeine (1:0:0:0, 1:10:0:0, 1:10:10:0, 1:10:10:1 1:25:10:10, 1:25:25:10, 1:25:25:25, 1:50:25:25, 1:50:50:25, 1:50:50:50, 1:100:50:50, 1:100:100:50, 1:1 1:200:100:100, 1:200:200:100, 1:200:200:200) at pH 4.7 and 20°C

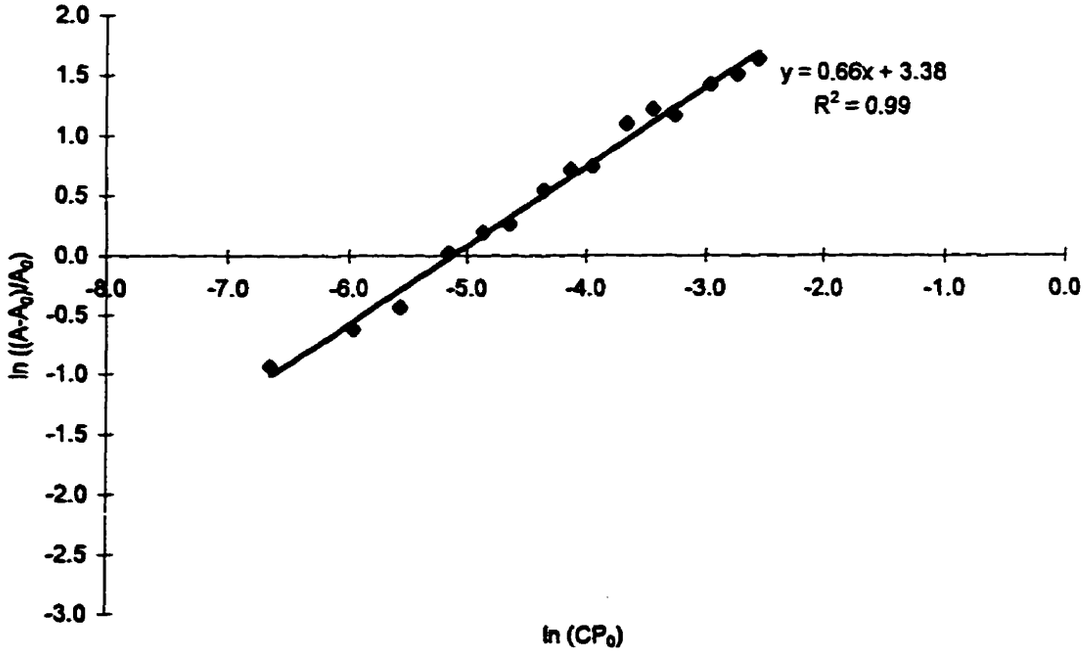


Figure 4.21: Copigmentation of cyanidin 3-glucoside (1.29×10^{-4} M) with multiple copigments: chlorogenic acid, protocatechuic acid and caffeine (1:0:0:0, 1:10:0:0, 1:10:10:0, 1:10:10:10, 1:25:10:10, 1:25:25:10, 1:25:25:25, 1:50:25:25, 1:50:50:25, 1:50:50:50, 1:100:50:50, 1:100:100:50, 1:100:100:100, 1:200:100:100, 1:200:200:100, 1:200:200:200) at pH 4.7 and 20°C

Figure 4.22: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) at pH 4.7 with no copigments (1:0, left) and with chlorogenic acid and caffeine (P:CP:CP = 1:100:100) (top right) and with chlorogenic acid, caffeine, and protocatechuic acid (P:CP:CP:CP = 1:200:200:200) (bottom right).

Cyanidin 3-glucoside (1.29×10^{-4} M) at pH 4.7

1:0

1:100:100
chlorogenic acid:caffeine

1:0

1:200:200:200
chlorogenic acid:caffeine:
protocatechuic acid

Table 4.13: CIE L*a*b* values for cyanidin 3-glucoside at pH 4.7 with no copigments and with chlorogenic acid and caffeine (1:100:100) and with chlorogenic acid, caffeine and protocatechuic acid (1:200:200:200).

Sample	Pigment:Copigment	L*	a*	b*	Hue angle (θ)
Control	1:0:0	92.2	11.4	0.0	0.0
Chlorogenic acid:caffeine	1:100:100	68.3	37.2	-11.0	-16.5
Control	1:0:0:0	91.4	13.3	0.0	0.0
Chlorogenic acid: caffeine:protocatechuic acid	1:200:200:200	58.1	47.3	-10.1	-12.1

L, 0-100 black to white; +a, red; -a, green; +b, yellow, -b, blue; Hue angle (θ), $\tan^{-1}(b^*/a^*)$.

4.3.7 Storage stability of copigmented anthocyanin solutions

Photostability of copigmented cyanidin 3-glucoside solutions was assessed over a 35 day period of continuous exposure to intense incandescent and fluorescent lights (418 μmol m^{-2} s^{-1}) at 20°C. A series of samples was also held in the dark at 20°C for the same time period to assess stability in the absence of light. Solutions of cyanidin 3-glucoside with the copigments chlorogenic acid, protocatechuic acid or caffeine at a pigment : copigment ratio of 1:200 were used. Cyanidin 3-glucoside (1.29×10^{-4} M) served as the control at the three pH values used (pH 3.7, 4.7, and 5.7). It was hypothesized that light would cause deterioration of the colour of the solutions in the absence of copigment. It was further hypothesized that addition of copigment would improve the stability of the colour of the resulting solutions. In order to compare the solutions, absorbance readings were taken at 525 nm to determine the stability of the flavylium cation concentration over time because 525 nm is the wavelength of maximum absorbance for the flavylium cation. The wavelength of maximum absorbance (λ_{max}) was also monitored over time.

Continuous intense artificial light (418 μmol m^{-2} s^{-1}) adversely affected the stability of the flavylium cation (Figures 4.23 and 4.25). All samples, which were stored in the presence of light, had lower absorbance readings at 525 nm and their colour degraded very rapidly compared to those stored in darkness (Figures 4.24 and 4.26). Solutions containing chlorogenic acid and protocatechuic acid became yellowish brown during exposure to light, and solutions containing protocatechuic acid developed a precipitate or flocculation under the same conditions. As shown in Figure 4.23 (top and middle), the absorbance at 525 nm of solutions containing chlorogenic and protocatechuic acid at pH 4.7 and 5.7 initially dropped but later increased. Visually, the solutions became less red in colour and became yellow-brown in colour. This was supported by a change in the absorption maximum from 525 to 400 nm. The apparent colour of each solution

based on the measured CIE L*a*b* values over 35 days is also illustrated in Figures 4.25 and 4.26 for the solutions exposed to light and unexposed to light, respectively.

The solutions containing cyanidin 3-glucoside and chlorogenic acid displayed good photostability at pH 3.7, since after 10 days of exposure to light, over 50% of its original absorbance at 525 nm remained (Figure 4.25). After 15 days, browning started to develop and became progressively worse until the spectra of the copigmented solution had the greatest absorbance in the 400 nm region and it decreased gradually until a fraction of the initial absorbance at 525 nm remained. All solutions that browned exhibited very similar changes in the spectrum over time. In contrast, solutions of cyanidin 3-glucoside and chlorogenic acid at pH 4.7 and 5.7 turned brown in colour within the first few days of storage under illumination (Figure 4.25). Control solutions (pH 3.7) containing only cyanidin 3-glucoside displayed colour stability (*i.e.*, maintained a red colour) for about two days before the wavelength of maximum absorbance decreased to approximately 400 nm. This shift in λ_{max} was accompanied by a change in colour of solution to tan/beige (Figure 4.25). As pH increased to 4.7 and 5.7, the number of days that elapsed before the shift in λ_{max} to 400 nm occurred was reduced to about 5 days. Similar storage stability and spectral changes were noted for solutions containing cyanidin 3-glucoside and caffeine as copigment at pH 3.7, 4.7, and 5.7. Although the purplish red colour of the pH 3.7 solution (λ_{max} = 532 nm) lasted for only 5 days, the purple pH 4.7 and 5.7 caffeine solutions (λ_{max} = 545 – 550 nm) retained their colour for less than 5 days. The spectral characteristics of solutions containing protocatechuic acid as copigment were difficult to assess due to flocculation and turbidity development in these solutions during the storage period.

When the copigmented pigment and control solutions were kept in the dark, they exhibit good colour stability, (Figures 4.24 and 4.26). All solutions at pH 3.7 had a relatively stable colour for the duration of the 35-day storage study. Generally, solutions at pH 4.7 and 5.7 had gradually decreasing readings at 525 nm but developed undesirable brown or tan/beige colours also. At pH 4.7 and 5.7, the desirable colour was only present for a maximum of 10 days before the colour deteriorated; this phenomenon was especially pronounced at pH 5.7. In contrast, solutions at pH 4.7 containing cyanidin 3-glucoside and caffeine showed no decrease in absorbance over 35 days of storage. The most stable solutions were those at pH 3.7. The copigmented solutions at pH 3.7 had the most intense colour compared to the control sample. But the pH 3.7 control still had a relatively stable colour after 35 days, even though it was not the most intense. At pH 4.7, caffeine prevented the solution from discolouring when compared to chlorogenic and protocatechuic acid (Figure 4.26).

Other than the exposure to light and the effect of different copigments on the hue of each copigmented solution, it was determined that the pH also has a marked effect on the stability and colour of the resulting solution. The effect of pH is evident in Figure 4.25 and 4.26. In Figure 4.26, it is apparent that pH 3.7 is the pH at which the solutions are most stable for long periods of time in the absence of light. Figure 4.26 also shows the stability of the solutions stored in the dark when one monitors the absorbance decrease. Figure 4.25 illustrates the same effect to a lesser extent since the effect of light is so detrimental to colour retention.

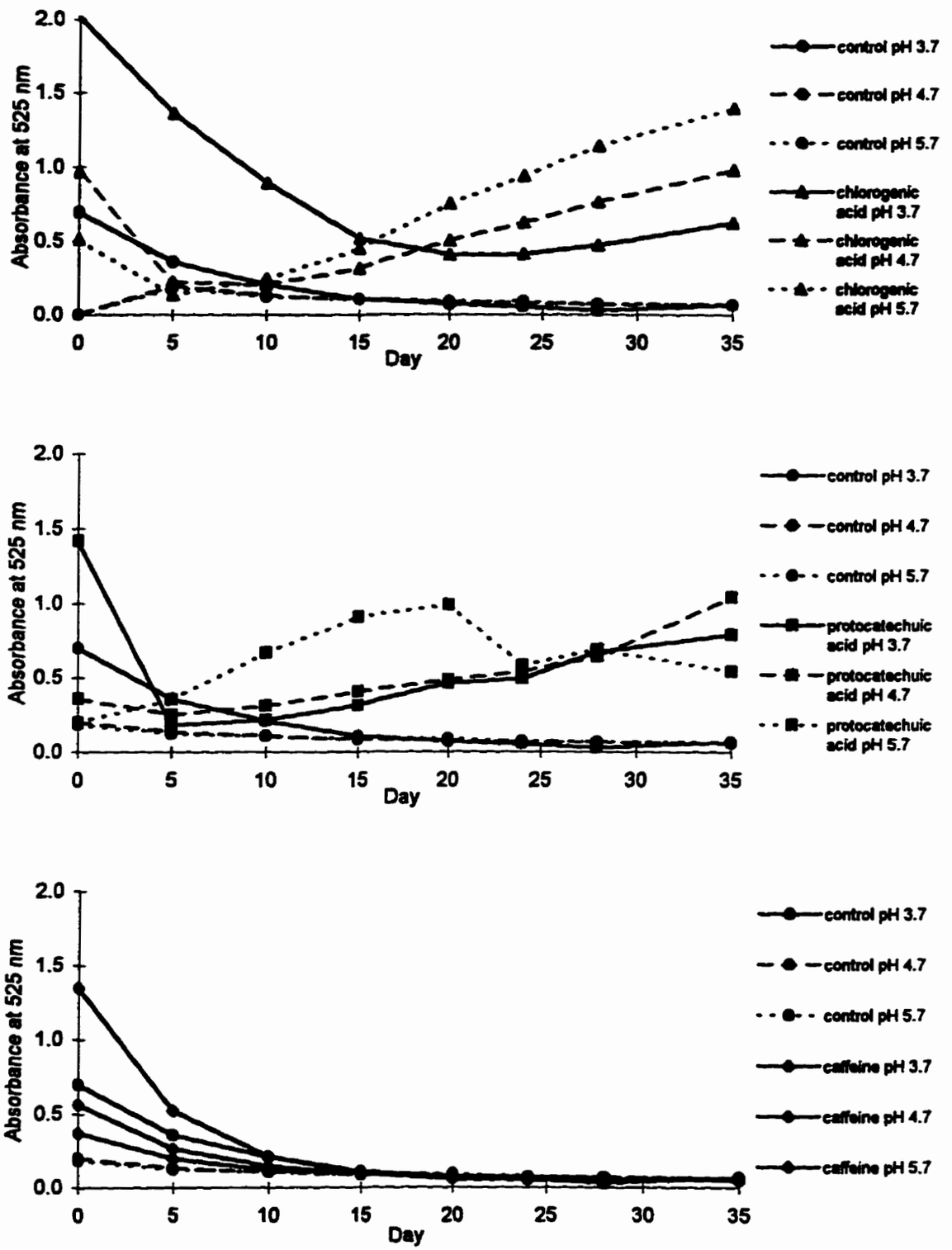


Figure 4.23: Absorbance change at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid (top), protocatechuic acid (middle) or caffeine (bottom) (1:200) at pH 3.7, 4.7, and 5.7 and 20°C for 35 days and exposed to light.

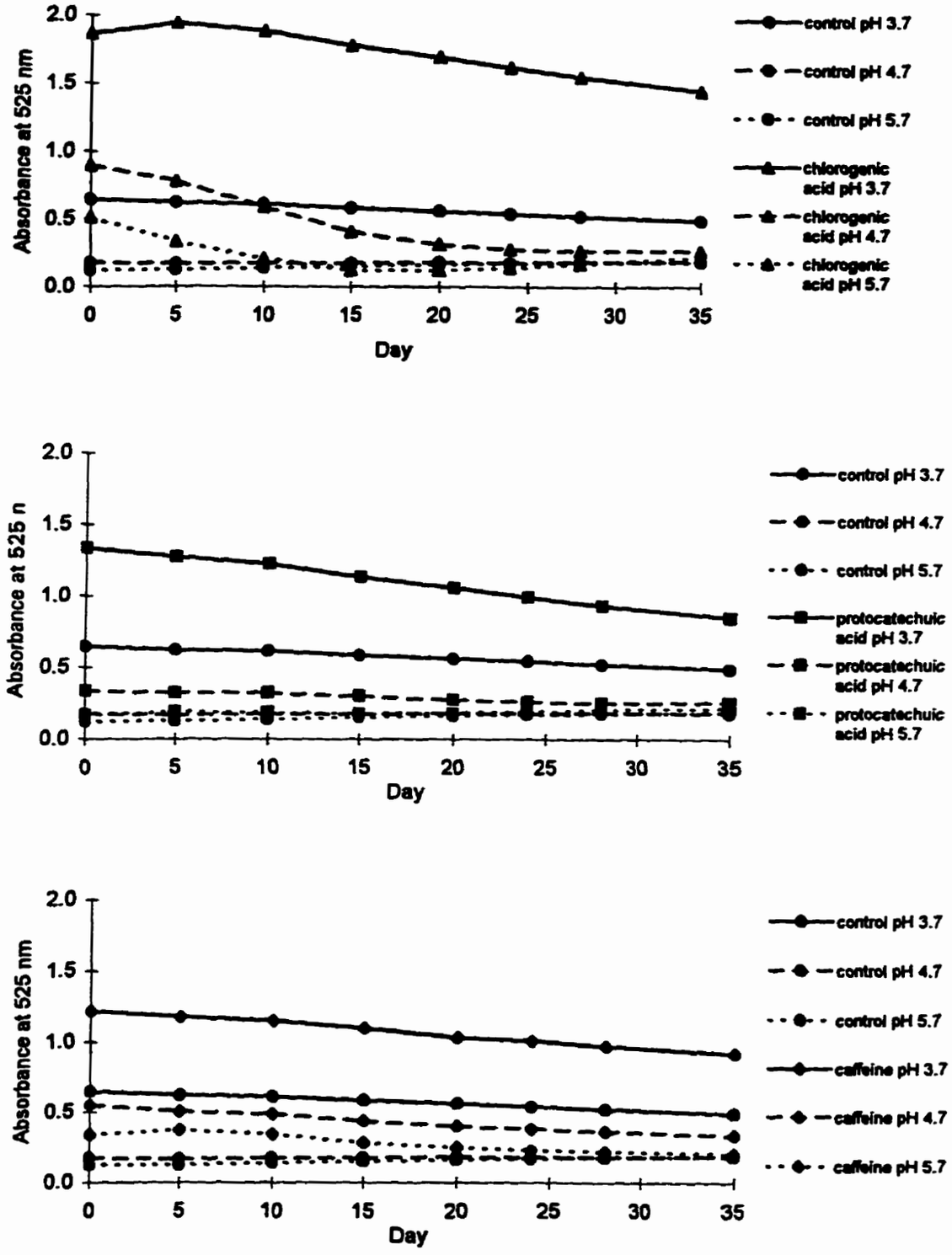


Figure 4.24: Absorbance change at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid (top), protocatechuic acid (middle) or caffeine (bottom) (1:200) at pH 3.7, 4.7, and 5.7 and 20°C for 35 days in the dark.

Figure 4.25: CIE L*a*b* values for cyanidin 3-glucoside solutions (1.29×10^{-4} M) as a control and with chlorogenic acid (1:200; 2.58×10^{-2} M), protocatechuic acid (1:200; 2.58×10^{-2} M) and caffeine (1:200; 2.58×10^{-2} M) at pH 3.7, 4.7 and 5.7 and 20°C for 35 days when exposed to light.

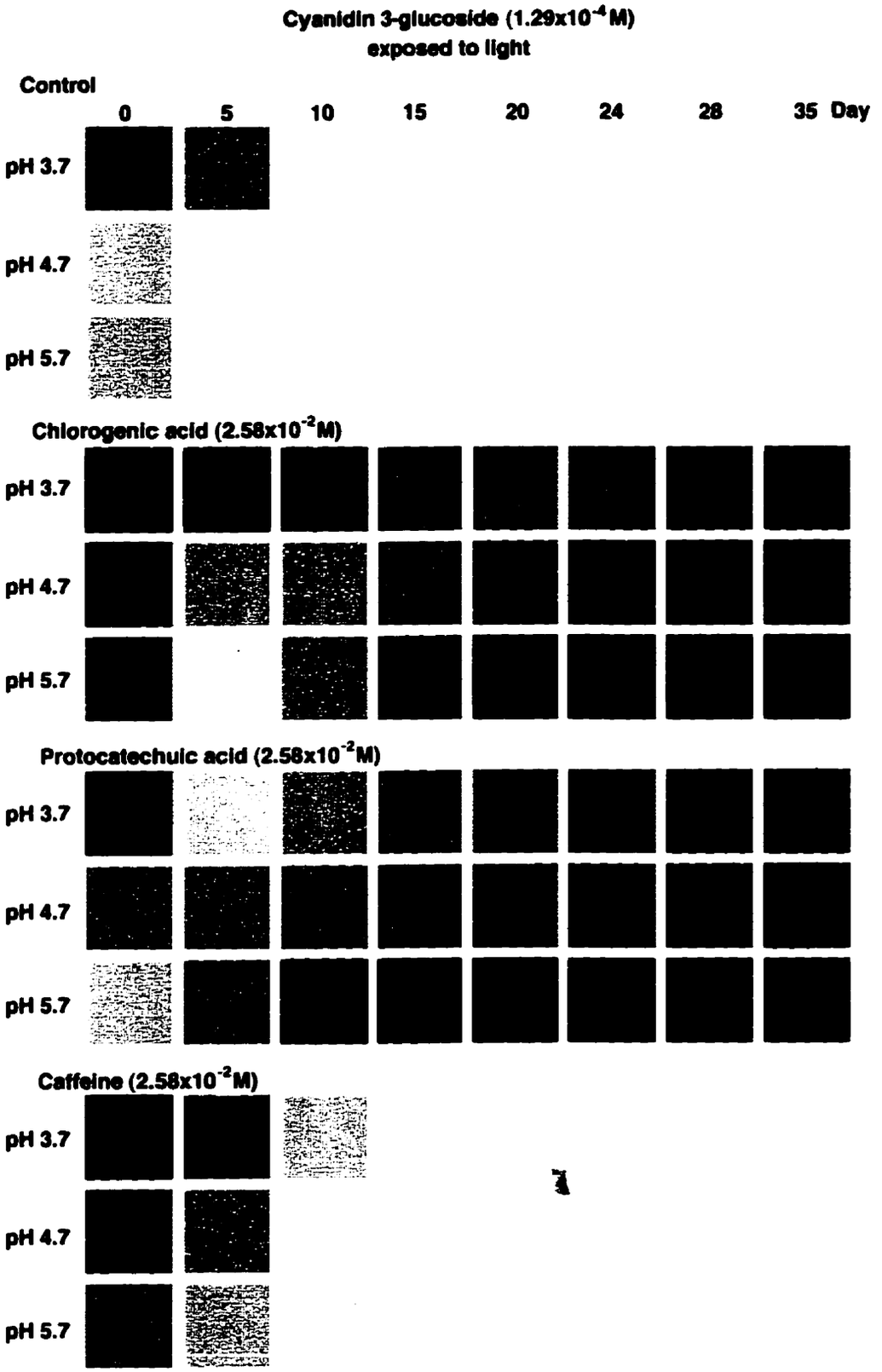
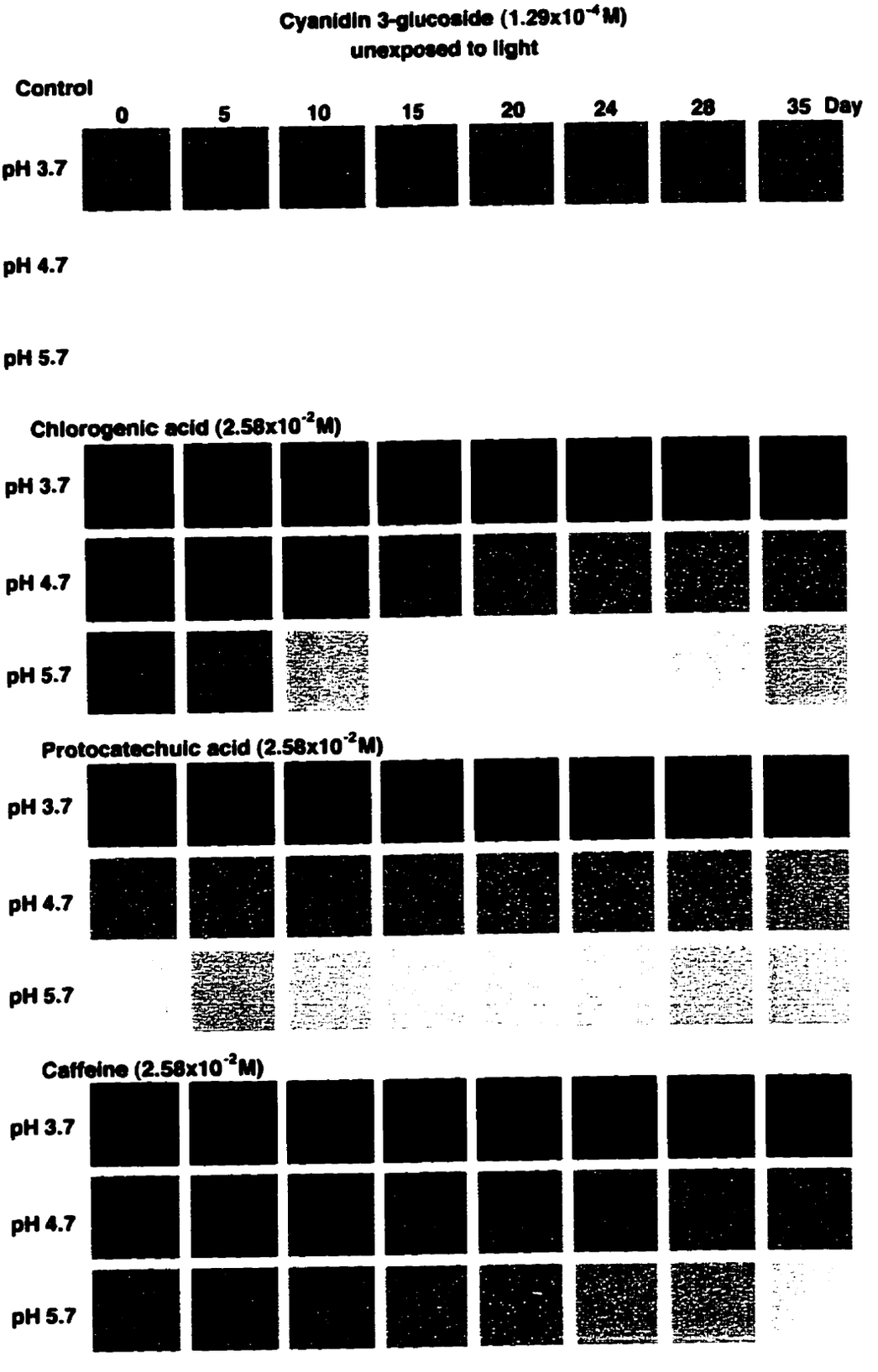

Cyanidin 3-glucoside (1.29x10^{-4}M)
exposed to light
Control
0
5
10
15
20
24
28
35 Day
pH 3.7
pH 4.7
pH 5.7
Chlorogenic acid (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7
Protocatechuic acid (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7
Caffeine (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7

Figure 4.26: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions as a control and with chlorogenic acid (1:200; 2.58×10^{-2} M), protocatechuic acid (1:200; 2.58×10^{-2} M) and caffeine (1:200; 2.58×10^{-2} M) at pH 3.7, 4.7 and 5.7 and 20°C for 35 days in the dark.


Cyanidin 3-glucoside (1.29x10^{-4}M)
unexposed to light
Control
0
5
10
15
20
24
28
35 Day
pH 3.7
pH 4.7
pH 5.7
Chlorogenic acid (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7
Protocatechuic acid (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7
Caffeine (2.58x10^{-2}M)
pH 3.7
pH 4.7
pH 5.7

4.4 Discussion

4.4.1 Influence of pigment structure

The cyanidin 3-glucoside solutions tend, in general, to have a more intense colour compared to the cyanidin and/or malvidin 3,5-diglucosides at equimolar concentrations and at the same pH (Mazza and Brouillard, 1990). Therefore, lower concentrations than those used by others (1.29×10^{-4} M) (Davies and Mazza, 1993; Mazza and Brouillard, 1990) had to be used at pH 3.7 to ensure absorbance readings between 0.2 and 0.8 as well as at other experimental pH values of 4.7 and 5.7. If a concentration of 2.58×10^{-4} M had been used, the copigmentation effect could not have been measured since the solution would have been deeply coloured with the absorbance being greater than 2.0. Initially it had been planned to determine the copigmentation at pH 2.7 also. However, at an anthocyanin concentration of 1.29×10^{-4} M, the solution was too deeply coloured to accurately read the absorbance on a spectrophotometer. Therefore, the anthocyanin concentration of 1.29×10^{-4} M was used at pH 3.7, 4.7, and 5.7.

From the results in Tables 4.1, and Figures 4.5, 4.6, and 4.7, it is evident that all three anthocyanins followed the same general copigmentation trend when subjected to the same pH and copigment conditions. These figures illustrate that under the same conditions, some combinations had a linear absorbance increase while others plateaued. Copigmentation was influenced more by the copigment involved and the pH of the solution than the anthocyanin. At low P:CP ratios of 1:5 to 1:50, the absorbance increase was linear and in agreement with the results reported by Wilska-Jeszka and Korzuchowska (1996). However, as the P:CP ratio increased, the absorbance increases differed depending in the copigment and pH (Table 4.1). Initially, it was hypothesized that the addition of malonic acid and an increase in the sugar moiety length on the anthocyanin structure (*i.e.*, cyanidin 3-glucoside, -3-malonylglucoside, -3-malonyllaminaribioside) would

result in a stronger copigmentation effect and colour stability. It was hypothesized that the acylation and/or length of the sugar molecule to which the organic acid is attached would result in different copigmentation characteristics. It was also hypothesized that the acylation might change how the copigment and anthocyanin would associate with each other. Figure 4.11 illustrates the spectra of each anthocyanin at pH 3.7, 4.7 and 5.7 while Table 4.10 lists the spectral characteristics. It was determined that there was no significant difference between wavelength of maximum absorbance among the anthocyanins at pH 3.7; however, at pH 4.7 and 5.7, there was a significant difference ($P \leq 0.05$). Since no statistically significant change in λ_{max} at pH 3.7 was observed in the present study, the tight packing of the acyl residues does not take place with these pigments as was determined by El Hajji *et al.* (1997) for more complex acylated anthocyanins from *Matthiola incana* (stock).

The three different anthocyanin pigments generally copigmented in a similar intermolecular fashion but they reacted slightly differently with the three copigments and pH values. Cyanidin 3-glucoside had larger increases in absorbance when combined with chlorogenic acid at pH 3.7, 4.7, and 5.7, than the two acylated cyanidin molecules with chlorogenic acid. The two acylated cyanidin molecules were similar to each other with respect to the absorbance increases induced by chlorogenic acid. When caffeine was added, cyanidin 3-glucoside and 3-malonylglucoside behaved similarly, while cyanidin 3-malonyllaminaribioside had less of an absorbance increase in the presence of caffeine (Figure 4.7). With protocatechuic acid, all pigments behaved similarly since the absorbance increase was always in a linear fashion and no one specific anthocyanin caused a greater absorbance increase (Figure 4.6). Therefore, with the anthocyanins used in this study, copigmentation was affected more by the type of copigment than the anthocyanin.

From Figures 4.5-4.7 it is evident that if an acylated anthocyanin is combined with a copigment such as chlorogenic acid, the absorbance change of this solution will not be as great as with a nonacylated anthocyanin. Therefore, the acid moiety of the anthocyanin may have a negative effect on copigmentation. If both malonylated and non-malonylated anthocyanins are combined with a poor copigment such as protocatechuic acid, the anthocyanin structure has no noticeable effect. Thus, under the same conditions, the anthocyanin's structure does not have a noticeable effect. On the contrary, Davies and Mazza (1993) observed that copigment complexes formed with monardaein, a diacylated pelargonidin derivative produced greater copigmentation magnitudes than the corresponding monoglucosides over a pH range of 2.7 to 5.7.

The similarity in n values for the same copigment at different pH values indicated, that the presence of malonic acid and/or laminaribioside does not generally aid nor hinder the copigmentation phenomenon. At pH 3.7 with chlorogenic acid, n values of 0.55 to 0.67 suggested 2:1 chlorogenic acid:anthocyanin complex formation. These values are in agreement with the value of 0.72 at pH 3.7 reported by Mazza and Brouillard (1990). Yet, as the pH increased to 4.7 and 5.7, n values increased to values close to 1. No n values for copigmented cyanidin 3-malonylglucoside and 3-malonyllaminaribioside have been reported. However, the higher n values at pH 4.7 and 5.7 suggest that there is one chlorogenic acid molecule associating with each cyanidin glycoside. At lower pH of 3.7, there are approximately 3 copigment molecules for 2 pigment molecules or 2 copigment molecules for each pigment. These results suggest that the anthocyanins form a 'sandwich' complex as was hypothesized by Goto *et al.* (1986).

4.4.2 Influence of copigment structure

Chlorogenic acid and protocatechuic acid are abundant phenolics found in plants. Chlorogenic acid (5-*O*-caffeylquinic acid) is a quinic ester of hydroxylated cinnamic acid. Quinic acid has a ringed structure similar to that of a sugar (hexose) (Figure 4.1). Chlorogenic acid has been used extensively in copigmentation studies and is a very good copigment (Mazza and Brouillard, 1990; Dangles and Brouillard, 1992; Davies and Mazza, 1993; Wilska-Jeszka and Korzuchowaska, 1996). Protocatechuic acid, also known as 3,4-dihydroxybenzoic acid has not been evaluated as a copigment, and was included in this study to assess its potential as a copigment. Caffeine, a *N*-methylated xanthine, has recently been studied for its copigmentation effect (Mistry *et al.*, 1991; Dangles and Brouillard, 1992; Dangles and El Hajji, 1994) and was included in this study to further evaluate its copigmentation potential.

Caffeine is a common food additive. Therefore, its ability to act as a copigment could have an impact on the use of anthocyanins as natural food colourants in products such as soft drinks. Caffeine is an abundant purine found in tea, coffee, and soft drinks and is important to the food and pharmaceutical industries. As a purine, the structure of caffeine favours molecular association by vertical stacking which is desirable for a copigment (Dangles and Brouillard, 1992). When copigmented with malvin 3,5-diglucoside, caffeine produced a shoulder at 670 nm in the absorbance spectrum, stabilized the quinonoidal base, and gave a red-violet solution at pH 3.42 (Mistry *et al.*, 1991). At pH values of 4.99, 6.20 and 6.85, quinonoidal bases and carbinol pseudobases were stabilized and violet and blue forms resulted (Mistry *et al.*, 1991). When caffeine was used as a copigmented in the present study, the same shoulder was observed (Figure 4.4 and 4.14).

Figures 4.2-4.4 and Tables 4.5, and 4.6 illustrate the important effect that the structure of the copigment had on the magnitude of copigmentation. At pH 3.7 and 4.7, chlorogenic acid exerted the greatest copigmentation effect, followed by caffeine and protocatechuic acid at these pH values. Chlorogenic acid and caffeine both resulted in a greater bathochromic shift (at a P:CP of 1:400, +22.8 and +31.9 nm, respectively) than did protocatechuic acid (+9.1 nm) (Table 4.7). Chlorogenic acid differs structurally from protocatechuic acid in that the latter lacks a quinic acid moiety. Quinic acid has a ringed structure similar to that of hexoses such as glucose and it may be the reason for the ability of chlorogenic acid to act as a good copigment. Caffeine is a purine and has a ringed structure that forms molecular associations with other molecules by vertical stacking (Mistry *et al.*, 1991). This ringed purine structure results in a larger bathochromic shift to a more purple/bluish red (magenta) colour when combined with anthocyanins (Table 4.7). Similar spectral and colour shifts were reported by Mistry *et al.* (1991) when they studied the effects of caffeine in the spectra of malvidin 3,5-diglucoside. Caffeine and chlorogenic acids produced solutions of different colours when added to cyanidin 3-glucoside. Solutions containing chlorogenic acid and cyanidin 3-glucoside were more red than the cyanidin 3-glucoside - caffeine solutions which were more bluish and appeared magenta-like. When protocatechuic acid was used as a copigment, the solutions were red in colour. The differences in colour with the various copigments can be attributed to the fact that each copigment stabilizes a different chemical form of the anthocyanin. At pHs of less than 1, when no copigment is present, the flavylium cation (AH^+) is the dominant form of anthocyanin present; this causes the solution to be intensely coloured. When a copigment is added to this solution, a bathochromic shift and small hypochromic shift in the visible region occurred in the spectra of the flavylium cation. This phenomenon was observed with flavylium cations and chlorogenic acid when determining the r_1 and K values. The hypochromic shift indicates that the complexed flavylium cation's molar absorption coefficient is smaller than that of the uncomplexed flavylium cation. At pH 3.5, the

colourless structures are predominant due to the hydration equilibrium. The resulting solution is poorly coloured if no copigment is present. When a copigment such as chlorogenic acid is added, a complex is formed between the flavylium cation (AH^+) and the quinonoidal base (A) and chlorogenic acid. This results in a shift of the hydration equilibrium to produce more flavylium cations. The copigment and flavylium cation complex produces a hyperchromic shift and colour increases. The flavylium cation is strongly solvated when no copigment is present; this might lead to nucleophilic attack by water at the C-2 position, resulting in the formation of hemiacetal B. When a copigment is added to the anthocyanin solution, it will compete with water to associate with the flavylium cation. The copigment molecules will stack vertically on the planar flavylium cation, causing desolvation of the flavylium cation, making it less susceptible to nucleophilic attack by water (Dangles and Brouillard, 1992).

At pH 3.7, uncopigmented cyanidin 3-glucoside molecules had an orange colour and a λ_{max} of 511 nm (Table 4.6 and 4.10). If the pH of the same solutions were reduced to 2.7 or lower, the solutions would have a dark red colour. This is because at a low pH, the red colour is due to the predominance of the flavylium cation (AH^+), which is red. As the pH is increased, the anthocyanin is hydrated and attacked by water molecules (nucleophilic attack) and the colourless hemiacetals (B) and chalcones (C) begin to dominate as the equilibrium is shifted to the right. At the same time, some of the flavylium cations are converted to the blue quinonoidal bases (Mazza and Brouillard, 1990). When the uncopigmented cyanidin glycosides are subjected to a pH increase, the colour changes from a dark orange-red to an orange colour at pH 3.7 and eventually a dull, faint pink colour at pH 5.7, due to the equilibrium favouring production of hemiacetals and chalcones. When different copigment structures are added, they are responsible for various hues of the solutions. It appears that chlorogenic acid effectively stabilizes the flavylium cation more than do protocatechuic acid or caffeine since the resulting solutions were magenta in colour at pH

3.7 but slightly duller at pH 4.7. At pH 4.7, the colourless chalcones should start to predominate (Mazza and Brouillard, 1987) accounting for the duller appearance. At pH 5.7, the chalcones predominate; consequently, solutions appear dull reddish yellow in colour (Table 4.7).

When used as a copigment, caffeine stabilizes different forms of anthocyanins in equilibrium than does chlorogenic acid, as evidenced by the different hues of solutions observed. At pH 3.7, the mixture of flavylium cations and quinonoidal bases become predominant and the caffeine will complex preferentially with the flavylium cation, resulting in a fuschia coloured solution. As the pH increases to 4.7 and 5.7, the solution becomes purplish in colour due to the growing predominance of the blue quinonoidal base. Blue quinonoidal bases combine with the red flavylium cations to produce purple solutions. In the spectral scans shown in Figure 4.6, a shoulder at approximately 580-600 nm becomes more prominent as caffeine is added at pH 4.7 and 5.7. This shoulder is due to accumulation of quinonoidal base. Mistry *et al.* (1991) observed that caffeine caused the same shoulder in a malvidin 3,5-diglucoside solution at pH 3.42.

Use of protocatechuic acid as the copigment resulted in a red colour at pH 3.7, yet an undesirable tan yellow hue at pH 4.7 and 5.7. Protocatechuic acid is therefore a poorer copigment than either caffeine or chlorogenic acid (Tables 4.6 and 4.8). At pH values of 4.7 and 5.7, the inability of protocatechuic acid to act as a copigment is pronounced, as evidenced by the yellow colour of the solutions due to the predominance of chalcones and hemiacetals.

The results of this study reinforce the view that for a copigment to be effective, it should have a planar π-electron-rich moiety capable of interacting with the coloured electrophilic forms of flavylium cations (AH^+) of anthocyanins (Dangles and Brouillard, 1994). The coloured flavylium cation and quinonoidal base consist of a planar chromophore in which the π-electrons

are strongly delocalized. The delocalized π-electrons of the flavylium cation would be stabilized by the planar π-electron-rich moiety present in a good copigment, such as chlorogenic acid. The copigment would compete with the water molecules for the flavylium chromophore, preventing nucleophilic attack and shifting the equilibrium towards the complexed coloured forms (Dangles and Brouillard, 1994).

By examining the copigment structures (Figure 4.1) and overlaying them with the anthocyanin, it is possible to hypothesize why some copigment structural attributes are more effective than others in forming coloured complexes with anthocyanins. Chlorogenic acid is a larger molecule that would allow it to cover one side of an anthocyanin better than protocatechuic acid. The quinic acid moiety of chlorogenic acid, absent in protocatechuic acid, would perhaps orient itself over the anthocyanin's A ring and 3,4-dihydroxycinnamate portion over the B ring. Protocatechuic acid would only be able to get good molecular contact with one of the anthocyanin's electrophilic ring structures like the B ring where it would have very little effect at higher pH's as was evident in Figure 4.3. This was because it could not prevent the nucleophilic attack of the water molecule at C-2 of the flavylium cation and the colourless chalcone and hemiacetal resulted. On the other hand, caffeine has a purine ring which would probably interact with the anthocyanin's A and C rings. It would not protect as much of the anthocyanin as chlorogenic acid does, although in a planar sense, caffeine is a larger molecule. This might explain the different hues, absorbance increases and presence of shoulders when these three different copigments were copigmented with the same anthocyanin. No H^1-NMR analysis, pH or temperature jump, or molecular interaction calculations were performed to validate this hypothesis.

Protocatechuic acid was originally hypothesized to be a poorer copigment than chlorogenic acid due to the lack of quinic acid in the former. However, at pH 3.7, two molecules of chlorogenic acid associated with one pigment molecule whereas one protocatechuic acid molecule associated with one pigment molecule even though it has less effect on the colour augmentation. Chlorogenic acid formed 1:1 complexes at higher pH values of 4.7 and 5.7, as was determined by Davies and Mazza (1993). Caffeine generally had n values of less than 1 yet they were close enough to 1 to lead one to believe that the association was 1:1. No published literature was found to which these could be directly compared to.

The equilibrium constant (K) describes the average strength and degree of association between the flavylium cation and the copigment as a function of pH (Brouillard *et al.*, 1989). The K values for each pigment and copigment were the highest for chlorogenic acid with any anthocyanin versus those for protocatechuic acid and caffeine with the same anthocyanin (Table 4.12). Generally, the K values for chlorogenic acid (8.3 to 197.0 M^{-1}) were double those for the remaining two copigments, and indicates a higher affinity of chlorogenic acid for the anthocyanin when compared to the affinity of protocatechuic acid and caffeine for the same anthocyanin. The high K values for chlorogenic acid reflects that it associates strongly with the flavylium cation rather than the quinonoidal base (Davies and Mazza, 1993). The lower K values for caffeine and protocatechuic acid indicate their preferential association with the quinonoidal base. This was reflected in the weak colour of the copigmented solutions. Dangles *et al.* (1993) reported K values of 76 (± 4) M^{-1} and 50 (± 3) and 81 (±5) M^{-1}, respectively, for nonacylated pelargonidin 3,5-diglycoside when copigmented with chlorogenic acid.

4.4.3 Influence of pH

The spectra of cyanidin 3-glucoside (1.29×10^{-4} M) at pH 3.7, 4.7 and 5.7 at 400 to 700 nm (Figure 4.11) with no added copigment were comparable to published data (Mazza and Brouillard, 1990). All three cyanidin derivatives showed classical hypochromic and bathochromic shifts in absorbance as a function of increasing pH. These spectral changes have been well documented and have been attributed to the equilibrium between the various anthocyanin species existing in an aqueous solution (Brouillard and Delaporte, 1977; Mazza and Brouillard, 1987; Brouillard *et al.*, 1989).

In general, addition of copigment produced hyperchromic and bathochromic shifts in the wavelength of maximum absorbance. Figures 4.2, 4.3, and 4.4 illustrate copigmentation of cyanidin 3-glucoside with chlorogenic acid, protocatechuic acid and caffeine at pH 3.7, 4.7 and 5.7. The hyperchromic and bathochromic shifts were observed with all cyanidin glycosides at all pH values as the copigment concentration increased. However, the magnitude of the bathochromic shift varied with each copigment. Comparison of the spectral scans in Figures 4.2, 4.3, and 4.4 illustrate this phenomenon at pH 4.7. The solution with chlorogenic acid had a greater magnitude due to the structural differences between the copigments. Figures 4.12, 4.13, and 4.14 illustrate that the pH does not have much of an effect between the pigment structures. The copigmentation effect was generally greatest at pH 4.7 when the three pigments and three copigments were compared. This is probably because the colour of the dilute solutions is due to the predominance of the colourless chalcones and hemiacetal at this pH value. The copigments are attracted to the few remaining flavylium ions and when they bind, the equilibrium is shifted so that more flavylium ions are created. At pH 3.7, the copigmentation is poorest due to the predominance of the flavylium ion lending it the intense colour, which can not be further accentuated. At pH 5.7, the copigmentation effect is greater than at pH 3.7 but not as large as

with pH 4.7. The effect is also dependent more on the type of copigment. Wilska-Jeszka and Korzuchowska (1996) found that pH 3.4 had the greatest copigmentation effect in chlorogenic acid copigmented solutions of strawberry and chokeberry juices. Therefore, the effect of pH is dependent upon factors such as the type of copigments and anthocyanins present.

4.4.4 Multiple copigment experiments

The copigmentation experiments with chlorogenic acid, caffeine and protocatechuic acid in Figures 4.18-4.22 illustrate the additive effect that copigments have. From Figures 4.18 and 4.20 of the absorbance change versus increasing copigment concentration, it is evident that the addition of the first copigment resulted in the largest absorbance increase. The first copigment was always chlorogenic acid followed by caffeine and then in the second experiment, protocatechuic acid. In both experiments, the addition of the second and third copigment usually resulted in less of an absorbance increase and eventually the absorbance increase changed from linear to a plateau as a dark coloured solution was produced. As the solution became more intensely coloured, the addition of more copigment could not produce a further increase, resulting in a plateau. Chlorogenic acid was the better copigment and produced greater absorbance increases than the others. If the order had been randomised, the high initial increase due to chlorogenic acid would perhaps have been absent along with the plateau present at higher concentrations with in each concentration series *e.g.*, 1:50:50:50 of chlorogenic acid, caffeine and protocatechuic acid.

The combination of multiple copigments resulted in different tints/hues for the final solution. For example, cyanidin 3-glucoside (1.29×10^{-4} M) at pH 4.7 has a faint pink colour (Figure 4.22). By adding chlorogenic acid and caffeine up to pigment:copigment molar ratios of 1:100:100 respectively, the solution was pinkish red. When chlorogenic acid, caffeine and

protocatechuic acid were added up to 1:200:200:200 pigment : copigment molar ratios, a very appealing dark red magenta solution was produced. At pH 4.7, these solutions had different hues and more appealing colours than any of the single copigmented solutions.

The chlorogenic acid and caffeine experiment had an n value of 0.83 (Figure 4.19) which is close enough to unity to assume that cyanidin 3-glucoside associates with one copigment on a one to one basis. Chlorogenic acid is probably the dominant copigment since it produces the largest absorbance increase and might be attracted to the cyanidin 3-glucoside's ring structure more. The caffeine may interact with the chlorogenic acid and cyanidin 3-glucoside in a complex. When chlorogenic acid, caffeine and protocatechuic acid were used, the n value was 0.66 (Figure 4.21). This n value is far away from 1 to suggest that the pigment and copigment do not interact on a 1:1 basis. The exact interaction mechanism could not be determined using the present methodology and further studies should determine the exact copigmentation mechanism in the presence of multiple copigments. Since no comparable work has been conducted in this area, these results could not be compared to any other studies. Miniati *et al.* (1992) studied self-association among pelargonin, cyanin, and malvin pooled samples. No similar work has been done with pooled copigments and single anthocyanins.

4.4.5 Stability of copigmented cyanidin 3-glucoside in the presence and absence of light

The detrimental effects of exposing anthocyanin solutions to light has been know for some time (Brouillard, 1982; Francis, 1989b). The objective of the stability study was to determine whether a simple, commonly found and readily obtainable anthocyanin such as cyanidin 3-glucoside, could be used in conjunction with copigments over a range of pH in the presence of light as a food colourant in an aqueous food system. It was originally hypothesized

that light would cause colour deterioration in the absence of copigment and that the copigment would improve colour stability.

From the data illustrated in Figures 4.21 – 4.29, it is apparent that factors such as light, pH and copigment have an effect on the stability of copigmented cyanidin 3-glucoside solutions. Light had the most detrimental effect on colour stability. Intense light (418 μmol m^{-2} s^{-1}) significantly reduce the colour stability of even the most stable coloured solution which was best at pH 3.7. The solutions copigmented with chlorogenic and protocatechuic acid all discoloured to a brownish yellow colour and eventually even flocculation developed which precipitated. The identity of the precipitate was not determined. One can hypothesize that it might perhaps be copigment condensing and polymerizing to form a precipitate. This might be true since a large concentration of copigment (2.58 x 10^{-2} M) was added. The large amount of copigment in solution might also polymerize with the various forms of anthocyanin in solution such as the chalcones, quinonoidal pseudobases and flavylium cations, with the intense light acting as the driving force for this reaction. Markakis *et al.* (1957) observed the formation of a brown, insoluble polyphenolic compound, formed when pelargonidin 3-glucoside in a buffer at pH 2.0 and 3.4 was heated from 45°C to 110°C. They proposed that the pyrilum ring is opened and a substituted chalcone is formed, degrading to form the brown preicpitate. When caffeine was used as the copigment in the presence of light, its solutions were visibly similar to the control samples stored under the same conditions. The only difference between the control and the caffeine solutions was that caffeine increased the initial hue and colour intensity of the solutions. This indicates that caffeine is a poor stabilizing copigment in the presence of light. Caffeine had little effect on stabilizing the different forms of anthocyanin present since its solutions were very similar to the controls. The fact that caffeine does not lead to brown colour development in the presence of light when compared to chlorogenic and protocatechuic acid, suggests that the light

induced browning reaction is due to the two phenolic acids. When one compares the CIE L*a*b* values of the samples kept in the light and in dark, one also notices that at pH 4.7 and 5.7 with the samples kept in the dark, that they also turned yellow or a beige/tan colour versus the brown colour for the same samples copigmented with chlorogenic and protocatechuic acid. This seems to indicate that perhaps polymerization among the copigments and anthocyanins is taking place and that light causes a more intense colour and a precipitate, and accelerates the process.

Besides the effect of light, pH was a significant factor. Figures 4.29 and 4.25 illustrate that the solutions with the most stable and well retained colour were at pH 3.7. Solutions at pH 4.7 and 5.7 were generally less stable. This can probably be explained by the changing concentrations of the different anthocyanin forms over the pH range 3.7 to 5.7. At pH 3.7, the flavylium cation is the predominant form being stabilized. It's characteristic red colour along with the copigment induced colour change and accentuation, results in the different hues illustrated in Figures 4.29 and 4.25. When the pH is increased to 4.7 and 5.7, a visible decrease in copigmentation and colour accentuation takes place. The lack of colour and colour retention is probably because the colourless chalcones and pseudobases are predominant at that pH. The same trend was observed by Miniati *et al.* (1992) over a pH range of 2.5 to 4.5.

The findings from the stability study were comparable to those conducted by others under similar conditions. A number of researchers have noted that under similar storage conditions, anthocyanins undergo a progressive decrease in absorbance, especially at the wavelength of maximum absorbance (Duhard *et al.*, 1997; Miniati *et al.*, 1992; Shi *et al.*, 1992).

4.4.6 General conclusions

The copigmentation study of cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside with chlorogenic acid, protocatechuic acid and caffeine at pH 3.7, 4.7 and 5.7 demonstrated that copigment structure and concentration, and pH have a dramatic influence on the copigmentation phenomenon. Pigment structure did have a slight effect but was minor relative to the effects of pH and copigment structure. The best copigment was determined to be chlorogenic acid, followed by caffeine, with protocatechuic acid being the poorest copigment investigated. The three copigments resulted in different colour hues and different magnitudes of copigmentation when combined with the cyanidin derivatives. In multiple copigment experiments, chlorogenic acid and caffeine without and with protocatechuic acid were combined with cyanidin 3-glucoside in increasing amounts. The magnitude in colour increase and copigmentation was greater when compared to a single copigment.

The investigation of the colour stability of copigmented anthocyanin solutions demonstrated that exposure to intense fluorescent and incandescent light (418 $\mu mol\ s^{-1}\ m^{-2}$) was detrimental to the photostability of a copigmented cyanidin 3-glucoside solution at any pH. The most photostable solution was cyanidin 3-glucoside with chlorogenic acid at pH 3.7. All other solutions discoloured and faded rapidly, and some developed a precipitate. The samples stored in the dark under the same conditions were found to be more stable, especially at pH 3.7. However, at pH 4.7 and 5.7, the solutions were also discoloured, although not to the same extent as the solutions exposed to intense light (418 $\mu mol\ m^{-2}\ s^{-1}$). This study demonstrated that light and pH have detrimental effects on the colour retention of anthocyanin solutions, while addition of copigment could provide colour stability under the right conditions.

5. CONCLUSIONS AND RECOMMENDATIONS

The anthocyanins and colourless phenolics present in 'Red Jumbo', 'Red Granex', 'Red Bone', and 'Mambo' red onions were identified and quantified using a rapid identification method based on spectrophotometric and chromatographic procedures. A total of four major and four minor anthocyanins and nine colourless phenolic compounds were found in all red onion cultivars analyzed. The anthocyanins were identified as cyanidin 3-glucoside, cyanidin 3-laminaribioside, cyanidin 3-(6"-malonylglucoside), cyanidin 3-(6"-malonyllaminaribioside), cyanidin 3-(3"-malonylglucoside), peonidin 3-glucoside, peonidin 3-malonylglucoside and cyanidin 3-dimalonyllaminaribioside. The other phenolic compounds were protocatechuic acid 4-glucoside, quercetin 7,4'-diglucoside, quercetin 3,4'-diglucoside, quercetin 3-glucoside, quercetin 4'-glucoside, and a 5,7-dihydroxy flavanone glucoside or a 5,7-dihydroxy dihydroflavanol glucoside.

The copigmentation reaction of cyanidin 3-glucoside, cyanidin 3-malonylglucoside and cyanidin 3-malonyllaminaribioside with chlorogenic acid, protocatechuic acid and caffeine at pH 3.7, 4.7 and 5.7 was studied. The results demonstrated that copigment structure, concentration and pH have a dramatic influence on the copigmentation phenomenon. Pigment structure did have a slight effect but was minor relative to the effects of pH and copigment structure. The best copigment was chlorogenic acid, followed by caffeine, with protocatechuic acid being the poorest copigment. All three copigments resulted in different colour hues and different magnitudes of copigmentation when combined with the pigments. When chlorogenic acid and caffeine without and with protocatechuic acid were combined with cyanidin 3-glucoside in increasing amounts, there was an increase in the range of colours generated and an enhanced copigmentation effect.

Colour stability measurements of cyanidin 3-glucoside solutions at pH 3.7, 4.7, and 5.7 without and with added chlorogenic acid, protocatechuic acid and caffeine in the presence and absence of light showed that exposure to intense fluorescent and incandescent light was detrimental to the photostability of a copigmented solutions at any pH. The most photostable solution was cyanidin 3-glucoside with chlorogenic acid at pH 3.7. All other solutions discoloured more rapidly and some developed a precipitate. The samples which were stored in the dark under the same conditions were found to be more stable, especially at pH 3.7. However, solutions at pH 4.7 and 5.7 also discoloured, although not to the same extent as those exposed to intense light.

A number of recommendations can be made from this research for future study. First, the structures of all anthocyanins and colourless phenolics should be verified using more advanced identification procedures such as mass spectrometry and nuclear magnetic resonance (NMR). Secondly, the copigmentation phenomenon should be studied using NMR so one can determine the nature of the association between the pigment and copigment. Thirdly, using the three red onion pigments, the presence of intramolecular copigment should be studied using NMR and pH jump experiments to determine the conformation in aqueous systems and the effect of sugar length on copigmentation. Fourth, the mechanism of degradation of copigmented solutions in the presence of light at high pH should be determined. Fifth, the mechanism of multiple copigmentation, along with the presence of more than one anthocyanin requires further studies. Last, the stability of copigmented and acylated anthocyanins need to be studied in food systems.

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APPENDICES

Appendix 1: Calculation of moisture content of red onions

Moisture content was determined with the following formula:

$$\text{Moisture content (\%)} = \frac{(\text{Initial weight of onion} - \text{Final weight of onion})}{\text{Initial weight of onion}}$$

Appendix 2: Preparation of spectral shift reagents.

Sodium methoxide (NaOMe)	Approximately 2.5 g of metallic sodium cut into small pieces and added to 100 mL methanol and mixed.
Sodium acetate (NaOAc)	Powdered, anhydrous sodium acetate.
Aluminum chloride ($AlCl_3$)	Approximately 5 g of aluminum chloride was added to 100 mL methanol and mixed.
Hydrochloric acid (HCl)	Concentrated reagent grade hydrochloric acid (50 mL) is added to 100 mL distilled water.
Boric acid (H_3BO_3)	Anhydrous, powdered boric acid.

(from Markham, 1982c)

Appendix 3: Preparation of buffers and pH adjusting solutions for copigmentation experiment.

Buffer preparation

0.2 M ortho-phosphoric acid (1.1%)

o-H_3PO_4 MW=98.00 g/mole

85 % o-H_3PO_4, specific gravity=1.70 44.6 Normality absolute

$$\frac{10\ mL}{1000\ mL} = 1\%$$

$$\frac{10\ mL}{85\%} = \frac{x}{100\%}$$

x mL = 11.76 mL of 85 % o-H_3PO_4 in 1000 mL water

molarity:

$$D = \frac{m}{V}$$

m=DV

$$m = \frac{1.70\ g\ conc.\ solt'n}{1\ mL\ conc'd\ solt'n} \times 11.76\ mL$$

m = 19.992 g o-H_3PO_4

wt A = (# mol A)(MW of A)

$$\#\ mol\ A = \frac{wt.\ A}{MW\ of\ A} = \frac{19.992\ g\ o-H_3PO_4}{98.00\ g/mole} = 0.204\ mole/L = 0.204\ M$$

0.2 M sodium acetate buffer

sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) MW=136.08

x g=(volume)(molarity)(MW)

= (1L)(0.2 mole/L)(136.08 g/mole)

= 27.216 g/L

pH adjusting solutions

5 M HCl

HCl MW=36.46 g/mol

specific gravity = 1.18 g/mol 36.5-38%

$$\frac{\text{wt. HCl}}{\text{vol. conc. solt'n}} = \frac{(1.18\ \text{g})(36.5\ \text{g})}{1\ \text{mL}} = \frac{0.4307\ \text{g/mL}}{100\ \text{g}}$$

$$\text{wt. HCl needed} = (1000\ \text{mL})\left(\frac{5\ \text{mol HCl}}{1\ \text{mL}}\right)\left(\frac{0.0365\ \text{g}}{\text{mol}}\right) = 182.5\ \text{g}$$

$$\text{volume} = (182.5\ \text{g HCl})\left(\frac{1\ \text{mL}}{0.437\ \text{g}}\right) = \frac{423.73\ \text{mL}}{1000\ \text{mL}}$$

$$\text{volume} = \frac{42.373\ \text{mL}}{100\ \text{mL}}$$

5 M NaOH

NaOH MW=40.00 g/mol

$$\left(\frac{5\ \text{mol}}{\text{L}}\right)\left(\frac{40\ \text{g}}{\text{mol}}\right) = 200\ \text{g/L}$$

5 M NaOH = 20 g/100 mL

(from Mazza and Brouillard, 1987, 1990; Davies and Mazza, 1993)

Appendix 4: Calculation of anthocyanin stock solution and volumes of buffer for specific pH values.

Cyanidin 3-glucoside MW = 449.2 g/mol

1.29 x 10-4 mol/L x 449.4 g/mol = 0.0579726 g/L

= (5.797 x 10-2 g/L)(1 L/1000 mL)(1000 mg/L g) = 0.057973 mg/mL

to get pH 2.7	need 6 mL o-H_3PO_4 + 4 mL NaOAc	pH ~ 2.6
	0.6 x 2 mL = 1.2 mL o-H_3PO_4	
	0.4 x 2 = 0.8 mL NaOAc	
to get pH 3.7	need 5 mL o-H_3PO_4 + 5 mL NaOAc	pH 3.83
	1 mL o-H_3PO_4	
	1 mL NaOAc	
to get pH 4.7	need 4 mL o-H_3PO_4 + 8 mL NaOAc	pH 4.70
	4/12 x 2 mL = 0.67 mL o-H_3PO_4	
	8/12 x 2 mL = 1.33 mL NaOAc	
to get pH 5.7	need 1 mL o-H_3PO_4 + 9 mL NaOAc	pH 5.58
	0.1 x 2 mL = 0.2 mL o-H_3PO_4	
	0.9 x 2 mL = 1.8 mL NaOAc	

pH 5.7 buffer has the least amount of o-H_3PO_4 buffer used . Therefore, it is the limiting volume. Dissolved anthocyanin in the minimum amount of o-H_3PO_4 buffer, since it would be more stable at this pH also in the refrigerator.

0.2 mL x 4 copigments x 4 pH's = 3.2 mL of o-H_3PO_4 to be used to dissolved stock solution of anthocyanin.

total volume of buffer used 2 x 4 x 4 = 32 mL

want 0.057973 x 10_{-2} mg/mL in each mL of buffer

therefore dissolve 5.7973 x 10^{-2} mg/mL x 32 mL = 1.855 mg

if weigh 2.00 mg, x mL needed to dissolve:

$$\frac{1.855 \text{ mg}}{3.2 \text{ mL}} = 2.00 \text{ mg}$$

$$x = \frac{(2.00\ \text{mg})(3.2\ \text{mL})}{1.855\ \text{mg}}$$

$x = 3.4499$ mL

$x = 3.45$ mL

so have excess, since might need to redo an experiment or two.

Stock solution was stored in the refrigerator at 4°C in a HPLC vial.

Note: the volumes used later on where reduced since pH 2.7 and sucrose were not factored into the experiment.

Appendix 5: Calculation for the amount of copigment to be used

chlorogenic acid MW = 354.3 g/mol

amount needed for 1:1 ratio:

$(1.29 \times 10^{-4}$ mol/L)(354.3 g/mol)(1000 mg/g)(1 L/1000 mL) = 4.571×10^{-2} mg/mL

1:5 (5)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0002285 g

1:10 (5)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0002285 g

1:15 (5)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0002285 g

1:20 (5)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0002285 g

1:50 (30)(4.571 x 10-2 mg/mL)(1 g/1000 mg) = 0.0013711 g

1:100 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:150 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:200 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:250 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:300 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:350 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

1:400 (50)(4.571×10^{-2} mg/mL)(1 g/1000 mg) = 0.0022852 g

Note: All amounts were added in succession to the cuvette. The amounts of copigment were weighed out while the anthocyanin solution was mixing in the closed sample compartment in the spectrophotometer. The preweighed amounts were stored on weighing paper in a metal dish, in a desiccator until they were added. The same calculations were performed for protocatechuic acid (MW = 154.1 g/mol) and caffeine (MW = 194.2 g/mol).

Appendix 6: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing chlorogenic acid concentration at pH 3.7, 4.7, and 5.7 (repetition number 1).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.704	511.7	0.8007	0.7245			0.67
5	6.45×10^{-4}	-7.35	3.696	512.7	0.8788	0.8176	0.1285	-2.0518	
10	1.29×10^{-3}	-6.65	3.699	513.3	0.9251	0.8716	0.2030	-1.5944	
15	1.94×10^{-3}	-6.25	3.693	515.1	0.9981	0.9524	0.3146	-1.1566	
20	2.58×10^{-3}	-5.96	3.700	516.1	1.0763	1.0396	0.4349	-0.8326	
50	6.45×10^{-3}	-5.04	3.700	520.7	1.3394	1.3285	0.8337	-0.1819	
100	1.29×10^{-2}	-4.35	3.695	527.3	1.5689	1.5652	1.1604	0.1488	
150	1.94×10^{-2}	-3.95	3.697	529.8	1.7859	1.7650	1.4362	0.3620	
200	2.58×10^{-2}	-3.66	3.698	531.7	1.8718	1.8411	1.5412	0.4326	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.702	516.3	0.1649	0.1611			1.19
5	6.45×10^{-4}	-7.35	4.700	518.6	0.1720	0.1691	0.0497	-3.0026	
10	1.29×10^{-3}	-6.65	4.704	519.5	0.1776	0.1755	0.0894	-2.4148	
15	1.94×10^{-3}	-6.25	4.696	520.6	0.1953	0.1938	0.2030	-1.5947	
20	2.58×10^{-3}	-5.96	4.704	521.9	0.2110	0.2098	0.3023	-1.1963	
50	6.45×10^{-3}	-5.04	4.704	525.9	0.3141	0.3123	0.9385	-0.0634	
100	1.29×10^{-2}	-4.35	4.700	531.4	0.4682	0.4629	1.8734	0.6277	
150	1.94×10^{-2}	-3.95	4.707	534.3	0.6125	0.5989	2.7176	0.9997	
200	2.58×10^{-2}	-3.66	4.704	535.5	0.7428	0.7189	3.4624	1.2420	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.700	526.9	0.1364	0.1353			1.06
5	6.45×10^{-4}	-7.35	5.706	532.1	0.1444	0.1429	0.0562	-2.8793	
10	1.29×10^{-3}	-6.65	5.701	530.8	0.1544	0.1517	0.1212	-2.1102	
15	1.94×10^{-3}	-6.25	5.703	527.3	0.1523	0.1499	0.1079	-2.2265	
20	2.58×10^{-3}	-5.96	5.707	530.8	0.1658	0.1636	0.2092	-1.5646	
50	6.45×10^{-3}	-5.04	5.695	535.9	0.2223	0.2168	0.6024	-0.5069	
100	1.29×10^{-2}	-4.35	5.698	539.3	0.3147	0.3005	1.2210	0.1997	
150	1.94×10^{-2}	-3.95	5.702	541.6	0.4126	0.3871	1.8611	0.6211	
200	2.58×10^{-2}	-3.66	5.701	544.3	0.5173	0.4788	2.5388	0.9317	

Appendix 7: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing chlorogenic acid concentration at pH 3.7, 4.7, and 5.7 (repetition number 2).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.706	511.6	0.7078	0.6432			0.68
5	6.45×10^{-4}	-7.35	3.696	512.7	0.7636	0.7102	0.1042	-2.2618	
10	1.29×10^{-3}	-6.65	3.693	513.8	0.8358	0.7895	0.2275	-1.4808	
15	1.94×10^{-3}	-6.25	3.7	514.8	0.8961	0.857	0.3324	-1.1014	
20	2.58×10^{-3}	-5.96	3.697	515.8	0.9548	0.9215	0.4327	-0.8378	
50	6.45×10^{-3}	-5.04	3.691	520.9	1.2600	1.2536	0.9490	-0.0523	
100	1.29×10^{-2}	-4.35	3.695	527.3	1.5843	1.589	1.4705	0.3856	
150	1.94×10^{-2}	-3.95	3.689	529.5	1.8030	1.7841	1.7738	0.5731	
200	2.58×10^{-2}	-3.66	3.695	532.3	1.9415	1.9028	1.9583	0.6721	
250	3.23×10^{-2}	-3.43	3.695	533.8	2.0313	1.974	2.0690	0.7271	
300	3.87×10^{-2}	-3.25	3.705	534.7	2.0972	2.0226	2.1446	0.7629	
350	4.52×10^{-2}	-3.10	3.701	536.3	2.1497	2.0601	2.2029	0.7898	
400	5.16×10^{-2}	-2.96	3.699	536.2	2.1854	2.0831	2.2387	0.8059	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.704	516.4	0.1712	0.1670			0.97
5	6.45×10^{-4}	-7.35	4.695	516.9	0.1905	0.1864	0.1162	-2.1527	
10	1.29×10^{-3}	-6.65	4.695	520.2	0.2056	0.2029	0.2150	-1.5373	
15	1.94×10^{-3}	-6.25	4.695	519.2	0.2262	0.2222	0.3305	-1.1070	
20	2.58×10^{-3}	-5.96	4.693	523.3	0.2390	0.2367	0.4174	-0.8738	
50	6.45×10^{-3}	-5.04	4.700	525.9	0.3794	0.3787	1.2677	0.2372	
100	1.29×10^{-2}	-4.35	4.696	531.4	0.5690	0.5623	2.3671	0.8617	
150	1.94×10^{-2}	-3.95	4.700	534.4	0.7445	0.7267	3.3515	1.2094	
200	2.58×10^{-2}	-3.66	4.703	535.1	0.9618	0.9310	4.5749	1.5206	
250	3.23×10^{-2}	-3.43	4.695	536.5	1.1082	1.0621	5.3599	1.6789	
300	3.87×10^{-2}	-3.25	4.697	537.7	1.2179	1.1582	5.9353	1.7809	
350	4.52×10^{-2}	-3.10	4.693	539.0	1.3151	1.2453	6.4569	1.8651	
400	5.16×10^{-2}	-2.96	4.693	539.2	1.4083	1.3257	6.9383	1.9371	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.696	528.8	0.1183	0.1174			1.03
5	6.45×10^{-4}	-7.35	5.693	532.0	0.1266	0.1251	0.0656	-2.7244	
10	1.29×10^{-3}	-6.65	5.694	531.6	0.1352	0.1329	0.1320	-2.0247	
15	1.94×10^{-3}	-6.25	5.696	531.2	0.1412	0.1397	0.1899	-1.6610	
20	2.58×10^{-3}	-5.96	5.699	533.0	0.1497	0.1473	0.2547	-1.3677	
50	6.45×10^{-3}	-5.04	5.704	536.5	0.2062	0.2054	0.7496	-0.2883	
100	1.29×10^{-2}	-4.35	5.704	540.6	0.3152	0.2988	1.5451	0.4351	
150	1.94×10^{-2}	-3.95	5.693	541.7	0.4285	0.3995	2.4029	0.8767	
200	2.58×10^{-2}	-3.66	5.695	543.4	0.5095	0.4691	2.9957	1.0972	
250	3.23×10^{-2}	-3.43	5.702	543.5	0.6116	0.5575	3.7487	1.3214	
300	3.87×10^{-2}	-3.25	5.704	544.1	0.7544	0.6515	4.5494	1.5150	
350	4.52×10^{-2}	-3.10	5.702	546.6	0.7747	0.6962	4.9302	1.5954	
400	5.16×10^{-2}	-2.96	5.706	546.4	0.8380	0.7494	5.3833	1.6833	

Appendix 8: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing protocatechuic acid concentration at pH 3.7, 4.7, and 5.7 (repetition number 1).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.707	512.0	0.6967	0.6340			0.90
5	6.45×10^{-4}	-7.35	3.702	512.0	0.7193	0.6584	0.0385	-3.2575	
10	1.29×10^{-3}	-6.65	3.700	512.3	0.7279	0.6698	0.0565	-2.8741	
15	1.94×10^{-3}	-6.25	3.696	512.8	0.7402	0.6832	0.0776	-2.5562	
20	2.58×10^{-3}	-5.96	3.705	513.4	0.7752	0.7199	0.1355	-1.9989	
50	6.45×10^{-3}	-5.04	3.670	514.5	0.8717	0.8269	0.3043	-1.1899	
100	1.29×10^{-2}	-4.35	3.695	516.1	1.0223	0.9893	0.5604	-0.5791	
150	1.94×10^{-2}	-3.95	3.700	518.4	1.1650	1.1426	0.8022	-0.2204	
200	2.58×10^{-2}	-3.66	3.696	519.2	1.3243	1.3078	1.0628	0.0609	
250	3.23×10^{-2}	-3.43	3.695	520.7	1.4534	1.4395	1.2705	0.2394	
300	3.87×10^{-2}	-3.25	3.700	522.8	1.5268	1.5151	1.3897	0.3291	
350	4.52×10^{-2}	-3.10	3.702	521.8	1.5438	1.4395	1.2705	0.2394	
400	5.16×10^{-2}	-2.96	3.695	524.1	1.7244	1.7145	1.7043	0.5331	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.704	514.6	0.1607	0.1572			0.91
5	6.45×10^{-4}	-7.35	4.702	515.6	0.1712	0.1677	0.0668	-2.7061	
10	1.29×10^{-3}	-6.65	4.698	517.4	0.1730	0.1696	0.0789	-2.5398	
15	1.94×10^{-3}	-6.25	4.695	516.3	0.1677	0.1642	0.0445	-3.1116	
20	2.58×10^{-3}	-5.96	4.694	516.5	0.1724	0.1693	0.0770	-2.5643	
50	6.45×10^{-3}	-5.04	4.700	517.5	0.1969	0.1939	0.2335	-1.4547	
100	1.29×10^{-2}	-4.35	4.697	518.4	0.2210	0.2186	0.3906	-0.9401	
150	1.94×10^{-2}	-3.95	4.705	519.7	0.2582	0.2565	0.6317	-0.4594	
200	2.58×10^{-2}	-3.66	4.697	521.8	0.2903	0.2894	0.8410	-0.1732	
250	3.23×10^{-2}	-3.43	4.701	522.8	0.3716	0.3709	1.3594	0.3071	
300	3.87×10^{-2}	-3.25	4.704	523.3	0.3947	0.3943	1.5083	0.4110	
350	4.52×10^{-2}	-3.10	4.693	524.2	0.4358	0.4357	1.7716	0.5719	
400	5.16×10^{-2}	-2.96	4.695	524.3	0.4979	0.4977	2.1660	0.7729	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.695	530.6	0.1435	0.1423			3.08
5	6.45×10^{-4}	-7.35	5.700	531.1	0.1381	0.1373	-0.0351	--	
10	1.29×10^{-3}	-6.65	5.702	531.8	0.1508	0.1373	-0.0351	--	
15	1.94×10^{-3}	-6.25	5.702	530.0	0.1245	0.1239	-0.1293	--	
20	2.58×10^{-3}	-5.96	5.702	528.8	0.1190	0.1184	-0.1680	--	
50	6.45×10^{-3}	-5.04	5.704	530.0	0.1220	0.1215	-0.1462	--	
100	1.29×10^{-2}	-4.35	5.704	530.2	0.1340	0.1335	-0.0618	--	
150	1.94×10^{-2}	-3.95	5.704	529.0	0.1477	0.1462	0.0274	-3.5970	
200	2.58×10^{-2}	-3.66	5.705	527.8	0.1616	0.1614	0.1342	-2.0083	
250	3.23×10^{-2}	-3.43	5.700	527.5	0.1862	0.1857	0.3050	-1.1875	
300	3.87×10^{-2}	-3.25	5.693	527.8	0.2012	0.2011	0.4132	-0.8838	
350	4.52×10^{-2}	-3.10	5.701	525.6	0.2184	0.2184	0.5348	-0.6260	
400	5.16×10^{-2}	-2.96	5.701	525.6	0.2291	0.2291	0.6100	-0.4943	

Appendix 9: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing protocatechuic acid concentration at pH 3.7, 4.7, and 5.7 (repetition number 2).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.702	510.7	0.6890	0.6266			0.97
5	6.45×10^{-4}	-7.35	3.693	511.6	0.5963	0.5496	-0.1229	--	
10	1.29×10^{-3}	-6.65	3.698	511.8	0.5985	0.5522	-0.1187	--	
15	1.94×10^{-3}	-6.25	3.703	512.7	0.5964	0.5527	-0.1179	--	
20	2.58×10^{-3}	-5.96	3.696	511.9	0.6087	0.5661	-0.0966	--	
50	6.45×10^{-3}	-5.04	3.700	513.7	0.6743	0.6375	0.0174	-4.0516	
100	1.29×10^{-2}	-4.35	3.702	515.7	0.8108	0.7809	0.2463	-1.4014	
150	1.94×10^{-2}	-3.95	3.698	516.3	0.9391	0.9158	0.4615	-0.7732	
200	2.58×10^{-2}	-3.66	3.698	519.2	1.0691	1.0520	0.6789	-0.3873	
250	3.23×10^{-2}	-3.43	3.700	520.1	1.1769	1.1624	0.8551	-0.1565	
300	3.87×10^{-2}	-3.25	3.699	520.9	1.2904	1.2822	1.0463	0.0452	
350	4.52×10^{-2}	-3.10	3.699	523.0	1.7016	1.6972	1.7086	0.5357	
400	5.16×10^{-2}	-2.96	3.706	523.9	1.8055	1.8034	1.8781	0.6302	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.695	516.1	0.1704	0.1662			1.10
5	6.45×10^{-4}	-7.35	4.693	517.5	0.1729	0.1689	0.0162	-4.1199	
10	1.29×10^{-3}	-6.65	4.694	516.5	0.1767	0.1723	0.0367	-3.3049	
15	1.94×10^{-3}	-6.25	4.700	517.4	0.1776	0.1733	0.0427	-3.1531	
20	2.58×10^{-3}	-5.96	4.705	517.3	0.1808	0.1769	0.0644	-2.7429	
50	6.45×10^{-3}	-5.04	4.695	517.0	0.2035	0.1992	0.1986	-1.6167	
100	1.29×10^{-2}	-4.35	4.700	519.2	0.2341	0.2315	0.3929	-0.9342	
150	1.94×10^{-2}	-3.95	4.693	520.8	0.2758	0.2741	0.6492	-0.4320	
200	2.58×10^{-2}	-3.66	4.695	521.0	0.3137	0.3123	0.8791	-0.1289	
250	3.23×10^{-2}	-3.43	4.696	522.8	0.3577	0.3566	1.1456	0.1359	
300	3.87×10^{-2}	-3.25	4.697	524.3	0.4004	0.4000	1.4067	0.3413	
350	4.52×10^{-2}	-3.10	4.705	523.3	0.4387	0.4381	1.6360	0.4922	
400	5.16×10^{-2}	-2.96	4.700	524.7	0.4774	0.4766	1.8676	0.6247	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.703	529.7	0.1260	0.1230			1.58
5	6.45×10^{-4}	-7.35	5.699	528.3	0.1244	0.1225	-0.0041	--	
10	1.29×10^{-3}	-6.65	5.697	530.3	0.1255	0.1234	0.0033	-5.7285	
15	1.94×10^{-3}	-6.25	5.695	528.5	0.1243	0.1235	0.0041	-5.5053	
20	2.58×10^{-3}	-5.96	5.700	527.0	0.1233	0.1228	-0.0016	--	
50	6.45×10^{-3}	-5.04	5.696	526.5	0.1292	0.1285	0.0447	-3.1074	
100	1.29×10^{-2}	-4.35	5.699	402.4	0.1632	0.1410	0.1463	-1.9218	
150	1.94×10^{-2}	-3.95	5.704	400.6	0.1879	0.1578	0.2829	-1.2626	
200	2.58×10^{-2}	-3.66	5.695	401.3	0.2068	0.1675	0.3618	-1.0167	
250	3.23×10^{-2}	-3.43	5.695	401.1	0.2328	0.1838	0.4943	-0.7046	
300	3.87×10^{-2}	-3.25	5.704	401.1	0.2566	0.1986	0.6146	-0.4867	
350	4.52×10^{-2}	-3.10	5.698	403.2	0.2790	0.2130	0.7317	-0.3124	
400	5.16×10^{-2}	-2.96	5.702	401.2	0.3026	0.2291	0.8626	-0.1478	

Appendix 10: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing protocatechuic acid concentration at pH 3.7 (repetition number 3).

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.698	511.5	0.7356	0.6679			1.33
5	6.45×10^{-4}	-7.35	3.699	511.3	0.7351	0.6706	0.0040	-5.5109	
10	1.29×10^{-3}	-6.65	3.693	511.4	0.7491	0.6860	0.0271	-3.6082	
15	1.94×10^{-3}	-6.25	3.697	512.1	0.7383	0.6785	0.0159	-4.1433	
20	2.58×10^{-3}	-5.96	3.696	512.0	0.7619	0.7025	0.0518	-2.9603	
50	6.45×10^{-3}	-5.04	3.694	514.5	0.8968	0.8464	0.2673	-1.3195	
100	1.29×10^{-2}	-4.35	3.701	516.0	1.0034	0.9709	0.4537	-0.7904	
150	1.94×10^{-2}	-3.95	3.695	520.0	1.2075	1.1811	0.7684	-0.2635	
200	2.58×10^{-2}	-3.66	3.695	519.8	1.3959	1.3761	1.0603	0.0586	
250	3.23×10^{-2}	-3.43	3.695	520.4	1.4442	1.4339	1.1469	0.1370	
300	3.87×10^{-2}	-3.25	3.704	523.0	1.5497	1.5412	1.3075	0.2681	
350	4.52×10^{-2}	-3.10	3.693	523.0	1.7137	1.7079	1.5571	0.4428	
400	5.16×10^{-2}	-2.96	3.690	524.3	1.8071	1.8045	1.7018	0.5317	

Appendix 11: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing caffeine concentration at pH 3.7, 4.7, and 5.7 (repetition number 1).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.692	511.3	0.6750	0.6150			0.68
5	6.45×10^{-4}	-7.35	3.705	512.8	0.7038	0.6555	0.0659	-2.7203	
10	1.29×10^{-3}	-6.65	3.696	514.1	0.6950	0.6574	0.0689	-2.6745	
15	1.94×10^{-3}	-6.25	3.705	515.0	0.7265	0.6959	0.1315	-2.0284	
20	2.58×10^{-3}	-5.96	3.696	516.2	0.7300	0.7042	0.1450	-1.9307	
50	6.45×10^{-3}	-5.04	3.698	520.3	0.8735	0.8672	0.4101	-0.8914	
100	1.29×10^{-2}	-4.35	3.705	525.8	1.0191	1.0186	0.6563	-0.4212	
150	1.94×10^{-2}	-3.95	3.687	529.7	1.0838	1.0760	0.7496	-0.2882	
200	2.58×10^{-2}	-3.66	3.700	529.7	1.1647	1.1461	0.8636	-0.1467	
250	3.23×10^{-2}	-3.43	3.702	534.3	1.2007	1.1700	0.9024	-0.1027	
300	3.87×10^{-2}	-3.25	3.696	535.7	1.2206	1.1797	0.9182	-0.0853	
350	4.52×10^{-2}	-3.10	3.707	537.1	1.2323	1.1832	1.0037	0.0037	
400	5.16×10^{-2}	-2.96	3.704	538.3	1.2418	1.1838	0.9249	-0.0781	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.698	517.2	0.1790	0.1752			0.82
5	6.45×10^{-4}	-7.35	4.699	517.8	0.1996	0.1973	0.1261	-2.0704	
10	1.29×10^{-3}	-6.65	4.698	520.7	0.1986	0.1974	0.1267	-2.0658	
15	1.94×10^{-3}	-6.25	4.705	521.0	0.1999	0.1995	0.1387	-1.9755	
20	2.58×10^{-3}	-5.96	4.691	524.0	0.2120	0.2119	0.2095	-1.5632	
50	6.45×10^{-3}	-5.04	4.705	531.0	0.2915	0.2889	0.6490	-0.4324	
100	1.29×10^{-2}	-4.35	4.703	538.8	0.3995	0.3839	1.1912	0.1750	
150	1.94×10^{-2}	-3.95	4.701	542.2	0.4844	0.4538	1.5902	0.4638	
200	2.58×10^{-2}	-3.66	4.705	544.7	0.5619	0.5185	1.9595	0.6727	
250	3.23×10^{-2}	-3.43	4.703	546.0	0.6104	0.5519	2.1501	0.7655	
300	3.87×10^{-2}	-3.25	4.701	547.1	0.6527	0.5831	2.3282	0.8451	
350	4.52×10^{-2}	-3.10	4.706	548.4	0.7011	0.6154	2.5126	0.9213	
400	5.16×10^{-2}	-2.96	4.705	549.4	0.7120	0.6263	2.5748	0.9458	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.696	530.5	0.1151	0.1145			0.85
5	6.45×10^{-4}	-7.35	5.693	532.5	0.1263	0.1246	0.0882	-2.4280	
10	1.29×10^{-3}	-6.65	5.694	536.4	0.1364	0.1335	0.1659	-1.7961	
15	1.94×10^{-3}	-6.25	5.704	537.2	0.1530	0.1489	0.3004	-1.2025	
20	2.58×10^{-3}	-5.96	5.695	538.1	0.1694	0.1640	0.4323	-0.8386	
50	6.45×10^{-3}	-5.04	5.704	543.8	0.2595	0.2432	1.1240	0.1169	
100	1.29×10^{-2}	-4.35	5.704	547.8	0.3472	0.3133	1.7362	0.5517	
150	1.94×10^{-2}	-3.95	5.700	550.4	0.4357	0.3827	2.3424	0.8512	
200	2.58×10^{-2}	-3.66	5.703	551.3	0.4831	0.4121	2.5991	0.9552	
250	3.23×10^{-2}	-3.43	5.697	551.9	0.5428	0.4609	3.0253	1.1070	
300	3.87×10^{-2}	-3.25	5.695	552.3	0.5876	0.4965	3.3362	1.2048	
350	4.52×10^{-2}	-3.10	5.703	552.7	0.6135	0.5155	3.5022	1.2534	
400	5.16×10^{-2}	-2.96	5.7	552.5	0.6285	0.5214	3.5537	1.2680	

Appendix 12: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing caffeine concentration at pH 3.7, 4.7, and 5.7 (repetition number 2).

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.698	511.2	0.7027	0.6399			0.68
5	6.45×10^{-4}	-7.35	3.700	513.6	0.7266	0.6756	0.0558	-2.8862	
10	1.29×10^{-3}	-6.65	3.698	513.8	0.7532	0.7107	0.1106	-2.2015	
15	1.94×10^{-3}	-6.25	3.697	514.5	0.7778	0.7432	0.1614	-1.8237	
20	2.58×10^{-3}	-5.96	3.697	515.6	0.7917	0.7630	0.1924	-1.6483	
50	6.45×10^{-3}	-5.04	3.695	520.1	0.9489	0.9403	0.4694	-0.7562	
100	1.29×10^{-2}	-4.35	3.696	525.2	1.0983	1.0970	0.7143	-0.3364	
150	1.94×10^{-2}	-3.95	3.700	530.2	1.1632	1.1546	0.8043	-0.2177	
200	2.58×10^{-2}	-3.66	3.697	532.3	1.2464	1.2258	0.9156	-0.0882	
250	3.23×10^{-2}	-3.43	3.700	533.9	1.3077	1.2715	0.9870	-0.0131	
300	3.87×10^{-2}	-3.25	3.703	536.7	1.3654	1.3174	1.0588	0.0571	
350	4.52×10^{-2}	-3.10	3.697	537.2	1.3970	1.3354	1.0869	0.0833	
400	5.16×10^{-2}	-2.96	3.698	538.8	1.4599	1.3855	1.1652	0.1529	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.704	516.2	0.1692	0.1655			0.80
5	6.45×10^{-4}	-7.35	4.704	517.6	0.1838	0.1811	0.0943	-2.3617	
10	1.29×10^{-3}	-6.65	4.704	520.1	0.1949	0.1932	0.1674	-1.7875	
15	1.94×10^{-3}	-6.25	4.696	518.6	0.2077	0.2068	0.2495	-1.3881	
20	2.58×10^{-3}	-5.96	4.703	522.7	0.2164	0.2157	0.3033	-1.1930	
50	6.45×10^{-3}	-5.04	4.704	529.5	0.2868	0.2856	0.7257	-0.3206	
100	1.29×10^{-2}	-4.35	4.698	537.9	0.4003	0.3869	1.3378	0.2910	
150	1.94×10^{-2}	-3.95	4.703	541.6	0.4863	0.4573	1.7631	0.5671	
200	2.58×10^{-2}	-3.66	4.699	544.7	0.5586	0.5143	2.1076	0.7455	
250	3.23×10^{-2}	-3.43	4.696	546.1	0.6220	0.5633	2.4036	0.8770	
300	3.87×10^{-2}	-3.25	4.700	546.7	0.6658	0.5960	2.6012	0.9560	
350	4.52×10^{-2}	-3.10	4.698	547.1	0.7076	0.6270	2.7885	1.0255	
400	5.16×10^{-2}	-2.96	4.704	547.7	0.7395	0.6505	2.9305	1.0752	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.703	530.7	0.1253	0.1238			0.86
5	6.45×10^{-4}	-7.35	5.699	532.3	0.1351	0.1329	0.0735	-2.6104	
10	1.29×10^{-3}	-6.65	5.705	534.8	0.1473	0.1441	0.1640	-1.8080	
15	1.94×10^{-3}	-6.25	5.696	535.6	0.1618	0.1575	0.2722	-1.3012	
20	2.58×10^{-3}	-5.96	5.703	538.2	0.1710	0.1656	0.3376	-1.0858	
50	6.45×10^{-3}	-5.04	5.708	542.5	0.2408	0.2255	0.8215	-0.1966	
100	1.29×10^{-2}	-4.35	5.704	547.7	0.3357	0.3018	1.4378	0.3631	
150	1.94×10^{-2}	-3.95	5.705	549.5	0.4104	0.3604	1.9111	0.6477	
200	2.58×10^{-2}	-3.66	5.704	550.8	0.4774	0.4118	2.3263	0.8443	
250	3.23×10^{-2}	-3.43	5.692	550.6	0.5444	0.4635	2.7439	1.0094	
300	3.87×10^{-2}	-3.25	5.702	550.7	0.5894	0.4967	3.0121	1.1026	
350	4.52×10^{-2}	-3.10	5.698	553.7	0.6177	0.5189	3.1914	1.1605	
400	5.16×10^{-2}	-2.96	5.695	552.0	0.6515	0.5436	3.3910	1.2211	

Appendix 13: Copigmentation experimental data for cyanidin 3-malonylglucoside (1.29 x 10^{-4} M) with increasing chlorogenic acid concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.697	510.3	0.9904	0.8988			0.55
5	6.45×10^{-4}	-7.35	3.694	513.5	1.0589	0.9844	0.0952	-2.3514	
10	1.29×10^{-3}	-6.65	3.704	513.7	1.1157	1.0524	0.1709	-1.7667	
15	1.94×10^{-3}	-6.25	3.703	515.3	1.1810	1.1300	0.2572	-1.3578	
45	5.81×10^{-3}	-5.15	3.695	521.5	1.3973	1.3891	0.5455	-0.6060	
50	6.45×10^{-3}	-5.04	3.695	521.6	1.4492	1.4441	0.6067	-0.4997	
100	1.29×10^{-2}	-4.35	3.700	528.5	1.6769	1.6711	0.8593	-0.1517	
150	1.94×10^{-2}	-3.95	3.697	530.6	1.8050	1.7828	0.9835	-0.0166	
200	2.58×10^{-2}	-3.66	3.702	532.7	1.8976	1.8554	1.0643	0.0623	
250	3.23×10^{-2}	-3.43	3.695	535.3	2.0529	1.9817	1.2048	0.1863	
300	3.87×10^{-2}	-3.25	3.700	536.6	2.1069	2.0169	1.2440	0.2183	
350	4.52×10^{-2}	-3.10	3.700	537.0	2.1673	2.0549	1.2863	0.2517	
400	5.16×10^{-2}	-2.96	3.700	537.7	2.2293	2.0889	1.3241	0.2807	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.696	518.6	0.2237	0.2187			0.85
5	6.45×10^{-4}	-7.35	4.705	517.0	0.256	0.2512	0.1486	-1.9065	
10	1.29×10^{-3}	-6.65	4.704	517.3	0.2715	0.2684	0.2273	-1.4817	
15	1.94×10^{-3}	-6.25	4.708	519.7	0.2998	0.2965	0.3557	-1.0336	
20	2.58×10^{-3}	-5.96	4.695	520.2	0.3192	0.3185	0.4563	-0.7845	
50	6.45×10^{-3}	-5.04	4.704	525.9	0.4630	0.4629	1.1166	0.1103	
100	1.29×10^{-2}	-4.35	4.704	531.6	0.6558	0.6485	1.9652	0.6756	
150	1.94×10^{-2}	-3.95	4.699	533.6	0.9024	0.8834	3.0393	1.1116	
200	2.58×10^{-2}	-3.66	4.710	535.8	1.0012	0.9742	3.4545	1.2397	
250	3.23×10^{-2}	-3.43	4.704	537.0	1.1472	1.1029	4.0430	1.3970	
300	3.87×10^{-2}	-3.25	4.694	539.0	1.2473	1.1840	4.4138	1.4847	
350	4.52×10^{-2}	-3.10	4.697	540.0	1.3294	1.2531	4.7298	1.5539	
400	5.16×10^{-2}	-2.96	4.697	539.5	1.4531	1.3725	5.2757	1.6631	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.703	532.5	0.1433	0.1429			0.96
5	6.45×10^{-4}	-7.35	5.704	528.3	0.1672	0.1670	0.1687	-1.7799	
10	1.29×10^{-3}	-6.65	5.699	530.8	0.1302	0.1293	-0.0952	--	
15	1.94×10^{-3}	-6.25	5.697	531.6	0.1433	0.1442	0.0091	-4.6998	
20	2.58×10^{-3}	-5.96	5.701	532.4	0.1562	0.1543	0.0798	-2.5285	
50	6.45×10^{-3}	-5.04	5.701	537.1	0.2049	0.1987	0.3905	-0.9404	
100	1.29×10^{-2}	-4.35	5.701	540.9	0.3267	0.3094	1.1652	0.1529	
150	1.94×10^{-2}	-3.95	5.693	542.8	0.4122	0.3835	1.6837	0.5210	
200	2.58×10^{-2}	-3.66	5.694	543.7	0.5180	0.4768	2.3366	0.8487	
250	3.23×10^{-2}	-3.43	5.694	544.5	0.6275	0.5717	3.0007	1.0989	
300	3.87×10^{-2}	-3.25	5.700	545.1	0.7257	0.6595	3.6151	1.2851	
350	4.52×10^{-2}	-3.10	5.702	545.3	0.7884	0.7108	3.9741	1.3798	
400	5.16×10^{-2}	-2.96	5.703	546.2	0.8316	0.7452	4.2148	1.4386	

Appendix 14: Copigmentation experimental data for cyanidin 3-malonylglucoside (1.29×10^{-4} M) with increasing protocatechuic acid concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.697	511.5	0.8299	0.7585			1.04
5	6.45×10^{-4}	-7.35	3.700	511.3	0.8491	0.7787	0.0266	-3.6257	
10	1.29×10^{-3}	-6.65	3.702	511.8	0.8435	0.7757	0.0227	-3.7864	
15	1.94×10^{-3}	-6.25	3.697	512.0	0.8632	0.7979	0.0519	-2.9576	
20	2.58×10^{-3}	-5.96	3.706	512.4	0.8689	0.8068	0.0637	-2.7539	
50	6.45×10^{-3}	-5.04	3.702	514.1	0.9751	0.9231	0.2170	-1.5278	
100	1.29×10^{-2}	-4.35	3.703	516.4	1.1729	1.1359	0.4976	-0.6980	
150	1.94×10^{-2}	-3.95	3.699	518.8	1.3211	1.2965	0.7093	-0.3435	
200	2.58×10^{-2}	-3.66	3.700	519.5	1.4217	1.4700	0.9380	-0.0640	
250	3.23×10^{-2}	-3.43	3.706	520.8	1.6023	1.5910	1.0976	0.0931	
300	3.87×10^{-2}	-3.25	3.705	522.8	1.6938	1.6869	1.2240	0.2021	
350	4.52×10^{-2}	-3.10	3.705	522.2	1.7681	1.7645	1.3263	0.2824	
400	5.16×10^{-2}	-2.96	3.701	523.2	1.8534	1.8544	1.4448	0.3680	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.703	516.3	0.1903	0.1858	0	0	0.73
5	6.45×10^{-4}	-7.35	4.694	516.9	0.2060	0.2020	0.0872	-2.4397	
10	1.29×10^{-3}	-6.65	4.702	516.7	0.2178	0.2125	0.1437	-1.9400	
15	1.94×10^{-3}	-6.25	4.694	517.3	0.2052	0.2008	0.0807	-2.5166	
20	2.58×10^{-3}	-5.96	4.694	518.8	0.2103	0.2052	0.1044	-2.2594	
50	6.45×10^{-3}	-5.04	4.701	517.8	0.2345	0.2307	0.2417	-1.4202	
100	1.29×10^{-2}	-4.35	4.701	517.7	0.2664	0.2633	0.4171	-0.8744	
150	1.94×10^{-2}	-3.95	4.702	517.6	0.3167	0.3135	0.6873	-0.3750	
200	2.58×10^{-2}	-3.66	4.698	521.3	0.3578	0.3555	0.9133	-0.0906	
250	3.23×10^{-2}	-3.43	4.696	522.1	0.3840	0.3824	1.0581	0.0565	
300	3.87×10^{-2}	-3.25	4.699	522.8	0.4056	0.4050	1.1798	0.1653	
350	4.52×10^{-2}	-3.10	4.708	524.2	0.4427	0.4426	1.3821	0.3236	
400	5.16×10^{-2}	-2.96	4.697	524.2	0.4945	0.4944	1.6609	0.5074	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.697	532.4	0.1357	0.1345			1.05
5	6.45×10^{-4}	-7.35	5.701	531.7	0.1367	0.1356	0.0082	-4.8063	
10	1.29×10^{-3}	-6.65	5.702	530.7	0.1358	0.1350	0.0037	-5.5947	
15	1.94×10^{-3}	-6.25	5.703	530.5	0.1378	0.1373	0.0208	-3.8719	
20	2.58×10^{-3}	-5.96	5.703	528.9	0.1589	0.1586	0.1792	-1.7194	
50	6.45×10^{-3}	-5.04	5.706	524.8	0.1615	0.1611	0.1978	-1.6207	
100	1.29×10^{-2}	-4.35	5.700	529.2	0.1628	0.1626	0.2089	-1.5658	
150	1.94×10^{-2}	-3.95	5.708	527.1	0.1706	0.1704	0.2669	-1.3208	
200	2.58×10^{-2}	-3.66	5.693	527.7	0.1860	0.1855	0.3792	-0.9697	
250	3.23×10^{-2}	-3.43	5.697	527.1	0.1881	0.1878	0.3963	-0.9256	
300	3.87×10^{-2}	-3.25	5.703	527.0	0.1981	0.1980	0.4721	-0.7505	
350	4.52×10^{-2}	-3.10	5.707	526.7	0.2204	0.2207	0.6409	-0.4449	
400	5.16×10^{-2}	-2.96	5.696	526.4	0.2350	0.2349	0.7465	-0.2924	

Appendix 15: Copigmentation experimental data for cyanidin 3-malonylglucoside (1.29×10^{-4} M) with increasing caffeine concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.701	511.5	0.8338	0.7624			0.68
5	6.45×10^{-4}	-7.35	3.700	512.6	0.8713	0.8084	0.0603	-2.8078	
10	1.29×10^{-3}	-6.65	3.705	513.1	0.8625	0.8108	0.0635	-2.7570	
15	1.94×10^{-3}	-6.25	3.704	513.7	0.8922	0.8461	0.1098	-2.2092	
20	2.58×10^{-3}	-5.96	3.693	514.7	0.9142	0.8743	0.1468	-1.9189	
50	6.45×10^{-3}	-5.04	3.698	520.5	1.0351	1.0249	0.3443	-1.0662	
100	1.29×10^{-2}	-4.35	3.704	525.6	1.1787	1.1786	0.5459	-0.6053	
150	1.94×10^{-2}	-3.95	3.697	528.6	1.2980	1.2909	0.6932	-0.3664	
200	2.58×10^{-2}	-3.66	3.705	531.7	1.3807	1.3617	0.7861	-0.2407	
250	3.23×10^{-2}	-3.43	3.695	533.9	1.3883	1.3497	0.7703	-0.2609	
300	3.87×10^{-2}	-3.25	3.700	535.7	1.4287	1.3796	0.8095	-0.2113	
350	4.52×10^{-2}	-3.10	3.687	536.5	1.4980	1.4381	0.8863	-0.1207	
400	5.16×10^{-2}	-2.96	3.695	536.3	1.5251	1.4506	0.9027	-0.1024	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.697	518.6	0.1857	0.1812			0.82
5	6.45×10^{-4}	-7.35	4.705	519.1	0.2024	0.1991	0.0988	-2.3148	
10	1.29×10^{-3}	-6.65	4.706	519.2	0.2110	0.2089	0.1529	-1.8782	
15	1.94×10^{-3}	-6.25	4.707	522.1	0.2240	0.2228	0.2296	-1.4715	
20	2.58×10^{-3}	-5.96	4.698	520.8	0.2227	0.2205	0.2169	-1.5284	
50	6.45×10^{-3}	-5.04	4.704	529.8	0.3318	0.3291	0.8162	-0.2031	
100	1.29×10^{-2}	-4.35	4.704	537.2	0.4281	0.4146	1.2881	0.2532	
150	1.94×10^{-2}	-3.95	4.705	541.2	0.5207	0.4888	1.6976	0.5292	
200	2.58×10^{-2}	-3.66	4.705	543.2	0.6012	0.5616	2.0993	0.7416	
250	3.23×10^{-2}	-3.43	4.707	544.7	0.6621	0.6102	2.3676	0.8619	
300	3.87×10^{-2}	-3.25	4.704	545.8	0.6976	0.6351	2.5050	0.9183	
350	4.52×10^{-2}	-3.10	4.708	546.7	0.7294	0.6574	2.6280	0.9662	
400	5.16×10^{-2}	-2.96	4.703	547.4	0.7732	0.6922	2.8201	1.0368	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.698	532.8	0.1394	0.1373			0.60
5	6.45×10^{-4}	-7.35	5.704	527.6	0.1724	0.1699	0.2374	-1.4379	
10	1.29×10^{-3}	-6.65	5.702	532.9	0.1971	0.1934	0.4086	-0.8950	
15	1.94×10^{-3}	-6.25	5.704	534.9	0.1932	0.1869	0.3613	-1.0182	
20	2.58×10^{-3}	-5.96	5.702	536.0	0.2076	0.2002	0.4581	-0.7806	
50	6.45×10^{-3}	-5.04	5.704	542.4	0.2953	0.2778	1.0233	0.0230	
100	1.29×10^{-2}	-4.35	5.697	547.6	0.3863	0.3500	1.5492	0.4377	
150	1.94×10^{-2}	-3.95	5.705	550.3	0.4477	0.3982	1.9002	0.6420	
200	2.58×10^{-2}	-3.66	5.700	551.9	0.5132	0.4482	2.2644	0.8173	
250	3.23×10^{-2}	-3.43	5.704	552.3	0.5527	0.4725	2.4414	0.8926	
300	3.87×10^{-2}	-3.25	5.707	552.8	0.5880	0.4995	2.6380	0.9700	
350	4.52×10^{-2}	-3.10	5.695	552.0	0.6193	0.5245	2.8201	1.0368	
400	5.16×10^{-2}	-2.96	5.700	552.1	0.6447	0.5439	2.9614	1.0857	

Appendix 16: Copigmentation experimental data for cyanidin 3- malonyllaminaribioside (1.29×10^{-4} M) with increasing chlorogenic acid concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.704	510.7	0.7463	0.6789			0.66
5	6.45×10^{-4}	-7.35	3.706	513.0	0.7725	0.7180	0.0576	-2.8544	
10	1.29×10^{-3}	-6.65	3.700	514.7	0.8661	0.8262	0.2170	-1.5280	
15	1.94×10^{-3}	-6.25	3.705	515.2	0.8953	0.8588	0.2650	-1.3281	
20	2.58×10^{-3}	-5.96	3.693	516.3	0.9437	0.9147	0.3473	-1.0575	
50	6.45×10^{-3}	-5.04	3.703	522.4	1.1778	1.1740	0.7293	-0.3157	
100	1.29×10^{-2}	-4.35	3.703	528.3	1.4171	1.4114	1.0790	0.0760	
150	1.94×10^{-2}	-3.95	3.698	532.2	1.5484	1.5259	1.2476	0.2212	
200	2.58×10^{-2}	-3.66	3.698	534.0	1.6267	1.5857	1.3357	0.2894	
250	3.23×10^{-2}	-3.43	3.693	535.5	1.7031	1.6455	1.4238	0.3533	
300	3.87×10^{-2}	-3.25	3.697	536.4	1.7374	1.6675	1.4562	0.3758	
350	4.52×10^{-2}	-3.10	3.696	537.1	1.7683	1.6876	1.4858	0.3959	
400	5.16×10^{-2}	-2.96	3.700	538.1	1.7827	1.6935	1.4945	0.4018	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.706	508.7	0.1940	0.1815			0.92
5	6.45×10^{-4}	-7.35	4.702	512.1	0.2126	0.2026	0.1163	-2.1520	
10	1.29×10^{-3}	-6.65	4.698	514.3	0.2276	0.2191	0.2072	-1.5743	
15	1.94×10^{-3}	-6.25	4.697	514.7	0.2423	0.2353	0.2964	-1.2160	
20	2.58×10^{-3}	-5.96	4.700	516.7	0.2580	0.2526	0.3917	-0.9372	
50	6.45×10^{-3}	-5.04	4.692	523.4	0.3624	0.3618	0.9934	-0.0066	
100	1.29×10^{-2}	-4.35	4.695	530.4	0.5712	0.5662	2.1196	0.7512	
150	1.94×10^{-2}	-3.95	4.700	534.8	0.7294	0.7130	2.9284	1.0744	
200	2.58×10^{-2}	-3.66	4.704	536.4	0.8918	0.8628	3.7537	1.3227	
250	3.23×10^{-2}	-3.43	4.693	536.6	1.0080	0.9659	4.3218	1.4637	
300	3.87×10^{-2}	-3.25	4.697	538.3	1.1047	1.0505	4.7879	1.5661	
350	4.52×10^{-2}	-3.10	4.696	538.6	1.1958	1.1313	5.2331	1.6550	
400	5.16×10^{-2}	-2.96	4.697	538.8	1.2632	1.1898	5.5554	1.7148	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.700	499.8	0.1634	0.1419			1.05
5	6.45×10^{-4}	-7.35	5.705	499.5	0.1691	0.1492	0.0514	-2.9672	
10	1.29×10^{-3}	-6.65	5.704	502.4	0.1730	0.1552	0.0937	-2.3674	
15	1.94×10^{-3}	-6.25	5.705	501.0	0.1790	0.1627	0.1466	-1.9202	
20	2.58×10^{-3}	-5.96	5.702	502.0	0.1885	0.1700	0.1980	-1.6194	
50	6.45×10^{-3}	-5.04	5.700	511.3	0.2261	0.2229	0.5708	-0.5607	
100	1.29×10^{-2}	-4.35	5.703	535.2	0.3178	0.3133	1.2079	0.1889	
150	1.94×10^{-2}	-3.95	5.697	539.7	0.4301	0.4138	1.9161	0.6503	
200	2.58×10^{-2}	-3.66	5.703	542.0	0.5226	0.4955	2.4919	0.9130	
250	3.23×10^{-2}	-3.43	5.710	543.5	0.6090	0.5689	3.0092	1.1017	
300	3.87×10^{-2}	-3.25	5.700	544.5	0.6836	0.6318	3.4524	1.2391	
350	4.52×10^{-2}	-3.10	5.705	544.6	0.7633	0.7011	3.9408	1.3714	
400	5.16×10^{-2}	-2.96	5.700	545.1	0.8127	0.7421	4.2297	1.4421	

Appendix 17: Copigmentation experimental data for cyanidin 3- malonyllaminaribioside (1.29×10^{-4} M) with increasing protocatechuic acid concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.699	511.0	0.7311	0.6647			0.99
5	6.45×10^{-4}	-7.35	3.703	511.3	0.7380	0.6730	0.0125	-4.3831	
10	1.29×10^{-3}	-6.65	3.701	510.6	0.7630	0.6973	0.0490	-3.0150	
15	1.94×10^{-3}	-6.25	3.702	511.2	0.7762	0.7117	0.0707	-2.6492	
20	2.58×10^{-3}	-5.96	3.698	511.9	0.7801	0.7182	0.0805	-2.5197	
50	6.45×10^{-3}	-5.04	3.696	513.3	0.8556	0.8042	0.2099	-1.5613	
100	1.29×10^{-2}	-4.35	3.707	516.0	0.9846	0.9470	0.4247	-0.8564	
150	1.94×10^{-2}	-3.95	3.699	518.0	1.0843	1.0584	0.5923	-0.5237	
200	2.58×10^{-2}	-3.66	3.702	519.1	1.2157	1.1976	0.8017	-0.2210	
250	3.23×10^{-2}	-3.43	3.695	520.1	1.3115	1.2983	0.9532	-0.0479	
300	3.87×10^{-2}	-3.25	3.702	520.9	1.3963	1.3882	1.0885	0.0848	
350	4.52×10^{-2}	-3.10	3.704	522.7	1.4762	1.4695	1.2108	0.1913	
400	5.16×10^{-2}	-2.96	3.696	523.0	1.5442	1.5401	1.3170	0.2753	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.705	510.9	0.1896	0.1785			1.31
5	6.45×10^{-4}	-7.35	4.705	508.6	0.1920	0.1799	0.0078	-4.8481	
10	1.29×10^{-3}	-6.65	4.697	509.8	0.1932	0.1808	0.0129	-4.3517	
15	1.94×10^{-3}	-6.25	4.704	510.0	0.1932	0.1807	0.0123	-4.3961	
20	2.58×10^{-3}	-5.96	4.704	510.6	0.1963	0.1845	0.0336	-3.3928	
50	6.45×10^{-3}	-5.04	4.698	511.8	0.2137	0.2027	0.1356	-1.9982	
100	1.29×10^{-2}	-4.35	4.704	513.0	0.2423	0.2335	0.3081	-1.1773	
150	1.94×10^{-2}	-3.95	4.699	515.0	0.2901	0.2825	0.5826	-0.5402	
200	2.58×10^{-2}	-3.66	4.693	516.7	0.3109	0.3055	0.7115	-0.3404	
250	3.23×10^{-2}	-3.43	4.704	515.9	0.3452	0.3391	0.8997	-0.1057	
300	3.87×10^{-2}	-3.25	4.703	517.8	0.3836	0.3798	1.1277	0.1202	
350	4.52×10^{-2}	-3.10	4.696	519.3	0.4277	0.4244	1.3776	0.3203	
400	5.16×10^{-2}	-2.96	4.694	519.9	0.4669	0.4636	1.5972	0.4683	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.696	498.9	0.1446	0.124			1.05
5	6.45×10^{-4}	-7.35	5.705	497.6	0.1452	0.1237	-0.0024	--	
10	1.29×10^{-3}	-6.65	5.695	498.7	0.1456	0.1246	0.0048	-5.3311	
15	1.94×10^{-3}	-6.25	5.693	497.1	0.1491	0.1267	0.0218	-3.8270	
20	2.58×10^{-3}	-5.96	5.684	498.1	0.1507	0.1280	0.0323	-3.4340	
50	6.45×10^{-3}	-5.04	5.696	503.5	0.1565	0.1330	0.0726	-2.6231	
100	1.29×10^{-2}	-4.35	5.709	499.1	0.1642	0.1406	0.1339	-2.0109	
150	1.94×10^{-2}	-3.95	5.704	405.3	0.2015	0.1508	0.2161	-1.5319	
200	2.58×10^{-2}	-3.66	5.704	400.4	0.2342	0.1613	0.3008	-1.2013	
250	3.23×10^{-2}	-3.43	5.693	405.8	0.2447	0.1751	0.4121	-0.8865	
300	3.87×10^{-2}	-3.25	5.698	404.6	0.2721	0.1894	0.5274	-0.6398	
350	4.52×10^{-2}	-3.10	5.704	402.3	0.2986	0.2001	0.6137	-0.4882	
400	5.16×10^{-2}	-2.96	5.706	406.3	0.3157	0.2121	0.7105	-0.3418	

Appendix 18: Copigmentation experimental data for cyanidin 3- malonyllaminaribioside (1.29 x 10^{-4} M) with increasing caffeine concentration at pH 3.7, 4.7, and 5.7.

pH 3.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	3.702	511.3	0.7440	0.6805			0.80
5	6.45×10^{-4}	-7.35	3.703	513.9	0.7548	0.6942	0.0201	-3.9054	
10	1.29×10^{-3}	-6.65	3.704	513.9	0.7747	0.7230	0.0625	-2.7733	
15	1.94×10^{-3}	-6.25	3.705	512.9	0.7760	0.7276	0.0692	-2.6706	
20	2.58×10^{-3}	-5.96	3.695	514.2	0.7865	0.7474	0.0983	-2.3196	
50	6.45×10^{-3}	-5.04	3.703	519.2	0.8786	0.8662	0.2729	-1.2987	
100	1.29×10^{-2}	-4.35	3.700	524.4	0.9887	0.9879	0.4517	-0.7947	
150	1.94×10^{-2}	-3.95	3.696	528.2	1.0595	1.0559	0.5517	-0.5948	
200	2.58×10^{-2}	-3.66	3.707	531.0	1.1262	1.1141	0.6372	-0.4507	
250	3.23×10^{-2}	-3.43	3.710	533.3	1.1735	1.1498	0.6896	-0.3716	
300	3.87×10^{-2}	-3.25	3.699	533.1	1.2036	1.1672	0.7152	-0.3352	
350	4.52×10^{-2}	-3.10	3.700	536.3	1.2225	1.1812	0.7358	-0.3068	
400	5.16×10^{-2}	-2.96	3.715	537.0	1.2417	1.1932	0.7534	-0.2831	

pH 4.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	4.704	509.9	0.1894	0.1782			0.83
5	6.45×10^{-4}	-7.35	4.699	512.9	0.1987	0.1898	0.0651	-2.7319	
10	1.29×10^{-3}	-6.65	4.695	512.6	0.2079	0.2005	0.1251	-2.0783	
15	1.94×10^{-3}	-6.25	4.704	513.4	0.2135	0.2075	0.1644	-1.8053	
20	2.58×10^{-3}	-5.96	4.700	517.3	0.2208	0.2159	0.2116	-1.5532	
50	6.45×10^{-3}	-5.04	4.692	525.1	0.2775	0.2766	0.5522	-0.5939	
100	1.29×10^{-2}	-4.35	4.695	534.4	0.3688	0.3643	1.0443	0.0434	
150	1.94×10^{-2}	-3.95	4.697	537.9	0.4378	0.4239	1.3788	0.3212	
200	2.58×10^{-2}	-3.66	4.693	541.1	0.4915	0.4678	1.6251	0.4856	
250	3.23×10^{-2}	-3.43	4.697	544.5	0.5325	0.4999	1.8053	0.5907	
300	3.87×10^{-2}	-3.25	4.700	545.0	0.5669	0.5254	1.9484	0.6670	
350	4.52×10^{-2}	-3.10	4.703	546.4	0.5958	0.5469	2.0690	0.7271	
400	5.16×10^{-2}	-2.96	4.712	547.1	0.6201	0.5649	2.1700	0.7747	

pH 5.7

P:CP	CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
					λ_{max}	525 nm			
0	0	0	5.702	498.5	0.1852	0.1513			0.86
5	6.45×10^{-4}	-7.35	5.694	501.7	0.1734	0.1596	0.0549	-2.9030	
10	1.29×10^{-3}	-6.65	5.696	501.0	0.1771	0.1654	0.0932	-2.3731	
15	1.94×10^{-3}	-6.25	5.694	503.2	0.1849	0.1754	0.1593	-1.8371	
20	2.58×10^{-3}	-5.96	5.697	506.0	0.1928	0.1860	0.2293	-1.4725	
50	6.45×10^{-3}	-5.04	5.706	527.5	0.2358	0.2348	0.5519	-0.5944	
100	1.29×10^{-2}	-4.35	5.698	545.3	0.3064	0.2933	0.9385	-0.0634	
150	1.94×10^{-2}	-3.95	5.696	549.1	0.3673	0.3412	1.2551	0.2272	
200	2.58×10^{-2}	-3.66	5.707	550.1	0.4194	0.3826	1.5288	0.4245	
250	3.23×10^{-2}	-3.43	5.704	550.8	0.4605	0.4143	1.7383	0.5529	
300	3.87×10^{-2}	-3.25	5.699	550.7	0.4974	0.4433	1.9299	0.6575	
350	4.52×10^{-2}	-3.10	5.704	551.1	0.5239	0.4636	2.0641	0.7247	
400	5.16×10^{-2}	-2.96	5.699	552.1	0.5518	0.4850	2.2056	0.7910	

Appendix 19: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing concentrations of chlorogenic acid and caffeine at pH 4.7.

P:CP:CP	CP (chlorogenic acid, M)	CP (caffeine, M)	Total CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
							λ_{max}	525 nm			
1:0:0	0	0	0		4.700	513.8	0.1717	0.1654	0	0	0.83
1:5:0	6.45×10^{-4}	0	6.45×10^{-4}	-7.35	4.702	513.8	0.1889	0.1846	0.1161	-2.1535	
1:5:5	6.45×10^{-4}	6.45×10^{-4}	1.29×10^{-3}	-6.65	4.700	514.0	0.2229	0.2207	0.3343	-1.0956	
1:10:5	1.29×10^{-3}	6.45×10^{-4}	1.94×10^{-3}	-6.25	4.705	520.2	0.2464	0.2424	0.4655	-0.7646	
1:10:10	1.29×10^{-3}	1.29×10^{-3}	2.58×10^{-3}	-5.96	4.700	522.0	0.2526	0.2516	0.5212	-0.6517	
1:25:10	3.23×10^{-3}	1.29×10^{-3}	4.52×10^{-3}	-5.40	4.698	524.4	0.3075	0.3075	0.8591	-0.1518	
1:25:25	3.23×10^{-3}	3.23×10^{-3}	6.45×10^{-3}	-5.04	4.697	527.0	0.3431	0.3427	1.0719	0.0695	
1:50:25	6.45×10^{-3}	3.23×10^{-3}	9.68×10^{-3}	-4.64	4.697	529.1	0.4455	0.4434	1.6808	0.5193	
1:50:50	6.45×10^{-3}	6.45×10^{-3}	1.29×10^{-2}	-4.35	4.697	533.9	0.4887	0.4714	1.8501	0.6152	
1:100:50	1.29×10^{-2}	6.45×10^{-3}	1.94×10^{-2}	-3.95	4.692	533.1	0.6446	0.6256	2.7823	1.0233	
1:100:100	1.29×10^{-2}	1.29×10^{-2}	2.58×10^{-2}	-3.66	4.698	538.7	0.6716	0.6400	2.8694	1.0541	

Appendix 20: Copigmentation experimental data for cyanidin 3-glucoside (1.29×10^{-4} M) with increasing concentrations of chlorogenic acid, caffeine, and protocatechuic acid at pH 4.7.

P:CP:CP:CP	CP (chlorogenic acid, M)	CP (caffeine, M)	CP (protocatechuic acid, M)	Total CP (M)	ln(CP)	pH	λ_{max}	Absorbance		$(A-A_0)/A_0$	$\ln((A-A_0)/A_0)$	n
								λ_{max}	525 nm			
1:0:0:0	0	0	0	0	0	4.696	516.5	0.1766	0.0172			0.66
1:10:0:0	1.29×10^{-3}	0	0	1.29×10^{-3}	-6.65	4.696	518.0	0.2455	0.2417	0.3902	-0.9412	
1:10:10:0	1.29×10^{-3}	1.29×10^{-3}	0	2.58×10^{-3}	-5.96	4.699	521.5	0.2715	0.2708	0.5374	-0.6211	
1:10:10:10	1.29×10^{-3}	1.29×10^{-3}	1.29×10^{-3}	3.87×10^{-3}	-5.55	4.697	522.2	0.2906	0.2897	0.6455	-0.4377	
1:25:10:10	3.23×10^{-3}	1.29×10^{-3}	1.29×10^{-3}	5.81×10^{-3}	-5.15	4.701	521.4	0.3562	0.3559	1.0170	0.0168	
1:25:25:10	3.23×10^{-3}	3.23×10^{-3}	1.29×10^{-3}	7.74×10^{-3}	-4.86	4.707	526.8	0.3915	0.3911	1.2169	0.1963	
1:25:25:25	3.23×10^{-3}	3.23×10^{-3}	3.23×10^{-3}	9.68×10^{-3}	-4.64	4.708	528.5	0.4066	0.4061	1.3024	0.2642	
1:50:25:25	6.45×10^{-3}	3.23×10^{-3}	3.23×10^{-3}	1.29×10^{-2}	-4.35	4.700	529.4	0.4799	0.4775	1.7174	0.5408	
1:50:50:25	6.45×10^{-3}	6.45×10^{-3}	3.23×10^{-3}	1.61×10^{-2}	-4.13	4.699	533.1	0.5374	0.5300	2.0430	0.7144	
1:50:50:50	6.45×10^{-3}	6.45×10^{-3}	6.45×10^{-3}	1.94×10^{-2}	-3.95	4.694	532.7	0.5490	0.5414	2.1087	0.7461	
1:100:50:50	1.29×10^{-2}	6.45×10^{-3}	6.45×10^{-3}	2.58×10^{-2}	-3.66	4.697	534.8	0.7060	0.6888	2.9977	1.0979	
1:100:100:50	1.29×10^{-2}	1.29×10^{-2}	6.45×10^{-3}	3.23×10^{-2}	-3.43	4.693	534.0	0.7721	0.7330	3.3720	1.2155	
1:100:100:100	1.29×10^{-2}	1.29×10^{-2}	1.29×10^{-2}	3.87×10^{-2}	-3.25	4.693	538.1	0.7449	0.7138	3.2180	1.1688	
1:200:100:100	2.58×10^{-2}	1.29×10^{-2}	1.29×10^{-2}	5.16×10^{-2}	-2.96	4.700	539.5	0.9048	0.8496	4.1234	1.4167	
1:200:200:100	2.58×10^{-2}	2.58×10^{-2}	1.29×10^{-2}	6.45×10^{-2}	-2.74	4.700	541.8	0.9752	0.9072	4.5221	1.5090	
1:200:200:200	2.58×10^{-2}	2.58×10^{-2}	2.58×10^{-2}	7.74×10^{-2}	-2.56	4.680	540.2	1.0753	1.0116	5.0889	1.6271	

Appendix 21: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) control solutions at pH 3.7, 4.7 and 5.7 over 35 days when exposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	77.1	37.6	12.2	18.0
	5	86.3	25.5	6.5	14.3
	10	90.4	14.0	5.5	21.4
	15	94.2	5.9	6.2	46.4
	20	93.5	2.1	7.3	74.0
	24	95.9	1.0	7.4	82.3
	28	96.0	0.4	7.1	86.8
	35	93.1	0.4	6.9	86.7
4.7	0	86.7	13.7	1.2	5.0
	5	92.4	8.2	4.2	27.1
	10	92.8	4.0	7.8	62.9
	15	93.8	1.4	10.3	82.3
	20	93.4	0.8	11.5	86.0
	24	95.1	0.3	10.8	88.4
	28	95.0	0.2	10.2	88.9
	35	93.5	0.4	9.9	87.7
5.7	0	85.8	10.6	-4.0	-20.7
	5	91.2	4.5	8.1	60.9
	10	91.9	1.5	13.1	83.5
	15	93.4	0.6	13.5	87.5
	20	90.7	-0.1	12.1	-89.5
	24	94.2	0.3	11.8	88.5
	28	95.0	0.1	10.9	89.5
	35	93.1	-0.1	9.4	-89.4

Appendix 22: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with chlorogenic acid (P:CP = 1:200; 2.58×10^{-2} M) at pH 3.7, 4.7 and 5.7 over 35 days when exposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	54.6	69.1	12.6	10.3
	5	60.5	62.7	6.2	5.6
	10	66.4	47.9	9.1	10.8
	15	74.5	26.4	22.7	40.7
	20	75.9	15.1	37.6	68.1
	24	75.6	12.9	47.9	74.9
	28	72.7	13.9	56.3	76.1
	35	65.5	18.6	62.5	73.4
4.7	0	62.0	51.5	-6.7	-7.4
	5	85.2	9.5	13.4	54.7
	10	84.7	3.0	32.2	84.7
	15	80.2	6.0	49.5	83.1
	20	69.7	13.5	62.5	77.8
	24	65.9	18.1	70.6	75.6
	28	60.7	22.5	74.2	73.1
	35	53.7	27.9	76.5	70.0
5.7	0	70.2	24.3	-4.8	-11.2
	5	90.2	-2.4	26.8	-84.9
	10	83.2	1.0	38.4	88.5
	15	72.9	10.5	64.2	80.7
	20	61.3	21.4	66.8	72.2
	24	54.0	27.1	78.7	71.0
	28	49.4	26.5	127.0	78.2
	35	43.5	25.0	127.0	78.9

Appendix 23: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with protocatechuic acid (P:CP = 1:200; 2.58×10^{-2} M)at pH 3.7, 4.7 and 5.7 over 35 days when exposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	6.4	59.4	19.2	17.9
	5	88.3	4.3	22.7	79.3
	10	85.3	3.4	31.7	83.9
	15	78.9	4.6	36.2	82.8
	20	69.5	6.8	40.3	80.4
	24	68.7	8.6	44.7	79.1
	28	59.8	9.6	44.0	77.7
	35	51.1	11.3	44.5	75.8
4.7	0	83.4	22.9	4.0	9.9
	5	83.4	5.1	29.9	80.3
	10	79.4	6.3	34.8	79.7
	15	73.7	6.8	38.3	79.9
	20	70.3	8.2	43.3	79.3
	24	65.9	9.1	45.2	78.6
	28	60.2	9.7	46.5	78.2
	35	41.2	11.1	39.8	74.4
5.7	0	86.5	10.4	2.7	14.6
	5	77.4	8.2	33.7	76.3
	10	43.3	6.4	22.3	74.0
	15	46.7	9.9	31.3	72.4
	20	44.5	7.1	17.1	67.5
	24	58.7	0.5	2.2	77.2
	28	54.1	1.3	4.5	73.9
	35	52.4	0.8	1.5	61.9

Appendix 24: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with caffeine (P:CP = 1:200; 2.58×10^{-2} M)at pH 3.7, 4.7 and 5.7 over 35 days when exposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	57.5	62.5	-9.8	-8.9
	5	75.1	34.1	-3.7	-6.2
	10	88.2	13.0	5.1	21.4
	15	93.7	3.3	10.0	71.7
	20	96.0	0.5	10.4	87.2
	24	96.4	0.1	9.8	89.4
	28	96.2	-0.1	8.8	-89.3
	35	95.3	0.1	8.0	89.3
4.7	0	68.6	32.7	-15.8	-25.8
	5	83.2	14.7	1.5	5.8
	10	90.3	5.0	10.8	65.2
	15	92.9	1.8	13.8	82.6
	20	94.6	0.6	13.1	87.4
	24	95.3	0.3	12.0	88.6
	28	95.5	0.1	10.9	89.5
	35	94.8	0.1	9.6	89.4
5.7	0	73.5	19.3	-13.3	-34.6
	5	85.7	6.7	11.3	59.3
	10	89.4	2.0	16.8	83.2
	15	93.0	0.8	15.8	87.1
	20	94.8	0.1	13.0	89.6
	24	95.5	-0.1	11.6	89.5
	28	96.0	-0.3	10.2	-88.3
	35	95.2	-0.1	8.1	-89.3

Appendix 25: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) control solutions at pH 3.7, 4.7 and 5.7 over 35 days when unexposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	80.1	38.7	11.8	17.0
	5	80.8	38.2	11.2	16.3
	10	80.3	37.2	11.2	16.8
	15	81.3	36.1	10.7	16.5
	20	81.3	34.8	10.5	16.8
	24	82.0	33.9	10.1	16.6
	28	82.0	32.6	10.3	17.5
	35	82.0	31.0	10.0	17.9
4.7	0	91.5	13.0	0.7	3.1
	5	91.5	12.7	1.6	7.2
	10	89.8	12.3	2.3	10.6
	15	90.8	11.4	2.7	13.3
	20	89.8	11.0	3.3	16.7
	24	89.7	10.6	3.9	20.2
	28	89.1	10.2	4.8	25.2
	35	88.8	9.6	5.4	29.4
5.7	0	91.7	7.8	0.2	1.5
	5	92.2	7.2	1.2	9.5
	10	90.8	6.9	2.7	21.4
	15	89.8	6.5	4.2	32.9
	20	88.7	6.3	5.3	40.1
	24	88.0	6.2	6.2	45.0
	28	87.1	6.0	7.2	50.2
	35	85.8	5.5	8.2	56.1

Appendix 26: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with chlorogenic acid (P:CP = 1:200; 2.58×10^{-2} M) at pH 3.7, 4.7 and 5.7 over 35 days when unexposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	55.9	69.6	10.3	8.4
	5	54.2	68.9	11.3	9.3
	10	55.3	69.1	10.9	9.0
	15	56.4	68.5	9.4	7.8
	20	56.7	67.3	8.8	7.4
	24	57.1	66.1	8.7	7.5
	28	57.6	64.7	8.5	7.5
	35	57.6	62.3	8.9	8.1
4.7	0	63.5	49.4	-6.8	-7.8
	5	66.5	43.3	-5.7	-7.5
	10	70.7	33.9	-1.3	-2.2
	15	77.7	21.7	7.6	19.3
	20	81.2	13.6	16.7	50.8
	24	82.6	9.9	22.6	66.3
	28	83.1	7.7	27.9	74.6
	35	81.8	6.3	35.6	80.0
5.7	0	72.3	22.8	-3.9	-9.7
	5	77.9	14.3	1.0	4.0
	10	85.4	4.8	8.7	61.1
	15	91.0	-2.0	20.4	-84.4
	20	91.1	-3.3	28.9	-83.5
	24	89.8	-3.3	34.0	-84.5
	28	88.1	-2.8	38.8	-85.9
	35	84.8	-0.6	47.0	-89.3

Appendix 27: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with protocatechuic acid (P:CP = 1:200; 2.58×10^{-2} M)at pH 3.7, 4.7 and 5.7 over 35 days when unexposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	67.2	58.0	17.9	17.2
	5	67.8	57.6	16.3	15.8
	10	67.4	55.8	15.4	15.4
	15	68.7	54.6	13.3	13.7
	20	69.2	52.6	11.7	12.5
	24	70.0	50.8	11.0	12.2
	28	70.0	48.5	10.4	12.1
	35	71.0	45.8	9.6	11.8
4.7	0	84.4	21.7	4.1	10.7
	5	83.9	20.3	5.3	14.6
	10	82.5	17.9	7.6	23.0
	15	82.3	14.3	11.7	39.3
	20	82.8	11.2	15.5	54.1
	24	83.4	9.4	18.3	62.8
	28	83.5	8.2	20.2	67.9
	35	83.2	7.2	23.4	72.9
5.7	0	89.1	8.9	4.5	26.8
	5	86.4	7.9	6.1	37.7
	10	86.6	5.3	9.9	61.8
	15	87.9	4.0	14.4	74.5
	20	87.6	4.1	16.5	76.0
	24	87.2	4.4	17.5	75.9
	28	85.9	4.7	19.0	76.1
	35	84.7	4.9	20.3	76.4

Appendix 28: CIE L*a*b* values for cyanidin 3-glucoside (1.29×10^{-4} M) solutions with caffeine (P:CP = 1:200; 2.58×10^{-2} M)at pH 3.7, 4.7 and 5.7 over 35 days when unexposed to light.

pH	Day	L*	a*	b*	Hue angle $\theta = \tan^{-1}(b^*/a^*)$
3.7	0	59.7	60.2	-10.5	-9.9
	5	60.5	59.4	-10.5	-10.0
	10	60.5	58.2	-9.9	-9.7
	15	61.8	57.1	-10.5	-10.4
	20	63.0	55.4	-10.0	-10.2
	24	63.6	54.0	-9.6	-10.1
	28	63.7	53.0	-9.1	-9.7
	35	64.6	50.8	-8.4	-9.4
4.7	0	69.2	31.9	-15.7	-26.2
	5	71.4	29.7	-14.2	-25.6
	10	72.3	27.8	-12.7	-24.6
	15	74.6	25.1	-10.3	-22.3
	20	76.0	22.5	-7.3	-18.0
	24	76.9	20.8	-5.3	-14.3
	28	77.7	18.9	-3.0	-9.0
	35	77.5	16.6	0.6	2.1
5.7	0	75.6	18.3	-12.1	-33.5
	5	74.2	17.6	-10.6	-31.1
	10	76.1	14.7	-7.7	-27.6
	15	79.6	10.3	-2.4	-13.1
	20	81.5	7.2	2.4	18.4
	24	83.1	5.6	5.1	42.3
	28	83.4	4.7	8.5	61.1
	35	84.8	3.6	11.5	72.6

Appendix 29: Relative absorbance at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days and exposed to light.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	1.00	0.51	0.30	0.15	0.10	0.08	0.04	0.10
	4.7	1.00	0.66	0.54	0.42	0.39	0.34	0.33	0.30
	5.7	1.00	0.68	0.58	0.49	0.48	0.38	0.37	0.33
Chlorogenic acid	3.7	1.00	0.67	0.44	0.26	0.20	0.20	0.24	0.31
	4.7	1.00	0.23	0.21	0.33	0.52	0.65	0.79	1.01
	5.7	1.00	0.26	0.48	0.89	1.48	1.83	2.23	2.74
Protocatechuic acid	3.7	1.00	0.13	0.15	0.22	0.33	0.35	0.47	0.56
	4.7	1.00	0.69	0.87	1.13	1.35	1.50	1.79	2.91
	5.7	1.00	1.78	3.35	4.58	4.97	2.93	3.46	2.74
Caffeine	3.7	1.00	0.39	0.16	0.07	0.04	0.04	0.04	0.05
	4.7	1.00	0.47	0.25	0.17	0.14	0.12	0.11	0.09
	5.7	1.00	0.53	0.32	0.27	0.19	0.16	0.15	0.13

Appendix 30: Relative absorbance at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days in the dark.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	1.00	0.96	0.94	0.90	0.87	0.83	0.80	0.76
	4.7	1.00	0.96	0.99	0.99	1.03	1.02	1.05	1.05
	5.7	1.00	1.01	1.13	1.23	1.32	1.38	1.45	1.50
Chlorogenic acid	3.7	1.00	1.04	1.01	0.95	0.91	0.87	0.83	0.77
	4.7	1.00	0.87	0.66	0.46	0.35	0.31	0.30	0.30
	5.7	1.00	0.65	0.40	0.24	0.24	0.27	0.33	0.42
Protocatechuic acid	3.7	1.00	0.95	0.92	0.85	0.79	0.74	0.70	0.64
	4.7	1.00	0.96	0.96	0.89	0.82	0.77	0.75	0.76
	5.7	1.00	1.13	1.10	1.03	1.07	1.12	1.21	1.28
Caffeine	3.7	1.00	0.97	0.94	0.90	0.85	0.83	0.80	0.75
	4.7	1.00	0.92	0.88	0.79	0.73	0.69	0.66	0.61
	5.7	1.00	1.10	1.02	0.84	0.74	0.68	0.65	0.60

Appendix 31: Change in λ_{max} for cyanidin 3-glucoside solutions (1.29 x 10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days and exposed to light.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	510.7	512.8	511.9	400.5	400.3	402.6	403.9	402.1
	4.7	516.4	514.8	400.3	405.5	402.1	411.1	400.5	402.9
	5.7	534.4	400.7	420.6	406.5	400.7	403.7	400.3	400.2
Chlorogenic acid	3.7	532.4	532.4	531.5	527.3	470.7	525.0	441.7	400.9
	4.7	535.6	457.9	520.0	401.1	400.5	404.2	415.0	406.3
	5.7	544.1	509.1	400.6	406.6	414.5	421.1	414.9	414.4
Protocatechuic acid	3.7	519.6	561.6	498.1	428.3	467.8	400.2	420.9	408.3
	4.7	522.1	480.7	525.0	479.9	0.0	0.0	408.1	400.6
	5.7	400.7	471.2	525.0	401.2	401.7	466.7	401.4	467.1
Caffeine	3.7	532.6	531.9	402.2	420.3	403.2	401.8	400.3	401.2
	4.7	544.0	539.9	401.9	421.7	402.9	402.4	405.4	400.3
	5.7	550.8	434.7	406.4	405.1	403.9	403.2	422.2	404.8

Appendix 32: Change in λ_{max} for cyanidin 3-glucoside solutions (1.29 x 10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days in the dark.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	510.9	511.6	510.5	511.9	510.1	511.4	511.7	511.4
	4.7	518.4	516.7	517.0	514.8	515.5	516.3	518.1	402.3
	5.7	530.3	464.1	400.8	402.0	400.6	402.8	402.2	405.4
Chlorogenic acid	3.7	532.2	531.9	532.0	532.7	532.4	531.5	532.8	531.9
	4.7	536.5	537.1	536.1	534.4	444.7	474.7	462.4	511.9
	5.7	543.9	545.2	545.3	450.0	483.4	431.8	454.1	400.8
Protocatechuic acid	3.7	519.4	520.5	520.2	520.0	519.8	519.6	520.8	521.8
	4.7	522.2	518.9	522.2	403.8	413.2	411.6	416.7	430.4
	5.7	411.0	402.8	404.6	413.7	465.0	418.2	425.4	416.4
Caffeine	3.7	532.6	532.0	532.2	530.4	531.5	531.1	532.5	532.0
	4.7	544.8	545.2	545.7	544.4	544.9	543.2	542.4	540.4
	5.7	551.2	551.3	551.3	475.0	400.7	403.2	412.5	409.1

Appendix 33: Absorbance change at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days and exposed to light.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	0.6975	0.3573	0.2066	0.1055	0.0719	0.0528	0.0279	0.0674
	4.7	0.2003	0.1325	0.1080	0.0842	0.0789	0.0674	0.0667	0.0596
	5.7	0.1840	0.1249	0.1066	0.0894	0.0886	0.0705	0.0676	0.0605
Chlorogenic acid	3.7	2.0130	1.3577	0.8913	0.5180	0.4079	0.4112	0.4734	0.6242
	4.7	0.9647	0.2230	0.2046	0.3163	0.5027	0.6260	0.7649	0.9789
	5.7	0.5106	0.1337	0.2463	0.4524	0.7542	0.9368	1.1392	1.3971
Protocatechuic acid	3.7	1.4167	0.1784	0.2145	0.3141	0.4619	0.4948	0.6701	0.7894
	4.7	0.3586	0.2488	0.3117	0.4049	0.4837	0.5395	0.6426	1.0421
	5.7	0.1984	0.3523	0.6653	0.9081	0.9860	0.5808	0.6865	0.5437
Caffeine	3.7	1.3457	0.5197	0.2103	0.0935	0.0572	0.0481	0.0475	0.0613
	4.7	0.5609	0.2649	0.1411	0.0971	0.0767	0.0659	0.0609	0.0480
	5.7	0.3693	0.1970	0.1173	0.0990	0.0720	0.0604	0.0558	0.0484

Appendix 34: Absorbance change at 525 nm for cyanidin 3-glucoside solutions (1.29×10^{-4} M) without and with chlorogenic acid, protocatechuic acid or caffeine (1:200) at pH 3.7, 4.7, and 5.7 and 20°C stored for 35 days in the dark.

Sample	pH	Day 0	5	10	15	20	24	28	35
Control	3.7	0.6457	0.6200	0.6097	0.5833	0.5604	0.5391	0.5179	0.4886
	4.7	0.1739	0.1664	0.1728	0.1723	0.1788	0.1773	0.1821	0.1827
	5.7	0.1228	0.1243	0.1385	0.1512	0.1625	0.1690	0.1782	0.1845
Chlorogenic acid	3.7	1.8628	1.9372	1.8807	1.7721	1.6886	1.6131	1.5428	1.4430
	4.7	0.8933	0.7770	0.5897	0.4068	0.3132	0.2758	0.2651	0.2647
	5.7	0.5135	0.3324	0.2073	0.1217	0.1222	0.1411	0.1697	0.2180
Protocatechuic acid	3.7	1.3333	1.2709	1.2233	1.1305	1.0544	0.9896	0.9278	0.8497
	4.7	0.3356	0.3225	0.3229	0.2991	0.2747	0.2582	0.2516	0.2548
	5.7	0.1681	0.1902	0.1846	0.1735	0.1801	0.1888	0.2033	0.2146
Caffeine	3.7	1.2194	1.1783	1.1501	1.0960	1.0319	1.0088	0.9702	0.9172
	4.7	0.5495	0.5030	0.4840	0.4362	0.3992	0.3784	0.3625	0.3328
	5.7	0.3375	0.3729	0.3431	0.2837	0.2488	0.2297	0.2196	0.2037