THE ANTHOCYANINS OF MONARDA FISTULOSA L.: CHARACTERIZATION, COMPLEXATION AND STABILIZATION

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by

Audra J. Davies

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AUDRA J. DAVIES

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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DEDICATION

This thesis is dedicated to my parents who have entrusted me with the gifts of confidence and determination with which to conquer my goals in life.

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ABSTRACT

Anthocyanins and other flavonoids in the petals of Monarda fistulosa, were separated on a reverse phase column by high performance liquid chromatography (HPLC), equipped with photodiode array detection to determine the UV–visible spectral characteristics of the pigments. Eighteen peaks were detected and separated in less than 55 minutes. Ten of the 18 peaks were collected from the column outlet for further characterization. The major anthocyanidin derivatives and the presence of acylation could be distinguished on the basis of retention times, and the UV–visible spectra. Auxiliary analytical techniques used for pigment characterization included acid hydrolysis, paper and thin layer chromatography, and paper electrophoresis. With the combination of these techniques, peak assignments for the major anthocyanins and other flavonoids of Monarda fistulosa, and their relative concentrations were confirmed. Colorless flavonoids included flavone 7–O–glucoside, flavone 8–C–glucoside, flavonol glucoside and hydroxyflavone compounds. The total anthocyanin concentration was 214.8 mg/100g of fresh petals. The main anthocyanin was pelargonidin 3,5 diglucoside acylated with coumaric and malonic acids, which accounted for 81% of the total anthocyanin content. Following characterization studies, the major anthocyanin of Marshall's Delight, monardaein, was quantified for subsequent complexation studies.

The copigmentation phenomenon was investigated using aqueous solutions of three anthocyanins of increasing structural complexity and three colorless phenolic compounds. By means of visible absorption spectrophotometry, buffered solutions of pelargonidin 3–glucoside, malvin, and monardaein were monitored at different pH and pigment concentration. Increasing the pH of the solution resulted in a bathochromic shift and a decrease in absorbance intensity. Addition of copigments, chlorogenic acid, caffeic acid and rutin, minimized nucleophilic attack of the flavylium cation and resulted in an increase in absorbance intensity of the pigment–copigment complex. It has been demonstrated that chlorogenic acid is an effective copigment at pH 3.2–3.7, while caffeic acid and rutin are effective copigments at pH 3.7–4.7 and 3.5–5.0, respectively.

The magnitude of copigmentation and subsequent stability has been shown to be related to the substitution pattern of the B ring, the degree of glycosylation and the presence of acyl substituents. Generally, the

magnitudes of copigmentation observed for the monardaein complexes were greater than the corresponding pelargonidin complexes, while the malvin solutions exhibited the greatest magnitude of copigmentation. The equilibrium constant for the reaction of complexation, K and the stoichiometric constants for the eight pigment–copigment complexes were determined and found to be dependent on the structure of the pigment and copigment, and unaffected by pH and the concentration of the anthocyanin.

Stability of pigment–copigment complexes formulated in pH 3.7 and 6.0 aqueous phosphoric acid–sodium acetate buffer stored at 20^{0}C were evaluated. The stability of the complexes in decreasing order was malvin, monardaein, and pelargonidin 3–glucoside. The complexes formed with chlorogenic acid exhibited the greatest stability at pH 3.7. At pH 6.0, an increase in absorbance intensity occurred in the 400nm range which can be attributed to the conversion of the pigment solution to the colorless chalcone species. It has been demonstrated that the acyl substituents confer increased color stability to the pigment–copigment complex however, in the pigment solutions investigated, the presence of methyl substituents had a greater influence on the color stability than the presence of acylation.

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CHAPTER 1
INTRODUCTION

There is increasing interest among food technologists and consumers alike, in the development of food colorants from natural sources. A consumer's sensory perception of a food begins with the overall appearance and eye appeal of the product. Food color dramatically influences a consumer's perception of a food's sensory characteristics affecting the judgement of such attributes as odour, flavor and texture. Experience has conditioned consumers to associate a particular food color with specific quality and sensory attributes (IFT, 1986). Therefore, if the color of a food is not appealing and attractive, it may lead to the premature rejection of the food product regardless of the acceptability of other sensory attributes.

Society as a whole is becoming increasingly more health conscious, and more emphasis has recently been placed on healthful diets and the safety of food ingredients. This recent interest stems from concern surrounding the controversial synthetic dyes through a perceived problem of food safety. Unfounded publicity about the safety of color additives has caused much public concern and controversy and has stimulated increased interest and research into the area of natural plant pigments for food colorant applications in the future.

The word "natural" has taken on economic importance, and its use in product advertising conveys an aura of wholesomeness and presumed healthfulness (Lauro, 1991). Colorants can be considered "natural" if:

1. they are from agricultural or biological sources;
2. they are extracted without chemical reaction;
3. they have had a long history of use.

There are many highly colored pigments occurring in nature which are potential food colorants. The anthocyanins are perhaps some of the best known of the natural food colorants as they account for many of the orange, red and blue pigments of flowers, fruits, vegetables, leaves and roots. The term anthocyanin, derived from the Greek words anthos and kyanos meaning flower

and blue, was coined by Marquart in 1835 to designate the blue pigments of flowers. These pigments are widespread in the higher plants of the plant kingdom and are found in all parts of the plant. The primary function of anthocyanins is to attract insects and birds to plants in order for pollination to occur (Harbourne, 1967). Since many fruits and flowers have very distinctive anthocyanin compositions, anthocyanin and flavonoid profiles have been found to be of particular importance in the chemotaxonomy of plants, successfully used to characterize families, genera, species or cultivars (Harbourne, 1967; Hong and Wrolstad, 1990). In addition, the anthocyanin content has been used as a quality control measure to monitor fruit maturity as anthocyanins appear in many fruits near maturity, and the anthocyanin content can be directly correlated with shelf-life (Kushman and Ballinger, 1975; Deubert, 1978).

Since the anthocyanin pigments are responsible for the appealing colors of many of nature's products including grapes, apples, strawberries, raspberries, cranberries, mangoes, and many flowers including roses, hibiscus, and iris, the anthocyanin pigments are a logical consideration for use as natural food colorants. The use of anthocyanins as natural red colorants may be widely accepted in the food industry as they possess the following advantages:

1. they have been consumed by man (and animals) for countless generations without apparent adverse effects to health;
2. they are water soluble, which simplifies their incorporation into aqueous food systems; and
3. they are brightly colored, especially in the red region (Markakis, 1982).

Despite obvious familiarity, the inherent chemical instability of anthocyanins over periods of extended storage and under typical food processing conditions has precluded the widespread application of anthocyanins as food colorants. Loss of color is accelerated under conditions of high temperature, high pH, and in the presence of normal co-occurring cellular constituents such as ascorbic acid, amino acids, sugars and sugar breakdown products. The major considerations in the use of anthocyanins as food colorants include potential supply and cost, ease of purification and utilization, efficiency of coloration, and aesthetic appeal of the colored products (Francis, 1975). Difficulty in purification and the unavailability of large quantities of pure anthocyanins commercially has hindered their widespread application. However, sample production technology combined with benefits other than color could make the isolation of natural pigments commercially feasible.

Research activities in the area of plant phenolics has lead to several interesting findings which may

facilitate the application of anthocyanins to use as colorants in the food industry. Recent discovery of the enhanced stability of anthocyanins with phenolic acid substitution has many positive implications in overcoming the inherent stability problem of anthocyanins in the past. Recognition of the contribution of the phenomenon of copigmentation to the vivid flower colors occurring in vivo, may also play a vital role in overcoming the stability problem of anthocyanins in the future. By complexing anthocyanins with co-occurring phenolic compounds, it is possible to enhance the coloration and simultaneously stabilize the anthocyanin solution. The copigmentation phenomenon paired with the widespread occurrence of acylated anthocyanins may result in the application of anthocyanins to the coloring of food products in the near future.

The objectives of this research were as follows:

1. To characterize the anthocyanin and other flavonoids of Monarda fistulosa L. by a combination of analytical techniques including:
    a. High performance liquid chromatography (HPLC);
    b. Paper chromatography;
    c. Thin layer chromatography;
    d. Paper electrophoresis;
    e. Spectral analysis.
2. To purify the major acylated anthocyanin of Monarda fistulosa L. hybrid Marshall's Delight and collect sufficient quantity to conduct complexation and stabilization studies.
3. To complex simple anthocyanins, malvidin diglucoside and pelargonidin monoglucoside and the corresponding acylated anthocyanin, isolated from Monarda by the copigmentation reaction with selected phenolic compounds in order to further elucidate the copigmentation mechanism occurring between anthocyanins and colorless copigments.
4. To evaluate the stability of the anthocyanin-copigment complexes in a model aqueous system.

CHAPTER 2
LITERATURE REVIEW

2.1 Anthocyanin Biosynthesis

The anthocyanins are part of a large group of compounds known collectively as flavonoids, based on a fifteen carbon atom structure where two aromatic rings are linked by a three carbon unit (Figure 2.01). It is estimated that about 2% of all carbon photosynthesized by plants is converted into flavonoids or closely related compounds (Wong, 1976). Thus, flavonoids constitute one of the largest groups of naturally occurring phenols. The anthocyanins can be differentiated from other flavonoid compounds by their characteristic absorbance of visible light which accounts for their vast array of attractive red, blue and violet colors.

2.1.1 Biosynthesis of Anthocyanins in the Context of Flavonoid Biosynthesis

The flavonoid variants are all based on a common biosynthetic pathway which incorporates precursors from both the "Shikimate" and "Acetate Malonate" pathways (Wong, 1976)(Figure 2.02), the first flavonoid being produced immediately following confluence of the two pathways. The key enzyme in the synthesis of the basic flavonoid structure is chalcone synthase (Grisebach, 1982) which catalyzes the condensation of 4–coumaroyl–CoA and three molecules of malonyl–CoA to form a chalcone (Figure 2.02). Isomerization of the chalcone by chalcone isomerase to form a flavanone precludes the synthesis of the dihydroflavonol, which is a biosynthetic intermediate for anthocyanin synthesis. The different types of anthocyanin molecules are differentiated by the addition or removal of hydroxyl groups or by methylation or glycosylation of the flavylium cation. Such modifications are known to be controlled by single gene substitutions in the flowers of many higher plants (Harbourne, 1967).

In the plant cell, flavonoids are thought to be synthesized outside the vacuoles and stored in the vacuoles as an aqueous solution which is slightly acidic or neutral (Asen et al., 1972). It is thought that the overall biosynthesis of the various flavonoid compounds occurs near the endoplasmic reticulum (ER) by cytoplasmic and ER–bound enzymes (Hrazdina, 1982). Upon production of the anthocyanins, they are then transferred by an unknown transport mechanism to

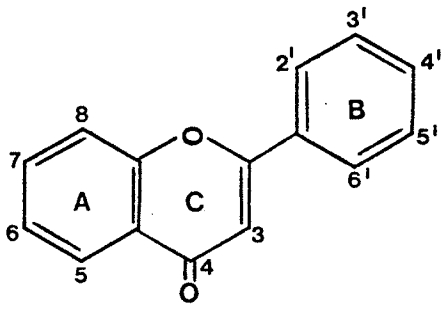


Figure 2.01 The fifteen carbon aglycone.

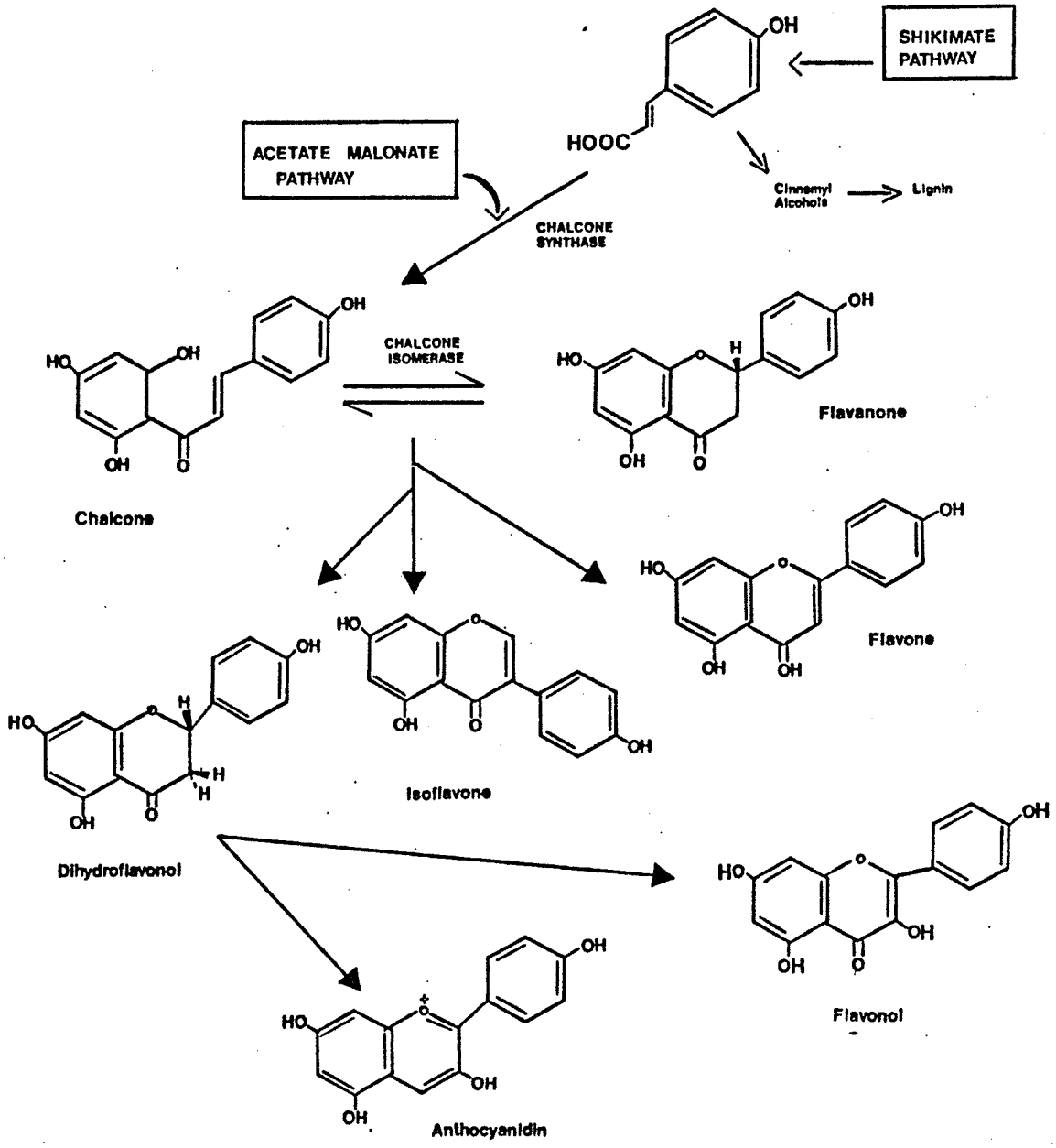


Figure 2.02 The interrelationships between flavonoid variants (adapted from Markham, 1982 and Wong, 1976).

the central vacuole of the cells.

2.1.2 Physiological Role of Anthocyanins

Anthocyanins have been implicated in a number of physiological roles in both plants and animals (Hrazdina, 1982). The presence of anthocyanins is thought to provide resistance to pathogens in many plants including sunflower (Burlov and Kostyuk, 1976) and brassica (Weisaeth, 1976). In addition, anthocyanins have been shown to enhance photosynthesis in leaves of tropical rain forest plants (Lee et al., 1979), and regulate photosynthesis in some woody plants grown in the Far East (Chernyshev, 1975). Anthocyanins are thought to have a phytoprotective role in their ability to provide protection against UV radiation (Glasgow, 1990). In mammals, anthocyanins play a physiological role and have been linked to the prevention of capillary fragility (Pourrat, 1977; Proserpio, 1977).

2.1.3 Hydroxylation and Methylation

The compounds within each of the different flavonoid classes are distinguished by the number and orientation of hydroxyl groups on the two benzene rings (A and B) and the extent to which the hydroxyl groups are modified by methyl and glycosyl substituents. The anthocyanin structure is characterized by its highly oxidized state of the C3 chain. The different substitution patterns of the two rings are determined by the different biosynthetic origins of the two aromatic nuclei, catalyzed by enzymes with high degrees of specificity (Wong, 1976). Oxygenation of the B ring is somewhat controversial among phytochemists. Firstly, it has been proposed that the oxygenation of the B ring may occur at the cinnamic acid stage, such that 4–coumarate acts as a precursor for pelargonin, caffeate for cyanidin, and ferulate for peonidin. The second possibility is that 4–coumarate is the precursor for all anthocyanidins, and the substitution of additional hydroxyl and methyl groups occurs later in the biosynthesis at the chalcone or flavanone stage (Grisebach, 1982). Although it is known that methylation is one of the last stages of anthocyanin biosynthesis, methyltransferases for anthocyanidins have not been found.

2.1.4 Glycosylation

Glycosylation is the last step in anthocyanin biosynthesis, using UDP–glucose as the glucosyl donor catalyzed by 3–O–glucosyltransferases (Saleh et al., 1976). Additional glycosylation occurs in a stepwise manner following the initial glycosylation at C3. Anthocyanins with acyl substituents

occur as a result of esterification of cinnamic acid derivatives to the sugar residue, specifically at the 6–hydroxyl group in the case of glucose and at the 4–hydroxyl group in the case of rhamnose (Grisebach, 1982; Cheminat et al., 1989). The transfer of the acyl moiety from the coenzyme A ester to the anthocyanidin–glycoside is catalyzed by the presence of an acyl transferase which is governed by a specific gene (Kamsteeg et al., 1980).

2.1.5 Biosynthetic Regulation

Flavonoid biosynthesis is regulated by a number of internal and environmental factors. Endogenous control by substrates, enzymes, end products, and hormones is greatly influenced by exogenous factors such as light, infection and stress. It has been suggested that the activity of the enzyme phenylalanine ammonia lyase (PAL), which is responsible for the conversion of phenylalanine to cinnamic acid, has regulatory properties (Wong, 1976), as this enzyme lies at the heart of the biosynthetic reactions and is the branching point of flavonoid synthesis,. It is known that PAL is sensitive to the physiology of the plant and its activity is dramatically influenced by light (Grisebach, 1982; Wong, 1976). However, it is difficult to correlate PAL activity with the production of anthocyanins as this enzyme has involvement in many other pathways (Hrazdina, 1982).

Some plant species, which are acyanic under normal conditions, produce anthocyanins in the presence of high amounts of sugars, or when deficient of certain metals. For example, the appearance of autumn coloration in leaves is thought to be due to the production of high amounts of sugars during photosynthetic periods followed by reduced transport during the cool autumn nights (Hrazdina, 1982). When attacked by a pathogen, some plants respond by increased production of flavonoid compounds, including anthocyanins, around the site of infection. The results of water stress on the plant also result in an increase in anthocyanin production as compared to the normal production of flavonoids (Spyropoulos and Mavrommatis, 1978). Deficiency in certain minerals also results in an increased anthocyanin content in plants. Recently, it has been shown in samples of moss, Bryum argenteum, that the production of flavonoid compounds increases in the presence of UV–B radiation (Glasgow, 1990). The increase in flavonoid production is thought to be due to a phytoprotective role of these compounds, as discussed previously.

2.2 Anthocyanin Chemistry

2.2.1 General Structural Considerations

The anthocyanins are water–soluble glycosylated pigments based on a single aromatic structure – 3,5,7,3',4'–pentahydroxyflavylium cation (Harbourne, 1967)(Figure 2.03). Typically, the anthocyanin molecule consists of two or three portions; the aglycone based on the flavylium nucleus arranged in a C6–C3–C6 configuration, a group of sugar(s), and often a group of acyl acid(s) (Harbourne, 1967; Timberlake, 1980; Francis, 1989). There are six common aglycones, own as anthocyanidins, which are all based on a common flavylium cation but are differentiated by their degree of methylation and hydroxylation. The six anthocyanidins which are most common in plant tissue are, in order of increasing substitution: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Harbourne, 1967)(Figure 2.04). The color of anthocyanins is altered by the addition or removal of hydroxyl groups or by methylation or glycosylation of the flavylium cation. The variety of different glycosyl (ie., mono–, di–, and trisaccharides) and acyl (mainly the phenolic acids, p–coumaric, caffeic, ferulic, and sinapic acids) constituents and their position of substitution result in the number of anthocyanins being 15–20 times greater than the number of anthocyanidins, resulting in approximately 140 anthocyanins which have been reported as occurring naturally in plants (Mazza and Brouillard, 1987).

Due to the electron deficient nature of the flavylium structure, the anthocyanins are stable only in very acidic solutions and form unstable, colorless pseudobases at neutral pH (Harbourne, 1967). The anthocyanidins are much less stable and less soluble than the anthocyanins which provides reason why they are seldom found in the free form in plant tissue. The most commonly found anthocyanins in nature are the glycosides of cyanidin, delphinidin and pelargonidin which account for 80% of the pigmentation of flowers, 69% of fruits and 50% of flowers (Swain, 1976). Pelargonidin– and delphinidin–type pigments occur more frequently in advanced plants replacing cyanidin which is considered to be the simplest and most primitive pigment (Harbourne, 1976).

2.2.2 Characteristic Anthocyanin Spectra

Both anthocyanins and their aglycones show two characteristic absorption maxima when in acid solution, a strong one in the visible region between 465 and 550 nm and a smaller one in the UV region at about 280 nm (Harbourne, 1967)(Figure 2.05). Variation within these ranges occurs as a result of changing hydroxylation patterns and the degree of substitution of the hydroxyl groups. The spectra of acylated anthocyanins differs from a non–acylated anthocyanin by the appearance of a weak absorption maxima in the 310–335 nm region, the actual maxima indicating the type of aromatic acylation present (Harbourne, 1967)(Figure 2.05).

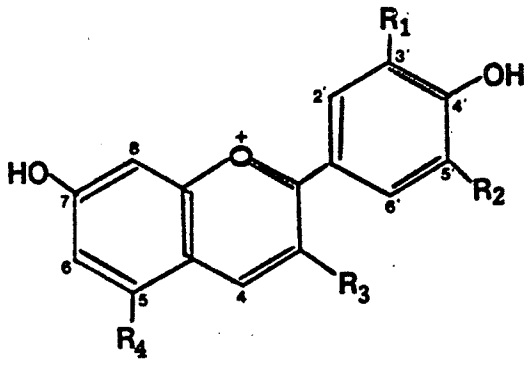
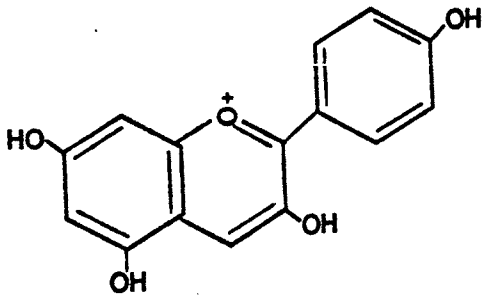
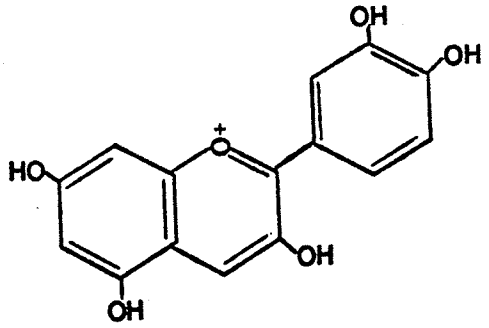


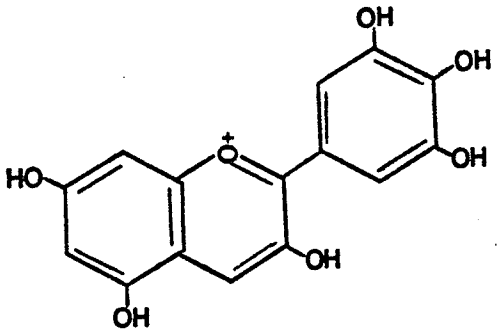
Figure 2.03 The anthocyanin flavylium nucleus.



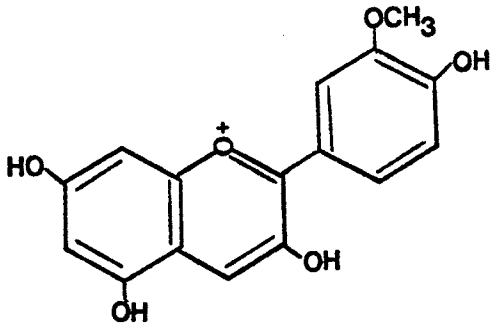
Pelargonidin λmax= 520



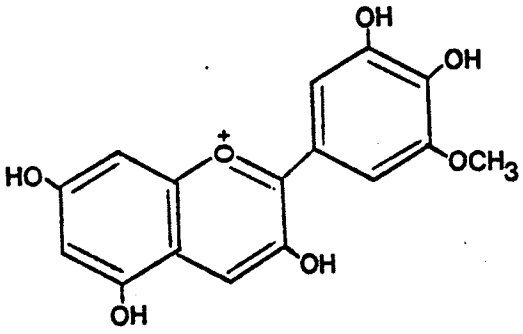
Cyanidin λmax= 535



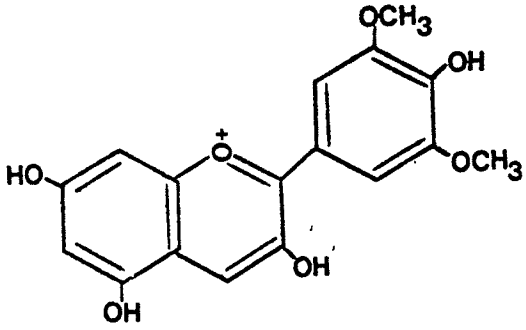
Delphinidin λ max= 546



Peonidin λmax= 532



Petunidin λmax= 543



Malvidin λmax= 543

Figure 2.04 The six common anthocyanins (adapted from Timberlake, 1980 and Markakis, 1982).

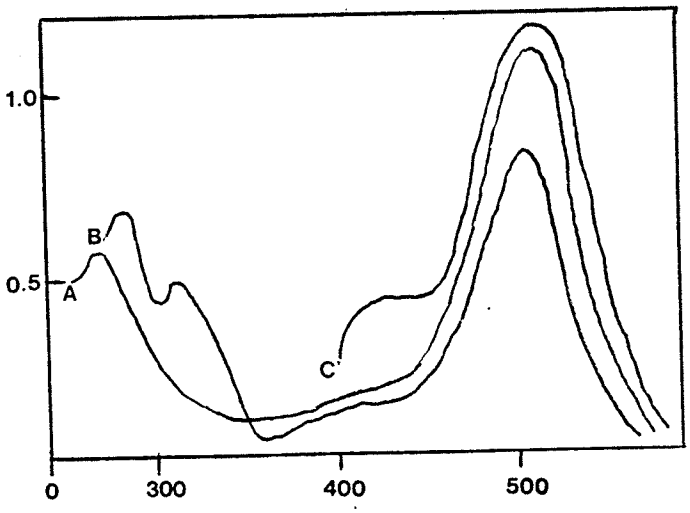


Figure 2.05 Characteristic absorption spectra of anthocyanins in MeOH/HCl: (A) pelargonidin 3,5-diglucoside; (B) pelargonidin 3-p-coumaroylglucoside-5-glucoside (monardaein); (C) pelargonidin 3-glucoside (adapted from Harbourne, 1967).

2.2.3 Effect of Substitution Patterns on Anthocyanin Color

Sugars, acylated sugars, methoxy and hydroxyl groups have a marked effect upon the color and reactivity of the anthocyanins. Generally speaking, as conjugation increases, flavonoid compounds absorb light at longer wavelengths (Figure 2.04). An increasing degree of hydroxylation has the same effect, as a result of an increase in the non–bonding electrons supplied by the hydroxyl group. Simply, as the number of phenolic hydroxyls increases, the color changes from pink to blue. However, the contribution of each additional hydroxyl group differs depending on the position of substitution (Harbourne, 1967). The hydroxyl group at C3 is particularly significant, as it shifts the color of the pigment from yellow–orange to red. Similarly, the presence of a hydroxyl group at C5 and substitution at C4 both stabilize the colored forms through the prevention of the hydration reactions which lead to the formation of colorless species. The degree of hydroxylation is important as anthocyanins which contain more hydroxyls are less stable.

Acylation or glycosylation of the hydroxyl groups in anthocyanins results in a hypsochromic shift due to the involvement of the non–bonded electrons in resonance with the acyl group itself (Harbourne, 1967; Francis, 1989). Methylation or glycoside formation has a much lower hypsochromic effect. Methoxy groups replacing hydroxyl groups tend to result in an increase in redness as well, a high degree of methylation increases the stability. Although it can be seen that changes in the B ring substituents result in changes in the color expressed by the anthocyanin, these changes are minimal and it is evident that additional mechanisms exist in vivo to account for the vast array of colors produced by anthocyanins.

2.2.4 Glycosylation Patterns

In general, the anthocyanidins do not accumulate in the plant tissue, and the pigments occur in flowers and fruits mainly in the glycosylated form known as anthocyanins. The sugars substituted on the aglycone are, in order of occurrence in nature: glucose, rhamnose, xylose, galactose, arabinose, and fructose. The nature of the individual sugars has little effect but their positions in the molecule can have a profound influence on the reactivity of the aglycone. They occur as monoglycosides, diglycosides, and triglycosides substituted directly on the aglycone. Firstly, the 3 position is occupied by a sugar followed by additional glycosylation in positions 5, 7, 3' and possibly 4' (Harbourne, 1967). When a second sugar is present, it is generally at C5 (Harbourne, 1967; Brouillard, 1982), with the resultant diglucoside exhibiting lower stability than a corresponding monoglucoside (Mazza and Brouillard, 1987). Since anthocyanidins have been

shown to be unstable in water and much less soluble than anthocyanins, glycosylation is assumed to confer stability and solubility to the pigment (Harbourne, 1967).

2.2.5 Acylation Patterns

The presence of anthocyanins acylated with dicarboxylic acids has recently been found to be widespread (Harbourne and Boardley, 1985). The main acylating groups occurring as anthocyanin substituents are the phenolic acids, which in order of occurrence, include p–coumaric, caffeic, ferulic, p–hydroxybenzoic, sinapic, malonic, acetic, succinic, oxalic, and malic acids (Francis, 1989). Due to the labile nature of the organic acid moieties commonly found as acyl substituents in anthocyanins, particularly malonic acid, there has been considerable difficulty in the isolation and characterization of these substituents. The use of methanolic hydrochloric acid traditionally used for the extraction of pigments is thought to have hydrolyzed the malonic acid esters resulting in the incomplete characterization of pigments containing malonic acid as the acyl substituent (Harbourne and Boardley, 1985).

Acylated sugars occur, without exception, bonded to the sugar at the 3 position of the aglycone in the case of mono–acylation (Harbourne, 1967), and additional acyl groups are bonded to the sugar at the C5 position in the case of di–acylation. Typically, the organic acid is attached to the C6 hydroxyl of the sugar moiety (Goto et al., 1982; Harbourne and Boardley, 1985; Idaka et al., 1987; Cheminat et al., 1989; Terahara et al., 1990a & b). Evidence of this substitution pattern is provided primarily from hydrolytic and oxidative cleavage of the acyl substituent from the aglycone (Harbourne, 1967), in addition to spectral analysis with particular attention to the E_{310}/E_{vis} ratio (Saito et al., 1985; Terahara et al., 1990b).

Examination of anthocyanin behavior by paper electrophoresis, as well as comparison to authentic standards using TLC, HPLC and spectral analysis have typically been used to determine the presence of acylation (Cornuz et al., 1980; Harbourne and Boardley, 1985; Harbourne, 1986; Takeda et al., 1986). Less commonly, gas chromatography has been used to detect acylation (Goto et al., 1983b). TLC and paper chromatography of acylated anthocyanins reveal high R_f values which can be attributed to the presence of acyl groups (Timberlake and Bridle, 1971). In reverse phase HPLC, elution rates of highly acylated compounds typically increase two–fold in comparison to non–acylated anthocyanin counterparts (Wulf and Nagel, 1978; Takeda et al., 1986; Hwa Kim et al., 1989; Terahara et al., 1990b). More sophisticated techniques used to elucidate

the anthocyanin structure, and determine the presence of acylation, have included IR (Timberlake and Bridle, 1971), FABMS, H–NMR and C–NMR (Saito et al., 1985; Kondo et al., 1985; Takeda et al., 1986; Cheminat et al., 1989; Terahara et al., 1989a and 1989b).

In an electrophoretic survey of anthocyanins in flowers of 81 species belonging to 27 plant families, Harbourne and Boardley (1985) found that malonated or similarly substituted zwitterionic anthocyanins occurred in half the sample, particularly in the more highly evolved angiosperm families. In such families as Compositae and Labiatae (including Monarda spp.), the presence of dicarboxylic acid substituents was found to be widespread. The attachment of malonic acid to the anthocyanin balances the cationic charge on the flavylium ion, such that the malonated anthocyanin is effectively a zwitterion and can easily be detected by its characteristic rapid mobility and high R_f value using paper electrophoresis techniques (Harbourne and Boardley, 1985).

2.2.6 Influence of acylation on anthocyanin stability

The significance of anthocyanins acylated with aliphatic dicarboxylic acids is related to the stabilization of these anthocyanins in the mildly acidic environment of the cell sap (Goto et al., 1979; Hoshino et al., 1980; Brouillard, 1981; Harbourne, 1986). It has been demonstrated in Zebrina pendula (Brouillard, 1981; Teh and Francis, 1988), Brassica oleracea (Sapers et al., 1981), and numerous flowers (Goto et al., 1979; 1982; 1983a; Saito et al., 1985) that anthocyanins with acyl group substitution exhibit unusual stability (Figure 2.06, 2.07). It has been established that the exceptional stability of the acylated anthocyanins is due to the interaction of the acyl groups with the positively charged pyrilium nucleus, such that addition of nucleophiles (such as water) to the pyrilium ring is prevented (Goto et al., 1979; Brouillard, 1981).

Comparison of acylated anthocyanin structures reveals that monoacylated anthocyanins do not show color stability when compared to anthocyanins substituted with two or more acyl residues linked to sugars. Hence, the presence of at least two acyl substituents is a fundamental requirement for good color stability in neutral solutions (Brouillard, 1981; Goto et al., 1982; 1983a; Saito et al., 1985). The color stability of these acylated anthocyanins appears to increase with increasing content of organic acids as well as an increasing degree of substitution on the aglycone (Saito et al., 1985).

A high acidity constant and an apparent lack of pseudobase and chalcone formation can be

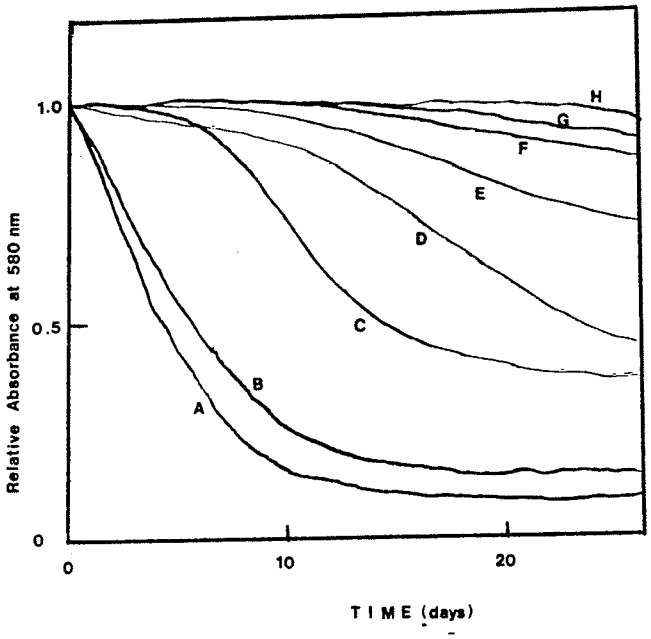


Figure 2.06 Stability of acylated anthocyanins in buffer solution (pH 6.95, ca 30mg/l, path length 10 mm, ca. 15-20°C). (A) platyconin; (B) gentiodelphin; (C) rubrocinerarin; (D) cinerarin; (E) Ipomea "Heavenly Blue" anthocyanin; (F) ternatin B; (G) ternatin A; (H) ternatin D (adapted from Saito et. al., 1985).

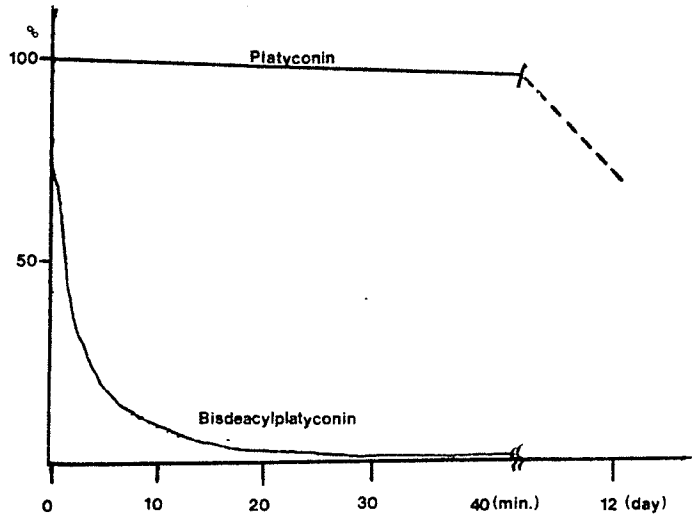


Figure 2.07 Enhanced stability of platyconin at pH 5.0, 4.0 X 10^{-5}M in acetate buffer at λmax (adapted from Goto et al., 1983a).

attributed to the enhanced color stability of Zebrina anthocyanins substituted with two acyl groups (Brouillard, 1981). It has been suggested that the acyl groups protect the flavylium cation through preferential ionization of the acyl groups, particularly in the case of malonic and glucuronic acid substitution (Saito et al., 1985; 1988). In order to protect the flavylium cation from hydration, the orientation of the acyl groups is thought to be such that one of the acyl groups is situated above the pyrilium ring and the other beneath it (Brouillard, 1981; Goto et al., 1979; 1982; 1983a)(Figure 2.08). It has been suggested that intramolecular hydrophobic interactions exist between the anthocyanidin and acyl moieties favoring the formation of a stacked structure which facilitates protection of the flavylium cation from nucleophilic attack (Goto et al., 1982; 1983a; Saito et al., 1985; Terahara et al., 1989b). This discovery of acylated anthocyanins may prove to be of particular importance to the technology of foods since, with new and better sources of these compounds and with better understanding of their physicochemical properties, they may find application to the coloring of food products (Mazza and Brouillard, 1987).

2.2.7 Structural Transformations of Anthocyanins in Aqueous Solutions

It is well known that the color of an anthocyanin solution is affected by the structure of the pigment. Non-acylated and monoacylated anthocyanins behave like pH indicators, being red at low pH, bluish at intermediate pH and colorless at high pH (Mazza and Brouillard, 1987). The generally accepted mechanism to explain the shift in color as a function of changing pH as described by Brouillard and colleagues (1977, 1978), is based on the interconversion of four structurally related species. Through a series of relaxation experiments, the authors were able to determine that four anthocyanin species exist in a fast equilibrium – quinonoidal base A, the flavylium cation AH+, the pseudobase B, and the colorless chalcone C (Figure 2.09). The only species absorbing light are AH+ and A, hence the degree of coloring of a solution at a given pH depends on the relative amounts of these two species (Brouillard and Dubois, 1977). As the pH increases, there is a shift or interconversion between these four species such that the predominant species at pH 2.0 (flavylium cation) is ionized to carbinol B or chalcone C as the pH increases to 4.5 and higher. There is very little color in anthocyanin solutions when the pH is increased beyond 4.0.

The main factor in the fading process is the great reactivity of the flavylium cation toward nucleophilic reagents such as the water molecule and the hydroxyl ion, which can be described on the basis of the hydration equilibrium constant (Brouillard and Dubois, 1977). By monitoring

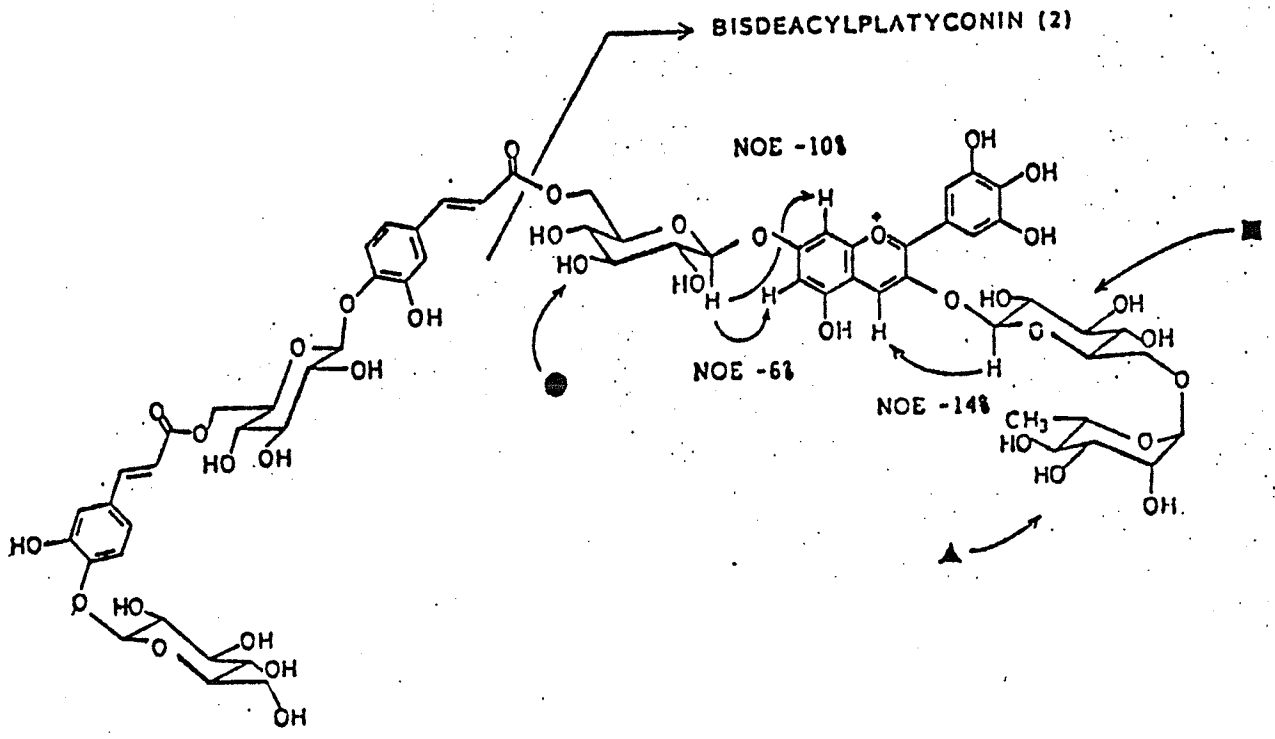


Figure 2.08 Structure of polyacylated platyconin (adapted from Goto et al., 1983a).

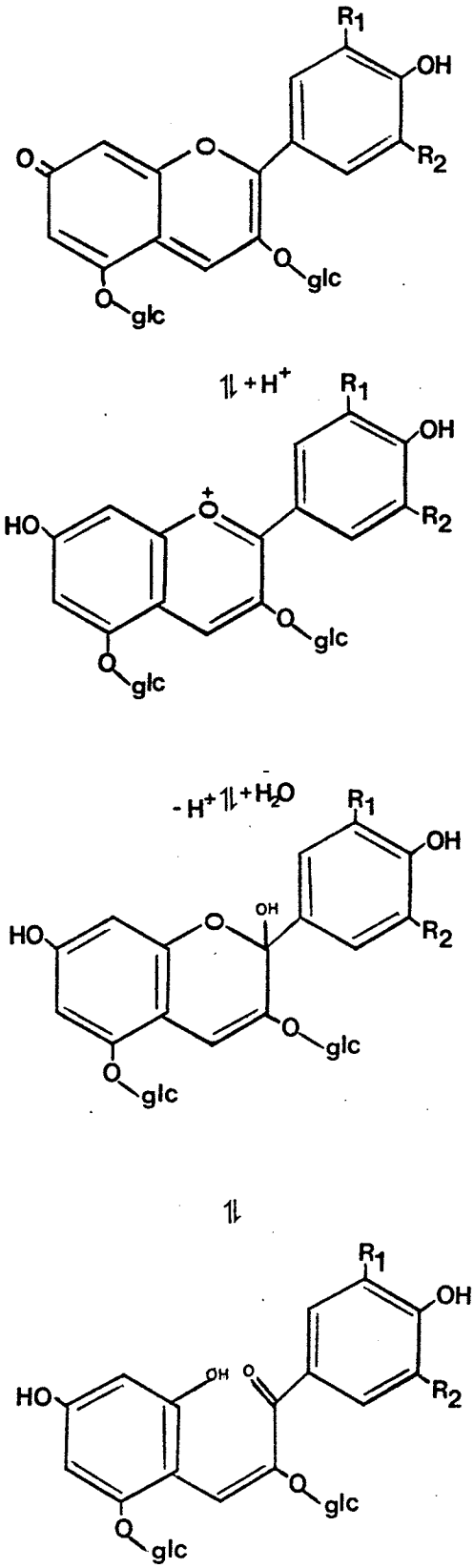


Figure 2.09 Structural transformations of anthocyanin species in equilibrated aqueous solution (adapted from Brouillard and Delaporte, 1977a).

a solution of anthocyanins at varying pHs by UV–vis spectrophotometry, the structural transformation process can be explained on the basis of three equilibrium constants, the proton transfer equilibrium represented as K_a', the hydration equilibrium, K_h', and the tautomeric equilibrium represented at K_T (Brouillard et al., 1978) (Figure 2.10). The concentrations at equilibrium can be described as $K_a' = ([A]/[AH+])a_{H+}$; $K_h' = ([B]/[AH+])a_{H+}$; $K_T = [B]/[C]$; where a_{H+} is the activity of the hydronium ion ($pH=-\log a_{H+}$). In studies monitoring the structural transformations of malvidin 3–glucoside and 3,5–diglucoside, by a series of relaxation experiments, Brouillard and Delaporte (1977) found that the hydration reaction of the diglucoside cation is much more rapid than that of the monoglucoside. It is known that the thermodynamic hydration constants of regular anthocyanins are close to 10^{-2} to 10^{-3} M (Timberlake and Bridle, 1967) which has been shown to be close to the upper limit of the free acidity concentration of the vacuole (Asen et al., 1975). Using the van't Hoff equation, Brouillard and Delaporte (1977) measured the enthalpy and entropy changes associated with the three equilibria characteristic of Figure 2.10 using malvidin 3–glucoside. The transformations from the flavylium cation to the colorless chalcone characterizing the pyrilium ring opening were shown to be endothermic (Brouillard and Delaporte, 1977). Thus, any rise in temperature strongly favors the ring opening to the chalcone form at the expense of the flavylium cation and the quinonoidal base. A subsequent study by Cheminat and Brouillard (1986) which monitored the structural transformations of malvidin 3–glucoside by PMR spectrometry supported this theory. Conversely, lowering the temperature to 5^0C strongly favors the formation of the quinonoidal base, A.

2.2.8 Expression of Anthocyanin Color in vivo

The color of the anthocyanins is therefore determined by the physico–chemical mileau in which they are viewed. Anthocyanins accumulate in the central vacuole of the plant cell as an aqueous solution dissolved in the slightly acidic cell sap (pH 3.5–5.5) (Asen et al., 1972). Since anthocyanins are known to exist in a colored form at pH values below 3.0, it can be said that anthocyanins primarily exist in colorless forms at pH values characteristic of the plant cell vacuole (Brouillard et al., 1990). Hence, anthocyanins appear more frequently as the quinonoidal form than as the flavylium cation in the plant itself due to the slightly acidic to neutral conditions of the vacuole. In order to explain the pigmentation of such media, the mechanisms whereby the quinonoidal bases are generated and stabilized are of great significance. It is evident that additional mechanisms exist within the plant tissue to retain the anthocyanin color, as it has been established that anthocyanins exhibit their most stable color at a pH below 3.0 in solution.

$$AH^+ \underset{k_a}{\rightleftharpoons} A + H^+$$

$$AH \underset{k_h}{\rightleftharpoons} B + H^+$$

$$B \underset{k_T}{\rightleftharpoons} C$$

Figure 2.10 Equilibrium constants describing the structural transformation process (adapted from Brouillard et al., 1978).

2.3 Stabilization of Anthocyanin Color

2.3.1 Self Association

Self association is characterized by a color increase which is more than proportional to an increase in the concentration of the anthocyanin pigment. Asen and colleagues (1972) found that at pH 3.16, anthocyanins exist as an equilibrium mixture of flavylium cation and carbinol base and contribute to flower color via self association.

The mechanism by which self association exhibits an increase in color can be attributed to the prevention of flavylium cation hydration (Scheffeldt and Hrazdina, 1978) whereby the higher the pigment concentration, the slower the hydration process. An anthocyanin concentration of 10^{-3}M has been shown to result in greater stability than a solution of 10^{-4} or 10^{-5}M, since the hydration reaction is much slower in a solution of higher pigment concentration (Hoshino et al., 1981). Since it is known that the anthocyanin concentration of the vacuole is generally higher than 10^{-2}M (Timberlake and Bridle, 1975), it may be implied that the self association mechanism may contribute significantly to flower color. In addition to the contribution to color at low pH, the self–associative chiral stacking of anthocyanins may account for much of the in vivo coloring occurring in flowers at higher pH in the range 5.7 – 7.0 (Hoshino et al., 1981; 1990; Goto et al., 1990).

Recently, Hoshino (1990, 1991) reported that quinonoidal bases are stabilized by a self–associative mechanism characterized by vertical stacking, determined by studies employing H–NMR and circular dichroism analysis. Circular dichroism has been found to be an effective probe for the conformational changes which occur during the molecular association of anthocyanins due to the sensitivity of this technique to molecular dissymmetry (Hoshino et al., 1981). Investigations into the effect of pH on the chiral stacking of anthocyanidin chromophores over a pH range of 6–10 revealed that both homo– (AA, A^-A^-) and hetero–association (AA^-)of neutral (A) and anionic (A^-) quinonoidal species, in the form of vertical stacking of the aromatic rings, occurred as a result of an increase in pigment concentration. As the pigment concentration increases, the ionization of AH^+ to A^- is suppressed. However, a decrease in the stacking interactions was observed as the pH increased (Hoshino, 1991). In addition, to a pH effect, an increase in temperature was shown to result in dissociation of the stacked anthocyanins which was attributed to an increased tumbling motion of the molecules (Goto et al., 1990; Hoshino, 1991).

It has been proposed that the self–association of anthocyanins occurs in a left or right handed helical conformation such that the anthocyanidins form a hydrophobic core surrounded by hydrophilic glucose moieties enabling solubilization in water (Hoshino et al., 1991). The presence of methylation has been shown to decrease the self association phenomenon, presumably due to stearic hinderance, while glycosylation has been shown to have little effect on the degree of self association. The driving force for self–association is the presence of hydrophobic bonds (Hoshino et al. 1981). In the resultant hydrophobic area, the ionization of the quinonoidal bases is stearically suppressed (Hoshino and Goto, 1990), as both sides of the pyrilium ring are protected from water attack, resulting in the kinetic stabilization of color (Hoshino, 1991).

2.3.2 Copigmentation

The color stabilization and augmentation effect, which is of greater interest to this research, is copigmentation. Observed as early as 1916 by Willstätter and Zollinger, and documented in 1931 by the Robinsons, the copigmentation phenomenon is widespread, and offers a logical explanation for the vast array of colors in vivo, where existing pH conditions normally prevent anthocyanins from producing colors (Asen et al., 1970). A copigment may be characterized as a molecule which usually has no color by itself, but when added to an anthocyanin solution, it greatly enhances the color of the solution (Asen et al., 1971). Copigments may be flavonols, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals, and anthocyanins themselves (Table 2.1)(Asen et al., 1972).

2.3.2.1 Factors influencing copigmentation

In the vacuoles of the colored cells of higher plants, at pH values ranging from pH 2 to neutrality, the copigmentation phenomenon has been shown to be responsible for the blueing (bathochromic effect) of the epidermal cells (Stewart et al., 1975). Using a microspectrophotometric method, Stewart et al. (1975), have shown that at approximately the same pH, azalea petals containing the same pigments differ in the maximum wavelength of visible absorbance (λmax) by 27nm. The differences in λ max can be explained by differences in the concentration and type of copigment present and ratio of copigment to pigment (Asen et al., 1972). Anthocyanin color is increased in intensity (hyperchromism) with a shift in the peak wavelength towards the blue upon addition of a copigment compound. Many factors influence the copigmentation phenomenon, the most important being the chemical structures of both the pigment and copigment, pH of the medium, the solvent, and the temperature at which copigmentation occurs (Mazza and Brouillard, 1989; Cai

et al., 1990).

Pigment structure has been shown to affect the magnitude of the copigmentation phenomenon. The copigment effect has been shown to increase with an increasing degree of methoxylation and glycosylation of the anthocyanin when studied under identical conditions of pH, pigment and copigment concentrations, temperature, ionic strength and solvent (Mazza and Brouillard, 1990). Copigment structure is also an important factor to consider in the evaluation of copigment effectiveness. In a study evaluating the copigmentation effectiveness of twenty five flavonoid compounds, Chen and Hrazdina (1981) noted several structural considerations. It was found that saturation of the C2–C3 bond markedly decreases the complex–forming properties of compounds, suggesting that electrostatic and configurational (stearic) effects are involved in the copigmentation phenomenon. The intensity of the complex formation depended mainly on the number of free hydroxyl groups in the flavonoid molecule, with the 7–OH group being the most important for complex formation. Methylation of the 3'– or 4'–OH groups was shown to decrease the complex forming ability of these compounds. A model illustrating the orientation of the anthocyanin and copigment molecules using cyanidin, rutin and caffeic acid, was put forth by Maccarone et al. (1985) as illustrated in Figure 2.11. The orientation of the anthocyanin and copigment molecules is such that hydrophilic attack of the anthocyanin molecule is minimized.

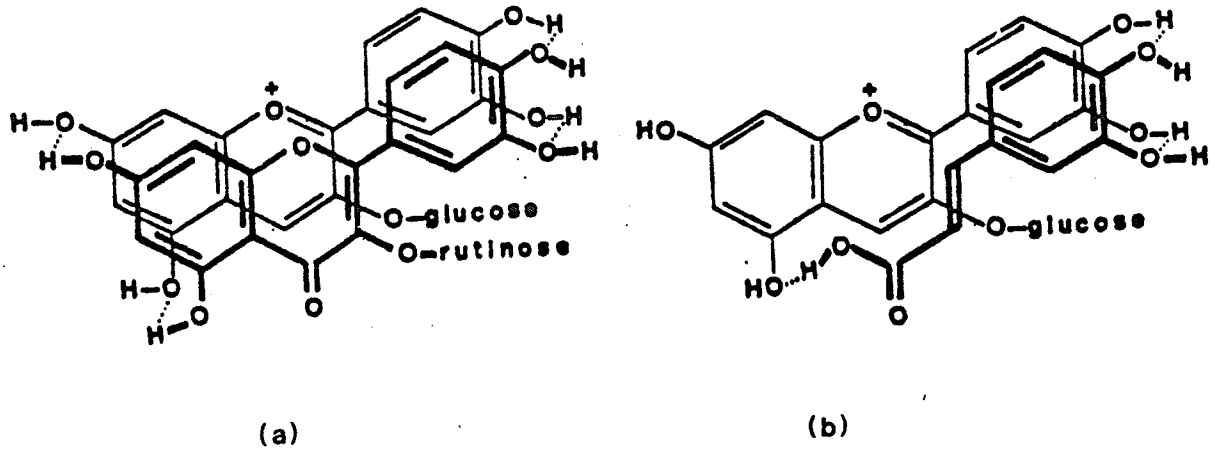
The copigmentation phenomenon has been shown to be sensitive to pH (Brouillard et al., 1989). It has been demonstrated that at a particular pH, an anthocyanin solution exists as a mixture of structural species in a fast equilibrium. Based on the previous discussion on self association, it is relevant to mention that in the presence of copigments, the self associated form of anthocyanins cannot form complexes with copigment compounds, which therefore results in a decrease in the effect of the copigmenting compound (Scheffeldt and Hrazdina, 1978).

In a study of malvidin– and cyanidin–diglucosides, Mazza and Brouillard (1990) demonstrated the copigmentation phenomenon to be most effective at pH values close to 3.6 in the case of chlorogenic acid however, the copigmentation phenomenon is not restricted to acidic solutions. Although the copigmentation effect has been shown to decrease under alkaline conditions, it is the structure of the copigment and the degree of affinity for the predominant pigment structure which governs the magnitude of the copigmentation effect. Brouillard et al. (in press) have shown that in the case of caffeine, a relatively large copigmentation effect is demonstrated at alkaline pH. It is important to note however, that the mechanism by which copigmentation has been shown to

Table 2.01 Copigmentation of Cyanidin 3,5-diglucoside (2 X 10^{-3}M) at pH 3.32 (from Asen et al., 1972).

Co-pigments (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				
Phloridzin	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 (isoquercitrin)	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.



(a) (b)

Figure 2.11 Anthocyanin–rutin complex (a) and anthocyanin–caffeic acid complex (b) (from Maccarone et al., 1985).

augment color in acidic solutions is not the same under conditions of alkali pH.

2.3.2.2 Experimental Assessment of the Copigmentation Phenomenon

The magnitude of the copigmentation phenomenon can be measured experimentally by spectrophotometry based on the parameter $((A-A_0)/A_0)$ where A and A_0 represent the absorbance of anthocyanin solutions at constant wavelength in the presence and absence of copigment, respectively. A plot of $\ln((A-A_0)/A_0)$ vs $\ln [CP]_0$ where CP_0 is the analytical concentration of the copigment, results in a straight line, the slope of which is equal to the stoichiometric constant, n. Determination of "n" provides a means of evaluating the molecular association between the pigment and copigment (Mazza and Brouillard, 1990). In a study examining the copigmentation phenomenon between the anthocyanins cyanidin– and malvidin–diglucoside, and chlorogenic acid, the stoichiometric constant was found to approximate 1.00 (Figure 2.12) indicating the association between chlorogenic acid and the diglucosides occurs in a 1:1 molar ratio (Mazza and Brouillard, 1990).

2.3.2.3 Mechanism of Copigmentation

A proposed mechanism by which copigmentation results in color augmentation was put forth by Brouillard and colleagues in 1989. It is felt by this group that the molecular interaction of pigment and copigment results in the reduction of the hydration reaction and an increased level of flavylium cation (AH^+) is in the complex. It corresponds to a protection of the flavylium nucleus against the nucleophilic attack of water at C2. Contrary to previous reports in the literature (Chen and Hrazdina, 1981), which ascribe copigmentation to hydrogen bonding between the carbonyl group of the anthocyanin anhydrobases and the aromatic hydroxyl group of flavonoids, Brouillard et al. (1989) attribute the copigmentation phenomenon predominantly to hydrophobic interactions.

The chemical mechanism of plant pigmentation has been described as a two stage decoloration and color stabilizing chemical mechanism (Brouillard et al., 1990). The first step corresponds to an almost complete loss of color by the anthocyanin present primarily in colorless structures. The second step, which involves the copigment molecule, permits a full recovery of the anthocyanin color. The first step has been shown to be a prerequisite to a full expression of plant

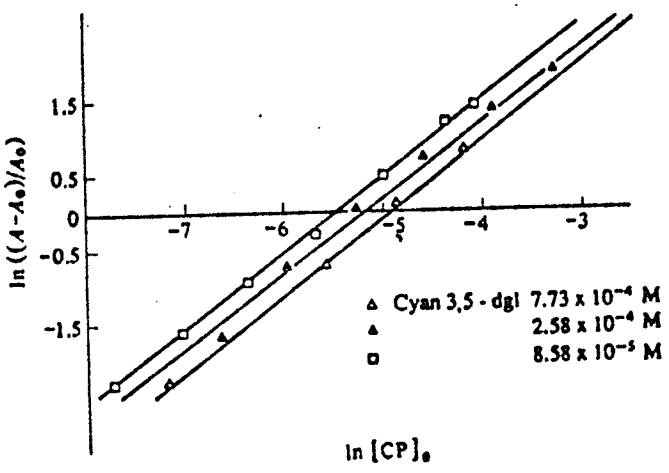


Figure 2.12 Plot of $\ln((A-A_o)/A_o)$ vs $\ln[Cp]_o$ for 7.73 X 10^{-4}M, 2.58 X 10^{-4}M, and 8.58 X 10^{-4}M cyanin solutions. Solvent: aqeous H_3PO_4-NaOAc buffer; I=0.20M; l=1cm; T=20°C; pH 3.65 (from Mazza and Brouillard, 1990).

pigmentation due to flavonoids. pH has been found to be an important factor in the production of color in vivo as it is known that the anthocyanins usually exist in stable colorless forms under the physicochemical conditions of the vacuole. The loss of color has been shown to be related to the presence of water which is the natural solvent of all flavonoids. The interaction of the copigment molecule with the colored structure of the anthocyanin, reduces the susceptibility of the anthocyanin to hydration and results in the production of new colors by a change in the chemical environment in which the pigment occurs (Brouillard et al., 1990).

Within the literature, there is some controversy as to whether the copigment stabilizes the flavylium cation or the quinonoidal base (Scheffeldt and Hrazdina, 1978; Timberlake, 1980; Brouillard et al., 1989). A recent report in this area by Brouillard and colleagues (1989), suggests that the flavylium cation is stabilized at low pH whereas the quinonoidal base is stabilized near neutrality. Hence, both forms are stabilized, but not in the same manner. Simply, it is known that the flavylium cation hydrates, whereas the quinonoidal base does not (Brouillard et al., 1989). Thus, the flavylium cation is stabilized by its interaction with the copigment such that hydration is prevented. It has been suggested that in the case of chlorogenic acid copigmentation, it is the stabilization of the AH^+ cation rather than the neutral base A which accounts for the magnitude of the copigmentation (Mazza and Brouillard, 1990), consequently, more flavylium ions are present at a given pH value.

The flavylium cation, when unprotected will strongly hydrate to a mixture of hemiacetal and chalcones. However, a reverse effect is observed in the case of caffeine, where the copigmentation effect is largely due to the association of the copigment with neutral base A, rather than the flavylium cation, AH^+ (Brouillard et al., in press). Similarly, in studies evaluating the effectiveness of rutin as a copigment, it has been shown that the rutin effectively stabilizes the quinonoidal bases of anthocyanins by hydrogen bonds between the keto group of anthocyanins in the 7 or 4 position by either the 7 or 3',4' hydroxyl group of rutin (Williams and Hrazdina, 1979; Maccarone et al., 1985) (Figure 2.11).

2.3.2.4 Copigmentation in Relation to Water Structure

It has been concluded that it is the hydrogen bonded molecular structure of liquid water which governs the non-covalent association between the copigment and the anthocyanin (Brouillard et al., 1989). The structure of liquid water is thought to be a random, three dimensional network

of hydrogen bonded water molecules arranged in a tetrahedral geometry (Stillinger, 1980). Due to the lattice structure of water, the anthocyanin and copigment are forced into close contact by hydrophobic interactions. Thus, the association of copigment and anthocyanins appears to be a solute–solvent interaction characteristic of aqueous solutions only (Brouillard et al., 1989).

Increasing the temperature has been shown to lead to a drastic weakening of the association of the flavylium cation and chlorogenic acid (Brouillard et al., 1989; Mazza and Brouillard, 1990; Cai et al., 1990). It has been concluded that the effect of temperature is only indirect, in that the association of pigment and copigment does not depend directly on the temperature but on the disruption of the structure of liquid water at higher temperature (Brouillard et al., 1989). It is the structure breaking effect of increasing temperature on the tetrahedral network of liquid water (Stillinger, 1980) that weakens the copigmentation complex. Copigmentation does not occur at temperatures near or above 100^0C (Mazza and Brouillard, 1990). Since the pigment:copigment complex is under the control of the hydrogen–bonded molecular structure of liquid water (Brouillard et al., 1989), a resultant decrease in the copigmentation effect is observed. Similarly, a change in the polarity of the solvent influences the structure of liquid water and consequently affects the effectiveness of the copigment complex. As the polarity is decreased (by addition of an alcohol), the structure of liquid water becomes disordered (Stillinger, 1980) and the pigment:copigment complex is weakened (Brouillard et al., 1989; Mazza and Brouillard, 1990).

2.3.2.5 Effect of Acylation on Copigmentation

In studies evaluating the effect of acyl group substitution on the copigmentation phenomenon occurring between the acylated anthocyanins, awobanin and tibouchinin and the flavone, flavycommelin, Hoshino et al. (1980) found that at 5.0 X 10^{-4}M and pH 6.0, the presence of acylation increased the strength of the complex formed and suppressed its dissociation. In comparison to complex formation with the corresponding deacylated anthocyanins at concentrations of 5.0 X 10^{-4}M and 5.0 X 10^{-3}M, it was shown that the unacylated complex is much more dependent upon anthocyanin concentration than the acylated anthocyanin complex.

Similar to the stacking mechanism put forth to explain the self association phenomenon, the complex formation between acylated anthocyanin and flavone copigment molecules has been described as a stacked structure whereby the aromatic rings of the pigment and copigment are forced together by hydrophobic interactions (Goto et al., 1979; 1986; Cai et al., 1990). The

stabilizing effect of the acyl moiety on complex formation has been attributed to a tightening of the bonding between the two flavonoid units, as a result of the hydrophobic nature of the acylglucose moiety at the 3–position of the anthocyanidin (Asen et al., 1977; Hoshino et al., 1980). In studies on the exceptional color stability of acylated anthocyanins of blue Hydrangea, Takeda et al. (1990) found that the acyl substituents must have the proper configuration or stereochemistry to bring their aromatic parts into proximity with the pyrilium nucleus, in order to prevent the addition of nucleophiles, especially water.

2.4 Monarda as a Source of Anthocyanins

Monarda, named after Nicholas Monardes, a 16th century Spanish physician who published on medical plant products, is widely distributed throughout North America (Marshall and Scora, 1972). Monarda is fairly closely related to Mentha or mint, and Salvia or sage, and has been commonly utilized by man as garden plants, as food and flavoring agents, and for medicinal purposes due to the antiseptic properties of the essential oil constituents (Scora, 1967). More, commonly known as bee–balm, Monarda is grown for its essential oils and as a winter hardy, drought tolerant ornamental perennial (Chubey, 1982). About 15 species are known (Marshall and Chubey, 1983), although Monarda fistulosa L. predominates in North America.

2.4.1 Plant characteristics

Varieties of Monarda fistulosa L. are very floriferous consisting of several color forms, ranging in color from deep purple to crimson to pink and white blooms and grow approximately 60–80cm in height. Characterized by pink flowers 50–60mm in diameter (Figure 2.13), Marshall's Delight is the primary hybrid which will be investigated in this study. The 'Marshall's Delight' cultivar was developed through a series of open pollination and selection cycles originating from the cultivar 'Souris' (Collicutt, 1989)(Figure 2.14). 'Souris' was selected from a population developed from a cross between Monarda didyma 'Cambridge Scarlet' and Monarda fistulosa var. menthaefolia.

2.4.2 Significance of Monarda Anthocyanins

The reasons for selecting the cultivar 'Marshall's Delight' as a source of anthocyanins for this study are several fold. Firstly, this cultivar has been chosen because of its availability at the Agriculture Canada Research Station, Morden, MB. Secondly, the anthocyanins and other phenolics

Figure 2.13 Marshall's Delight flowers.

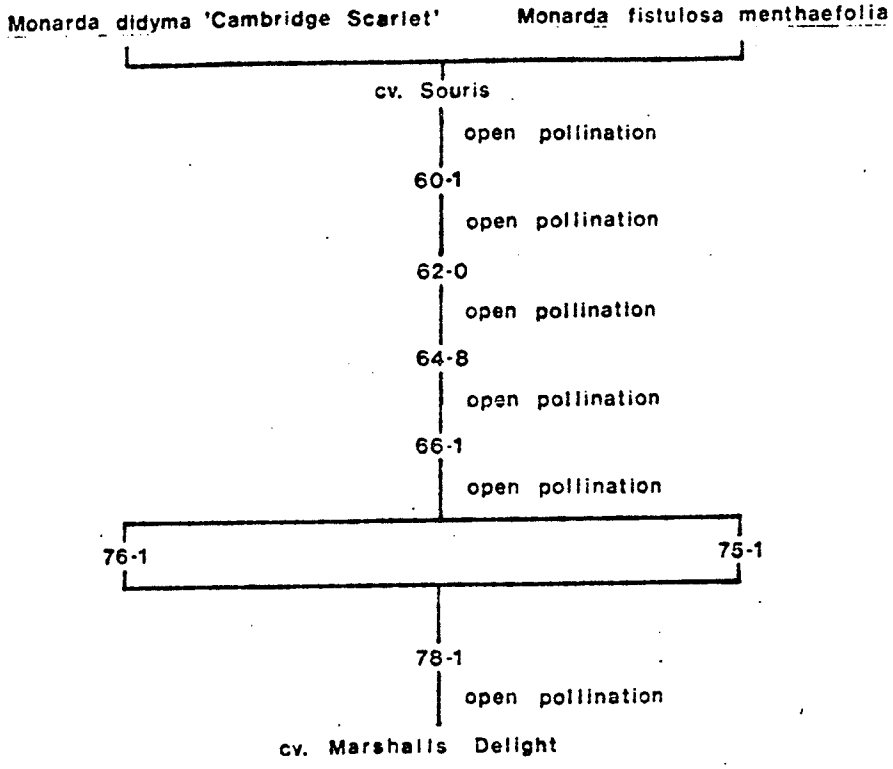


Figure 2.14 Parentage of `Marshalls Delight' Monarda (from Collicut, 1989).

of Monarda fistulosa L. flowers have not been characterized. It is of interest to investigate the anthocyanin composition of 'Marshall's Delight' as it is hypothesized that the flavonoid composition will resemble that of Monarda didyma, particularly by the presence of an acylated anthocyanin, based on the origin of this cultivar. Thirdly, and most significant, it is believed that the major anthocyanins of 'Marshall's Delight' are acylated as the flowers of Monarda didyma have been characterized as containing a polyacylated anthocyanin with coumaric and malonic acid substitution (Figure 2.15) (Harbourne, 1967; Kondo et al., 1985). Since it has been discussed in a previous section that anthocyanins with acyl group substitution exhibit unusual stability (Teh and Francis, 1988; Sapers et al., 1981; Goto et al., 1982; 1983a; Saito, 1985), acylated compounds isolated from 'Marshall's Delight' will be used as means to study the copigmentation phenomenon of acylated compounds. Also, to compare the copigmentation effect of simple and acylated anthocyanins.

2.5 Analytical Techniques Used in the Isolation and Characterization of Anthocyanins

A systematic approach to the qualitative analysis of anthocyanins first involves their extraction, followed by specific purification and identification procedures. Since the study of anthocyanins is not new, the protocol for the extraction, purification and characterization of anthocyanins is fairly well established (Harbourne, 1967; Mabry et al., 1970; Markham, 1982). Recent years has seen the increased use of more sophisticated and efficient means of characterization including such techniques as HPLC, FAB–MS, H^1–NMR and C^{13}–NMR.

2.5.1 Extraction

Since the anthocyanin pigments are located in the vacuoles of the pigmented cells in most fruits, flowers, leaves and vegetables, the extraction procedure generally involves the use of acidic solvents. The solvent acts to denature the membranes of cell tissue and simultaneously dissolve the water soluble pigments, which is followed by filtration and concentration under vacuum. The most commonly used solvent is methanol, since its low boiling point allows for easier concentration of the extracted material. Acidification with acetic acid, formic acid or trifluoroacetic acid serves to maintain a low pH, thus stabilizing the anthocyanin. Since it is known that the organic acid substitution of many anthocyanins may include malonic acid, the use of hydrochloric acid is avoided throughout the extraction and characterization phases as these acyl groups are very labile and may be lost under such harsh conditions (Harbourne and Boardley, 1985; Takeda et al.,

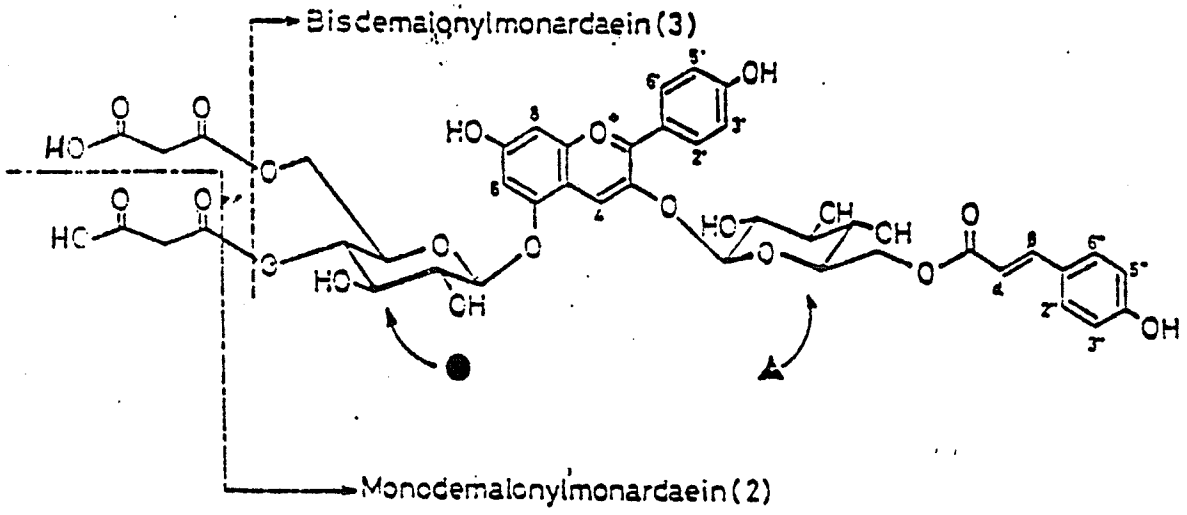


Figure 2.15 Structure of monardaein, isolated from Monarda didyma (from Kondo et al., 1985).

1986). Some researchers (Tamura et al., 1983; Kondo et al., 1985) have avoided the use of methanol on the premise that esterification of the free carboxyl group of the acid may promote hydrolysis of the other end of the acid.

Following extraction, the anthocyanin extract is concentrated under vacuum with the temperature maintained at or below 30^0C to minimize pigment deterioration (Francis, 1982). It is important to concentrate the crude extract to dryness as it is known that acylated pigments will decompose in aqueous solution (Takeda et al., 1986).

2.5.2 Quantification

Quantification of the anthocyanins is typically based on a differential spectrophotometry method which relies on the structural transformations of the pigments and subsequent color changes that occur as a result of changes in the pH of the solution. A commonly used method, established in 1968 by Francis and Fuleki, measures the spectra of anthocyanin solutions in pH 4.5 and 1.0 buffers. The difference in the absorbance between the two pH levels at a defined wavelength gives a measure of the anthocyanin concentration, since the absorbance due to interfering substances is cancelled out in the subtraction. This information, paired with peak area and area percentages of the crude extract obtained by HPLC analysis enables the determination of specific information on the quantity of a particular component.

2.5.3 Separation

A combination of column, paper and high performance liquid chromatography (HPLC) techniques are typically employed to isolate the major components of an extract in order to purify individual peaks for subsequent characterization. Classically, purification of flavonoids has been carried out by basic chromatographic techniques, in particular paper and column chromatography. Depending upon the degree of separation achieved using these techniques individually, a combination of these two techniques may be used in order to achieve separation of the major pigments in the extract. Other methods of separation which have not gained as much popularity or success as column and paper chromatography techniques are paper electrophoresis and thin–layer chromatography.

2.5.3.1 Column Chromatography

Column chromatography is a very useful technique in the preliminary purification of flavonoids or preparative scale separation of a crude extract. A variety of column adsorbant materials have been used for the separation of flavonoids including ion–exchange, polyamide, PVP, silica and Sephadex gels (Wrolstad and Putnam, 1969; Van Teeling et al., 1971; Torre and Barritt, 1977; Andersen, 1989; Pouget et al, 1990). Although all are useful in the separation of flavonoids, they have different applications and characteristics which are relevant depending upon the particular type of compound to be separated. For example, silica is primarily used in the separation of less polar aglycones such as flavones and flavonols, whereas polyamide is suitable for the separation of all flavonoids, although ideal for glycosides (Markham, 1982).

Sephadex gel is widely used in the separation and purification of flavonoid compounds. Sephadex gels are available in two series, G and LH, where the G series separates primarily on the basis of molecular weight. In the LH series, Sephadex LH–20 separates compounds on the basis of both molecular weight and polarity. Specifically designed for use with organic solvents, LH–20 is particularly well suited for final purification of flavonoid aglycones and glycosides.

Depending on the flavonoid profile of the crude extract to be separated, it may be desirable to combine several types of columns based on the selectivities of the various types of matrices available. For example, following the separation of an extract into distinctive bands using a Sephadex matrix, one of the bands may be applied to a second matrix, such as ion exchange. Based on the properties of an ion exchange column, if the extract contains an acylated compound, it can be selectively removed as it is known that an acylated compound would pass straight through the column, while the remainder of the extract would be retained in the usual manner.

2.5.3.2 Paper Chromatography

Paper chromatography has been considered by many researchers to be the most useful technique available for both the separation and identification of flavonoids. Paper chromatography may be used on a preparative scale to separate the major components of a crude extract by application of the extract in the form of a strip, running the entire width of the page, to a paper chromatogram. Following development of the chromatogram in an alcoholic solvent such as BAW (butanol–acetic acid–water; 4:1:5), the separated bands may be cut from the chromatogram and the flavonoid eluted from the strips, using a solvent such as 15% acetic acid. In order to assess the

purity and permit preliminary characterization, the flavonoid may then be subjected to a combination of analytical techniques including paper chromatography, in order to assess spot color and R_f, as well as spectral and HPLC analysis.

2.5.3.3 Final Purification using HPLC

Following preliminary purification by column or paper chromatography techniques, either individually or in combination, the isolated pigment fractions may then subjected to HPLC for final purification. Although time consuming, collection of a peak from the HPLC column outlet provides an excellent means of final purification. Once sufficient quantity of a particular peak is collected from the HPLC, the pure compound may be subjected to a combination of techniques including paper chromatography for assessment of spot color and R_f value, spectral analysis in the presence of shift reagents, and hydrolysis in order to characterize and identify the compound.

2.5.4 Characterization

2.5.4.1 High Performance Liquid Chromatography

In the identification of anthocyanins, spectrophotometry and HPLC are the most widely used techniques as they are rapid and provide accurate identification of these pigments by comparison to standard compounds and established literature identifications. HPLC is a powerful tool in the characterization of anthocyanins, providing fast and sensitive analysis, coupled with the capacity for purification on a preparative scale, as previously discussed.

2.5.4.1.1 Column Matrix

Typically, flavonoid analysis employs reverse–phase HPLC. A reverse phase column is characterized by a hydrocarbon chain of a specific length bonded to the surface of a silica matrix. Various chain lengths are available, ranging from C22 to C1. The retention of a compound by the reverse phase column varies depending on the length of the hydrocarbon chain. Typically, a C18 column (octadecyl chain) is used in flavonoid analysis, although octadecylsilyl (ODS) has been widely used as well (Goto et al., 1982: Kondo et al., 1985; Terahara et al., 1990; Yabuya, 1991). In reverse phase chromatography, the material passing through the column is separated on the basis of a compound's polarity, generally eluting in order of decreasing polarity (Wulf and Nagel, 1978).

Typically the elution profile of flavonoids using a gradient elution profile is such that monoglucosides elute early in the elution profile, followed by aglycones, di-glucosides and finally, acylated anthocyanins (Wulf and Nagel, 1978; Takeda et al., 1986; Velioglu and Mazza, 1991). The gradient normally consists of an acidified water solution combined with increasing amounts of methanol in order to elute the less polar compounds. The use of an acidic solution is important in order to maintain the pigment exclusively in the flavylium structure.

2.5.4.1.2 Detection

The use of photodiode array detection in the analysis of flavonoids has proven useful in the preliminary characterization of compounds. By interfacing the HPLC detector to a computer, it is possible to monitor retention times and spectral data on each of the peaks and compare to authentic standard data, analyzed under identical conditions, enabling preliminary identification. The spectral characteristics of the anthocyanins yield specific information on the nature of the aglycone and the sugar and acyl substitution patterns. The retention characteristics yield information regarding the degree of sugar substitution. The use of dual wavelength detection in the 280 and 500–555nm ranges, has proven useful in the preliminary identification of anthocyanins as it is known that absorbance in the visible region is characteristic of anthocyanins.

2.5.4.2 Paper Chromatography

2.5.4.2.1 R_f Values

The mobility of a pigment in four or more solvents has been considered to be the most important data for identification of a compound (Harbourne, 1967). A compound's mobility on a chromatographic medium such as paper or thin layer, developed in a specific solvent is referred to as its R_f value. The R_f value is defined as the distance travelled by the compound (from the origin) relative to the distance travelled by the solvent front (Markham, 1982). Comparison of R_f values obtained experimentally to literature values obtained under similar conditions of solvent, paper thickness, and temperature is useful in the identification of flavonoid compounds. Based on the relative behavior of a particular compound in several solvents, insight into the B ring oxidation pattern can be obtained (Mabry et al., 1970). Viewing the chromatograms under UV light is often helpful in spot detection.

2.5.4.2.2 Spot Color

Spot color is a valuable means of identifying flavonoid structure. If a spot is not visible under UV light, exposure of the chromatogram to NH_3 vapors will heighten the resolution of the spot. The relationship between spot color and flavonoid structure is well established in the literature (Markham, 1982), both under conditions of UV light and upon exposure to NH_3, as it is known that many flavonoids change color upon exposure to UV light and NH_3 vapor. Typically anthocyanins appear as orange, pink, red or magenta spots under visible and UV light, and upon exposure to NH_3 vapor, the compound changes to a blue color. Conversely, flavones typically exhibit a fluorescent blue color under UV light and turn yellow–green or fluorescent blue–green upon exposure to NH_3.

2.5.4.3 Paper Electrophoresis

Paper electrophoresis provides an excellent means for distinguishing malonated pigments (Cornuz et al., 1981; Harbourne, 1986; Borger and Barz, 1988; Takeda et al., 1986; Harbourne and Boardley, 1985). At pH values above 3, the malonated pigments which are zwitterionic can be clearly distinguished by their movement from the origin in comparison to cationic anthocyanins which exhibit little movement from the origin (Harbourne and Boardley, 1985). In the case of dimalonylation, the pigments will be more mobile, moving twice as far towards the anode as compared to monomalonates when electrophoresed in acetate buffer at pH 4.4.

2.5.4.4 Spectroscopy

UV spectroscopy has proven to be one of the most valuable criterion in the identification of flavonoids. Paired with the use of shift reagents such as sodium acetate, sodium methoxide, aluminum chloride, and boric acid, the position of unsubstituted phenolic hydroxyl groups on the flavonoid nucleus can be established (Markham, 1982). Comparison to spectral data reported in the literature (Harbourne, 1967; Mabry et al., 1970), provides assistance in the identification of the substitution patterns of the unknown flavonoid.

2.5.4.4.1 Shift Reagents

Following measurement of the spectrum in weakly acidified methanol, the flavonoid is subjected

to a variety of shift reagents which are added in a stepwise manner. Depending upon the behavior of the compound upon addition of a shift – either a change in absorption intensity or shift in maximum wavelength absorption – the flavonoid can be characterized as to the hydroxylation and methylation patterns. When a sodium methoxide shift reagent is added to a solution of flavonoid, the principal hydroxylation pattern as well as the presence of unsubstituted acidic hydroxyl groups can be determined. Monitoring the spectrum as a function of time yields information as to the presence of alkali sensitive groupings. The sodium acetate shift reagent is used primarily to detect the presence of a 7–hydroxyl group, on the premise that sodium acetate causes significant ionization of the most acidic of the flavonoid hydroxyl groups (Markham, 1982). Sodium acetate coupled with boric acid results in the bridging of two hydroxyls in an ortho–dihydroxyl group and is used to detect their presence. More commonly used to detect the presence of ortho–dihydroxyl groups, particularly in anthocyanins, $AlCl_3$ forms acid–stable complexes between hydroxyls and neighboring ketones, and acid–labile complexes with ortho–dihydroxyl groups. Upon addition of HCl to the solution, the presence of the acid–stable hydroxy–keto complexes can be detected (Figure 2.16). The interpretation of the spectra upon addition of shift reagents has been well established in the literature by Harbourne (1967) and Mabry et al. (1970).

2.5.4.5 Hydrolysis

After determining the chromatographic mobility and spectral characteristics of an anthocyanin, its structure is normally confirmed by a more destructive technique – hydrolysis. Hydrolysis procedures utilize hot acid to break apart the sugar and aglycone, cold alkali to separate the acyl constituent from the glycosylated aglycone or enzyme treatments.

2.5.4.5.1 Acid Hydrolysis

Typically, hydrolysis involves boiling the flavonoid in 2N HCl for 30 minutes to 1 hour, such that the sugar can be cleaved from the aglycone. Following a series of extractions, the aglycone and sugar can be chromatographed against standards and identified on the basis of their relative mobility in Formic and Forestal solutions and BBPW (butanol–benzene–pyridine–water) and phenol solutions, respectively. Additional techniques which are useful in identification of the portions of the flavonoid molecule following hydrolysis, include thin–layer chromatography, spectroscopy and high performance liquid chromatography techniques.

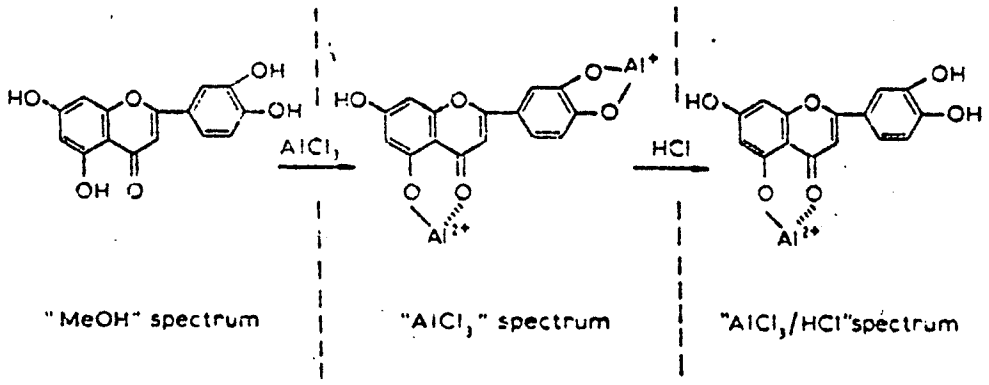


Figure 2.16 Complex formation accounting for the $AlCl_3$ and $AlCl_3$/HCl induced shifts in the spectrum of luteolin (Markham, 1982).

If sufficient quantity of the flavonoid is available, a useful technique for elucidation of the structure is controlled hydrolysis. A similar system is used to that which has been described with samples removed from the boiling water bath at regular intervals such as 0, 1, 2, 4, 6, 8, 12, 16, 20, and 32 minutes. The samples are spotted on paper chromatography and based on the migration of the spots, the intermediate products of hydrolysis can be identified. For example, a monoglucoside will only exhibit two spots on paper chromatography whereas a diglycoside will exhibit up to six spots if it is substituted with two different sugars.

2.5.4.5.2 Alkali Hydrolysis

If the flavonoid compound to be hydrolyzed contains an acyl substituent, a mild alkali hydrolysis procedure is typically employed to aid in the identification of the acyl group. The use of 2N NaOH selectively removes the acyl substituent from the aglycone–sugar moiety which can then be subjected to acid hydrolysis as described previously. The exclusion of oxygen is crucial in the removal of the acyl group as pigments with ortho–dihydroxyl groupings are unstable in alkali media (Francis, 1982). Therefore, upon addition of 2N NaOH, the solution is typically kept under nitrogen for 15–30 minutes prior to extraction of the acyl substituent(s). Following extraction of the acid with ethyl ether, the acid can be compared to standards on either paper or thin–layer chromatography in a number of solvents (BAW, BuN – butanol, ammonium hydroxide) to confirm its identity. Peroxide hydrolysis, which utilizes 3% peroxide and ammonia, specifically hydrolyzes sugars on the C3 position, and it is therefore typically used in the identification of acyl substituents attached to the C3 sugar (Takeda et al., 1986; Terahara et al., 1989). Following removal of the acyl substituent, it is subjected to thin layer chromatography against standards as described previously.

2.5.4.5.3 Enzymatic Hydrolysis

Enzyme hydrolysis may be used to determine a specific linkage between the sugar and the flavonoid. Theoretically, it provides a method with which a specific sugar can be cleaved from an agylcone, however, due to difficulties in obtaining pure enzyme preparations and limited commercial availability of specific enzymes, this type of specificity is difficult to achieve (Markham, 1982). This method of hydrolysis also has limited applicability to flavonoids as acylated sugars and C–glycosides are resistant to enzymic hydrolysis.

CHAPTER 3
CHARACTERIZATION OF ANTHOCYANINS AND OTHER PHENOLICS OF MONARDA FISTULOSA L.

3.1 Introduction

Monarda fistulosa L. or bee balm is an erect perennial native to the Canadian prairies. Plants are very floriferous and produce white, purple, red or pink blooms. While the anthocyanins and other phenolics of Monarda fistulosa have not been characterized, the major anthocyanin of Monarda didyma has been reported as a polyacylated anthocyanin containing coumaric and malonic acids (Kondo et al., 1985). The acylated anthocyanins are of interest as it has been demonstrated that anthocyanins with acyl group substitution exhibit increased stability (Brouillard, 1981; Sapers et al., 1981; Teh and Francis, 1988). Due to this enhanced degree of stability of acylated anthocyanins, it is of interest to investigate the composition of the anthocyanins of Monarda fistulosa L., as it is hypothesized that the flavonoid composition of Monarda fistulosa will resemble that of Monarda didyma.

At the onset of this experiment, a comparison of the phenolic profiles of the different cultivars of Monarda fistulosa L. available at the Morden Agriculture Canada Research Station was of interest. The four cultivars were compared on a qualitative basis with a primary interest in the characterization of a cultivar with a distinctive separation in order to facilitate purification of the primary anthocyanin. In addition, it was hypothesized that comparison of the HPLC flavonoid profiles of the four cultivars of Monarda fistulosa may provide insight into the wide variation in color hues exhibited by the cultivars in vivo.

The colorless flavonoid constituents occurring in conjunction with the anthocyanins of Monarda fistulosa were characterized as it is known that many of the spectacular colors occurring in vivo can be attributed to the presence of copigments (Asen et al, 1972). By characterization of the colorless flavonoids of Monarda fistulosa, coupled with the literature reports of copigmentation in vivo, insight into the compounds responsible for the copigmentation of Monarda fistulosa

anthocyanins may be attained. Subsequent to purification and characterization of the major anthocyanin occurring in Monarda fistulosa, the copigmentation phenomenon of selected anthocyanins and copigments will be studied in a model system.

The objectives of the present study were several fold:

1. to compare the flavonoid profiles of several cultivars of Monarda fistulosa L. by high performance liquid chromatography;
2. to isolate and characterize the anthocyanins and other flavonoids of Monarda fistulosa L. using a combination of spectrophotometry, high performance liquid chromatography, column, paper and thin–layer chromatography techniques; and
3. to quantify the major anthocyanin of Monarda fistulosa L. by a combination of column chromatography and high performance liquid chromatography for subsequent complexation studies.

3.2 Materials

The flower petals of Monarda fistulosa L. plants (hybrids 80–1b red, 80–1b purple, Marshall's Delight), were collected in July of 1990 from a plantation at Agriculture Canada Research Station, Morden, Manitoba, when the plants were in approximately 80% bloom. The petals were picked from the plant and immediately submersed into an insulated cylinder containing liquid nitrogen. The petals of Morden #3 were collected from a 1986 plantation at the same location, freeze dried and stored under identical conditions. The name, purity and sources of the reagents used for this study are listed in Appendix 1.

3.3 Methods

A schematic of the methodology employed for the isolation and characterization of the anthocyanins and colorless flavonoids of Monarda fistulosa L. cv. Marshall's Delight is presented in Appendix 2.

3.3.1 Moisture Determination

Petals were drained of liquid nitrogen and stored at -20^0C until freeze–drying (approximately 14 days). The weight of the petals was recorded immediately prior to placing in the freeze–drier (Labconco Freeze Dry–5). The petals were freeze–dried at -50^0C for five days, removed, weighed and freeze–dried for an additional 24 hours to ensure that the petals were completely dry. The petals were stored in plastic bags at -20^0C in the dark to minimize anthocyanin degradation. The moisture content was expressed as a percent of the original weight of the petals (Appendix 3).

3.3.2 Extraction

Freeze–dried petals (5g, 20g fresh weight equivalent) were extracted at room temperature, overnight in the dark by continuously stirring in 400 ml of methanol–acetic acid–water (10:1:9 MAW). The extract was filtered under vacuum through a Whatman No.2 filterpaper and the residue washed with 3 X 100 ml of 10:1:9 MAW. The extract was concentrated to dryness, in a Büchi rotary

evaporator at 30°C. Since malonated pigments may decompose slowly in solution, they were routinely stored at 0°C as solids, following evaporation to dryness. The dried pigment extract was re-dissolved in MAW (10:1:9) just prior to high performance liquid chromatography and filtered (0.45 um Millipore filter). This extract was denoted as MAW crude extract (methanol-acetic acid-water (10:1:9) crude extract).

3.3.3 Total Anthocyanins

Total anthocyanins were determined by the pH differential method of Fuleki and Francis (1968) and Wrolstad (1976) and calculated based on the extinction coefficient (31,900) of the predominant polyacylated anthocyanin of Monarda in 0.01% HCl methanol (Kondo et al., 1985). Immediately following extraction of the petal pigments, the extract was diluted with the same solvent, resulting in a total volume of 1000 ml of pigment extract. From this volume, two - 10 ml aliquots were removed for total anthocyanin determination.

Each 10ml aliquot of pigment extract was diluted to 50ml using pH 1.0 and 4.5 buffers, as described in Appendix 4. The extracts were diluted such that the absorbance magnitude of the sample at pH 1.0 was in the range of 0.4 - 0.6 absorbance units. The dilution strength of the two samples was identical. The absorbance of each sample was determined using a Beckman DU-50 Spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Div., Irvine, CA). Readings from each sample were done in triplicate and the mean value was used for calculation of total anthocyanin content.

The difference in absorbance between the two samples was calculated as follows:

$$\text{Absorbance} = (A_{510nm}\ \text{pH 1.0} - A_{700nm}\ \text{pH 1.0}) - (A_{510nm}\ \text{pH 4.5} - A_{700nm}\ \text{pH 4.5})$$

The absorbance at 700nm was subtracted from the absorbance at 510nm to correct for any turbidity in the sample. The total anthocyanins were calculated as described in Appendix 5.

3.3.4 Isolation and Purification of Anthocyanins and Other Flavonoids

The concentrated crude extract, after dissolution in MAW, was passed through a Sephadex LH20 column (30 X 800 mm) and eluted with MAW (5:1:14). The separated bands were collected, concentrated to dryness, re-dissolved, and analyzed by HPLC. If further preparative scale purification was necessary, the pigment fractions were applied to a second Sephadex LH20 column and eluted with MAW (5:1:14).

3.3.5 High Performance Liquid Chromatography

The HPLC equipment used for separation and characterization of petal pigments consisted of an LKB liquid chromatograph system (LKB – Produkter, Bromme, Sweden) equipped with a Model 2156 solvent conditioner, two Model 2150 pumps, a Model 2152 controller, and a Rheodine 7125 injector valve with a 100 ul loop, and an Ultropac prepared column (250 X 4.6 mm) of Spheri 10-RP18 (10um), (Brownlee Labs, Santa Clara, CA). An LKB Model 2140 photodiode array detector was used at 190-370nm interfaced with an IBM personal computer and a Canon A-1210 color printer. For detection in the visible range, a Pharmacia Model 2141 Variable Wavelength Detector was used at 280 and 510nm interfaced with an IBM personal computer and a Nelson Data Acquisition Interface.

The following solvent systems and elution profile were used for the separation of flavonoids by HPLC: Solvent A, formic acid-water (5:95 w/v); solvent B, methanol. Elution profile: 0-10 min., 17-22% B (linear gradient); 10-12 min., 22-27% B; 12-33 min., 27-37% B; 33-39 min., 37-55% B; 39-49 min., 55-59% B; 49-54 min., 59-65% B; 54-55 min., 65-17% B (Appendix 6). The injection volume was 100 ul with a solvent flow rate of 1.0 ml/min. and the column pressure was 50-60 bar. All separations were performed at 22 $\pm$ 1^{0}C and all solvents were HPLC grade filtered through a 0.45 um Millipore filter before use. The retention times were calculated with an IBM personal computer equipped with a Wavescan Spectral Detector program (LKB 2140-202). The UV maxima were determined from the spectrum of each HPLC peak displayed on the IBM personal computer equipped with the Wavescan Spectral Detector program. The capacity factor (k') was calculated by the following equation (Kirkland, 1971):

$$k' = (t_r - t_0)/t_0$$

where t_r= retention time of compound, t_0= the time of zero retention measured as the time of

the nonretained solvent peak, k'= capacity factor of compound.

The peak area and area percent were calculated with the same IBM computer equipped with Model 2600 chromatography software, revision 3.1 (Nelson Analytical, Inc., Cupertiono, CA). Fractions of selected HPLC peaks were collected, concentrated under reduced pressure by a rotary evaporator at 30^0C and subjected to subsequent analysis for characterization.

3.3.6 Paper Chromatography

R_f values of the purified pigments were obtained by descending chromatography using Whatman No.1 paper and the following solvent systems (Francis, 1982; Mabry et al., 1970): BAW – n–butanol–glacial acetic acid–water, 4:1:5, upper phase, aged 3 days; Bu–HCl – n–butanol–2N hydrochloric acid, 1:1, upper phase, paper equilibrated 24 hours after spotting and before running, in tank containing aqueous phase of Bu–HCl mixture; 1% HCl –concentrated hydrochloric acid in water 3:97; HOAC–HCl – water–glacial acetic acid – 12N hydrochloric acid, 82:15:3; 15% HOAc –glacial acetic acid–water, 15:85. The preparation of chromatography solvents is detailed in Appendix 7.

3.3.7 Spectral Analysis

Purified anthocyanins were dissolved in 2ml methanolic 0.01% HCl; other flavonoids were dissolved in 2 ml methanol. UV spectrum of the anthocyanins and flavonoids were measured from 200–700nm in a Beckman DU–50 spectrophotometer connected to an Epson RX–80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft–Pak, Beckman Instruments Inc., Scientific Instruments Div., Irvine, CA). For colorless flavonoids, sodium methoxide (NaOMe), sodium acetate (NaOAc) and sodium acetate + boric acid (NaOAc + H_3BO_3) shifts were recorded over the same wavelengths and interpreted as described by Mabry et al. (1970) and Markham (1982). After measurement of the spectrum in methanol, 3 drops of sodium methoxide were added to the cuvette, mixed and the spectrum taken. To allow sufficient time for degradation, the spectrum was measured again following 5 minutes. For the sodium acetate spectrum, powdered NaOAc was added to cuvette such that a 2mm layer formed at the bottom of the cuvette. The cuvette was shaken and the spectrum was recorded. After 5 minutes, the spectrum was measured again to monitor for decomposition. Boric acid was then

added to the cuvette in approximately one half of the quantity of the sodium acetate and the spectrum was measured.

For the anthocyanin solutions, the spectrum was measured in methanolic 0.01% HCl initially and six drops of aluminum chloride ($AlCl_3$) were added to the cuvette and a second spectrum measured. Three drops of hydrochloric acid were then added to the same solution, mixed and a third spectrum measured.

The preparation of shift reagents is detailed in Appendix 8.

3.3.8 Hydrolysis of Flavonoids

Purified flavonoids were subjected to acid hydrolysis as described by Francis (1982). Two ml of 2N HCl were added to 1 to 2 mg of flavonoid dissolved in a minimal amount of methanol. The aglycone and sugar portion of the flavonoid were obtained by heating the pigment–solvent mixture in a water bath at 100^0C for 1 hour. After cooling, the aglycone was extracted with 3 X 2 ml of amyl alcohol and chromatographed against reference standards on Whatman No.1 paper with Forestal (glacial acetic acid–concentrated hydrochloric acid–water, 30:3:10) and Formic acid (formic acid–water–hydrochloric acid, 5:3:2) solvent systems. Prior to paper chromatography, the aqueous solution containing the sugar was washed with 3 X 0.5ml 10% di–n–octylmethylamine in chloroform to remove the acid. A final rinse of 0.5ml chloroform was used to remove all traces of amine, and the sugar solution was subsequently dried under vacuum. The sugar was dissolved in two drops of water and chromatographed on Whatman No.1 paper against reference sugars, arabinose, xylose, galactose, glucose and rhamnose in the solvent BBPW (n–butanol–benzene–pyridine–water, 5:1:3:3). For viewing of sugar spots, the paper chromatograms were dried and sprayed with aniline hydrogen phthalate sugar reagent (Appendix 9). The chromatograms were dried and heated at 105^0C for 3 minutes. The position of the sugar in the flavonoid molecule was determined from spectral shifts (Markham, 1982; Mabry et al., 1970) and by comparing HPLC retention times of Monarda flavonoids with those of authentic compounds analyzed under the same conditions.

3.3.9 Acyl Hydrolysis

Acylated anthocyanins were subjected to cold alkali hydrolysis as described by Terahera et al. (1990a). The acylated anthocyanin was dissolved in 2 ml methanol and 1 ml of 2N NaOH was added. The solution was kept under nitrogen for 30 minutes at room temperature, acidified with 1 ml 2N HCl and taken to dryness under vacuum. The residue was dissolved in methanol and the liberated acid was extracted from the residue by the addition of 1 ml of ethyl ether. The acyl group was chromatographed on Whatman microcrystalline cellulose (250u) TLC plates and detected using glucose aniline (Appendix 9) as described by Takeda et al. (1986). Two solvents, EtOH–H_2O–NH_4OH (16:3:1) and EtOAc–HOAc–H_2O (3:1:1) were used for development of the TLC plates. Following hydrolysis of the acyl group, the deacylated pigment was subjected to HPLC, spectral analyses, and acid hydrolysis as described previously.

3.3.10 Paper Electrophoresis

Electrophoresis was conducted on Whatman No.3 paper strips (2.5 X 35cm) in pH 4.4 acetate buffer (prepared as described in Appendix 4 and adjusted to pH 4.4) for 2 h at 10 v cm^{-1}, 2 mA cm^{-1}. The equipment consisted of a Gelman electrophoresis unit (Gelman Instrument Company, Ann Arbor, Michigan) equipped with power supply (Gelman Model 38200) and an electrophoresis chamber (Gelman Model 51170). The papers were removed, dipped briefly in 1% aqueous HCl, dried and viewed under UV light.

3.4 Results

3.4.1 Varietal Differences

On the basis of the total anthocyanin determinations, the cultivars of Monarda studied were quantitively different (Table 3.01). The moisture contents of the three cultivars range from 80.6 to 83.4% (Table 3.01). The mean moisture content of 'Marshall's Delight' Monarda was 82.5%. Since fresh petals of Morden #3 were not available at the time of freeze-drying and moisture determinations, the moisture content is not reported. The total anthocyanin contents of the four cultivars differ significantly, with the mean values ranging from 58.0-220.4 mg/100g fresh petals. 'Marshall's Delight' Monarda represented the highest anthocyanin content with 214.8 mg/100g fresh petals.

The chromatograms of the crude extracts of the five cultivars of Monarda fistulosa L. presented in Figures 3.01-3.05 reveal qualitative similarities/differences between the cultivars. Quantitatively however, the four cultivars exhibit differences. Tables 3.02 and 3.04 reveal that Morden #3 exhibits a higher proportion of the compound with a retention time (t_R) of 22 minutes, while t_R 29 and 43 are notably lower than the other cultivars examined. The anthocyanin profiles of the four cultivars presented in Tables 3.03 and 3.04 reveal quantitative differences exist. Notable differences are apparent in the extract of the hybrid 80-1b red characterized by the absence of the anthocyanin with a retention time of 29 minutes, which was present in the other extracts, and the large proportion of the total area represented by peak t_R 31 as compared to the other extracts. The hybrid 80-1b red also exhibited the presence of a colorless flavonoid (t_R 37) closely eluting with the major anthocyanin, while the other extracts did not exhibit this peak.

All of the cultivars evaluated exhibited a major anthocyanin peak characterized by an elution time of 39 minutes (Table 3.03 and 3.04), which accounted for 71-88% of the total peak area. Comparison to the elution profile of Monarda didyma cv. 'Cambridge Scarlet', reveals the major anthocyanin is commmon to Monarda didyma and Monarda fistulosa. Based on the similarities in the flavonoid profiles of Monarda didyma and Monarda fistulosa L. cv. 'Marshall's Delight', and the availability of Marshall's Delight as a source of petals, complete characterization of the flavonoid profile of Marshall's Delight was pursued. In addition, comparison of the resolution of the flavonoid profiles of the four Monarda fistulosa cultivars, led to the conclusion that Marshall's Delight would yield the most easily purified source of anthocyanins.

Table 3.01 Average moisture content and total anthocyanin content of Monarda fistulosa L. cultivars (n=3).

Cultivar	Moisture Content (%)	Total Anthocyanin Content (mg/100g fresh petals)
Marshall's Delight	82.5 ± 1.0	220.4 ± 60.9
80-1b (purple)	82.3 ± 1.0	85.8 ± 15.1
80-1b (red)	83.1 ± 0.9	58.0 ± 18.9
Morden #3	-	142.1 ± 26.6

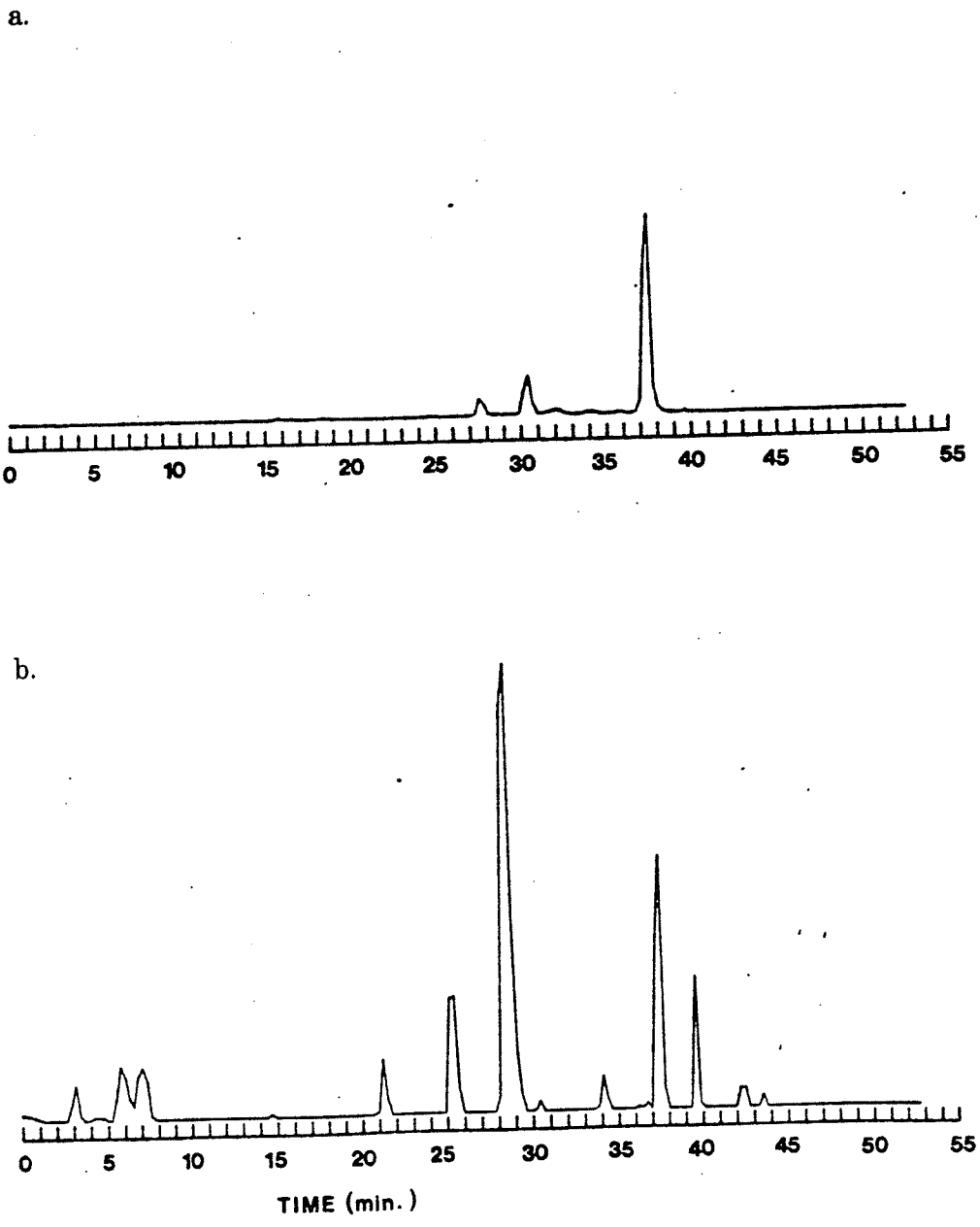


Figure 3.01 HPLC chromatogram of Monarda 80-1b (red) crude extract separated on Spheri 10-RP18, detection at (a) 510 and (b) 280 nm.

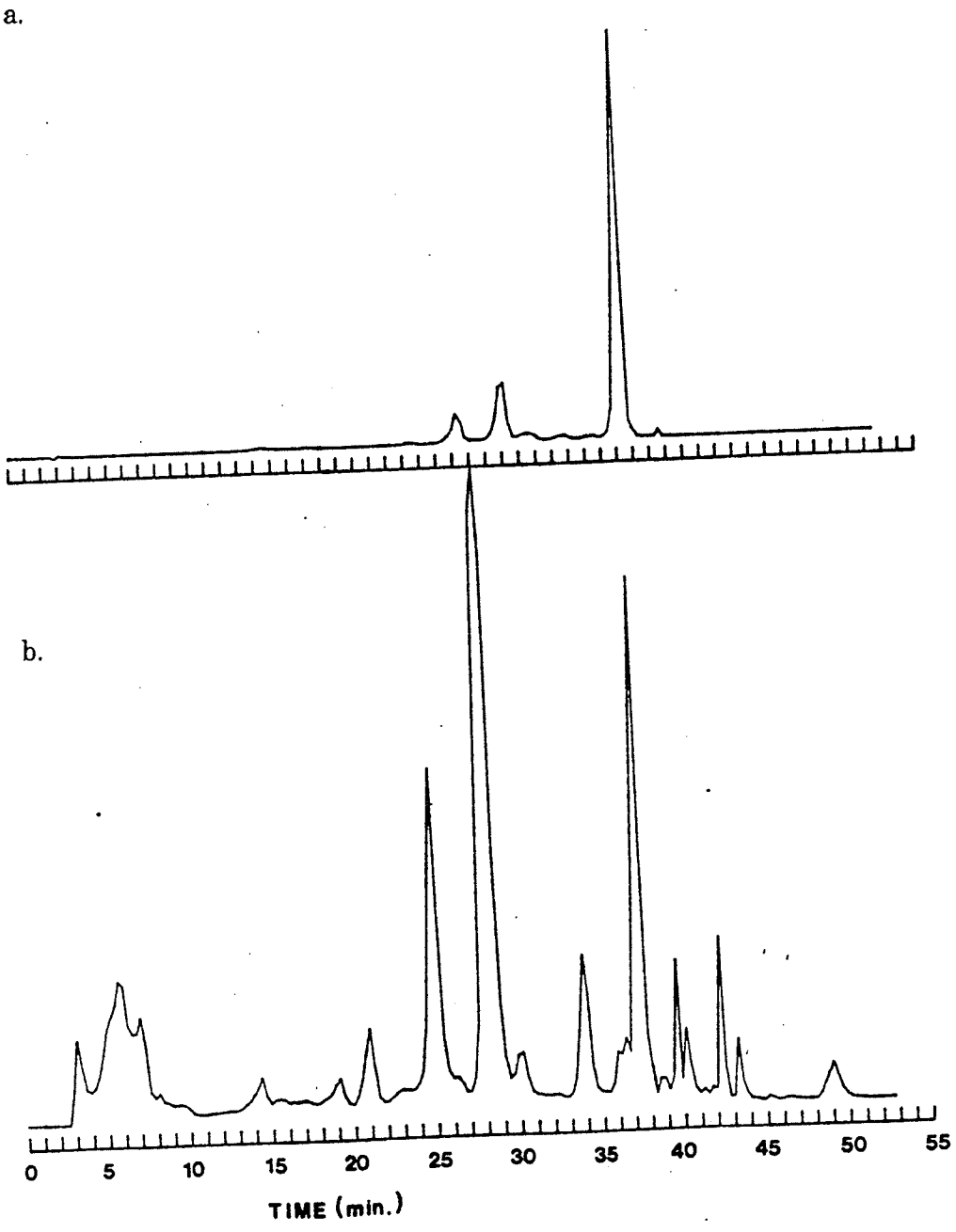


Figure 3.02 HPLC chromatogram of Marshall's Delight crude extract separated on Spheri 10-RP18, detection at (a) 510 and (b)280 nm.

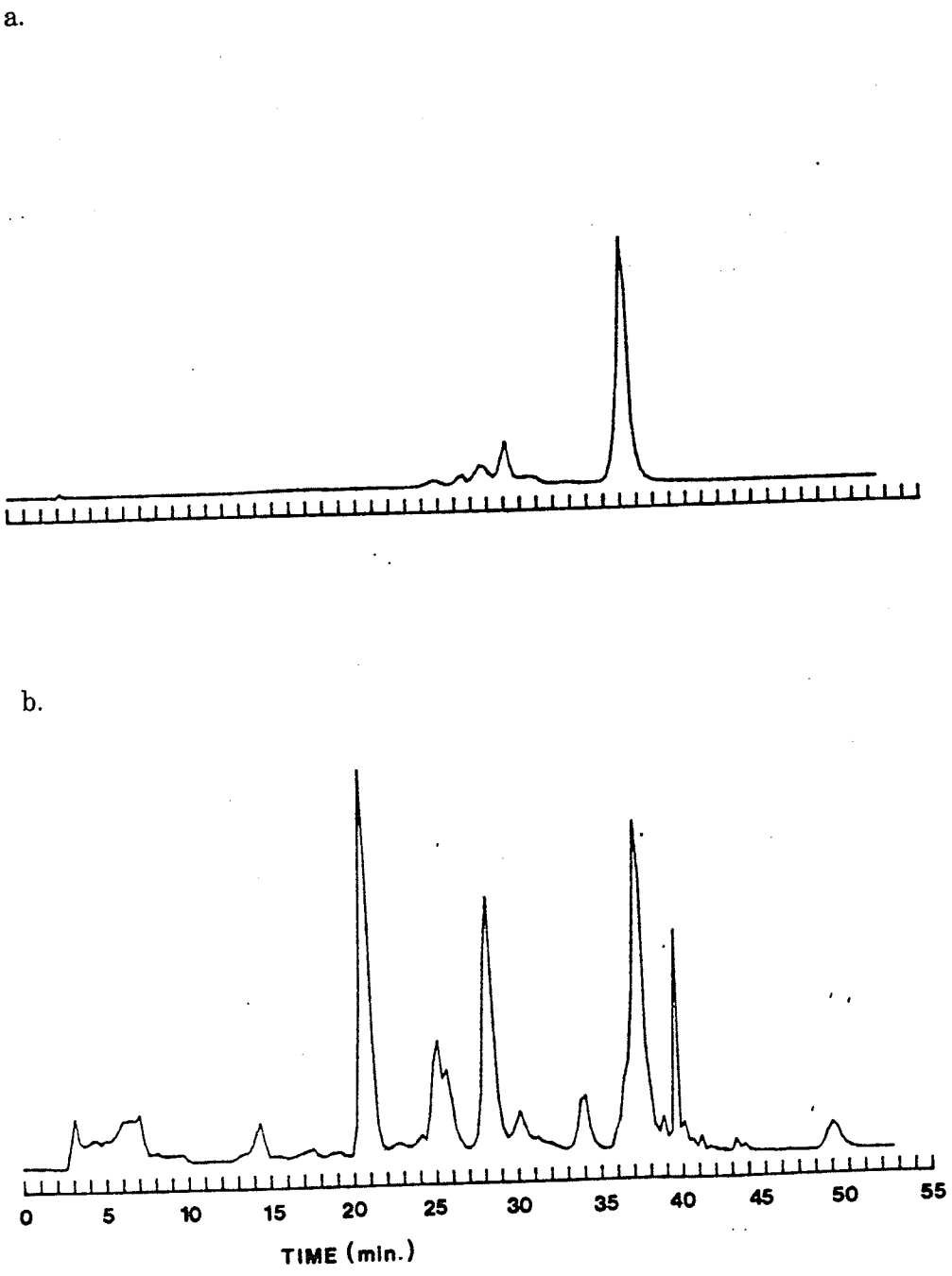


Figure 3.03 HPLC chromatogram of Morden #3 crude extract separated on Spheri 10-RP18, detection at (a) 510 and (b) 280 nm.

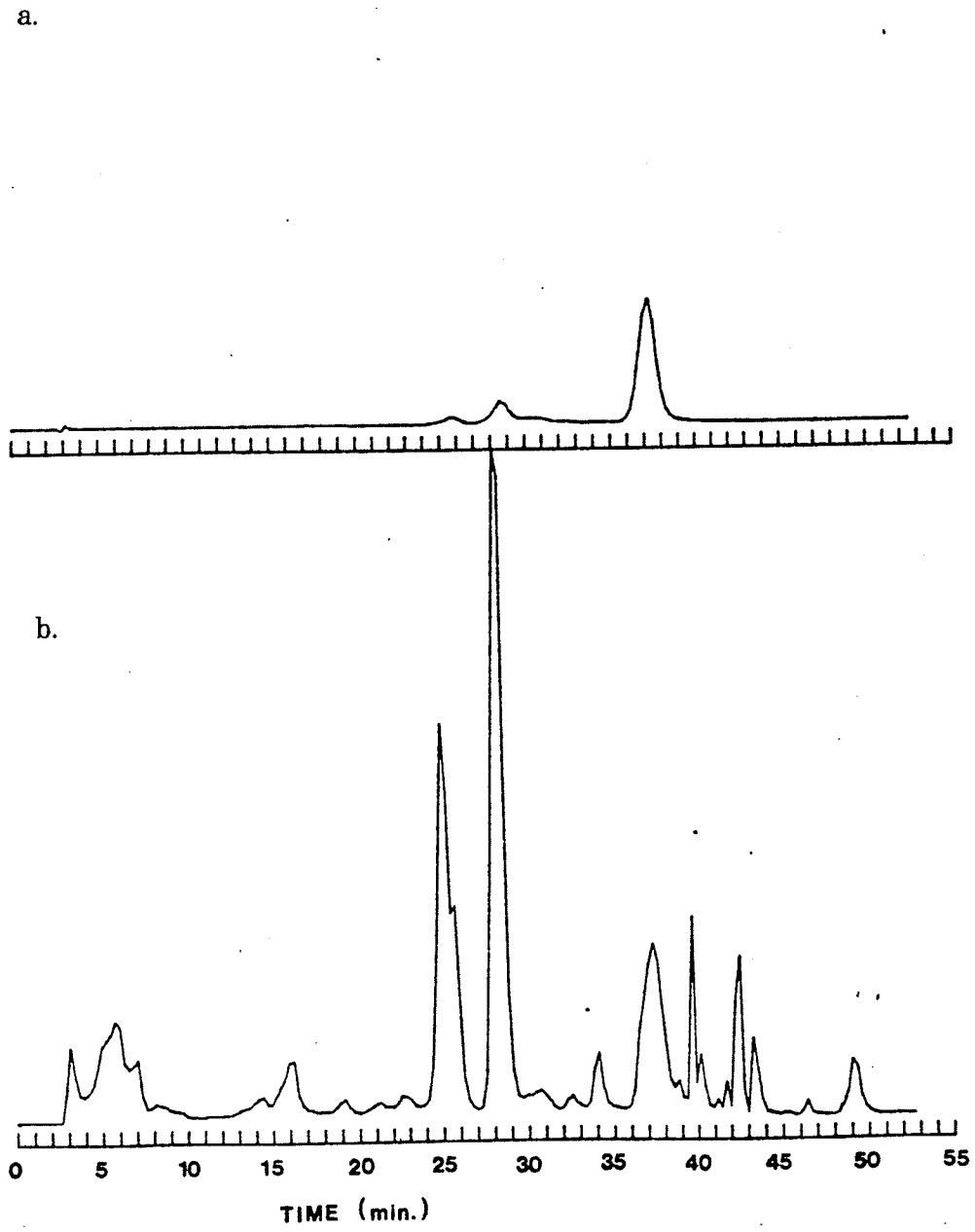


Figure 3.04 HPLC chromatogram of Monarda 80-1b (purple) crude extract separated on Spheri 10-RP18, detection at (a) 510 and (b) 280 nm.

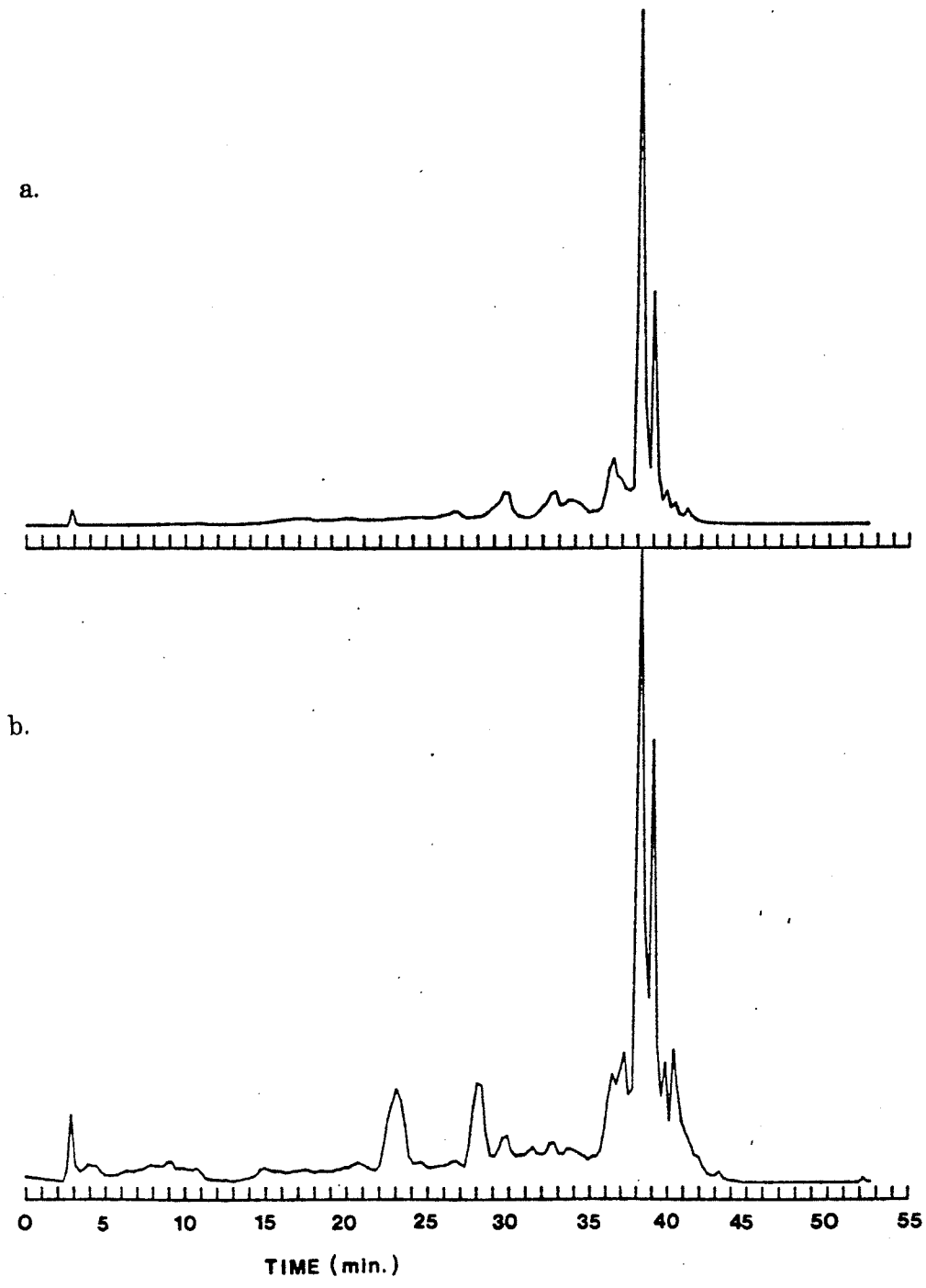


Figure 3.05 HPLC chromatogram of Monarda didyma cv. 'Cambridge Scarlet' crude extract separated on Spheri 10-RP18, detection at (a) 510 and (b) 280 nm.

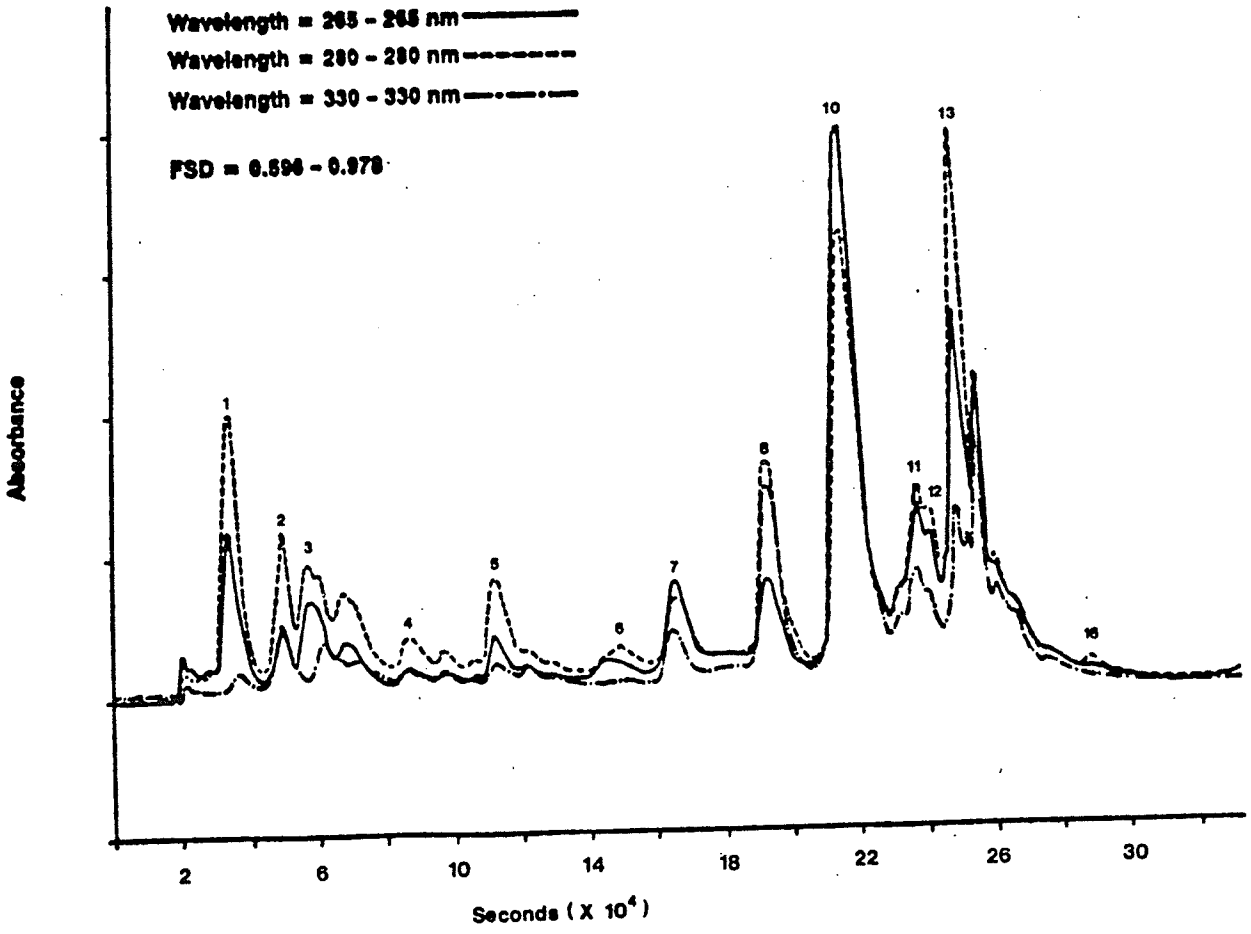


Figure 3.06 HPLC chromatogram of Marshall's Delight crude extract separated on Spheri 10-RP18, detection at 265, 280, and 330 nm.

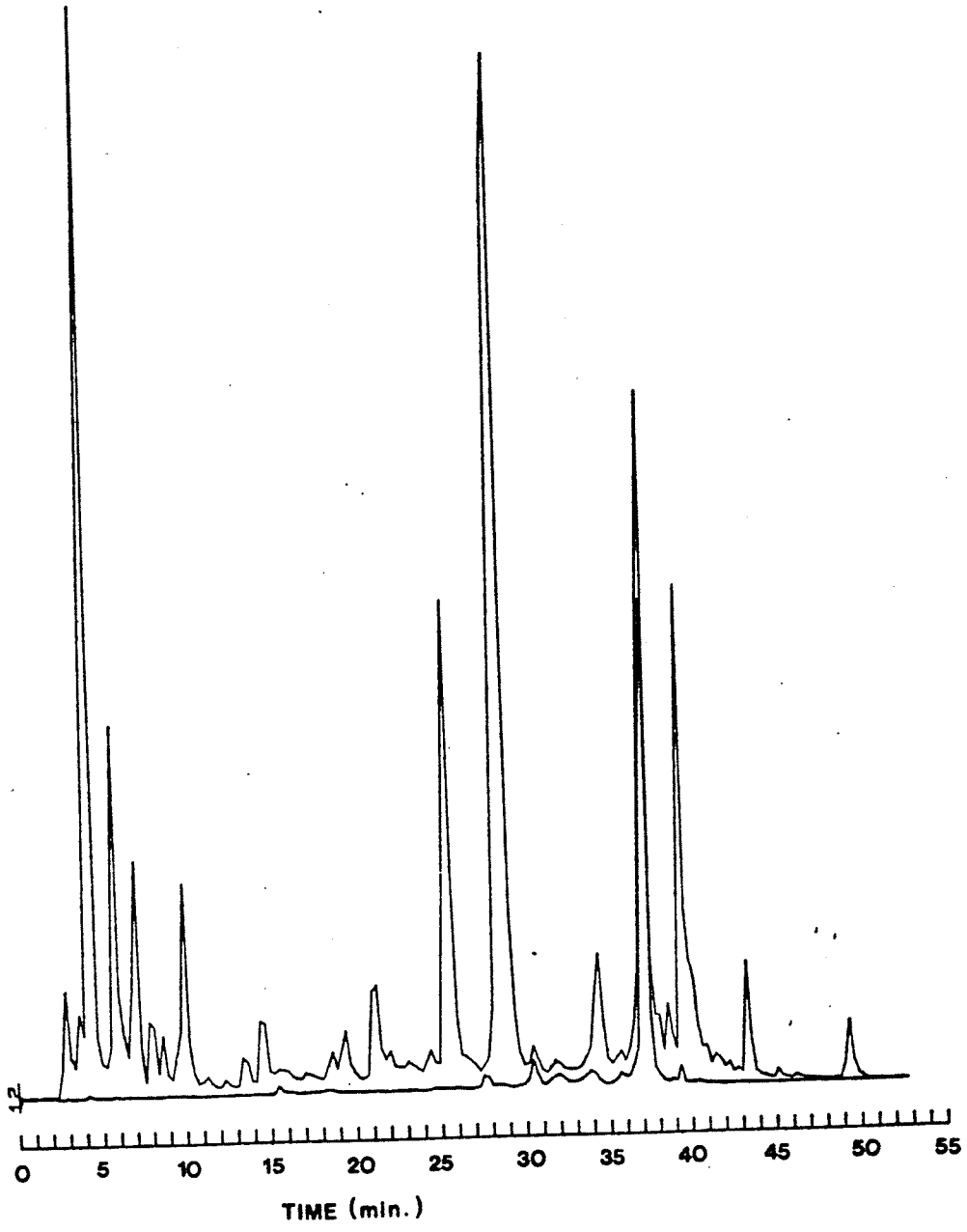


Figure 3.07 HPLC chromatogram of Marshall's Delight crude extract separated on Spheri 10-RP18, superimposed detection at 280 and 510 nm.

Table 3.02 Peak area and area percent of crude extracts of Monarda fistulosa L. cultivars separated on Spheri 10-RP18 at 280 nm detection.

t_R	Morden #3		80-1b red		80-1b purple	
	Peak Area ($X10^4$)	Area %	Peak Area ($X10^4$)	Area %	Peak Area ($X10^4$)	Area %
4.5	2.9	0.5	2.1	0.1	28.0	3.5
6.1	11.8	1.9	8.1	2.8	24.7	3.1
7.4	12.2	1.9	9.9	3.5	20.9	2.6
15.1	13.2	2.2	0.3	0.1	30.2	3.7
22.0	129.9	21.2	9.3	3.3	1.4	0.2
25.8	38.9	6.3	-	-	135.2	16.7
26.2	25.9	4.2	30.8	10.8	51.2	6.3
29.4	100.5	16.4	135.3	47.5	264.6	32.7
31.4	15.0	2.5	1.5	0.5	3.7	0.5
35.4	20.8	3.4	5.7	2.0	18.1	2.2
37.4	3.9	0.7	0.2	0.1	-	-
37.8	-	-	1.3	0.4	-	-
38.6	144.2	23.5	53.3	18.7	57.8	7.2
40.9	34.7	5.7	13.1	5.6	25.3	3.1
43.8	1.3	0.2	6.1	2.1	36.3	4.5
44.9	1.0	0.2	2.7	0.9	16.1	1.9

Table 3.03 Peak area and area percent of crude extracts of Monarda fistulosa L. cultivars separated on Spheri 10-RP18 at 510 nm detection.

t_R	Morden #3		80-1b red		80-1b purple	
	Peak Area ($X10^4$)	Area %	Peak Area ($X10^4$)	Area %	Peak Area ($X10^4$)	Area %
28.6	3.4	2.9	4.4	6.8	0.1	0.1
29.7	5.7	5.0	-	-	6.2	7.9
31.4	7.1	6.3	10.0	15.5	0.2	0.2
38.7	92.8	82.3	46.1	71.0	68.3	87.6

Table 3.04 Retention times (t_R), UV-vis maxima (λmax), capacity factors (k') and relative retention times (α), peak areas and area percents of Marshall's Delight petal pigments on a Spheri 10-RP18 column using methanol-formic acid-water as eluent[a].

Peak No.	t_R	λ max(nm)[b]	k'	α	Peak Area (X 10^5) 280nm	Peak Area (X 10^5) 510nm	Area % 280nm	Area % 510nm
1	4.9	295, 320	0.7		0.7		1.6	
				1.7				
2	6.2	307	1.2		1.0		2.1	
				1.1				
3	6.6	240, 295, 322	1.3		2.4		5.0	
				1.0				
4	8.2	290	1.3		3.2		6.6	
				3.6				
5	16.4	306	4.7		0.9		1.8	
				1.4				
6	22.0	280	6.7		0.4		0.8	
				1.1				
7	24.0	262, 330	7.4		0.7		1.5	
				1.2				
8	29.0	235, 290sh, 325	9.1		5.6		11.6	
				1.1				
9	30.8	279, 313, 513	9.7		0.3	0.3	0.8	2.8
				1.1				
10	32.4	264, 325	10.3		14.7		30.8	
				1.0				
11	34.0	273, 312, 511	10.8		0.9	1.6	1.9	14.9
				1.1				
12	38.1	280, 313, 511	12.3		2.1		4.3	0.2
				1.0				
13	39.3	280, 320, 511	12.7		8.0	8.6	16.7	81.4
				1.1				
14	41.7	280, 315, 511	13.5		3.5	0.1	1.1	

Table 3.04 continued

Peak No.	t_R	λ max(nm)[b]	k'	α	Peak Area (X 10^5) 280nm	Peak Area (X 10^5) 510nm	Area % 280nm	Area % 510nm
15	45.3	265, 325	14.8	1.0	0.9		1.8	
16	46.4	265, 333	15.2		0.3		0.7	

[a] Flow rate, 1.0 ml/min.; t_o=2.87

[b] sh=shoulder

3.4.2 Characterization of Anthocyanins and Other Flavonoids of Marshall's Delight

The HPLC retention data and UV maxima of Marshall's Delight crude extract in methanol–formic acid–water are presented in Table 3.04. A typical HPLC chromatogram of the methanolic petal extract of M. fistulosa cv. 'Marshall's Delight' monitored using photodiode array detection, at 265, 280 and 330 nm, and dual wavelength detection at 280 and 510nm are presented in Figures 3.06 and 3.07, respectively. Ten of the 16 peaks separated in the crude extract of Marshall's Delight, indicated in Figure 3.06 were collected as separate fractions from the HPLC column, concentrated and subsequently analyzed by paper chromatography as described in the methods section. It is known that the principle ultraviolet absorption for anthocyanins is at 270–280 nm while that of hydroxycinnamic acids is at 290–300 nm, and the absorbance of other flavonoids is at 310–370 nm (Appendix 10). Table 3.04 presents the retention times and spectral properties, as determined by computer integration with the detector, and reveals that peaks 1, 2, and 4 and 5 exhibit the spectral characteristics of hydroxycinnamic acids, and peaks 7, 8, 10, 15, and 16 exhibit characteristics of flavones, while peaks 9, 11, 12, 13, and 14 exhibit the spectral characteristics of anthocyanins. From comparison to standard values reported in the literature (Mabry et al., 1970; Kondo et al., 1985; Velioglu and Mazza, 1991), peaks 9, 11, 12, and 13 correspond to acylated pelargonidin glycosides.

3.4.2.1 High Performance Liquid Chromatography Separation

Acylation is known to produce a two fold increase in the retention time of anthocyanins when using reverse phase HPLC (Takeda et al., 1986; Kim, 1989). Comparison of the retention data of peak 13 (Table 3.04) to retention data of pelargonidin 3,5–diglucoside (t_r 15.5 min.) as determined by Velioglu and Mazza (1991) under identical experimental conditions, reveals a 2.5 fold difference in the retention times of pelargonidin 3,5–diglucoside and peak 13. The longer retention times of peaks 11, 12, and 13 compared to pelargonidin 3,5–diglucoside, and the UV–vis absorbance in the 310–335 nm range are indicative of the presence of acyl moieties attached to the anthocyanidin glycosides. These three acylated anthocyanins account for approximately 10% of the total flavonoids. Peak 13 accounted for 81% of the total anthocyanin concentration. Based on the total anthocyanin content determined for Monarda fistulosa L. var. 'Marshall's Delight', the concentration of peak 13 was determined to be approximately 18.5 mg/100g fresh Monarda petals. The concentration was determined based on the molecular weight of monardaein (881.23) as isolated from Monarda didyma by Kondo et al. (1985).

Comparison to selected flowers reveals that Monarda petals contain a slightly higher anthocyanin concentration than roselle (Du and Francis, 1973), but a considerably higher concentration (7 fold) than that of rose petals (Velioglu and Mazza, 1991)(Table 3.05). Although other flower sources containing pelargonidin as the primary anthocyanidin derivative have been characterized in recent years, the total anthocyanin concentrations have not been reported. Of the fruits investigated, the anthocyanin concentration of Monarda is comparable to raspberry (Torre and Barritt, 1977), but approximately 2 fold higher than that of cranberry (Fuleki and Francis, 1968) and Saskatoon berries (Mazza, 1986).

3.4.2.2 Paper Chromatography

Table 3.06 presents the R_f values and colors of 9 peaks before acid hydrolysis. Peaks 1, 2 and 3 all exhibited a fluorescent blue color in UV light before and after exposure to NH_3. The chromatographic color and early elution time from the HPLC column suggests that peaks 1, 2 and 3 are hydroxycinnamic acid derivatives. The principal absorbance in the 290 nm range (Table 3.07) provides further evidence to support this suggestion. The presence of a glucose molecule attached to p-coumaric acid of peak 2 was confirmed by chromatography against sugar standards. Peak 5 had the spectral characteristics of a flavonol-glycoside, however limited quantity prevented complete characterization of this peak.

Peaks 6, 7, 8, 10, and 16 all exhibited fluorescent blue colors when exposed to UV light and changed to fluorescent yellow green or blue green upon exposure to NH_3 (Table 3.06). Based on these colors and color changes, it is suggested that peaks 7, 8, 10 and 16 are glycosylated flavones (Mabry, et al., 1970). Peak 6 appears to be a flavone however, limited quantity prevented complete characterization.

Table 3.05. Total anthocyanin contents of some fruits and flowers reported in the literature.

	Total anthocyanin content (mg/100g)	Source
FLOWERS:		
Rose (Rosa Damascena)	28	Velioglu and Mazza, 1991
Roselle (Hibiscus sabdariffa, L.)	150	Du and Francis, 1973
FRUITS:		
Cowberry (Vaccinium vites idaea L.)	174	Andersen, 1985
Cranberry (Vaccinium oxycoccus L.)	78	Andersen, 1989
Cranberry (Vaccinium macrocarpon Ait.)	101	Fuleki and Francis, 1968
Crowberry - Northern (Empetrum nigrum coll.)	460	Karppa, 1984
- Southern	300-420	Linko et al., 1983
Raspberries (Rubus occidentalis)	213-427	Torre and Barritt, 1977
Saskatoon Berries (Amelanchier alnifolia Nutt.)	86-125	Mazza, 1986
Sunflower Hulls	1216	Mok and Hettiarachchy, 1991

Table 3.06 Chromatographic characteristics of Marshall's Delight petal pigments and colorless flavonoids.

Peak No.	Color[b] in Vis.	UV	UV/NH_3	R_f (X 100)[a] 1% HCl (3:97)	BAW (4:1:5)	H_2O:HoAc:HCl (82:15:3)	BuOH:HCl (1:1)
1	inv.	fl.blue	fl.blue	63	45	74	59
2	inv.	fl.blue	fl.blue/gr.	80	47, 63	65, 78	50, 87
3	inv.	fl.blue	green	72	48	74	76
6	inv.	fl.blue	green & purple	52	20, 51	8, 52	
7	inv.	fl.blue	y.green	39	17	72	28
8	inv.	yellow	fl.blue	20	67		42
10	inv.	fl.blue	green	15	38	49	41
12	inv.	yellow	pink	42	41		30
13	pink	pink	magenta	28	25	65	33
16	inv.	fl.blue		23			26
Standards:							
Apigeninidin	org.	yellow	pink	8	76	38	69
Pg 3,5-diglu	pink	yellow	pink	29	23	11, 60	8

Table 3.06 continued

Peak No.	Vis.	Color[b] in UV	UV/NH_3	1% HCl (3:97)	R_f (X 100)[a] BAW (4:1:5)	H_2O:HoAc:HCl (82:15:3)	BuOH:HCl (1:1)
Quercitin		yellow	yellow		69	4	74
Rutin	inv.	y.green	yellow	31	40	67	42

[a] Whatman No.1 paper
[b] Color: inv. = invisible; fl.blue = fluorescent blue; fl.blue.gr.= fluorescent blue-green; y.green = yellow-green; org. = orange

Table 3.07 Spectral characteristics of Marshall's Delight flavonoids and authentic standards in methanol with the addition of shift reagents.

Peak No.	λmax (nm)	NaOMe Δ Band I	$AlCl_3$ Δ Band I	$AlCl_3$/HCl Δ Band I
1	269, 323sh,374	0		
2	309	+47	0	
3	204, 249, 332	+43	+5	-3
5	291	+ 7, +38*		
7	257, 320, 376	+38	+60	-4
8	267, 331	+57		
10	276, 296, 328, 358	+66		
16	268, 328	+45	+49	+2
Standards:				
Rutin	256, 357	+50	+52	+43
Myricetin	253, 375	+49	+76	+54
Quercitin	254, 306sh, 372	+33	+80	+51
Apigeninidin	265, 423, 482	+55	+48	+45

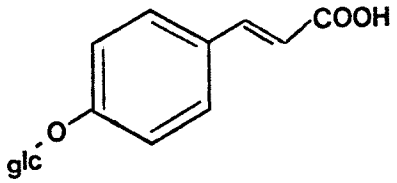
* appearance of new peak at 330 nm upon addition of shift

3.4.2.3 Spectrophotometry

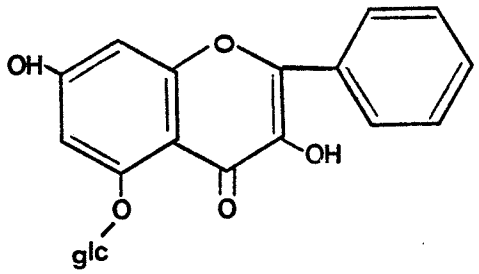
Absorption spectra of the anthocyanins and colorless flavonoids were obtained in the 200 to 700 nm range, and shift reagents were used to elucidate detailed structural information concerning the substitution patterns of the compounds. The use of sodium methoxide as a shift reagent is valuable for the detection of free 3 and 4' hydroxyl groups in flavones and flavonols and the degradation of the spectrum over time is indicative of the presence of alkali sensitive groupings (Mabry et al., 1970). The addition of aluminum chloride and hydrochloric acid shifts yield information concerning the hydroxylation pattern of the B ring whereas sodium acetate is used primarily to detect the presence of a free hydroxyl group on the A ring. Based on the shape, magnitude and stability of the spectrum upon addition of shift reagents, as well as the chromatographic color and R_f values, the chemical structure of the major flavonoids in the pigment extract of Monarda fistulosa petals was determined as shown in Figure 3.08.

3.4.2.3.1 Flavonoids

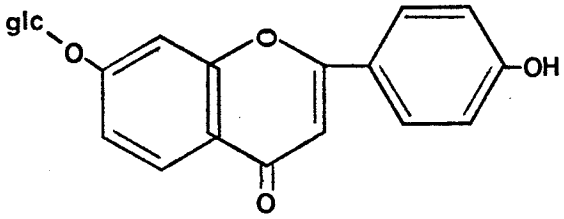
The spectral characteristics of peaks 7, 8, 10 and 16 in the presence of sodium methoxide, (Table 3.07) indicated that these peaks are flavone glycosides with free 4' hydroxyl substitution. The addition of a milder alkali shift, provides evidence for the absence of free 7 hydroxyl groups on peaks 7 and 10, and differentiates the glycosyl site of these three peaks leading to the identification of peaks 7 and 10 as flavone 7–O–glycosides and peak 8 as a dihydroxyflavone 8–C–glucoside. The shift behavior of peak 7 in the presence of boric acid indicates the absence of a dihydroxyl group on the A ring whereas the shift observed in peak 8 indicates the presence of alkali sensitive groups on the A ring. Peak 7 was assigned the structure of flavone 7–O–glycoside based on this information. The absence of a band I shoulder following the addition of sodium acetate provides evidence for a free 7 hydroxyl group on peak 8. Following acid hydrolysis, the UV–vis spectra and the chromatographic behavior of the aglycones of peaks 8 and 10 exhibited close similarity to apigenin, thus confirming the suggestion that these two peaks are apigenin derivatives. Based on this information, peak 8 can be identified as 7,4' dihydroxyflavone–8–C–glucoside and peak 10 as apigenin 7–O–glycoside (Figure 3.08). Based on the magnitude of the shift and the appearance of two new peaks on the spectrum of peak 16 in the presence of $AlCl_3$ and HCl, it is suggested that peak 16 is a 5–hydroxyflavone.



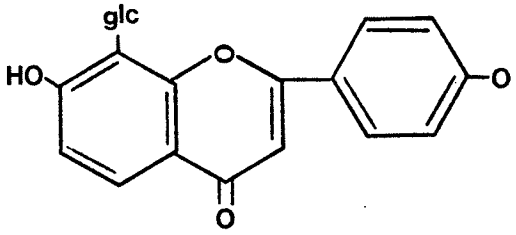
Peak 2: p-Coumaric acid glucoside



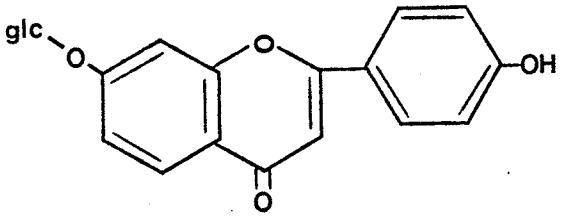
Peak 5: Flavonol-glycoside



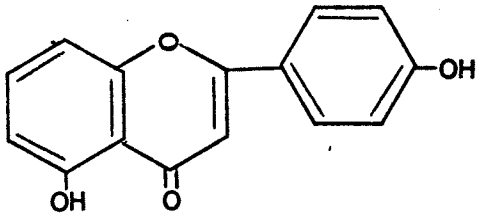
Peak 7: Flavone 7-O-glucoside



Peak 8: 7,4′ Dihydroxyflavone 8-C-glucoside



Peak 10: Apigenin 7-O-glucoside



Peak 16: 5-Hydroxyflavone

Figure 3.08 Structural characteristics of the major flavonoids of Monarda fistulosa L. cv. 'Marshall's Delight'.

3.4.2.3.2 Anthocyanins

Peaks 12 and 13 exhibited yellow and pink spots, when viewed in UV light and pink spots following exposure to NH_3 (Table 3.06). According to Markham (1982), these colors and color changes are indicative of anthocyanidin 3,5–diglucosides. The presence of aromatic organic acids attached to peaks 12 and 13 is evident by comparison of R_f values in four solvents to the literature values (Harborne, 1967; Takeda et al., 1986). The greater mobility of these two peaks as compared to the pelargonidin 3,5–diglucoside standard confirms the presence of acyl groups in these three compounds (Table 3.06). Characterization of peaks 9, 11 and 14 was not complete as these compounds could not be adequately separated from co–eluting colorless flavonoids (Figure 3.07). Nonetheless, their chromatographic and spectral characteristics indicate that they are acylated pelargonidin glycosides.

Detailed structural information regarding peaks 12 and 13 can be obtained from the examination of the UV–vis spectral data. Examination of the UV–vis spectra of these peaks reveal a peak absorbance in the 280 nm range, λ max at 506 nm, an absorption peak at 306–311 nm and the lack of a distinct shoulder in the 410–440 nm range, which are characteristic of acylated anthocyanins with a sugar residue at both the 3–and 5–OH positions (Harborne, 1967) (Table 3.08; Figure 3.09). Since these three anthocyanin pigments did not produce a spectral shift upon addition of $AlCl_3$, the absence of free ortho–hydroxyl groups on the B ring is evident. From the similarities in retention times, spectral data, and chromatographic behavior both before and after hydrolysis it is suggested that peaks 12 and 13 are both pelargonidin 3,5–diglycosides acylated to different degrees (Figure 3.10). The strong intensity of the UV absorbance of peak 13 at 306 nm as compared to the lower intensity of the UV absorbance in peak 12, suggests complex polyacylation of peak 13 by phenolic acids (Saito et al., 1985). The different degree of acylation is apparent from comparison of the E_{acyl}/E_{vismax} absorbance ratios (Table 3.08). As is the case with peaks 12 and 13, Saito and co–workers (1985), observed the appearance of a smaller peak closely preceding the major peak, and suggested that this close association may be the result of a hydrolysis reaction (loss of an unstable malonyl group) or an isomer differing in some slight structural detail from the major peak. It is suggested that peak 12 is acylated to a lower degree than peak 13 from the lower $E_{acyl}/E_{vis\ max}$ ratio and the slightly earlier retention times.

Table 3.08 Spectral characteristics of Marshall's Delight anthocyanins in methanolic 0.01% HCl solution.

Peak No.	λ max (nm)	E_{440}/E_{vismax} (%)	E_{358}/E_{vismax} (%)	E_{uvmax}/E_{vismax} (%)	E_{257}/E_{vismax} (%)	E_{acyl}/E_{vismax} (%)
12	273, 310, 507	28	32	128	118	92
13	283, 306, 506	20	13	179	98	144
Standards:						
Pelargonidin	257, 359, 520	82	832	1014	1014	
Pg 3,5-diglu	265, 304, 511	22	31	71	68	

Table 3.09 Chromatographic data for the aglycone and sugar moieties of Marshall delight petal pigments following hydrolysis.

Peak No.		R_f Values (X 100)[a]		Aglycone Color	
	BBPW	Formic	Forestal	UV	UV/NH_3
2	25	71, 88			Fl.blue
3	22				
5	22	86			yl.green
6	29				
7	29	39	70	Fl.blue	Fl.blue
8	29	87			Green
13	28	44	61	Pink/Or.	Magenta
16	28	49	81	Fl.blue	Fl.blue

[a] Whatman No.1 paper

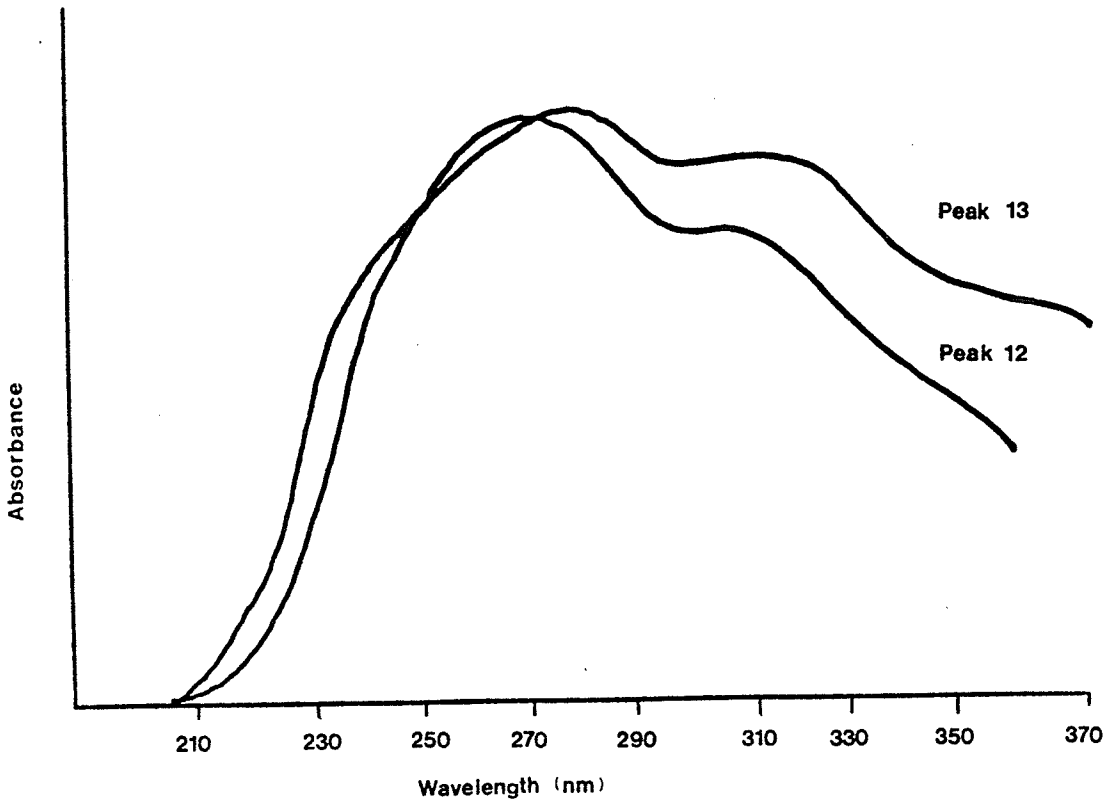


Figure 3.09 Normalized UV photodiode array spectra of peaks 12 and 13 of Marshall's Delight.

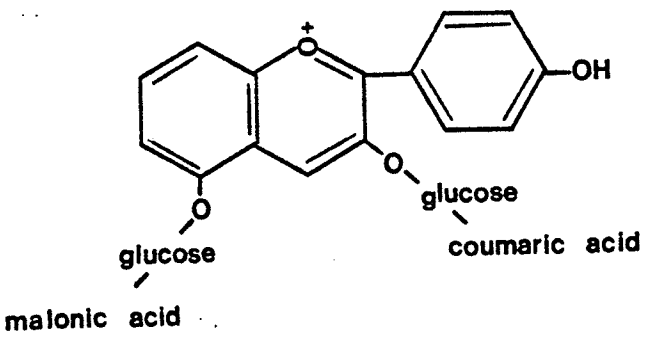


Figure 3.10 Structure of the acylated anthocyanin of Marshalls delight (peak 13).

3.4.2.4 Hydrolysis of Anthocyanins

3.4.2.4.1 Acid Hydrolysis

Following acid hydrolysis of peaks 12 and 13, the aglycones and sugars were confirmed to be pelargonidin and glucose, respectively, by paper (Table 3.09) and thin–layer chromatography techniques. The presence of coumaric acid was confirmed by comparison to authentic standards using TLC, however malonic acid was not detected using this technique. The presence of malonylation was however, confirmed by the electrophoretic mobility of peak 13 toward the anode in pH 4.4 acetate buffer. It has been well established in the literature (Harborne and Boardley, 1985; Takeda et al., 1986) that zwitterionic anthocyanins can be clearly distinguished from cationic anthocyanins by their mobility towards the anode upon electrophoresis.

3.4.2.4.2 Alkali Hydrolysis

It is believed that malonic acid is attached to the C6 of glucose, as this represents the most widely occurring site of acyl attachment (Harborne, 1986; Borger and Barz, 1988). Evidence to support this was obtained by subjecting the pigment to spectral analysis following alkali hydrolysis. Since there was no change in the absorbance, it is evident that the acyl group cleaved from the molecule during alkali hydrolysis was attached to the sugar molecule of the anthocyanin (Markham, 1982).

In comparison to the findings of Kondo et al. (1985) regarding Monarda didyma, it has been established that several acylated forms of pelargonidin diglucoside also exist in Monarda fistulosa which possess similar UV–vis spectra and E_{440}/E_{510} ratios to those of Monardaein isolated from Monarda didyma (Table 3.10).

Table 3.10 Comparison of spectral and chromatographic characteristics of peak 13, monardaein and pelargonidin 3,5-diglucoside.

	Peak 16	Monardaein[a]	Pelargonidin 3,5-diglucoside[b]
UV (0.01% HCL-MeOH)	507,310,287	509,316,287[c]	504, 269
E_{440}/E_{510}	0.20	0.23	0.21

	R_f (X 100)			
	BAW	BuHCl	1% HCl	HoAc:HCl
Peak 12	25	33	28	65
Monarda didyma petals[b]	40	46	19	53

[a] Kondo et al., 1985
[b] Harbourne, 1967
[c] Solvent 3%TFA-MeOH

3.5 Discussion

On the basis of high performance liquid chromatography, paper chromatography, spectral analysis and paper electrophoresis, nine flavonoid compounds were characterized in the Marshall delight Monarda hybrid. The major anthocyanin was identified as pelargonidin 3,5–diglucoside acylated with malonic and coumaric acids. Three other acylated anthocyanins were tentatively identified. Nonanthocyanin phenolic compounds identified included flavone 7–O–glucoside, flavone 8–C–glucoside, flavonol glucoside and hydroxyflavone compounds. The total anthocyanin concentration was 215mg/100g fresh petals with a diacylated pelargonidin 3,5–diglucoside accounting for 17% of the total flavonoids and 81% of the total anthocyanins.

It is believed that the major anthocyanins of Monarda fistulosa L. cv. Marshall's Delight are acylated as a polyacylated anthocyanin containing coumaric and malonic acids has been found in the flowers of Monarda didyma (Kondo et al., 1985). Due to the enhanced degree of stability of acylated anthocyanins, it is of interest to investigate the influence of these di–acylated anthocyanins on the strength and stability of the copigmentation phenomenon and the mechanism by which stabilization occurs. Quantification of the diacylated form of pelargonidin 3,5–diglucoside will provide a unique opportunity to elucidate the copigmentation mechanism in flowers and food products.

CHAPTER 4
COPIGMENTATION AND STABILIZATION OF ANTHOCYANINS IN AQUEOUS SOLUTION

4.1 Introduction

It is well known that the structure of a pigment affects its color in solution. A generally accepted model to describe the expression of anthocyanin color as a function of pH was put forth by Brouillard et al. (1977a,b). Based on this model, it is has been established that anthocyanins exist as a mixture of hemiacetal and colorless chalcone species in solutions approaching neutrality. It is also generally accepted that anthocyanins accumulate in the plant cell vacuole, where the pH ranges from 3.5–5.5 (Asen et al., 1972). Based on the pH of the plant vacuole and the vast array of vivid colors expressed by anthocyanins, it is evident that additional mechanisms exist to account for the broad range of anthocyanin colors in vivo.

A phenomenon which plays a major role in the expression of such a wide range of brilliant colors by anthocyanins in plants is copigmentation, a molecular interaction which occurs between anthocyanins and copigments. A copigment can be described as a molecule which usually has no color by itself, but when added to an anthocyanin solution, it greatly enhances the color of the solution (Asen et al., 1971). The expression of plant color by copigmentation is characterized by an increase in intensity (hyperchromic effect) with a shift in the peak wavelength towards the blue (bathochromic effect). Copigments may be flavonols, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals, and anthocyanins themselves (Asen et al., 1972).

The vast array of pigmentation occurring in vivo can be explained on the basis of differences in the concentration of anthocyanin and copigment, and the type of copigment present (Asen et al., 1972). Other factors which have been shown to influence the copigmentation phenomenon include the chemical structures of both the pigment and copigment, pH of the medium, solvent, and the temperature at which copigmentation occurs (Mazza and Brouillard, 1989; Cai et al., 1990).

In the case of the diglucosides, malvin and cyanin, the copigmentation phenomenon has been shown to be most effective at pH values close to 3.6 using chlorogenic acid (Mazza and Brouillard, 1990) however, the copigmentation phenomenon is not restricted to acidic solutions. Although the copigmentation effect has been shown to decrease under alkaline conditions, it is the structure of the copigment and the degree of affinity for the predominant pigment structure which governs the magnitude of the copigmentation effect. Therefore, in order to further elucidate the mechanism by which the copigmentation phenomenon occurs, the effectiveness of three different phenolic copigments were investigated in the present study. The copigments were individually complexed with solutions of malvin, pelargonidin 3-glucoside, and monardaein, the acylated anthocyanin isolated from Monarda petals as described in the previous chapter.

In studies examining the effect of acyl group substitution on the copigmentation phenomenon occurring between the acylated anthocyanins, awobanin and tibouchinin and the flavone, flavycommelin, Hoshino et al. (1980) found that the presence of acylation increased the strength of the complex formed and suppressed its dissociation. Brouillard (1981) attributed the exceptional color stability of acylated anthocyanins isolated from Zebrina pendula to a high value of the acidity constant and to the total absence of formation of the colorless pseudobase and chalcone.

The copigments used in this study, chlorogenic acid, caffeic acid and rutin were selected on the basis of their availability, solubility and copigmentation effect (Asen et al., 1972). The anthocyanins were selected on the basis of their physico-chemical characteristics, as well as their availability and purity. Due to the difficulty in obtaining a pure commercial source of pelargonidin 3,5-diglucoside, a monoglucoside was used to study the copigmentation phenomenon. It is known that the copigmentation phenomenon is less effective in the case of monoglucoside substituted anthocyanidins, however, since pelargonidin 3-glucoside and monardaein are based on the same aglycone, the monoglucoside was studied such that the influence of the acyl substituents of Monardaein on the copigmentation magnitude and subsequent stability could be assessed.

The objectives of the present study were:

1. to evaluate the effectiveness of three copigments, chlorogenic acid, caffeic acid, and rutin when complexed with malvidin 3,5-diglucoside and pelargonidin 3-glucoside by spectrophotometry;
2. to evaluate the copigmentation effectiveness and subsequent stability of a di-acylated form of pelargonidin (monardaein) complexed with chlorogenic acid, caffeic acid and

rutin;

3. to elucidate the mechanism by which the copigments chlorogenic acid, caffeic acid, and rutin interact with the pigments malvidin–diglucoside, pelargonidin–monoglucoside, and monardaein; and
4. to assess the stability of the pigment:copigment complexes as a function of time and pH.

4.2 Materials

The reagents used for preparation of the buffer system used to study the copigmentation reaction were generally reagent grade, purchased from Fisher Scientific (Winnipeg, MB), J.T. Baker (Phillipsburg, NJ), and Merck and Co., (Montreal, PQ) (Appendix 1). Double distilled water was used to prepare the phosphoric acid and sodium acetate solutions. Chlorogenic acid (Lot no. 397-0668) and caffeic acid (Lot no. 109F3733) were purchased from Sigma (St. Louis, MO). Malvin chloride (Lot no. 1396141) and pelargonidin 3-glucoside chloride (Lot no. 0990797) were purchased from Carl Roth (Karlsruhe). Monardaein was purified from petals of Monarda fistulosa L. as previously described. All pigments and copigments were analyzed by HPLC for assessment of purity.

4.3 Methods

4.3.1 Chlorogenic acid complexation

Pure anthocyanins (pelargonidin 3-glucoside, malvidin 3,5-diglucoside, and monardaein) were dissolved in 1% (0.06M) aqueous phosphoric acid at concentrations of 5.16×10^{-4} to 1.55×10^{-3}M. Immediately after preparation, each solution was thoroughly mixed in the dark at 20^0C for 30-45 minutes, and diluted to half the original concentration by addition of 0.2M aqueous NaOAc. Absorption spectra of buffered solutions with and without copigment were monitored in the visible range from 400-700nm with a Beckman DU-50 spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Div., Irvine, CA). A and Ao absorbance values, representing the absorbance with and without added copigment, were recorded at 525nm. At this wavelength, no interference by the copigment in the absorbance occurs. The spectrophotometer was fitted with a magnetic stirring device (GFS Chemicals, Columbus, OH) and a temperature controlled 1 cm quartz cuvette connected to a 20^0C $\pm$ 1^0C water bath. A 2 ml aliquot of anthocyanin solution was placed in a cuvette and after recording its spectrum, a known weight of chlorogenic acid was added.

Following 15-20 minutes of mixing, the pH of each copigment:pigment solution was adjusted to the desired level and the spectrum of the pigment-copigment was recorded. The pH of each solution of anthocyanin was adjusted to the desired level by injection into the sample cells of a

few microlitres of 10N HCl or 10M NaOH. The pH of the solution was measured directly in the cuvette with a Fisher Accumet pH Meter Model 825 MP and Fisher Accu–pHast combination glass electrode. The pH meter was calibrated using Fisher pH 4.00 and 7.00 standard buffers at room temperature. Detailed procedures used to prepare the buffers and anthocyanin stock solutions are given in Appendix 11 and 12.

4.3.2 Caffeic acid complexation

Due to the low solubility of caffeic acid and rutin in the mildly acidic H_3PO_4–NaOAc solution, it was necessary to dissolve the caffeic acid in the sodium acetate buffer prior to addition to the phosphoric acid anthocyanin solution. The anthocyanin solution was prepared as described in Section 4.3.1, however the solution was not diluted immediately with sodium acetate. A known weight of copigment in twice the required molality was dissolved in 0.2M NaOAc. Once thoroughly dissolved, 1 ml pigment solution in 0.06M H_3PO_4 and 1 ml copigment solution in 0.2M NaOAc were added to the cuvette and mixed thoroughly for 15–20 minutes. The pH was adjusted as previously described.

4.3.3 Rutin complexation

In the case of rutin, a higher degree of solubility was obtained by dissolution of rutin in 0.08M NaOH followed by partial neutralization with 0.01M HCl prior to adding to the 0.06M H_3PO_4 anthocyanin solution. The anthocyanin and copigment solutions were prepared in twice the required molarity and the pH adjusted as described previously. A sample calculation used for the preparation of copigment solutions is presented in Appendix 13.

4.3.4 Color stability of pigment–copigment complexes

The anthocyanin–copigment complexes were prepared and analyzed as described in Sections 4.3.1–4.3.3. Once the solutions had been mixed for 15 minutes and the pH adjusted to the desired level, the spectrum was recorded and this was considered as time zero. The solutions were mixed continually for 1 hour and the spectrum was recorded every 10 minutes. The temperature was kept constant at $20^0C \pm 1^0C$ using the temperature controlled 1 cm quartz cuvette as described in the complexation section. The spectrum was then recorded once every hour for six hours and

at intervals of 24, 48, 72 hours and 5 and 7 days followed by measurements every 7 days for a period of 35 days. Between readings, the samples were stored in amber vials in a Model I–24 Conviron controlled environmental storage chamber (Controlled Environments, Winnipeg, MB), kept in 20^0C.

The stability readings were recorded as relative absorbance at 525nm,

ie., Absorbance 525nm time=n/Absorbance 525nm time=0

4.3.5 Equilibrium Constant (K) Determination

Pure anthocyanins (pelargonidin 3–glucoside, malvidin 3,5–diglucoside, and monardaein) were dissolved in 0.2 M aqueous HCl solution at a concentration of 5.16 X 10^{-4} M as described in Section 4.3.1. Absorption spectra of buffered solutions without copigment and with an excessive amount of copigment were monitored in the visible range from 400–700nm. A and Ao absorbance values, representing the absorbance with and without added copigment, were recorded at 525nm. In the 0.2M aqueous HCl solution, A corresponds to the completely complexed flavylium cation. In order to determine K, r_1 must first be determined where $r_1=A/A_0$ at 525nm. From the value of the intercept of the linear relationship and the value of r_1 at 525nm, K can be estimated according to the following equation:

$$\ln((A-A_0) = \ln(Kr_1) + n \ln[Cp]_0$$

4.3.6 Data Analysis

Stoichiometric constants were determined using Statistical Analysis System (SAS) Version 6.06 (SAS Institute Inc., Cary, NC, USA) and the data was plotted using Harvard Graphics Version 2.30 (SPC Software Publishing Corp., Mountain View, CA, USA).

4.4 Results

4.4.1 Magnitude of copigmentation

4.4.1.1 Influence of copigment structure

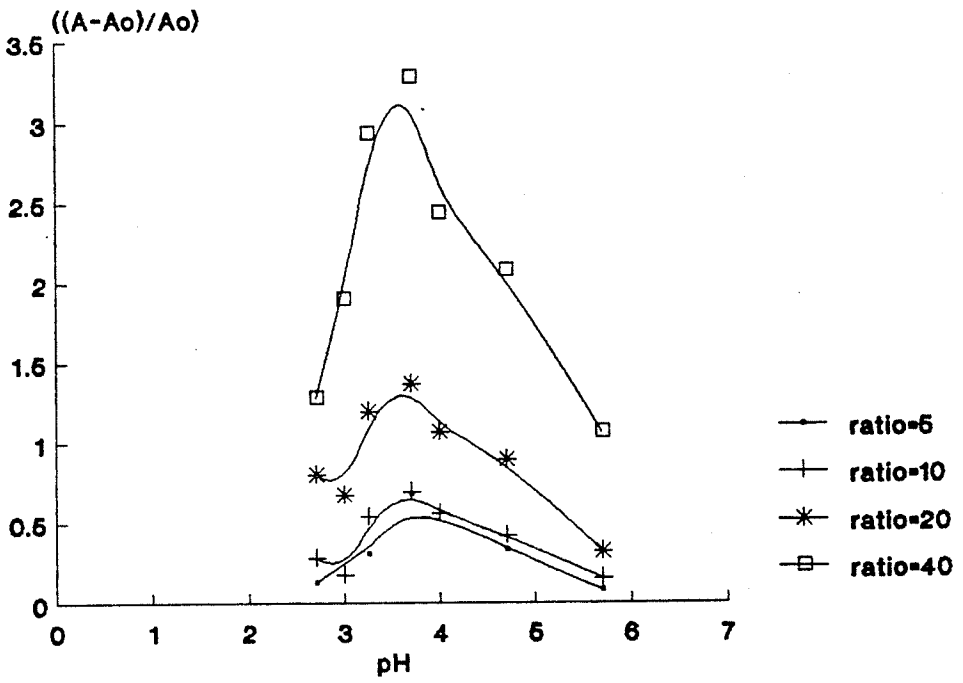
The magnitude of the copigmentation effect has been found to be dependent upon a variety of factors, including the nature of both the pigment and copigment, and can be measured by the parameter $((A-A_0)/A_0)$. Copigmentation data describing the effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at λmax and at 525 nm of pigment solutions are presented in Appendices 14–21. Figures 4.01 – 4.04 illustrate the general trends observed for the eight copigment:pigment complexes studied. At a pigment concentration of 2.58 X 10^{-4}M and a copigment concentration of 5.16 X 10^{-3}M, the chlorogenic and caffeic acid complexes formed with malvin exhibited similar patterns in the copigmentation magnitude over the entire pH range studied (Figure 4.01). However, the pH for the maximum copigmentation effect by the two phenolic acids was different. As summarized in Table 4.01, the chlorogenic acid complexes formed with the three pigments, malvin, pelargonidin 3–glucoside, and monardaein, generally exhibited a maximum copigmentation effect in the pH range 3.2–3.7. Caffeic acid however, exhibited a maximum copigmentation effect at a slightly higher pH range, approximately pH 3.5–4.7. Rutin exhibited differences between the two pigments, malvin and pelargonidin, exhibiting a maximum copigmentation effect at approximately pH 3.5–4.0 for pelargonidin and pH 4.0–5.0 for malvin.

Generally the malvin–caffeic acid and pelargonidin 3–glucoside–caffeic acid complexes exhibited greater copigmentation magnitudes than the chlorogenic acid based complexes (Figures 4.05, 4.06). Likewise, the copigmentation effectiveness of the two copigments was notably different when complexed with monardaein (Figure 4.05b). The caffeic–monardaein complexes exhibited 40–90% greater copigmentation magnitude at pH 2.7–4.7 than the corresponding chlorogenic acid complexes. The copigmentation trends exhibited by pelargonidin 3–glucoside as a function of pH were similar to the complexes formed with monardaein however, the magnitude of copigmentation exhibited by the pelargonidin complexes was considerably lower.

Dramatic increases in the magnitude of copigmentation as a function of pH were observed when rutin was complexed with malvin (Figure 4.06a). At pH 2.7 – 5.7, and a copigment:pigment molar ratio=1.0, the rutin–malvin solutions exhibited copigmentation magnitudes comparable to

Figure 4.01 Effect of pH and copigment concentrations of (a) chlorogenic acid and (b) caffeic acid on the magnitude of copigmentation ((A–Ao)/Ao) of malvin solutions (2.58 X 10^{-4}M)(solvent: aqueous H_3PO_4–NaOAc buffer; l=1cm; ionic strength=0.20M; T=20^0 $\pm$ 0.5^0C).

a.


((A-Ao)/Ao)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
pH
ratio=5
ratio=10
ratio=20
ratio=40

b.

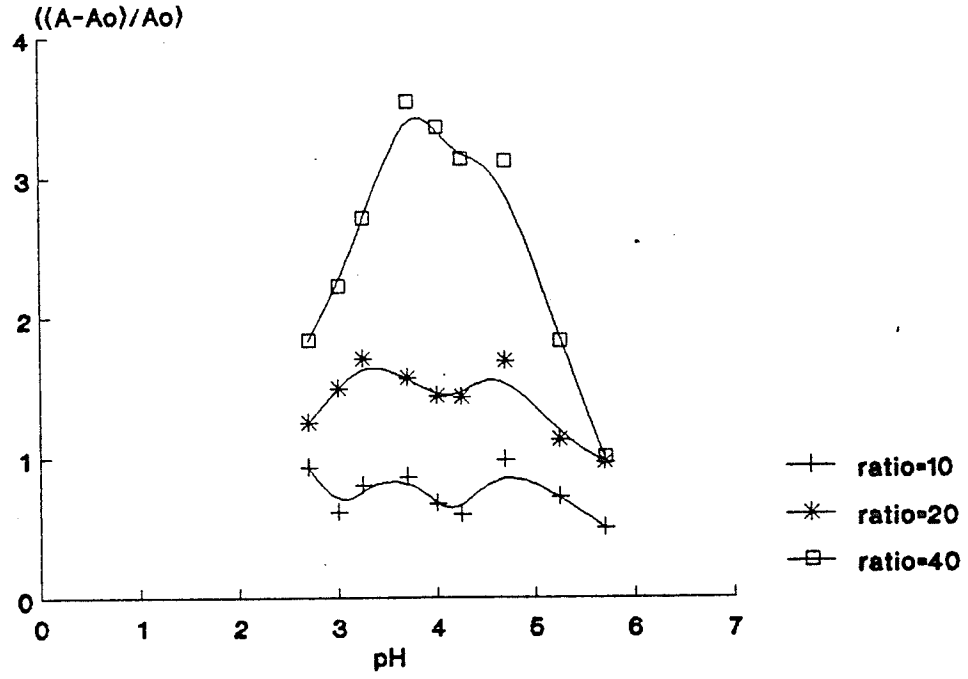
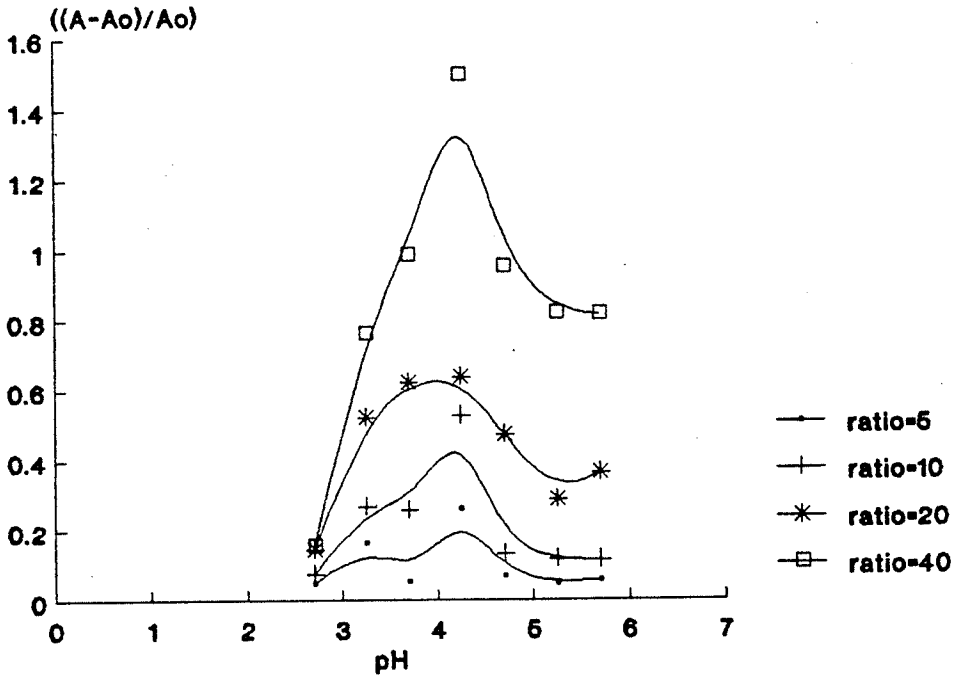

((A-Ao)/Ao)
4
3
2
1
0
0
1
2
3
4
5
6
7
pH
ratio=10
ratio=20
ratio=40

Figure 4.02 Effect of pH and copigment concentrations of (a) chlorogenic acid and (b) caffeic acid on the magnitude of copigmentation ((A–Ao)/Ao) of pelargonidin 3–glucoside solutions (2.58 X 10^{-4}M)(solvent: aqueous H_3PO_4–NaOAc buffer; l=1cm; ionic strength=0.20M; T=20^0 $\pm$ 0.5^0C).

a.


((A-Ao)/Ao)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
pH
ratio=5
ratio=10
ratio=20
ratio=40

b.

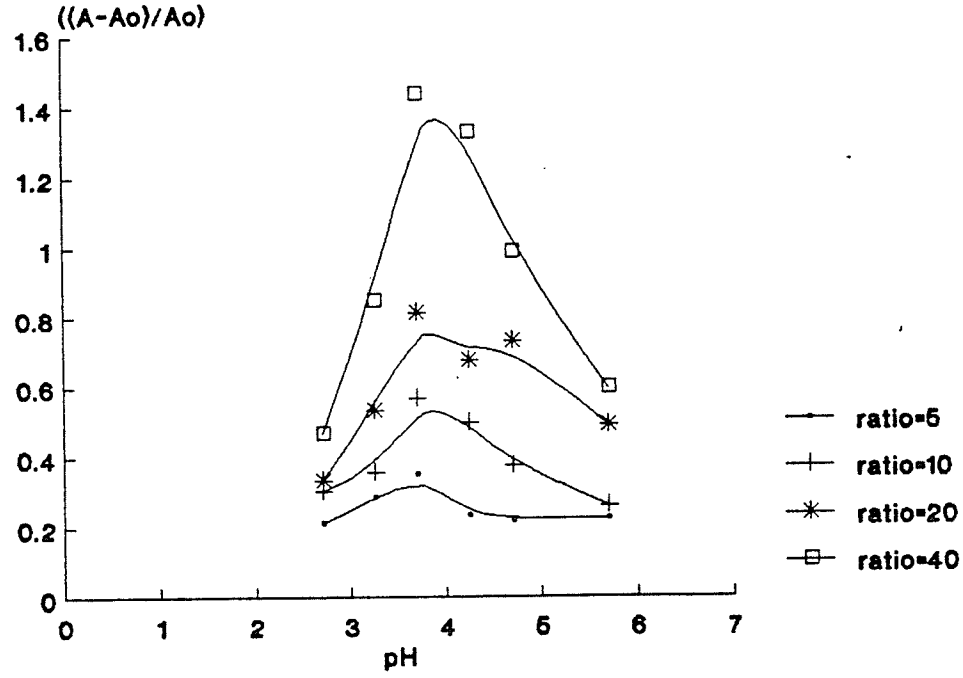
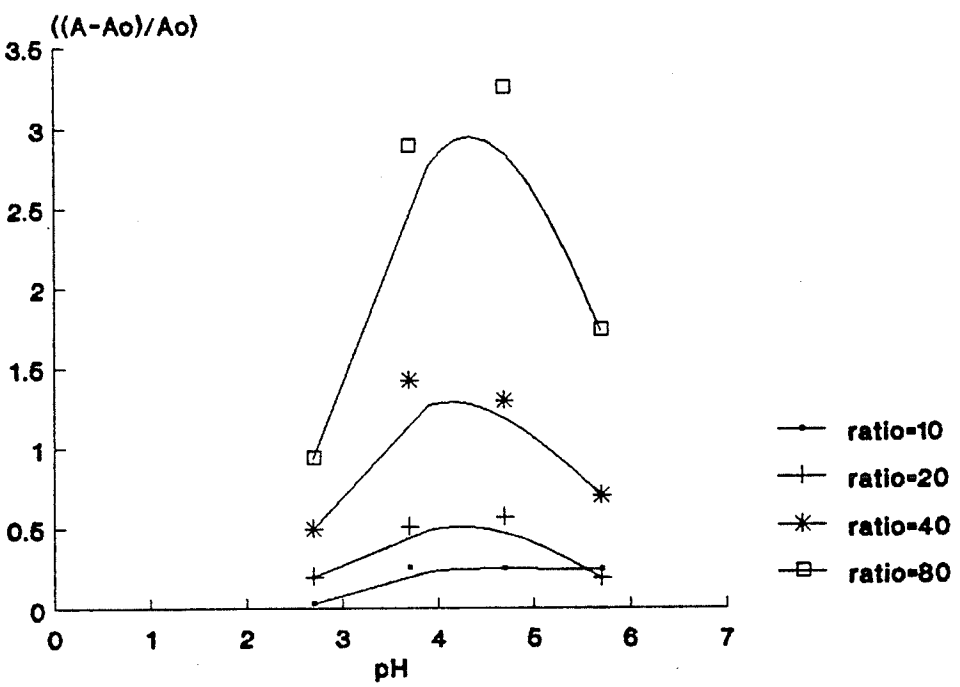

((A-Ao)/Ao)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
pH
ratio=5
ratio=10
ratio=20
ratio=40

Figure 4.03 Effect of pH and copigment concentrations of (a) chlorogenic acid and (b) caffeic acid on the magnitude of copigmentation ((A–Ao)/Ao) of monardaein solutions (2.58×10^{-4}M)(solvent: aqueous H_3PO_4–NaOAc buffer; l=1cm; ionic strength=0.20M; T=$20^0 \pm 0.5^0$C).

a.


((A-Ao)/Ao)
3.5
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7
pH
ratio=10
ratio=20
ratio=40
ratio=80

b.

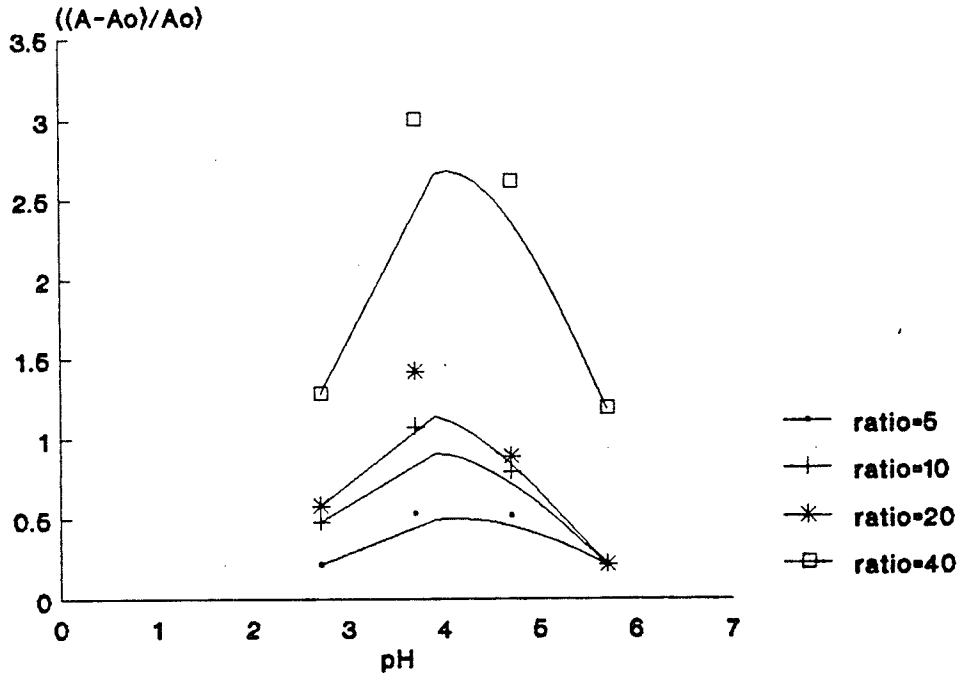
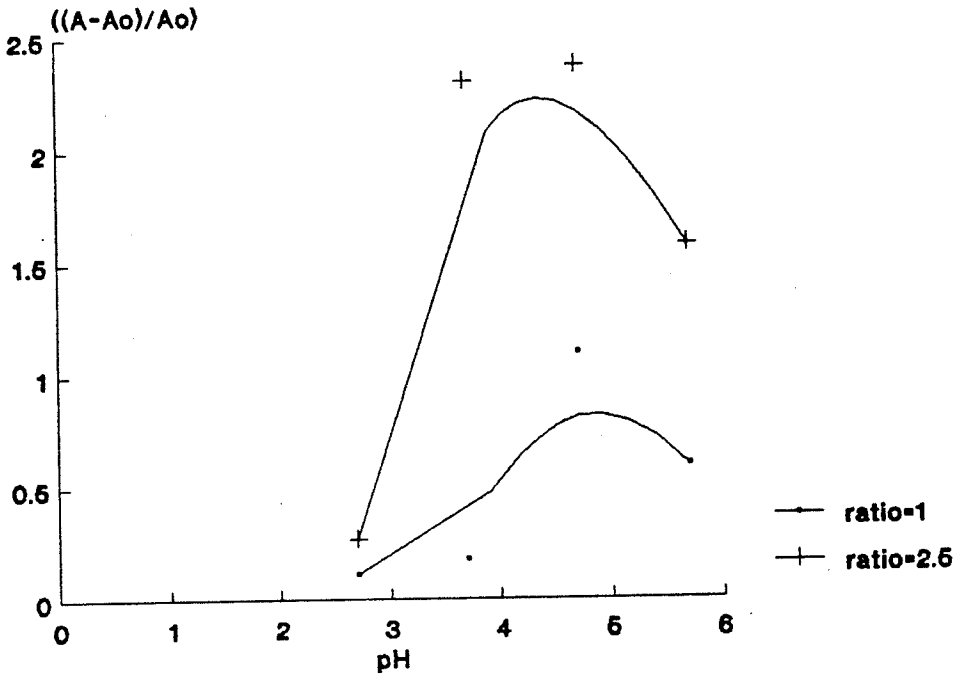

((A-Ao)/Ao)
3.5
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7
pH
ratio=5
ratio=10
ratio=20
ratio=40

Figure 4.04 Effect of pH and copigment concentrations of rutin on the magnitude of copigmentation ((A–Ao)/Ao) of (a) malvin and (b) pelargonidin 3–glucoside solutions (2.58 X 10^{-4}M)(solvent: aqueous H_3PO_4–NaOAc buffer; l=1cm; ionic strength=0.20M; T=20^0 $\pm$ 0.5^0C).

a.


((A-Ao)/Ao)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
pH
ratio=1
ratio=2.5

b.

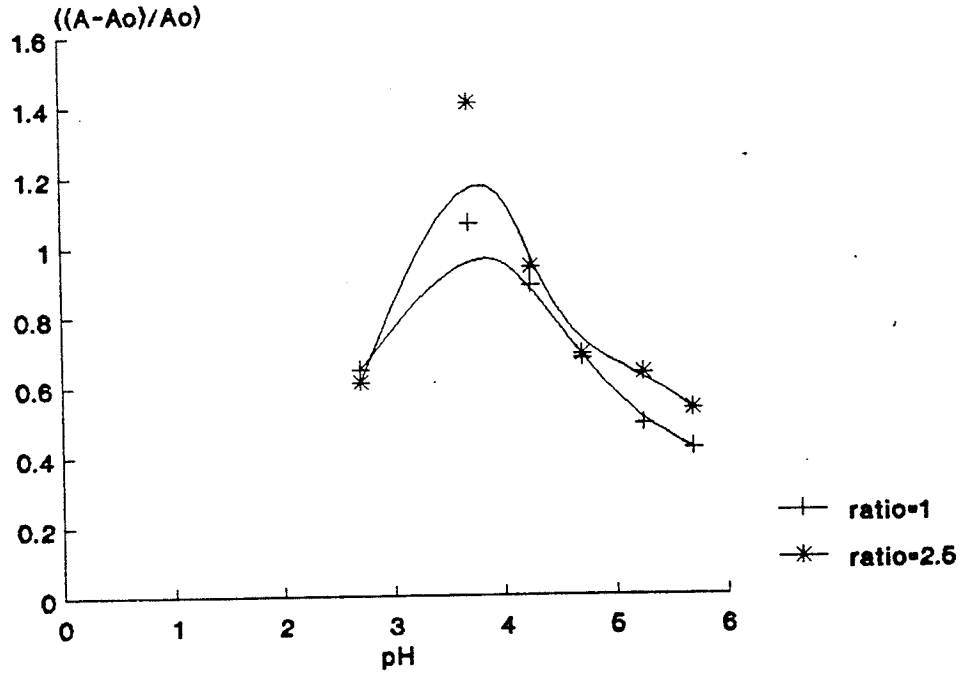

((A-Ao)/Ao)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
pH
ratio=1
ratio=2.5

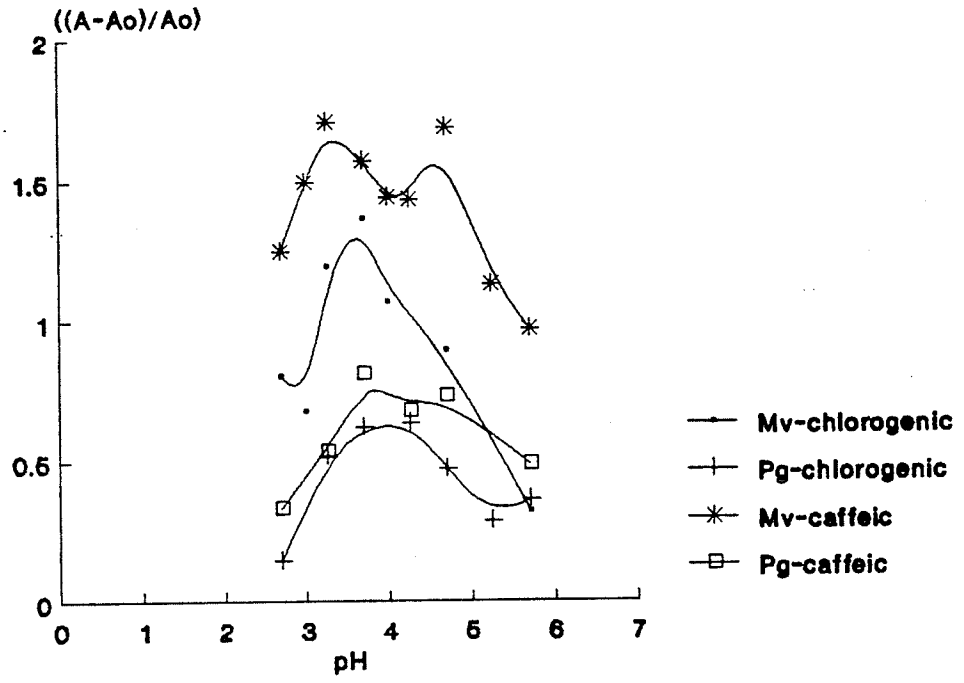
Table 4.01 Influence of pigment and copigment structures on pH of maximum copigmentation effect.

Pigment	Copigment	pH of maximum copigmentation effect
Malvin	Chlorogenic acid	3.2–3.7
	Caffeic acid	3.7–4.7
	Rutin	4.7–5.0
Pelargonidin 3–glucoside	Chlorogenic acid	3.2–3.5
	Caffeic acid	3.5–4.5
	Rutin	3.5–4.0
Monardaein	Chlorogenic acid	3.7–4.7
	Caffeic acid	3.2–4.7

Figure 4.05 (a) Plot of ((A–Ao)/Ao) versus pH for malvin and pelargonidin 3–glucoside solutions (2.58 X 10^{-4}M) copigmented with chlorogenic acid (5.16 X 10^{-3}M) and caffeic acid (5.16 X 10^{-3}M); solvent, aqueous H_3PO_4– NaOAc buffer, ionic strength=0.20M; l=1cm; T=20^0 + 0.5^0C.

(b) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside and monardaein solutions (2.58 X 10^{-4}M) copigmented with chlorogenic acid (5.16 X 10^{-3}M) and caffeic acid (5.16 X 10^{-3}M); solvent, aqueous H_3PO_4– NaOAc buffer, ionic strength=0.20M; l=1cm; T=20^0C $\pm$ 0.5^0C.

a.


((A-Ao)/Ao)
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7
pH
Mv-chlorogenic
Pg-chlorogenic
Mv-caffeic
Pg-caffeic

b.

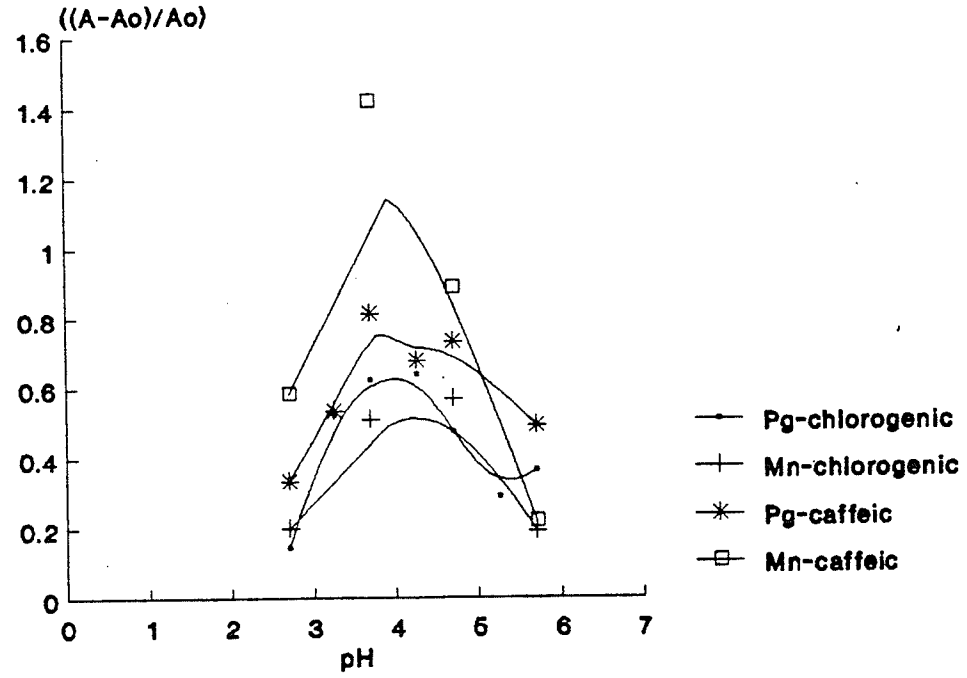
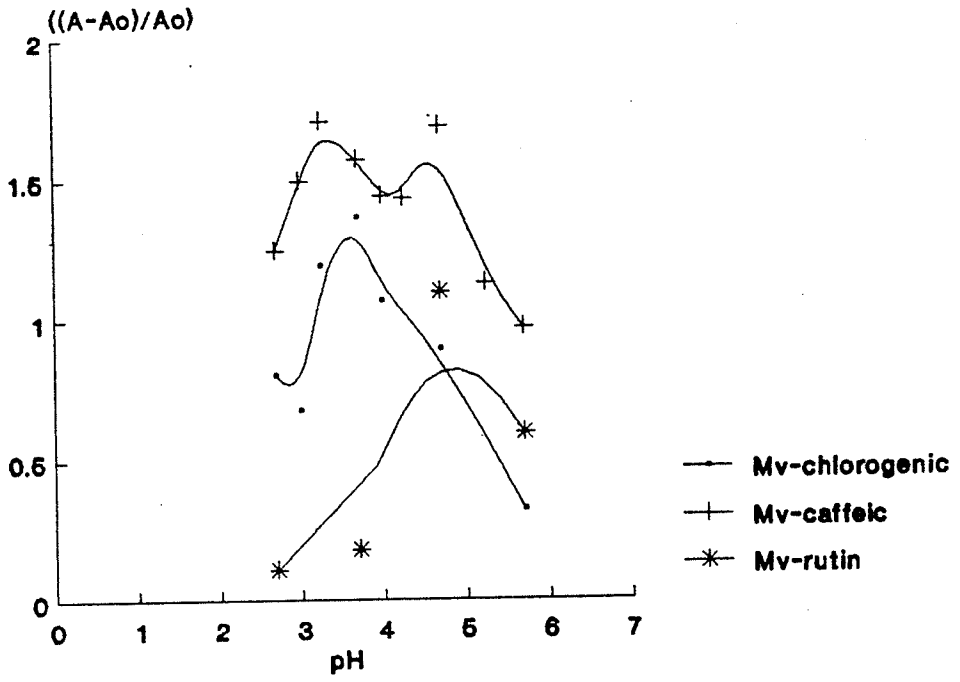

((A-Ao)/Ao)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7
pH
Pg-chlorogenic
Mn-chlorogenic
Pg-caffeic
Mn-caffeic

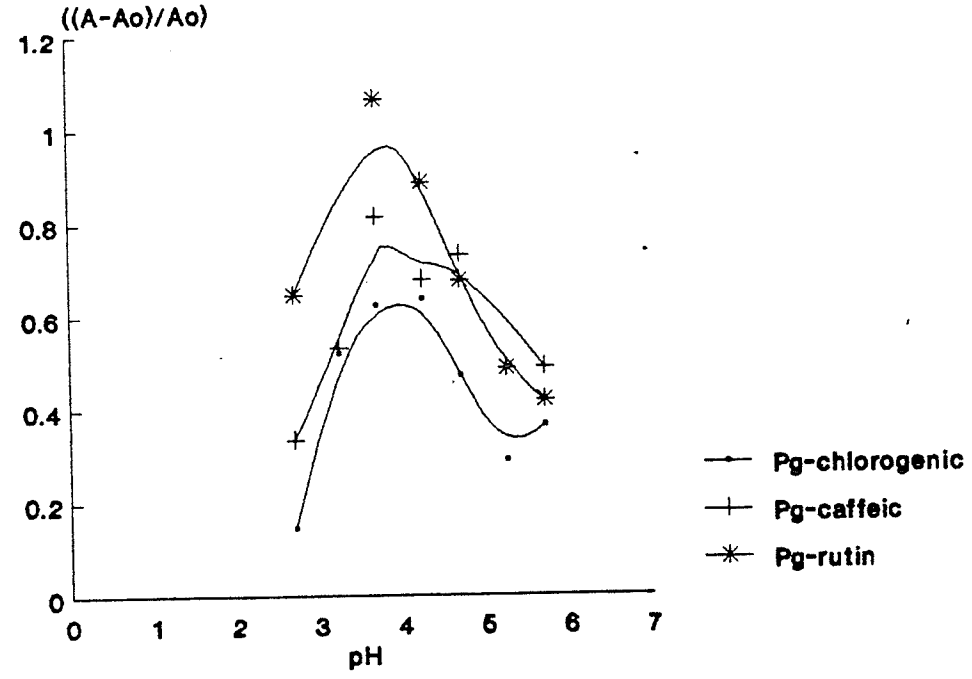
Figure 4.06 (a) Plot of (A–Ao)/Ao versus pH for malvin solutions (2.58×10^{-4}M) copigmented with chlorogenic acid (5.16×10^{-3}M), caffeic acid (5.16×10^{-3}M) and rutin (2.58×10^{-4}M); solvent, aqueous H_3PO_4–NaOAc buffer; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(b) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside solutions (2.58×10^{-4}M) copigmented with chlorogenic acid (6.45×10^{-4}M), caffeic acid (6.45×10^{-4}M) and rutin (6.45×10^{-4}M); solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength=0.20M; l=1cm; T=$20^0 + 0.5^0$C.

a.


((A-Ao)/Ao)
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7
pH
Mv-chlorogenic
Mv-caffeic
Mv-rutin

b.


((A-Ao)/Ao)
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7
pH
Pg-chlorogenic
Pg-caffeic
Pg-rutin

those of chlorogenic and caffeic acid complexes with malvin at a copigment:pigment molar ratio=20. Also, as the pH increased, the copigmentation magnitude of the rutin solutions increased, and at the pH of 5.7, the copigmentation effect exhibited by rutin–malvin solutions at the copigment:pigment molar ratio=1 was approximately two–fold that of chlorogenic–malvin at the copigment:pigment molar ratio of 20 (Figure 4.06a). The complexation of rutin with pelargonidin 3–glucoside (copigment:pigment molar ratio=2.5) at pH 3.5–4.0 resulted in approximately 6.5 fold the copigmentation magnitude of the caffeic acid–pelargonidin 3–glucoside complex (copigment:pigment molar ratio=2.5), and approximately 12 fold the copigmentation magnitude of the chlorogenic acid–pelargonidin 3–glucoside complex (copigment:pigment molar ratio=2.5) (Figure 4.06b). As the pH increased, the magnitude of the rutin complex remained consistently higher than the chlorogenic acid and caffeic acid complexes.

4.4.1.2 Influence of pigment structure

From the data presented in Figure 4.07(a), the acylated and corresponding non–acylated pelargonidin pigment complexes formed with chlorogenic acid (copigment:pigment molar ratio=20) exhibited similar behavior in maximum copigmentation as a function of pH. Pelargonidin 3–glucoside exhibited a maximum degree of copigmentation at approximately pH 4.0 when complexed with chlorogenic acid. The complex formed with monardaein exhibited slightly lower magnitudes of copigmentation than the complex formed with pelargonidin, and exhibited a maximum copigmentation at pH 4.2–4.3. The chlorogenic acid complexes formed with malvin, at the same copigment:pigment molar ratio, exhibited a maximum $((A-A_0)/A_0)$ value at approximately pH 3.5–3.7. These results are in agreement with the chlorogenic acid–malvin copigmentation reported by Brouillard et al. (1989) and Mazza and Brouillard (1990). The copigmentation magnitude exhibited by the chlorogenic acid–malvin complexes were considerably higher (1.5–3 fold) than the corresponding monardaein complexes.

Figure 4.07(b) reveals that at the copigment:pigment molar ratio=20, all three pigments exhibited similar trends in the maximum magnitude of copigmentation when complexed with caffeic acid The maximum copigmentation effect exhibited by the anthocyanin solutions was approximately pH 3.0–3.7. Figure 4.07(b) illustrates that the copigmentation magnitudes exhibited by the caffeic acid–monardaein complexes were consistently 2–fold higher than the corresponding pelargonidin complexes. The copigmentation magnitudes exhibited by the malvin complexes were however

PAGINATION ERROR.

TEXT COMPLETE.

ERREUR DE PAGINATION.

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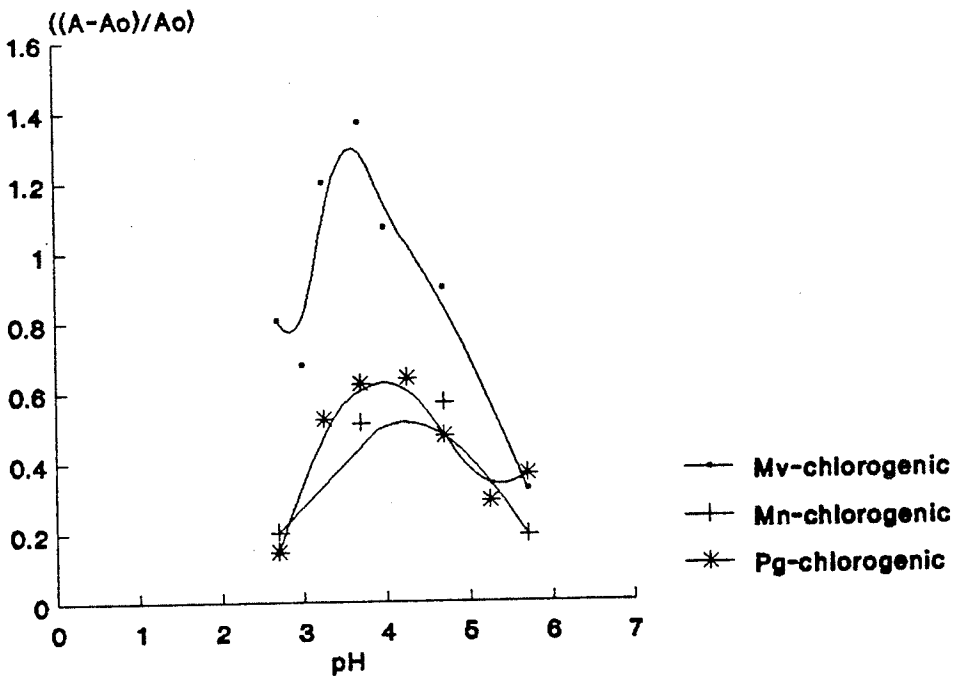
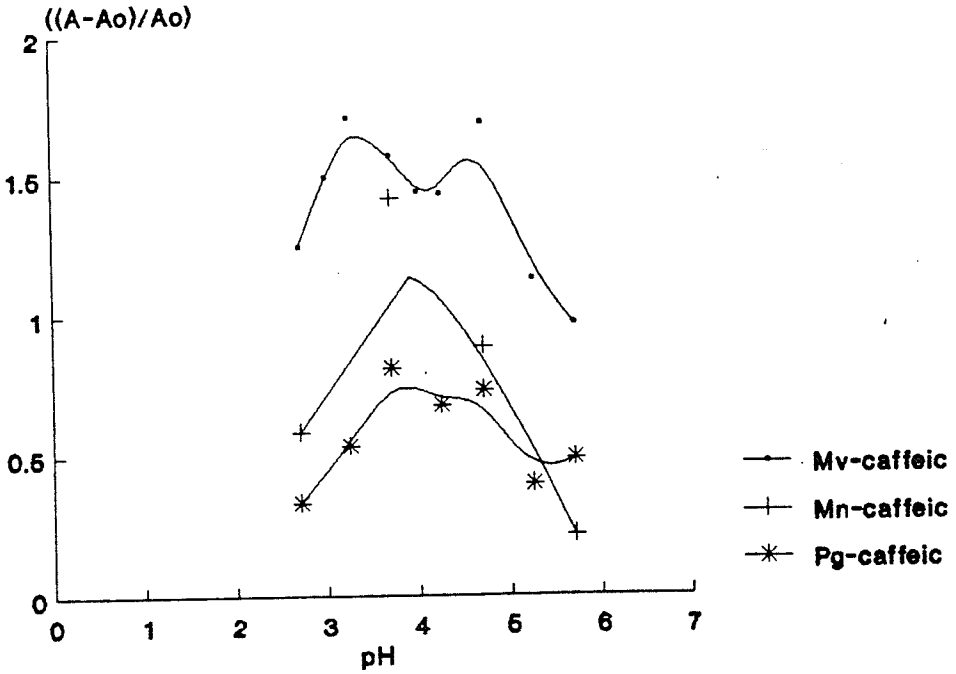
Figure 4.07 (a) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside, malvin and monardaein solutions (2.58 X 10^{-4}M) copigmented with chlorogenic acid (5.16 X 10^{-3}M); solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength=0.20M; l=1cm; T=20^0 + 0.5^0C.

(b) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside, malvin and monardaein solutions (2.58 X 10^{-4}M) copigmented with caffeic acid (5.16 X 10^{-3}M); solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength=0.20M; l=1cm; T=20^0 + 0.5^0C.

a.


((A-Ao)/Ao)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
pH
Mv-chlorogenic
Mn-chlorogenic
Pg-chlorogenic

b.


((A-Ao)/Ao)
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
pH
Mv-caffeic
Mn-caffeic
Pg-caffeic

greater than both the monardaein and pelargonidin complexes.

Complexes formed between monardaein and caffeic acid resulted in generally higher stoichiometric constants over all pH levels studied (0.71–1.23) as compared to the values determined for the complexes formed with pelargonidin and malvin (Table 4.02).

Comparison of the $((A-A_0)/A_0)$ values for the rutin complexes formed with malvin and pelargonidin reveal that maximum copigmentation occurred at approximately pH 4.7–5.0 and 3.7, respectively (Figure 4.08a, b). The copigmentation magnitude for the two pigment complexes formed with rutin were similar although malvin exhibited a slightly greater magnitude of copigmentation.

4.4.2 Influence of pH on maximum absorbance

The spectral analysis (400–700nm) of malvin (2.58×10^{-4} M) without the addition of copigment (Figure 4.09), illustrates the hyperchromic and bathochromic shifts observed in the maximum absorbance as a function of pH. It is well established in the literature (Brouillard and Delaporte, 1977; Mazza and Brouillard, 1987; Brouillard et al., 1989), that an aqueous solution of anthocyanins contains a mixture of anthocyanin species which exist in a fast equilibrium. The structural transformation illustrated in Figure 4.09 is characterized by a dramatic shift in the equilibrium towards the quinonoidal base species as the pH increases from 5.7 to 6.7, from the predominant flavylium cation species found in the pH 2.7–3.30 range. The very low absorbance of the pigment solution in the pH 4.7–5.7 range can be attributed to the transformation to the colorless chalcone followed by the subsequent shift to the quinonoidal base at higher pHs.

Generally speaking, all of the complexes studied (8 copigment–pigment combinations) exhibited bathochromic and hyperchromic shifts in the maximum absorbance as a result of the copigmentation reaction. Figure 4.10 illustrates the bathochromic and hyperchromic effects observed for the complexes formed between monardaein and chlorogenic acid as the copigment concentration increased at pH 3.7. The bathochromic and hyperchromic effects were observed in all pigment solutions as the concentration of the copigment increased. However, the magnitude of the bathochromic shift in λmax for each of the copigment–pigment combinations differs dramatically depending on both the pigment and copigment chemical structures. Figure 4.11 illustrates the variation in the magnitude of the bathochromic shift as a function of copigment structure in

Table 4.02 Stoichiometric constants for pelargonidin 3–glucoside, malvin and monardaein solutions (2.58×10^{-4}M) complexed with chlorogenic acid, caffeic acid and rutin in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; $T=20^0 \pm 0.5^oC$.

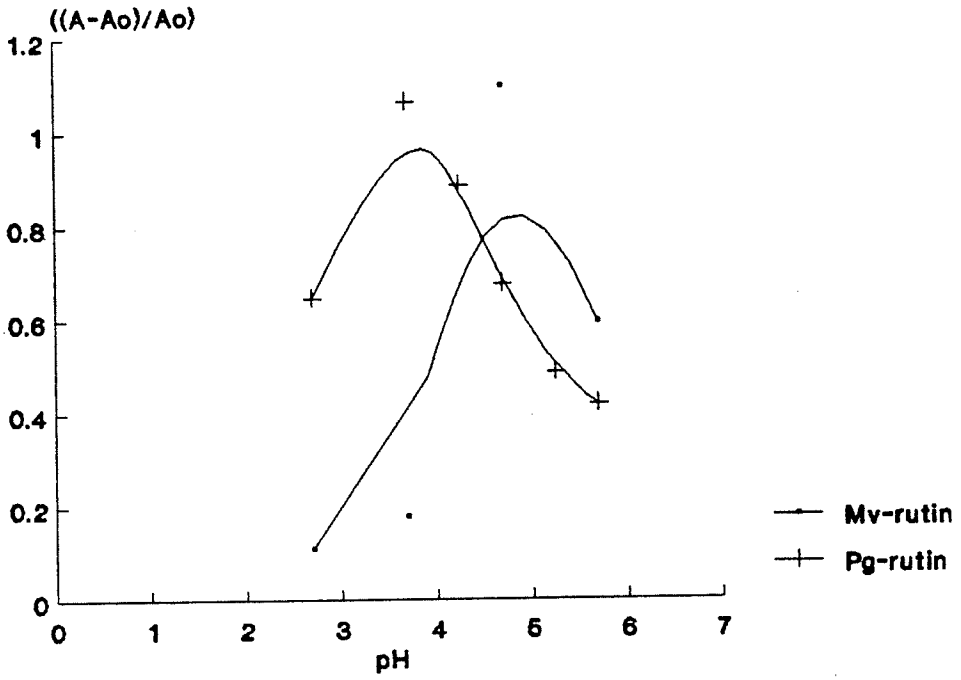
		Anthocyanin		
Copigment	pH	Malvin	Pelargonidin 3–glucoside	Monardaein
Chlorogenic Acid	2.7	0.94 (0.28*)	0.67	1.46
	3.7	0.97 (1.06*)	1.14 (1.17*)	0.99
	4.7	1.05	1.25	1.38
	5.7	1.17	1.22	0.90
Caffeic Acid	2.7	0.63 (0.53*)	0.46	0.79
	3.7	0.86 (1.48*)	0.66	0.79
	4.7	0.49	0.67	0.71
	5.7	0.38	0.53	1.23
Rutin	2.7	1.10	0.14	–
	3.7	1.14	0.31	–
	4.7	0.76	–	–
	5.7	–	0.26	–

* concentration = 7.73×10^{-4}M

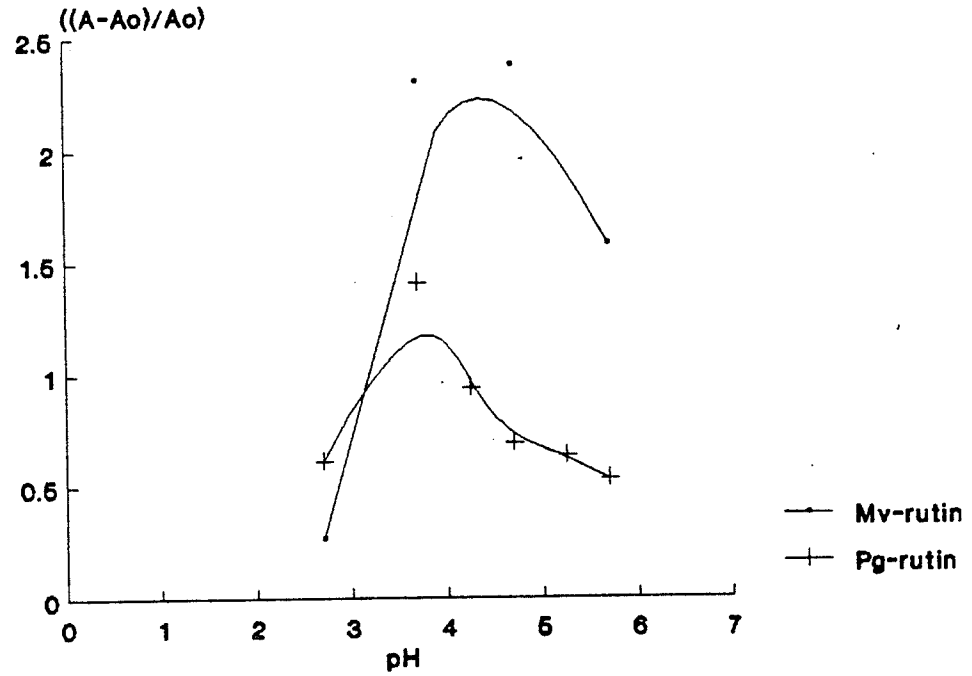
Figure 4.08 (a) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside and malvin solutions (2.58 X 10^{-4}M) copigmented with rutin (2.58 X 10^{-4}M); solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(b) Plot of ((A–Ao)/Ao) versus pH for pelargonidin 3–glucoside and malvin solutions (2.58 X 10^{-4}M) copigmented with rutin (6.45 X 10^{-4}M); solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


((A-Ao)/Ao)
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
pH
Mv-rutin
Pg-rutin

b.


((A-Ao)/Ao)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
pH
Mv-rutin
Pg-rutin

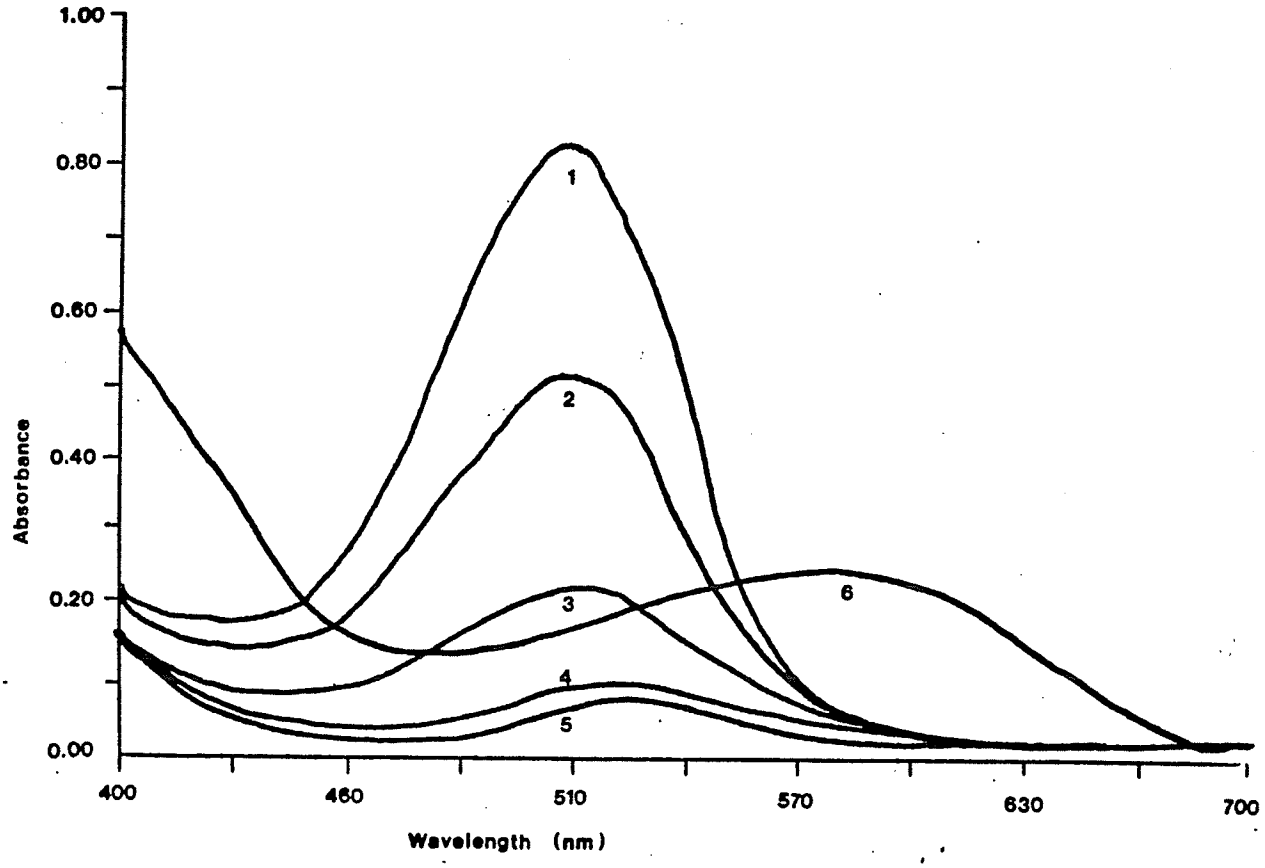


Figure 4.09 Visible spectra of malvin (2.58×10^{-4}M) solutions in aqueous H_3PO_4–NaOAc buffer at varying pH: (1) 2.70, (2) 3.01, (3) 3.30, (4) 4.71, (5) 5.69, (6) 6.71; ionic strength=0.20M; l=1cm; $T=20^0 \pm 0.5^0$C.

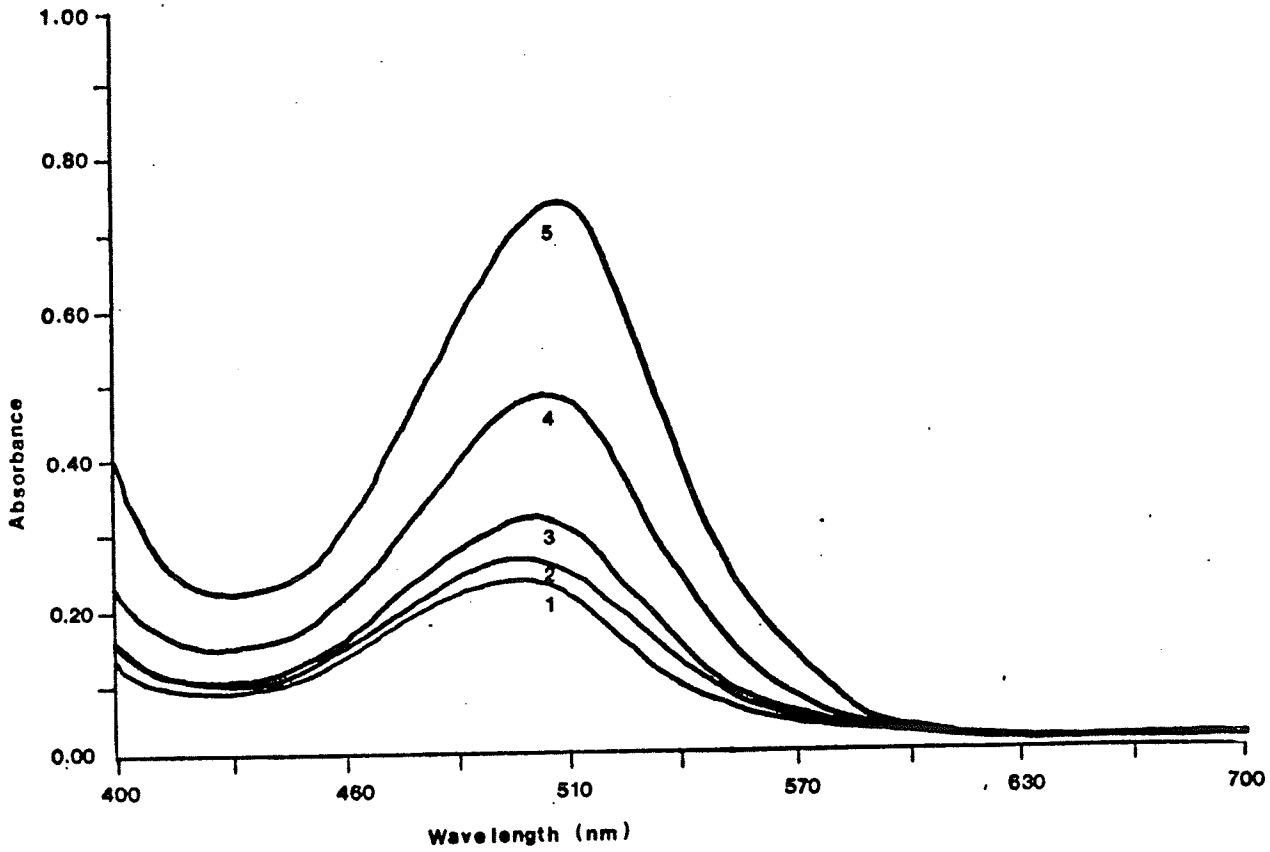


Figure 4.10 Visible spectra of monardaein (2.58×10^{-4}M) solutions copigmented with chlorogenic acid at (1) 0, (2) 5, (3) 20, (4) 40 and (5) 80 copigment:pigment molar ratios in aqueous H_3PO_4–NaOAc buffer, pH 3.7, ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

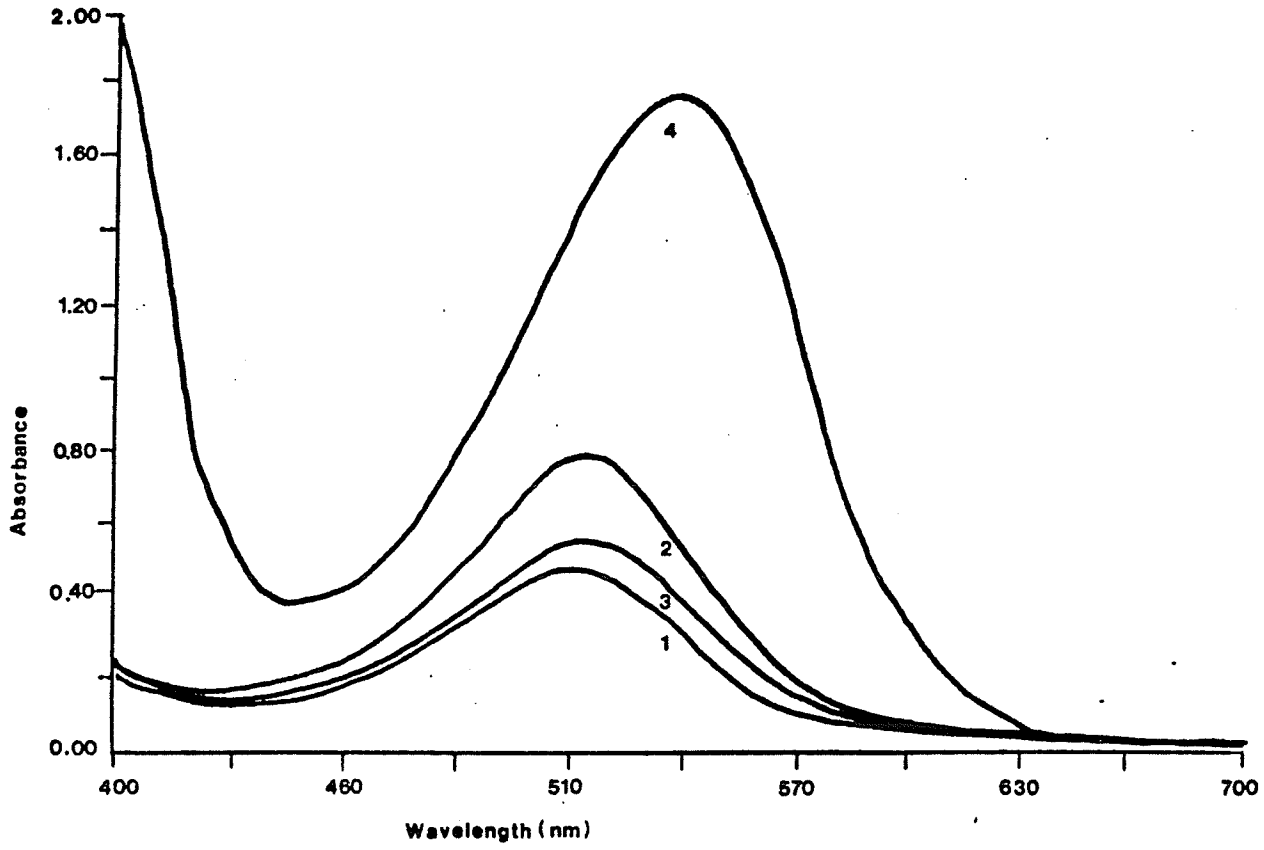


Figure 4.11 Visible spectra of (1) malvin (2.58×10^{-4}M) solutions copigmented with (2) chlorogenic acid, (3) caffeic acid and (4) rutin (copigment concentration 1.29×10^{-3}M) in aqueous H_3PO_4–NaOAc buffer, pH 3.0, ionic strength=0.20M; l=1cm; $T=20^0 \pm 0.5^0$C.

Table 4.03 Bathochromic shift magnitudes for pigment solutions complexed with chlorogenic acid, caffeic acid and rutin at pH 3.7.

Pigment	Copigment	λmax no copigment	λmax copigment ratio=20	Magnitude of bathochromic shift
Pg 3–glu	Chlorogenic acid	496	503	+ 4
	Caffeic acid	497	501	+ 7
Malvin	Chlorogenic acid	520	526	+ 6
	Caffeic acid	520	535	+15
	Rutin	522	558	+36
Monardaein	Chlorogenic acid	503	510	+ 7
	Caffeic acid	503	515	+12

Table 4.04 Bathochromic shift magnitudes for pigment solutions complexed with chlorogenic acid, caffeic acid and rutin at pH 4.7.

Pigment	Copigment	λ max no copigment	λ max copigment ratio=20	Magnitude of bathochromic shift
Pg 3-glu	Chlorogenic acid	500	514	+14
	Caffeic acid	508	511	+ 3
	Rutin	508	508*	0
Malvin	Chlorogenic acid	528	533	+ 5
	Caffeic acid	521	533	+12
	Rutin	515	527*	+12
Monardaein	Chlorogenic acid	511	519	+ 8
	Caffeic acid	511	516	+ 5

* copigment:pigment molar ratio=1

complexes formed with malvin at pH 3.0. The λ max for solutions of malvin (both copigmented and noncopigmented) are consistently higher than solutions of pelargonidin 3–glucoside and monardaein at all pH levels observed (Tables 4.03 – 4.05). This can primarily be attributed to the presence of methyl substituents on the B ring of the malvin molecule as well as the presence of diglycosylation. Generally, the bathochromic shifts observed for pelargonidin and monardaein complexes formed with caffeic acid were slightly greater than those observed for complexes with chlorogenic acid, while the shifts observed with the malvin–chlorogenic complexes were greater than the caffeic acid complexes. The rutin complexes exhibited considerably larger (2–4X) shifts than both caffeic and chlorogenic acid, for all pigment solutions studied (Figure 4.11, Tables 4.03–4.05).

At pH 2.7, little difference in the bathochromic shift magnitudes were found between the pigment solutions complexed with caffeic and chlorogenic acids. However, notable differences between the 2 copigments were observed at pH 3.7. For both the malvin and monardaein solutions, the shifts for complexes formed with caffeic acid were larger than the corresponding complexes formed with chlorogenic acid (Table 4.03). Conversely, the shift observed for the pelargonidin–caffeic acid complex is slightly less than that observed in the pelargonidin–chlorogenic acid complex.

In the higher pH range, a definite effect of pigment structure on the shift behavior can be observed. At pH 4.7, malvin and monardaein exhibited similar trends in the bathochromic shift magnitudes of chlorogenic and caffeic acid complexes (Table 4.04). However, in the case of pelargonidin, the shift magnitude observed with chlorogenic acid was much greater (+14nm) than that observed with caffeic acid (+3nm). The large bathochromic shift was also accompanied by a large hyperchromic shift for the chlorogenic acid–pelargonidin complex at pH 4.7. At pH 5.7, the shift magnitudes observed for the complexes between chlorogenic and caffeic acid and malvin do not differ (Table 4.05). However, the magnitude of the shifts observed for the chlorogenic acid complexes formed with malvin and pelargonidin differ dramatically (+17nm and +3nm, respectively). The caffeic acid complexes with monardaein and pelargonidin did not differ in shift magnitude at pH 5.7. However, notable differences in the shift magnitude were observed for the two pigment complexes formed with chlorogenic acid (Table 4.05).

From the data presented in Table 4.03, rutin exhibits a large (+36 nm) bathochromic and hyperchromic shifts in the λmax when complexed with malvin. Figure 4.12 illustrates the large bathochromic shift observed for a solution of malvin complexed with rutin at copigment:pigment

Table 4.05 Bathochromic shift magnitudes for pigment solutions complexed with chlorogenic acid, caffeic acid and rutin at pH 5.7.

Pigment	Copigment	λmax no copigment	λ max copigment ratio=20	Magnitude of bathochromic shift
Pg 3–glu	Chlorogenic acid	517	520	+ 3
	Caffeic acid	516	518	+ 2
	Rutin	516	521*	+ 5
Malvin	Chlorogenic acid	535	556	+21
	Caffeic acid	523	540	+17
	Rutin	526	525*	– 1
Monardaein	Chlorogenic acid	513	522	+ 9
	Caffeic acid	513	515	+ 2

* copigment:pigment molar ratio=1

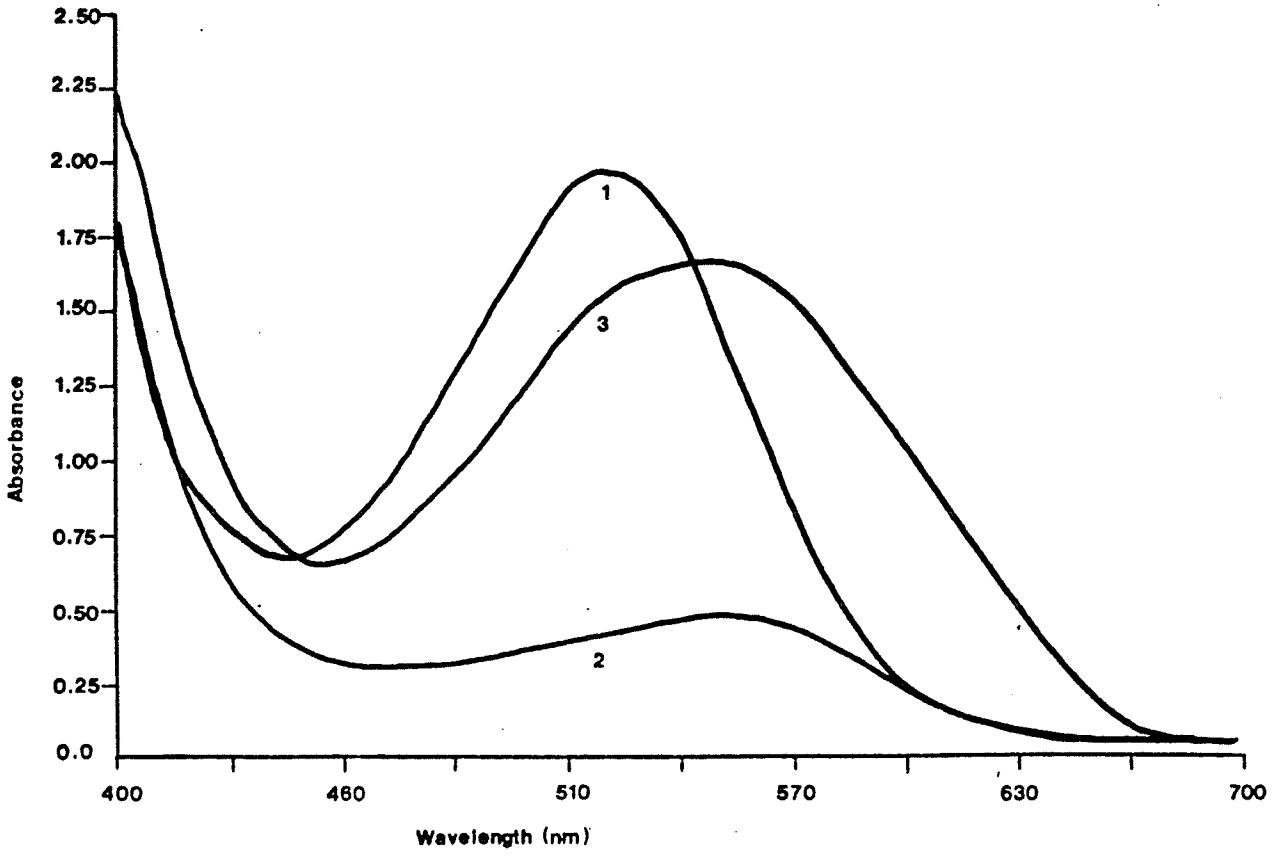


Figure 4.12 Visible spectra of malvin (2.58×10^{-4}M) solutions copigmented with rutin (1.29×10^{-3}M) in aqueous H_3PO_4–NaOAc buffer, at pH 2.7(1), 4.7(2), and 5.7(3); ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

molar ratio=5, as the pH shifts from 2.7–5.7. Conversely, complexation with pelargonidin results in small (+3–5nm) bathochromic shifts (Table 4.05). It has been discussed previously that the copigmentation magnitude of the pelargonidin solutions was found to be lower than that of the malvin solutions and the copigment:pigment molar ratio of rutin incorporated into the pelargonidin 3–glucoside solutions was lower than in the malvin solutions. The differences between the two pigment solutions may be attributed to the presence of methyl substituents attached to the malvin molecule.

4.4.3 Influences on the stoichiometric constant

4.4.3.1 The effect of pigment structure

On the basis of the n values determined from the plots of $\ln[Cp]_0$ vs $\ln((A-Ao)/Ao)$, it can be seen that generally the pigment structure and pH had little influence on the stoichiometry describing the association between chlorogenic acid and the anthocyanin pigments studied (Table 4.02). From pH 3.7–5.7, the chlorogenic acid and caffeic acid complexes formed with the three pigments, exhibit n values approximating 1.00 (Figure 4.13a, 4.14a and Table 4.02). It has been established previously in the literature that a stoichiometric constant close to unity is characteristic of a 1:1 association between pigment and copigment (Mazza and Brouillard, 1990). The results presented in Table 4.02 are in agreement with this theory. However, at pH 2.7 the stoichiometric relationships for the chlorogenic and caffeic acid complexes remain linear but their slopes do not correspond to the n values close to unity (Figure 4.13 and 4.14, b–d). The malvin and pelargonidin 3–glucoside complexes with chlorogenic acid exhibited similar n values, ca. 0.65 while the acylated pigment exhibited a much larger n value, ca. 1.5 (Table 4.02). It is apparent from these results that an additional model is required to account for the variation in the stoichiometric constants at pH 2.7.

The stoichiometric constants for the three pigment complexes formed with caffeic acid at pH 2.7 exhibited an increasing trend as the complexity of the pigment structure increased, such that the n value observed for pelargonidin 3–glucoside was 0.46, malvin 0.63, and the n value for the monardaein–caffeic acid complex was 0.79 (Table 4.02). Similarly, the acylated anthocyanin exhibited the highest n value at pH 4.7 and 5.7. Similar trends were observed in the stoichiometric constants for the chlorogenic acid complexes with malvin and pelargonidin.

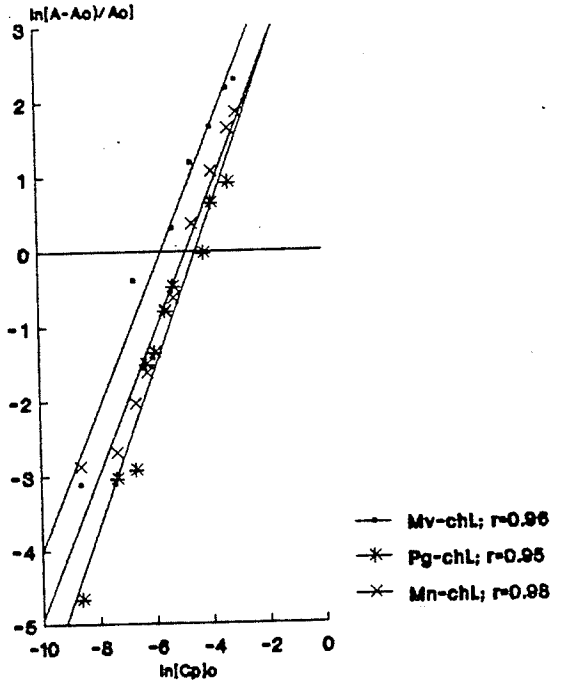
Figure 4.13 (a) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M pelargonidin 3–glucoside, malvin, and monardaein solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer, at pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(b) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M malvin solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

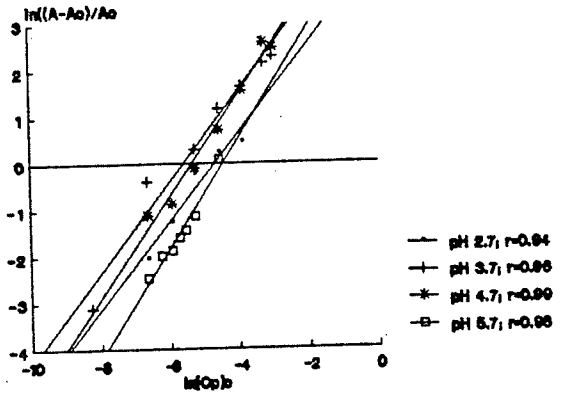
(c) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M pelargonidin 3–glucoside solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(d) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M monardaein solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

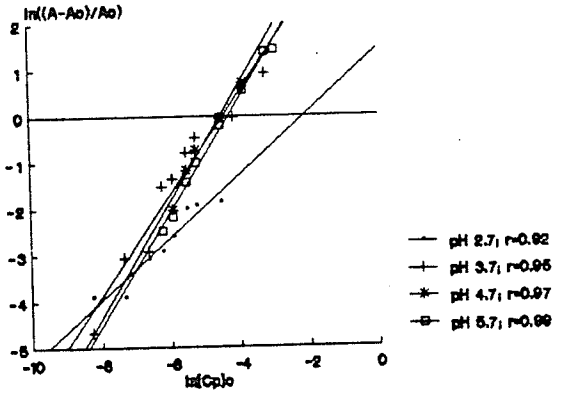
a.


ln[A-Ao)/Ao]
ln[Cp]o
Mv-chL; r=0.96
Pg-chL; r=0.95
Mn-chL; r=0.98

b.


ln((A-Ao)/Ao
ln[Cp]o
pH 2.7; r=0.94
pH 3.7; r=0.96
pH 4.7; r=0.99
pH 5.7; r=0.98

c.


ln((A-Ao)/Ao)
ln[Cp]o
pH 2.7; r=0.92
pH 3.7; r=0.95
pH 4.7; r=0.97
pH 5.7; r=0.99

d.

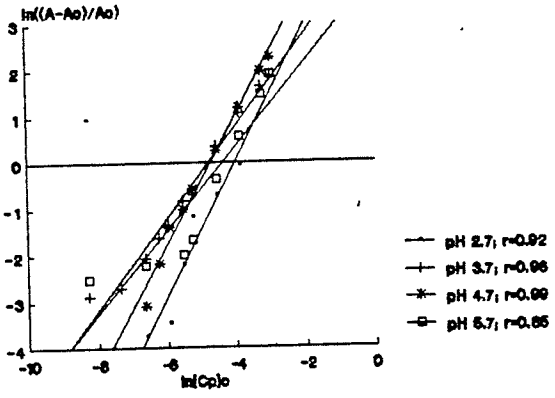

ln((A-Ao)/Ao)
ln[Cp]o
pH 2.7; r=0.92
pH 3.7; r=0.96
pH 4.7; r=0.99
pH 5.7; r=0.95

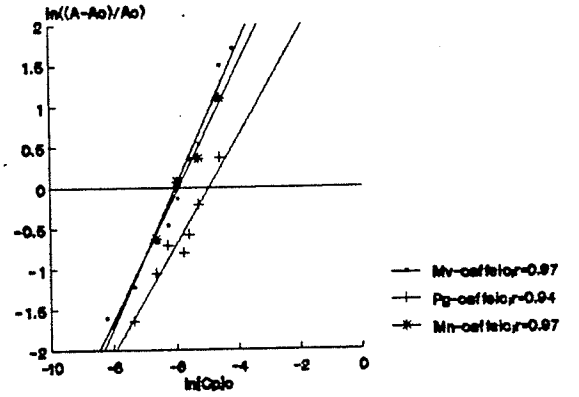
Figure 4.14 (a) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M pelargonidin 3–glucoside, malvin, and monardaein solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer, at pH 3.7; ionic strength=0.20M; l=1cm; T=20^0 $\pm$ 0.5^0C.

(b) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M malvin solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=20^0 $\pm$ 0.5^0C.

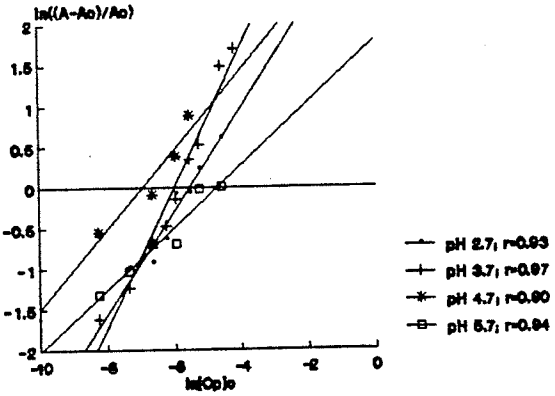
(c) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M pelargonidin 3–glucoside solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=20^0 $\pm$ 0.5^0C.

(d) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M monardaein solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=20^0 $\pm$ 0.5^0C.

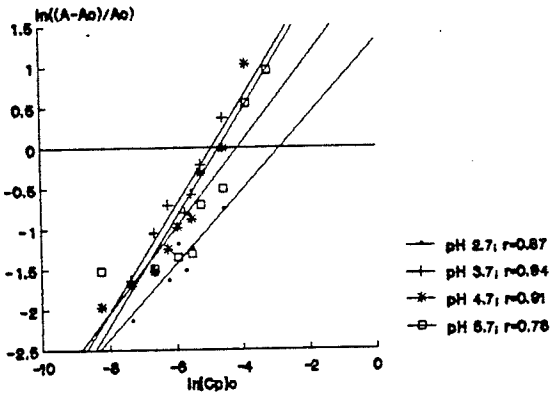
a.


ln((A-Ao)/Ao)
ln(Cp)o
Mv-caffeine r=0.97
Pg-caffeine r=0.94
Mn-caffeine r=0.97

b.


ln((A-Ao)/Ao)
ln(Cp)o
pH 2.7; r=0.93
pH 3.7; r=0.97
pH 4.7; r=0.90
pH 5.7; r=0.94

c.


ln((A-Ao)/Ao)
ln(Cp)o
pH 2.7; r=0.87
pH 3.7; r=0.94
pH 4.7; r=0.91
pH 5.7; r=0.78

d.

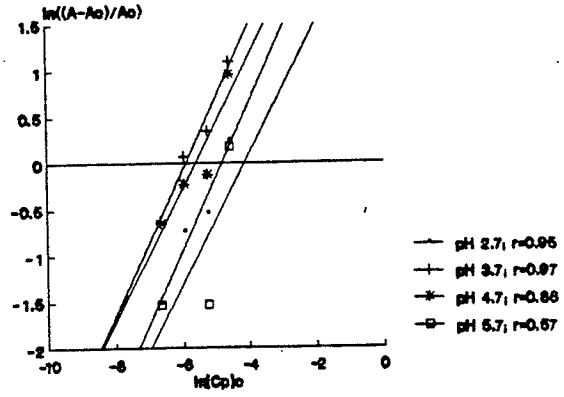
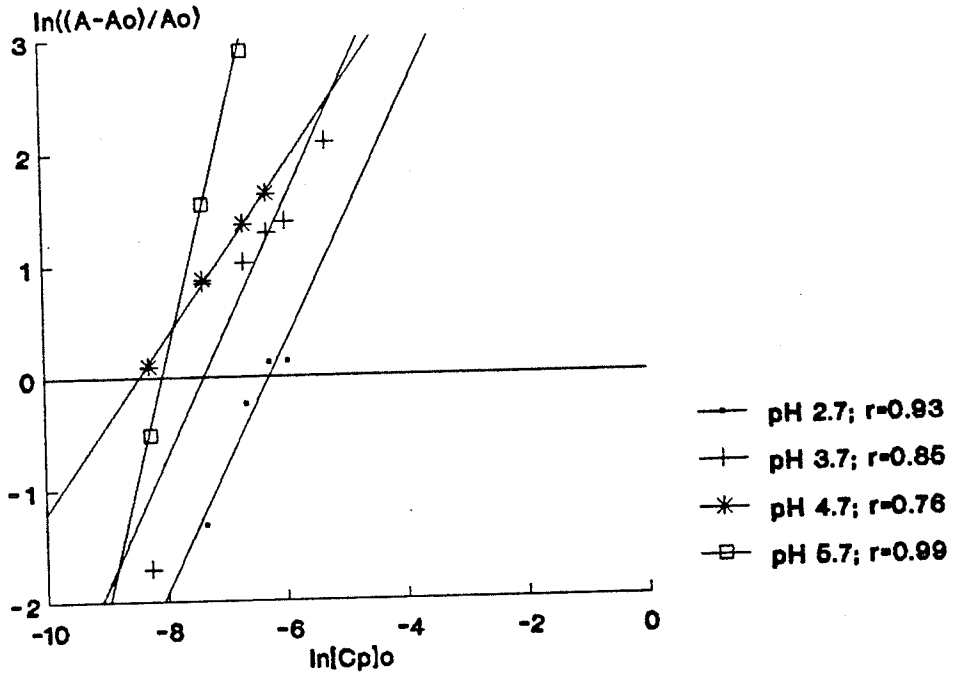

ln((A-Ao)/Ao)
ln(Cp)o
pH 2.7; r=0.95
pH 3.7; r=0.97
pH 4.7; r=0.86
pH 5.7; r=0.57

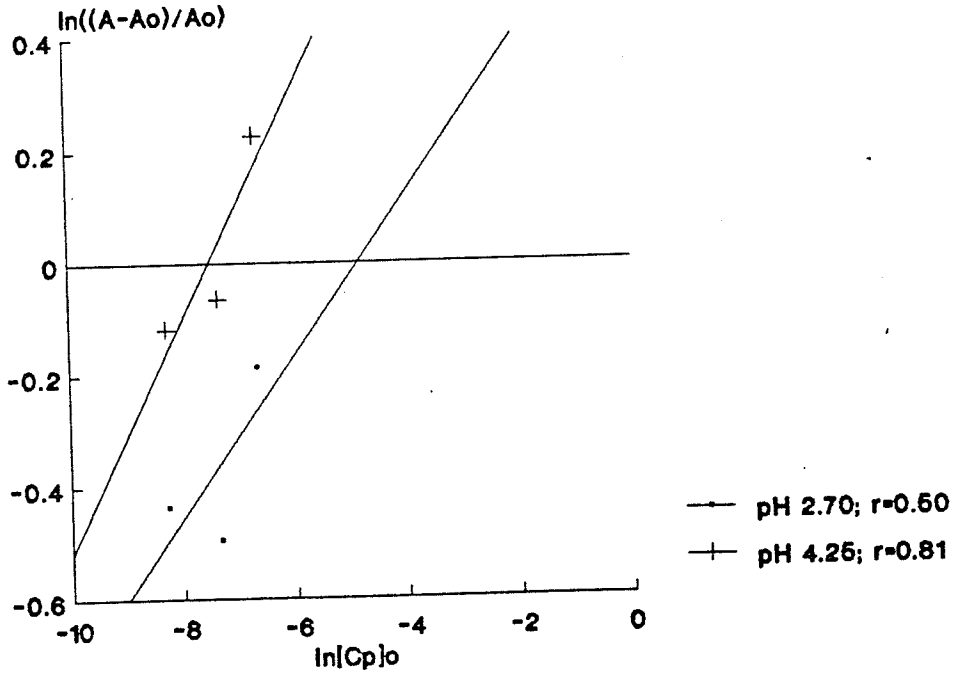
Figure 4.15 (a) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M malvin solutions complexed with rutin in aqueous H_3PO_4–NaOAc buffer, in the pH range 2.7–5.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(b) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for 2.58 X 10^{-4}M pelargonidin 3–glucoside solutions complexed with rutin in aqueous H_3PO_4–NaOAc buffer, at pH 2.70 and 4.25; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


ln((A-Ao)/Ao)
3
2
1
0
-1
-2
-10
-8
-6
-4
-2
0
ln[Cp]o
pH 2.7; r=0.93
pH 3.7; r=0.85
pH 4.7; r=0.76
pH 5.7; r=0.99

b.


ln((A-Ao)/Ao)
0.4
0.2
0
-0.2
-0.4
-0.6
-10
-8
-6
-4
-2
0
ln[Cp]o
pH 2.70; r=0.60
pH 4.25; r=0.81

The association between rutin and the two pigments, malvin and pelargonidin at low pH appears to be 1:1 however beyond pH 3.7, the slope remains linear but the stoichiometric constant does not correspond to the linear line depicted in Figure 4.15.

4.4.3.2 Influence of copigment structure

Comparison of the stoichiometric constants for the 3 copigment:pigment complexes reveal distinct differences as a function of pH (Table 4.02). At pH 2.7, rutin exhibits a stoichiometric constant close to unity while the corresponding complexes with malvin and the two phenolic acids exhibit low n values, ca. 0.65. This suggests that at low pH, rutin effectively interacts with the flavylium cation of malvin in a 1:1 ratio while the copigmentation exhibited by chlorogenic and caffeic acids cannot be described by this model. Based on the similarities in stoichiometric constants, shift magnitudes, and copigmentation magnitudes observed for the malvin solutions complexed with caffeic and chlorogenic acid, there does not appear to be any significant differences in the effectiveness of these two phenolic compounds as copigments at pH values of 2.7–3.7. However, at pH 4.7–5.7, notable differences between the three copigments become apparent. Generally, the stoichiometric constants for the chlorogenic acid containing solutions were higher than the values for the caffeic acid and rutin complexes formed with all three of the pigment solutions. In the pH range 3.7–5.7, the chlorogenic acid complexes formed with the three pigment solutions can be described as a 1:1 association. The caffeic acid complexes, particularly those with malvin and pelargonidin exhibited stoichiometric constants closer to 0.50 than 1.0. This suggests that the complexation between these molecular species is not a 1:1 copigment:pigment formation but perhaps a 2:1 association. The monardaein solutions exhibited similar stoichiometric constants for chlorogenic acid and caffeic acid complexes, but once again, only the chlorogenic acid based complexes can be described as a 1:1 complex. The caffeic acid based complexes do not fit the 1:1 model. It is thus suggested that the lack of the quinic acid moiety in caffeic acid may be conducive to the association of two molecules of this copigment for each molecule of anthocyanin. As shown in Figure 2.11b, this type of association (two molecules of caffeic acid with one molecule of anthocyanin) appears feasible and is likely to occur.

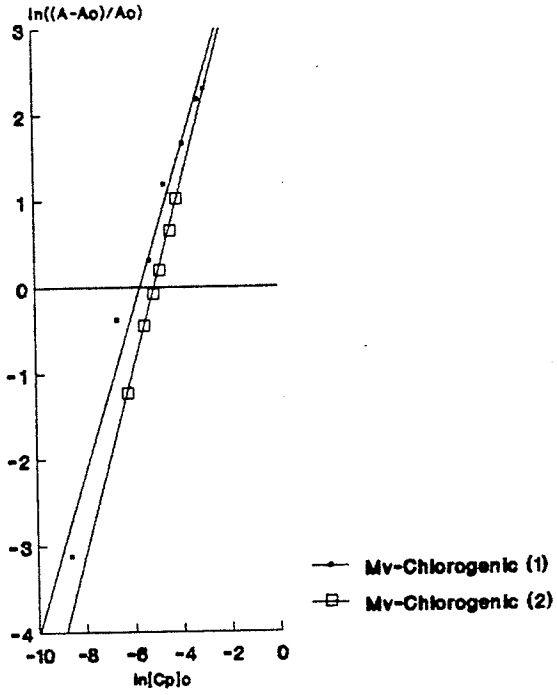
4.4.4 Influence of pigment concentration

Figure 4.16 illustrates that pigment concentration had little effect on the stoichiometry of the malvin complexes formed with the copigments, chlorogenic acid and caffeic acid at pH 3.7.

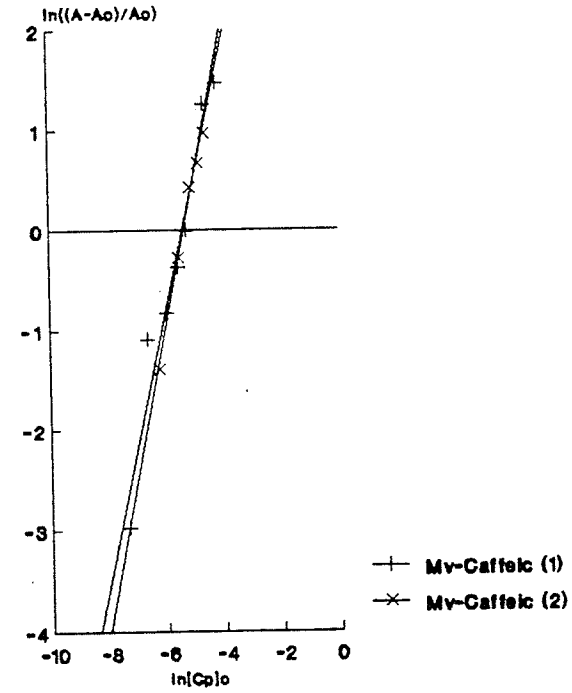
Figure 4.16 (a) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for (1) 2.58 X 10^{-4} M and (2) 7.73 X 10^{-4}M malvin solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer, at pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

(b) Plot of $\ln((A-A_0)/A_0)$ versus $\ln[Cp]_0$ for (1) 2.58 X 10^{-4}M and (2) 7.73 X 10^{-4}M malvin solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer, at pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


ln((A-Ao)/Ao)
3
2
1
0
-1
-2
-3
-4
-10
-8
-6
-4
-2
0
ln[Cp]o
Mv-Chlorogenic (1)
Mv-Chlorogenic (2)

b.


ln((A-Ao)/Ao)
2
1
0
-1
-2
-3
-4
-10
-8
-6
-4
-2
0
ln[Cp]o
Mv-Caffeic (1)
Mv-Caffeic (2)

Similar findings for complexes between malvin and chlorogenic acid have been reported by Mazza and Brouillard (1990). Although the increased anthocyanin concentration resulted in increases in the absolute absorbance at 525nm and λmax, the copigmentation effect ((A–Ao)/Ao) observed for the 7.73 X 10^{-4}M malvin solutions was low when complexed with 3.87 X 10^{-3}M chlorogenic acid. However, as the copigment concentration increased (1.55 X 10^{-2}M), the copigmentation magnitude observed was approximately two fold that observed for the corresponding complex at the low pigment concentration (2.58 X 10^{-4}M). This is in agreement with previously published results (Mazza and Brouillard, 1990) showing increasing copigmentation with increasing concentration of pigment.

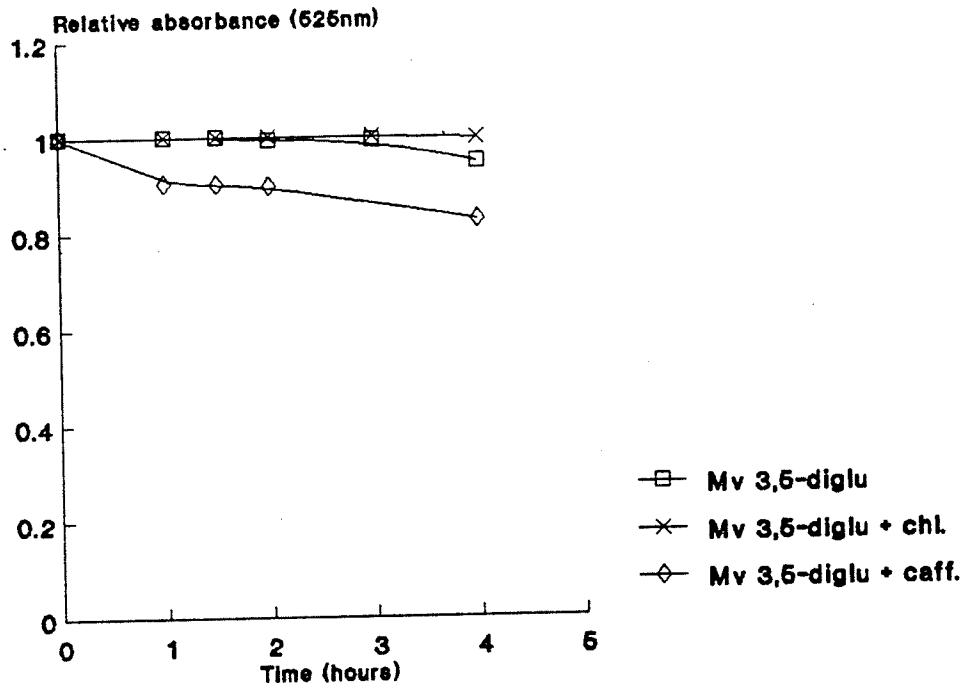
4.4.5 Stability

Comparison of the relative color stability of malvin solutions complexed with chlorogenic and caffeic acids at pH 3.7, expressed as the relative absorbance at 525nm at time=n, reveals that the relative stability of the solutions complexed with chlorogenic acid was greater than that of the caffeic acid complexes (Figure 4.17). Conversely, the pelargonidin solutions complexed with the two phenolic acids revealed that the relative stability of solutions complexed with caffeic acid was greater than that of the chlorogenic acid complexes at pH 3.7 (Figure 4.18b) Interestingly, both the malvin and pelargonidin complexes formed with caffeic acid exhibited lower degrees of stability than the corresponding non–copigmented anthocyanin solutions. At pH 3.7, the two pigment solutions complexed with rutin began to precipitate and measurements ceased as the precipitate could not be redissolved upon additional mixing. Up to 3 hours however, the solutions exhibited similar stability to the complexes formed with chlorogenic acid. The malvin–chlorogenic acid complex exhibited the most stability of the complexes studied at pH 3.7, decreasing only by 15% of the original absorbance after ten days (Figure 4.17b).

The monardaein solutions complexed with chlorogenic and caffeic acids at pH 3.7 (Figure 4.19 a and b) exhibited a higher degree of color deterioration than the corresponding malvin and pelargonidin complexes (Figure 4.17, 4.18). The chlorogenic acid complex exhibited a high degree of deterioration in the first five days and decreased during the later stages of the storage period such that a plateau was reached, maintaining the absorbance at approximately 50% of the original absorbance for the remainder of the storage period (Figure 4.19b).

Figure 4.17 Stability of non–copigmented and chlorogenic acid and caffeic acid (copigment concentration 5.16 X 10^{-3}M) complexed malvin (2.58 X 10^{-4}M) solutions as a function of time (a) hours; (b) days; in aqueous H_3PO_4–NaOAc buffer, pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Time (hours)
Mv 3,5-diglu
Mv 3,5-diglu + chl.
Mv 3,5-diglu + caff.

b.

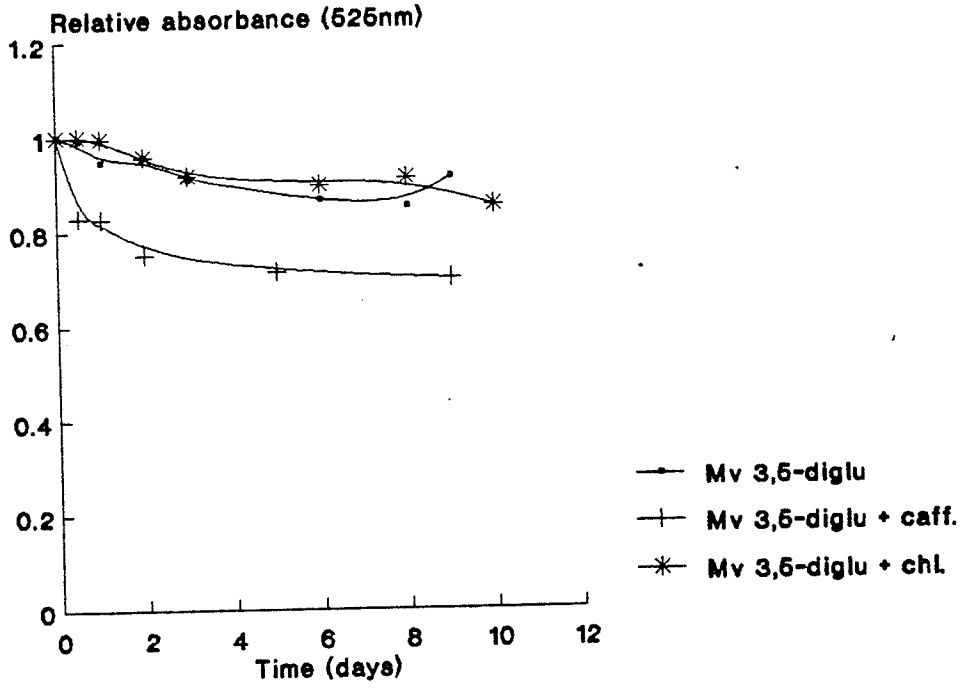
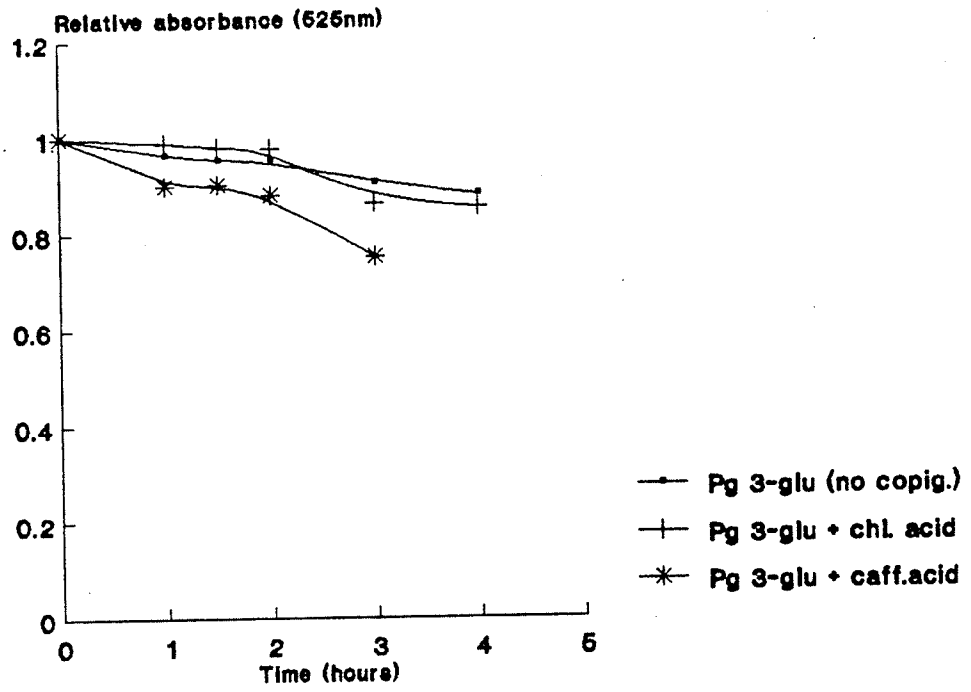

Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
Time (days)
Mv 3,5-diglu
Mv 3,5-diglu + caff.
Mv 3,5-diglu + chl.

Figure 4.18 Stability of non–copigmented and chlorogenic acid and caffeic acid (copigment concentration 5.16 X 10^{-3}M) complexed pelargonidin 3–glucoside (2.58 X 10^{-4}M) solutions as a function of time (a) hours; (b) days; in aqueous H_3PO_4–NaOAc buffer, pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Time (hours)
Pg 3-glu (no copig.)
Pg 3-glu + chl. acid
Pg 3-glu + caff.acid

b.

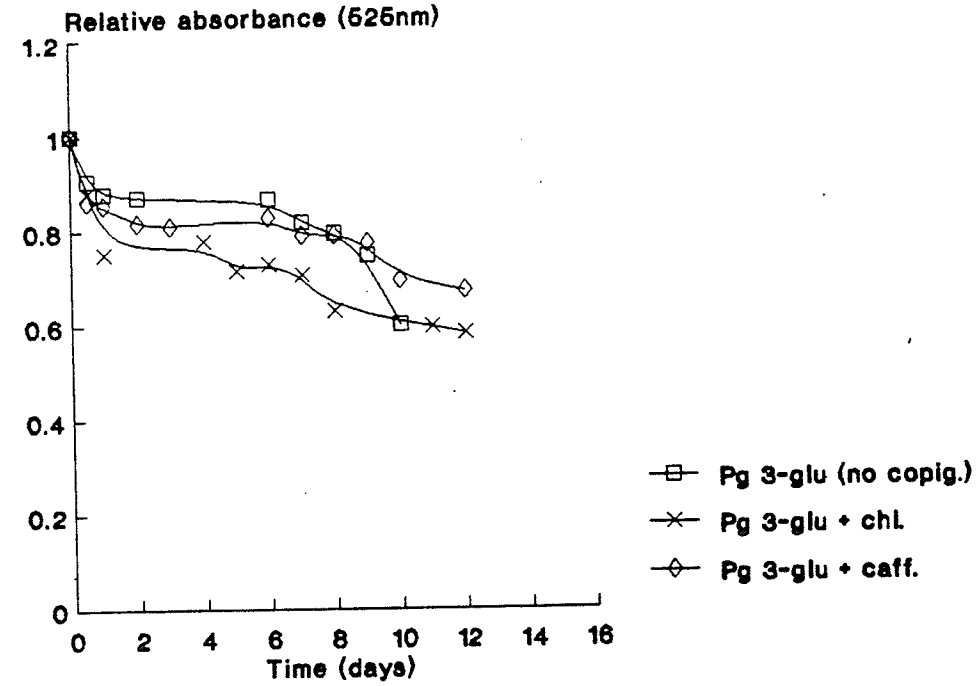
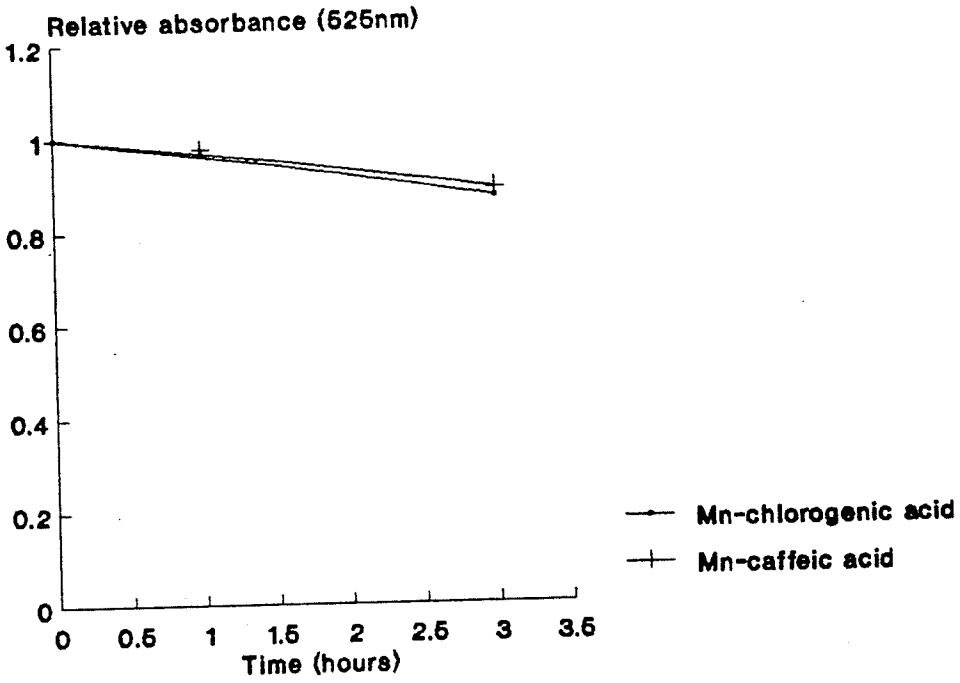

Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
Time (days)
Pg 3-glu (no copig.)
Pg 3-glu + chl.
Pg 3-glu + caff.

Figure 4.19 Stability of chlorogenic acid and caffeic acid (copigment concentration 5.16 X 10^{-3}M) complexed monardaein (2.58 X 10^{-4}M) solutions as a function of time (a) hours; (b) days; in aqueous H_3PO_4–NaOAc buffer, pH 3.7; ionic strength=0.20M; l=1cm; T=$20^0 \pm 0.5^0$C.

a.


Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
Time (hours)
Mn-chlorogenic acid
Mn-caffeic acid

b.

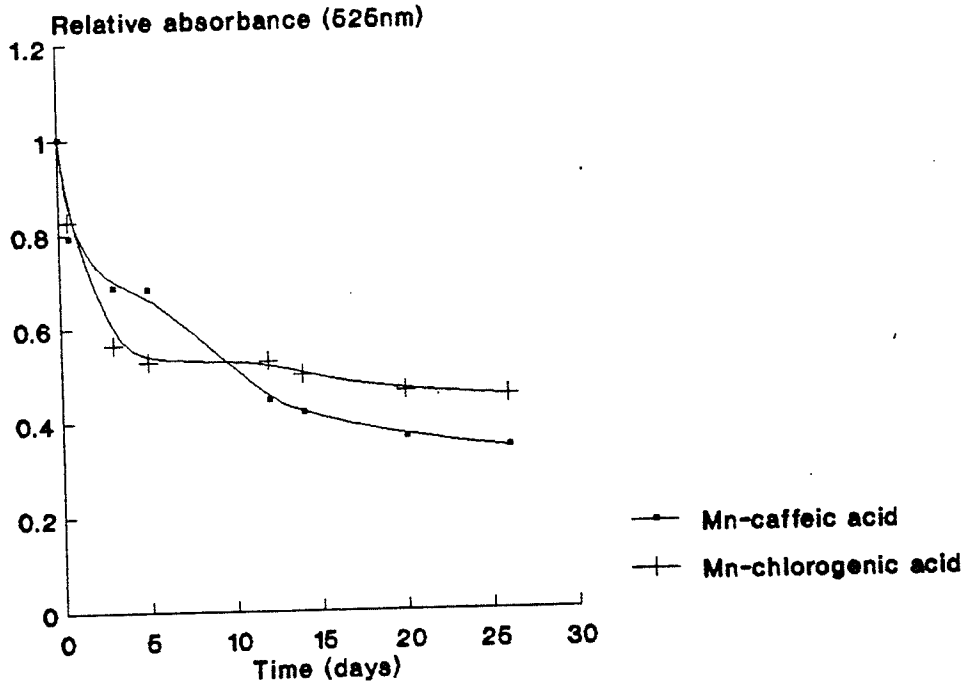
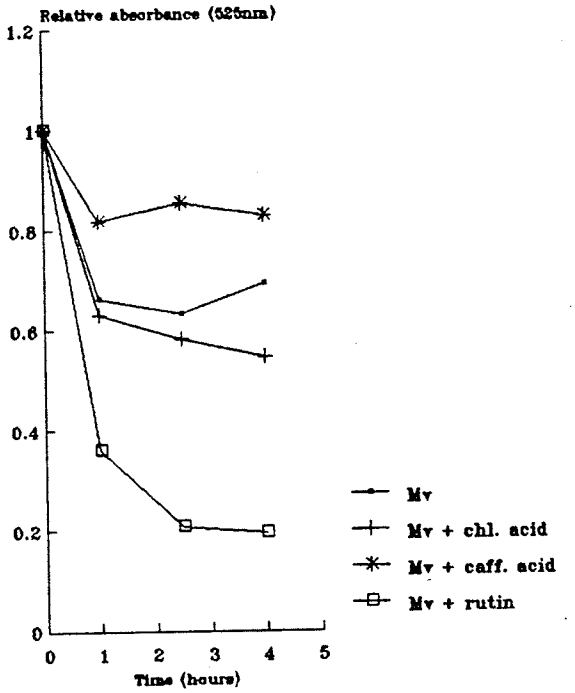

Relative absorbance (525nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Time (days)
Mn-caffeic acid
Mn-chlorogenic acid

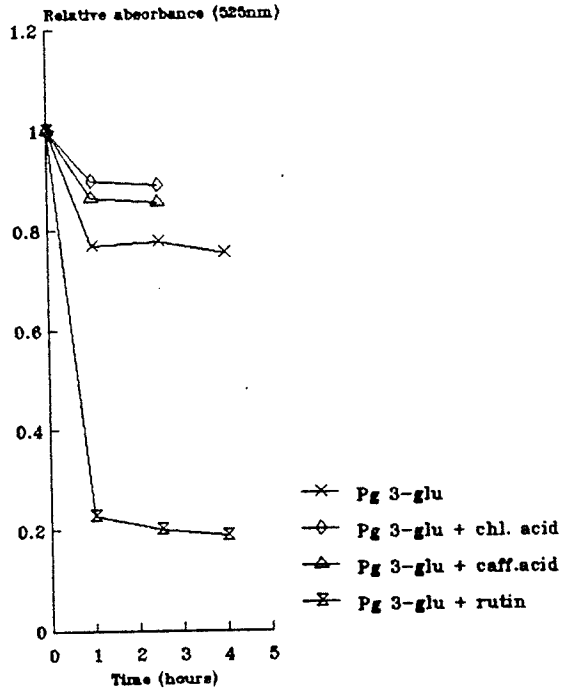
Figure 4.20 (a) Stability of non–copigmented and chlorogenic acid and caffeic acid (copigment concentration 5.16 X 10^{-3}M) and rutin (6.45 X 10^{-4}M) complexed malvin (2.58 X 10^{-4}M) solutions in aqueous H_3PO_4–NaOAc buffer, pH 6.0; ionic strength=0.20M; T=20^0 $\pm$ 0.5^0C.

(b) Stability of non–copigmented and chlorogenic acid and caffeic acid (copigment concentration 5.16 X 10^{-3}M) and rutin (6.45 X 10^{-4}M) complexed pelargonidin 3–glucose (2.58 X 10^{-4}M) solutions in aqueous H_3PO_4–NaOAc buffer, pH 6.0; ionic strength=0.20M; T=20^0 $\pm$ 0.5^0C.

a.


Relative absorbance (520nm)
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5
Time (hours)
Mv
Mv + chl. acid
Mv + caff. acid
Mv + rutin

b.


Relative absorbance (520nm)
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5
Time (hours)
Pg 3-glu
Pg 3-glu + chl. acid
Pg 3-glu + caff.acid
Pg 3-glu + rutin

At pH 6.0, the complex formed between pelargonidin 3–glucoside and chlorogenic acid exhibited greater color stability than the caffeic and rutin complexes (Figure 4.20b). However, in the malvin solutions, the complex formed with caffeic acid exhibited the greatest stability (Figure 4.20a). Both pigment complexes formed with rutin resulted in rapid decreases in absorbance, decreasing to less than 50% of the original absorbance in 1 hour. The malvin–rutin complex was slightly more stable than the pelargonidin 3–glucoside complex. From the results presented previously, it was established that rutin results in a very dramatic increase in absorbance in the pH range approaching neutrality, however from the stability data presented it can be seen that the large magnitude of copigmentation is forfeited by the apparent instability. For all pigments and copigments combinations examined, solutions at pH 3.7 displayed considerably more color stability than solutions at pH 6.0.

4.5 Discussion

From the data presented in Figure 4.09, it can be seen that as the pH increased, the absorbance of the pigment solutions in the absence of added copigments decreased, accompanied by a simultaneous bathochromic shift in the maximum absorbance. As discussed in the literature review, the decrease in absorbance and bathochromic shifts reflect the conversion of the red flavylium cation (AH^+) to the colorless carbinol pseudobase (B) followed by the formation of the blue quinonoidal bases (A, A^-) and the colorless–yellow chalcone forms (C, C^-). The copigmentation reaction reduces the extent of the covalent hydration of the pyrillium nucleus of the flavylium cation. In the present study, it has been shown that the extent to which the copigmentation reaction reduces the hydration reaction is dependent upon pH, copigment and pigment structure and concentrations of both copigment and pigment. Previous reports have shown that temperature, ionic strength and solvent also influence the degree of association between pigment and copigment (Mazza and Brouillard, 1990).

It has been established in the literature that chlorogenic acid effectively stabilizes both the flavylium cation and the quinonoidal bases (Mazza and Brouillard, 1987), forming a stronger association with the flavylium cation, as the pK_a of chlorogenic acid is approximately 3.5 (Timberlake, 1959). The data illustrating the pH of the maximum copigmentation effect of the malvin–chlorogenic acid complexes (Figure 4.01) is in agreement with this theory. It has been established that hydrophobic interactions are the major driving force in the complex formation, governed by the

tetrahedral geometry of liquid water which, in addition to van der Waals forces and hydrogen bonding between pigment and copigment, contributes to the stability of the copigmentation complex (Brouillard et al., 1989; Stillinger, 1980).

The present study reveals that the copigmentation magnitudes exhibited by the caffeic acid complexes are generally higher than the corresponding chlorogenic acid complexes. In the pH range 3.7–5.7, it can be seen that the stoichiometric constants for the all the pigment complexes formed with chlorogenic acid approximate 1.00. Similarly, Mazza and Brouillard (1990) found the stoichiometric constants for solutions of malvin and cyanin complexed with chlorogenic acid approximate 1.00. The authors concluded that a stoichiometric constant close to unity was indicative of a 1:1 association between the copigment and pigment. It has been reported previously in the literature that the intensity of the complex formation depends mainly on the number of free aromatic hydroxyl groups in the flavonoid molecule (Chen and Hrazdina, 1981). On a molecular basis, Figure 4.21 reveals that the structure of chlorogenic acid is approximately two fold that of caffeic acid. The stoichiometry of the copigment and pigment molecules may be such that the alignment of the hydroxyl groups of the chlorogenic acid molecule results in a stronger affinity with the pigment molecule, than the caffeic acid complex. Ultimately, the configuration of the complex formed between the two molecules is such that the site(s) of nucleophilic attack is blocked in order to minimize the hydration reaction. Based on the results presented, it appears that the interaction of chlorogenic acid with the pigment molecule in the pH range 3.2–3.7 is such that the hydration reaction is effectively blocked.

Self association may be attributed to the low degree of copigmentation and the low stoichiometric constants observed for the chlorogenic acid and caffeic acid complexes at pH 2.7, as some of the pigment binding sites would be unavailable for complexation with the copigment. Mazza and Brouillard (1987) have established that the values of K_h', K_a', and K_c' reflect the equilibrium distribution of the structural species AH^+, A, B, and C. From the pK_h' value of 1.85 for solutions of malvin, it can be concluded that both AH^+ and A species exist above this pH. Due to the high concentration of AH^+ species, the copigmentation effect observed at pH 2.7 is lower than that observed at higher pH where the species equilibrium distribution is shifted in favor of the quinonoidal base, A, and chalcone, C.

The pigment structure has also been shown to influence the degree of copigmentation exhibited by

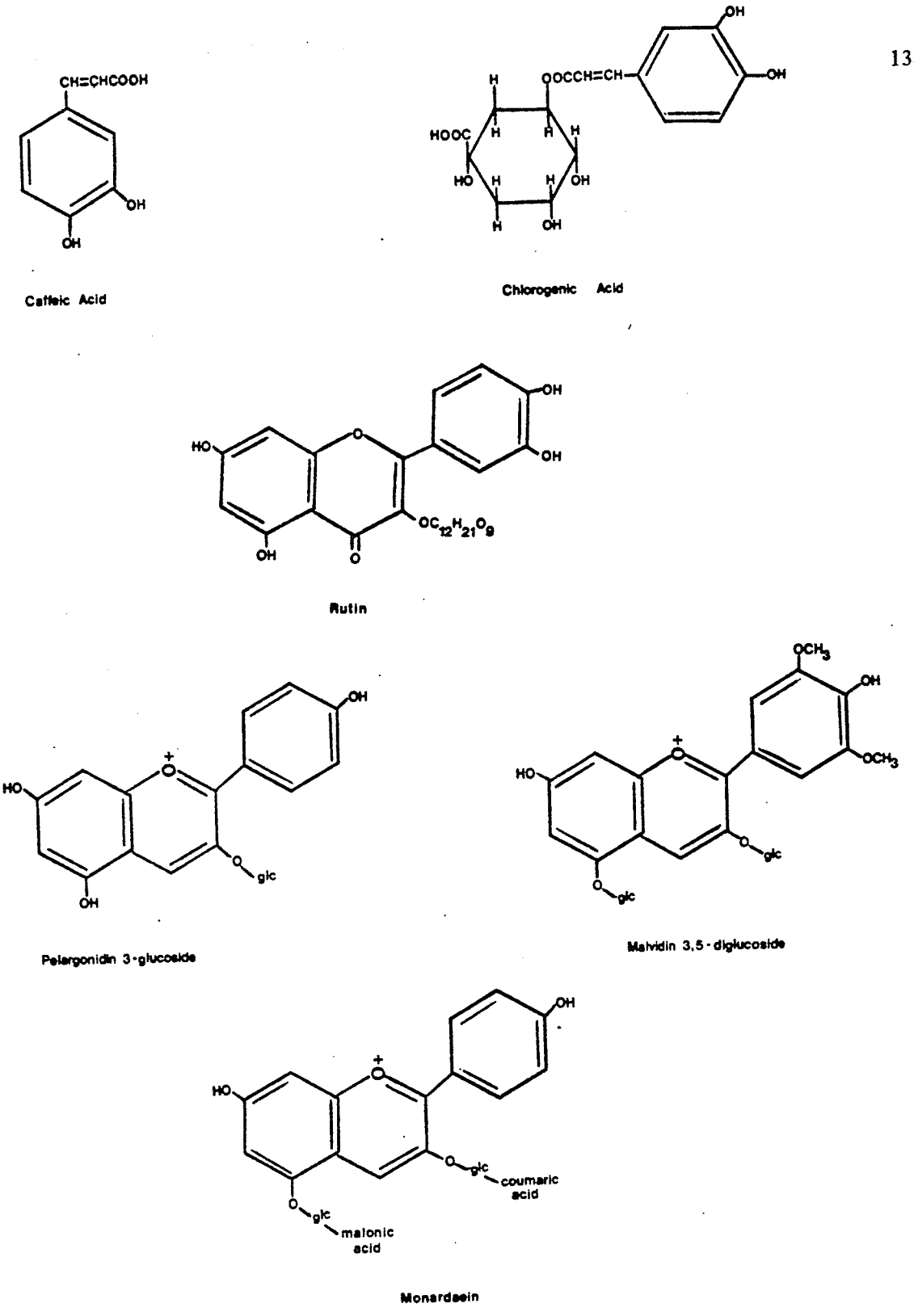


Figure 4.21 Structures of pigments and copigments used for complexation studies.

chlorogenic acid and the predominant form which is stabilized by the copigment in the present study. Solutions of non–acylated and acylated pelargonidins exhibited maximum degree of copigmentation at approximately pH 3.2–3.5 and 3.7–4.7, respectively when complexed with chlorogenic acid. From this, it can be concluded that chlorogenic acid effectively stabilizes the quinonoidal forms (A and A^-) of both pelargonidin 3–glucoside and monardaein. Since it has been established that the substitution pattern of the B ring of the pigment affects the interaction between copigment and pigment, the difference in chlorogenic acid copigmentation observed between the three pigment solutions can be attributed to the structural differences between the three pigments. It can be concluded that methyl substitution has a greater influence on the copigmentation magnitude than the presence of acylation. Generally, the copigmentation observed with pelargonidin 3–glucoside solutions was lower than the other pigments studied and can be attributed to the value of the hydration constant, K_h which has been reported as being approximately 10 fold smaller than that of the corresponding diglucosides (Mazza and Brouillard, 1987). Thus, on the basis of the low hydration constant of the mono–glucoside, the recovery of color by the copigmentation reaction is lowered since the hydration reaction occurs at a much higher pH than in the diglucoside and acylated pigment solutions. Since the hydration reaction occurs at a much lower rate in the monoglucosides, the copigmentation reaction occurs to a lesser extent resulting in lower magnitudes of copigmentation.

Similarly, the trends in copigmentation magnitude exhibited by the caffeic acid complexes formed with the three pigments illustrate the influence of pigment structure on the copigmentation effectiveness of caffeic acid. In solutions of pelargonidin 3–glucoside and monardaein, it has been illustrated that caffeic acid also has a stronger affinity for the quinonoidal bases (A and A^-) than for the flavylium cation (AH^+) in the copigmentation reaction. The maximum copigmentation magnitude observed for the caffeic acid complexes coincides with the pH values 3.5–4.5 where the quinonoidal bases (A and A^-) predominate. The enhanced degree of copigmentation observed with caffeic acid at high pH, ie., the stronger association formed between caffeic acid and the quinonoidal base form of the anthocyanins, can be explained on the basis of the pK_a value of caffeic acid which is approximately 4.5 (Timberlake, 1959).

The difference in stoichiometric constants observed for the eight pigment–copigment combinations over the pH range 2.7–5.7, can be described on the basis of the hydration constants. As illustrated by the results presented, the low stoichiometric constants observed for the caffeic acid–

pelargonidin complexes indicate a 2:1 copigment:pigment formation. It is thus suggested that the lack of the quinic acid moiety in caffeic acid may be conducive to the association of two molecules of this copigment for each molecule of anthocyanin. As shown in Figure 2.11b, this type of association (two molecules of caffeic acid with one molecule of anthocyanin) appears feasible and is likely to occur. Interestingly, the stoichiometric constant exhibited by monardaein is approximately 2 fold greater than the value exhibited by the pelargonidin 3–glucoside solution complexed with chlorogenic acid at pH 2.7. It seems apparent from the results of this study and the reported low acidity constant of the tri–acylated cyanidin anthocyanin of Zebrina pendula (Brouillard, 1981), that the hydration constant of monardaein is much lower than both the di–glucoside and mono–glucoside. On the basis of a low acidity constant and consequent less efficient hydration reaction, the copigmentation magnitudes observed for the monardaein complexes can be explained. Also, the stoichiometric relationship as described by the plot of $\ln((A-Ao)/Ao)$ vs $\ln[Cp]_0$ appears to remain valid at low pH for the acylated anthocyanin. Generally speaking, when compared to the corresponding monoglucoside, the monardaein complexes result in an enhanced degree of copigmentation due to the presence of acylation which decreases the value of the hydration constant and therefore minimizes the extent to which the hydration reaction occurs at a high pH. The end result is a greater copigmentation magnitude observed for the monardaein solutions over all the pH levels studied, as compared to the pelargonidin 3–glucoside solutions.

The results presented reveal that caffeic acid copigmentation results in greater color augmentation than complexes formed with chlorogenic acid. These results are contrary to the report of Chen and Hrazdina (1981) which stated that simple phenols are not effective as copigments. Based on the literature reports of Maccarone et al (1985) and Takeda et al (1990), caffeic acid may have the requisite stereochemistry or configuration to bring its aromatic parts into proximity with the pyrillium nucleus. More significantly, the pK_a value of caffeic acid at 4.5 can be attributed to the stabilization of the quinonoidal base species and hence to the enhanced copigmentation effect observed at high pH. The high pK_a value of caffeic acid paired with the postulated high pK' of monardaein results in effective copigmentation at high pH, as illustrated in Figure 4.07b.

The complex formed between malvin and rutin exhibited a dramatic change in the magnitude of copigmentation as a function of pH (Figure 4.04). From the previous discussion concerning the the large bathochromic shift (+12 nm) observed for the rutin–malvin complex at high pH, it is apparent that there is a displacement of the hydration equilibrium in favor of the flavylium cation.

The results reported are in agreement with those of Sadlowski (1985) which show that the rutin copigmentation observed with solutions of malvin in the pH range 1.3–4.1, occur as a result of complexation with the flavylium cation. Contrary to these findings are the reports of Scheffeldt and Hrazdina (1978) and Williams and Hrazdina (1979), which suggest that the anhydro–base species are stabilized by the complexation reaction with rutin.

The degree of copigmentation exhibited by the malvin solutions complexed with rutin was considerably larger than the effect exhibited by the pelargonidin solutions over the entire pH range studied. Similar trends were observed by Scheffeldt and Hrazdina (1978). As discussed previously, it is the small K_h value characteristic of monoglucosides which results in a lower copigmentation effect than that observed for the di–glucoside solutions. In agreement with the results of Mazza and Brouillard (1990), stating that an increasing degree of glycosylation and methylation of the anthocyanin results in a greater copigmentation effect, the rutin complexes formed with malvin resulted in an enhanced degree of copigmentation. In addition, the absolute configuration of the pigment:copigment complex may provide some explanation for the enhanced stability observed at high pH. Rutin has two additional hydroxyl groups in comparison to chlorogenic acid which are available for bonding to the anthocyanin molecule such that increased protection of the flavylium cation at C2 is provided. Similar to the mechanism describing the enhanced degree of stability of acylated anthocyanins proposed by Goto et al. (1982), it may be the very configuration of the rutin molecules once bound to the anthocyanin which account for the enhanced degree of protection. Rutin, a large structure in comparison to the other copigments investigated, may contribute to the enhanced copigmentation effect observed by stacking in such a way as to prevent nucleophilic attack of the pyrilium ring at high pH. Sadlowski (1985) attributed the hyperchromic copigmentation effects observed with rutin–malvin complexes to a displacement of the cation hydration equilibrium as a result of a hydrophobic stacking interaction of the flavylium cation and the copigment. Thus, the complex stoichiometry of the rutin–pigment complex with the flavylium cation (AH^+) prevents or suppresses the hydration reaction resulting in enhanced pigmentation at high pH.

The equilibrium constant (K) describes the strength of the association between the copigment and the flavylium cation of the pigment (Brouillard et al., 1989). From the K values presented in Appendix 22 it is postulated that the association between caffeic acid and the flavylium form of malvin is stronger than that of the chlorogenic acid association with malvin, while rutin has been shown to form a significantly stronger association than either the chlorogenic or caffeic acid.

Conversely, chlorogenic acid forms a stronger association with the flavylium cation of both pelargonidin 3–glucoside and monardaein than caffeic acid.

From the stability data presented, the pelargonidin 3–glucoside solutions exhibited a more rapid deterioration in absorbance intensity than the corresponding malvin solutions. This substantiates the previous discussion of the effect of methylation and glycosylation on the stability of anthocyanin solutions. From the data presented in Figure 4.20 (a and b), it appears that rutin is very unstable in dilute solution approaching neutrality. However, it is interesting to note that beyond two hours, the relative absorbance of the solution begins to plateau. This may be indicative of a longer equilibration time required for the rutin copigmented solutions. Based on the solubility of rutin, which has been reported as 2.13×10^{-4}M (Sadlowski, 1985), the rapid deterioration in relative absorbance may be as a result of a metastable solution preceding rutin precipitation. The rutin solutions at pH 3.7 exhibited precipitation following 3 hours of mixing however, at pH 6.0, precipitation was not evident until after 72 hours.

In comparison to literature reports of enhanced stability of acylated anthocyanins in neutral solution, monardaein does not appear to be stable in dilute solutions. This low stability can be attributed to the presence of only two acyl substituents. Literature reports of Saito et al. (1985, 1988) state that the stability of acylated anthocyanins increases with increasing organic acid contents, and also with increased substitution of the aglycone. The present study has shown that the presence of methyl substituents on the malvin molecule has a larger effect on the stability of the complexes formed than the presence of acylation on the monardaein molecule. The proposed mechanism of stabilization of the acylated anthocyanins is one of stacking of the aromatic residues of the acyl substituents with the pyrilium ring. Thus, the greater the number of acyl substituents to contribute to the stacking phenomenon, the greater the stability of the anthocyanin.

The results presented illustrate that the addition of a phenolic compound, such as chlorogenic acid or caffeic acid may enhance the stability of a solution of anthocyanin at a given pH. Depending upon the application and the pH of the food product or model system, the effectiveness of these two phenolic compounds in enhancing the stability of anthocyanins varies depending upon the pH and the particular anthocyanin composition of the system. Since the copigments used in this study are also widely occurring plant phenolics in nature, their incorporation into a food in conjunction with an anthocyanin may not be as controversial as a synthetic compound for the enhancement of color.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Results of the characterization of 'Marshall's Delight' Monarda have shown that the flavonoid profile of this Monarda fistulosa X Monarda didyma hybrid contains several acylated anthocyanins and other flavonoids which were identified by spectrophotometry, hydrolysis, and electrophoretic analysis. The predominant anthocyanin was identified as pelargonidin 3,5–diglucoside acylated with coumaric and malonic acids. The colorless flavonoids identified included hydroxycinnamic acids, flavone and apigenin 7–0–glycosides, 5–hydroxyflavone and a dihydroxyflavone 8–C–glucoside.

Quantitative differences were found between the four cultivars of Monarda examined, with Marshall's Delight and Morden #3 containing the highest levels of anthocyanins. The total anthocyanin concentration of Marshall's Delight was found to be higher than most flowers and many of the fruits reported in the literature.

Results of the complexation study demonstrated that pH, copigment and pigment structure and concentration of both copigment and pigment have a dramatic influence on the copigmentation phenomenon. The three phenolic compounds were shown to interact differently with each of the pigments studied, indicating an influence of both copigment and pigment structure on the magnitude of copigmentation. Rutin was shown to result in a dramatic increase in copigmentation magnitude at very low levels of incorporation however, the dramatic copigmentation effect was forfeited by a rapid deterioration in the complex color intensity. Chlorogenic acid and caffeic acid exhibited similar magnitudes of copigmentation, although the caffeic acid complexes exhibited slightly greater copigmentation effects.

Differences were observed in the pH of maximum copigmentation between the two copigments, chlorogenic acid and caffeic acid. Caffeic acid demonstrated a maximum copigmentation effect at a higher pH than chlorogenic acid, which exhibited a maximum copigmentation magnitude in the pH range 3.2–3.7. These differences can be explained on the basis of the acidity constants of the phenolic copigments.

Complexes formed with monardaein resulted in greater copigmentation magnitudes than the corresponding monoglucoside complexes over all pH levels studied. These differences can be attributed to the presence of acyl substituents attached to the monardaein molecule which result in a higher value of the hydration constant as compared to the monoglucoside anthocyanin. Comparison to complexes formed with malvin however, reveal that the presence of methyl substituents has a greater effect than the presence of acylation on the copigmentation phenomenon.

From the results of this study, implications for further research include:

1. isolation of polyacylated anthocyanins from other plant sources with a greater degree of acylation and methylation than the anthocyanin isolated from Monarda, followed by subsequent complexation and stabilization studies;
2. study of the complexation phenomenon in vivo such that the mechanism of the copigmentation phenomenon can be more thoroughly understood;
3. complexation of anthocyanins with phenolic compounds which are co-occurring cellular constituents in vivo, such that the copigmentation phenomenon can be more closely replicated to that which occurs in the plant tissue itself; and
4. evaluation of the copigmentation phenomenon and subsequent stability in a model system which more closely resembles the chemical mileau of an actual food system.

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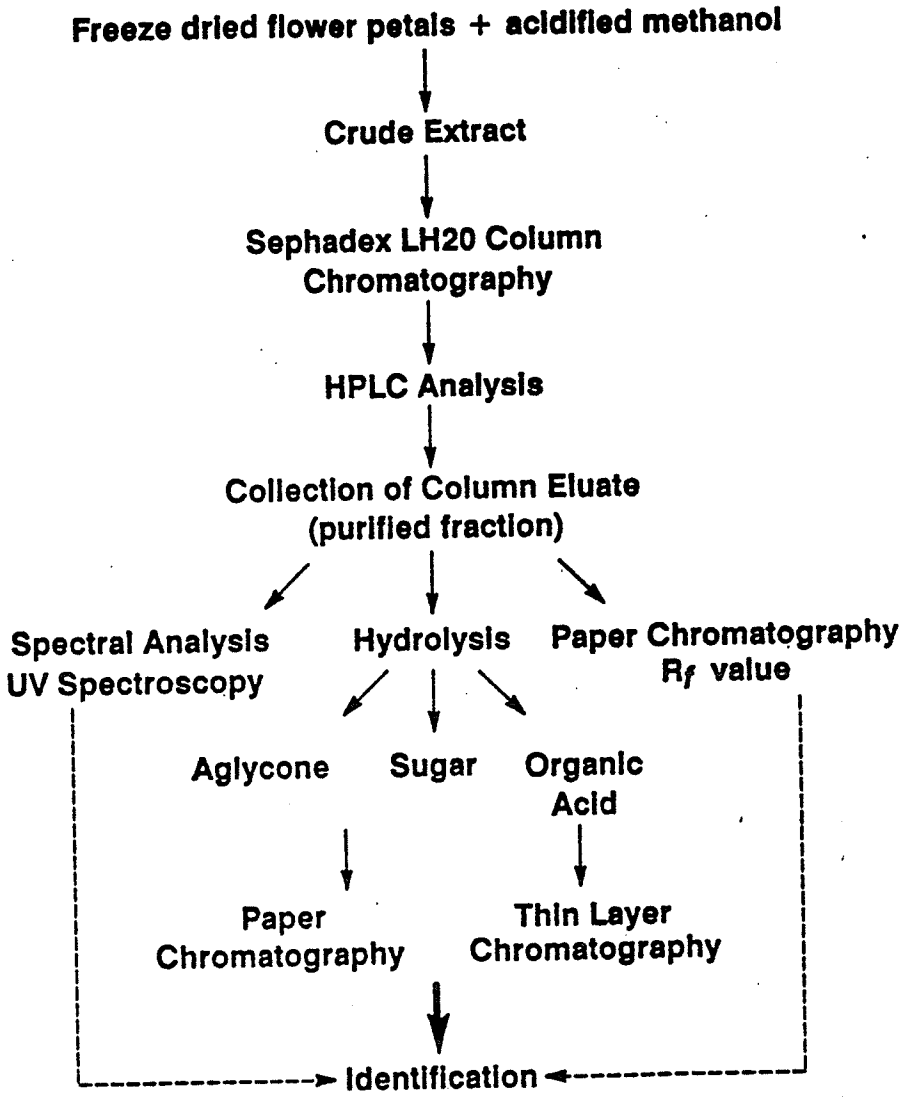
APPENDICES

Appendix 1: Chemical reagents (grade and supplier) used in the structural characterization of flavonoid compounds.

Chemical	Grade	Supplier
Methanol	HPLC grade	Fisher Scientific, Winnipeg, MB
n–Butanol	HPLC grade	Fisher Scientific, Winnipeg, MB
Ethyl ether	Spectral grade	Fisher Scientific, Winnipeg, MB
Formic acid	Reagent grade	Fisher Scientific, Winnipeg, MB
Acetic acid	Reagent grade	Fisher Scientific, Winnipeg, MB
Hydrochloric acid	Reagent grade	Fisher Scientific, Winnipeg, MB
Ammonium hydroxide	Reagent grade	Fisher Scientific, Winnipeg, MB
Potassium chloride	Reagent grade	Fisher Scientific, Winnipeg, MB
n–Amyl alcohol	Reagent grade	Fisher Scientific, Winnipeg, MB
Sodium methoxide	Reagent grade	J.T. Baker, Phillipsburg, NJ
Boric acid	Reagent grade	J.T. Baker, Phillipsburg, NJ
Benzene	Reagent grade	J.T. Baker, Phillipsburg, NJ
Aniline	Reagent grade	J.T. Baker, Phillipsburg, NJ
Phosphoric acid	Reagent grade	J.T. Baker, Phillipsburg, NJ
Coumaric acid	Reagent grade	J.T. Baker, Phillipsburg, NJ
Phthalic acid	Reagent grade	Eastman Organic Chemicals, Rochester, NY
Ferulic acid	Reagent grade	Eastman Organic Chemicals, Rochester, NY
Sodium acetate	Reagent grade	Merck and Co., Montreal, PQ
Pyridine	Reagent grade	Anachemia, Winnipeg, MB
Ethanol	Reagent grade	Stanchem, Winnipeg, MB
Reference sugars	Reagent grade	Sigma Chemical Co., St.Louis, MO

Appendix 2: Methodology schematic for the isolation and characterization of the anthocyanins and colorless flavonoids of Monarda fistulosa L.



Appendix 3: Calculation of moisture content of Monarda petals.

Moisture content was determined using the following formula:

$$\frac{\text{(initial weight of petals} - \text{final weight of petals)}}{\text{initial weight of petals}}$$

Appendix 4: Preparation of buffers for total anthocyanin determination.

Preparation of pH 4.5 buffer:

400 ml of 1M sodium acetate (136g NaOAc/L distilled water)

240 ml of 1N HCl (83.0ml concentrated HCl/L distilled water)

360 ml of distilled water

Preparation of pH 1.0 buffer:

125 ml of 0.2N KCl (14.9g/L distilled water)

335 ml of 0.2N HCl (16.57ml/L distilled water)

The pH of each of the buffers was determined using a Fisher 825 MP Accumet pH meter and were adjusted as required to obtain final pH values of 1.0 and 4.5.

For electrophoresis, the sodium acetate buffer was prepared as described above and adjusted to pH 4.4.

Reference: Wrolstad (1978)

Appendix 5: Sample calculation of total anthocyanin determination.

Based on Lambert–Beer's Law: $A = \varepsilon CL$

where A = absorbance measured spectrophotometrically

ε = molar absorbance

C = molar concentration

L = pathlength of spectrophotometer cell in cm

The difference in absorbance between the two samples was calculated as follows:

$$\text{Absorbance} = (A_{510nm}\ \text{pH 1.0} - A_{700nm}\ \text{pH 1.0}) - (A_{510nm}\ \text{pH 4.5} - A_{700nm}\ \text{pH 4.5})$$
$$= (0.334 - 0) - (0.023 - 0)$$
$$= 0.334 - 0.023$$
$$= 0.311$$

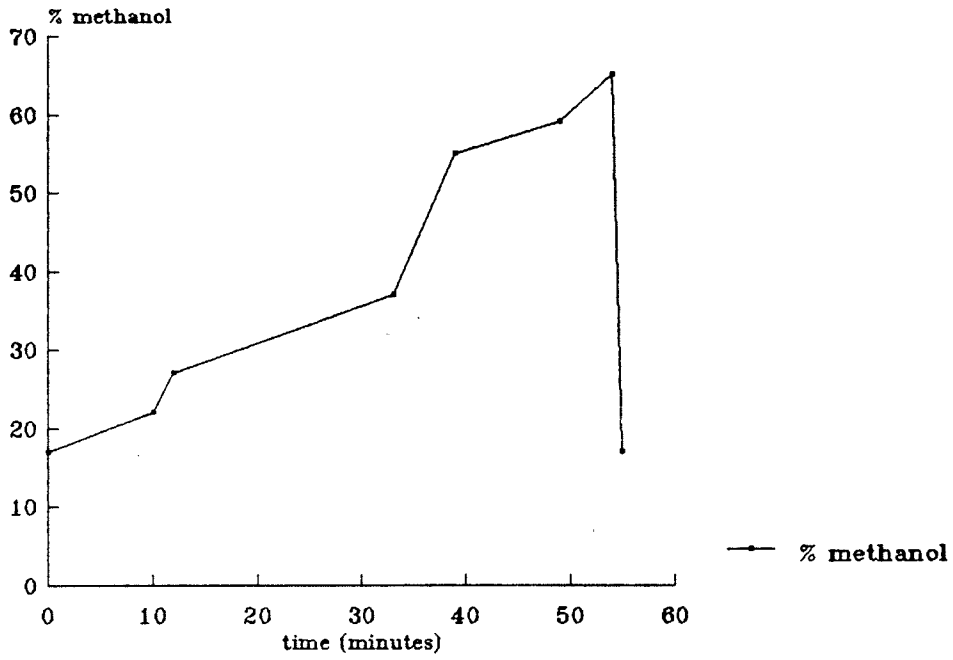
Since 5g of freeze–dried sample is equivalent to 20g of fresh petals, the concentration of total anthocyanin per 100g of fresh petals can be determined as follows:

$$C(mg/100g) = A/\varepsilon L \times MW \times 10^3 \times \text{dilution factor} \times \text{fresh weight equivalent factor}$$

$$= \frac{0.311}{31,900(1)} \times 881.23 \times 10^3 \times 5 \times 5$$

$$= 214.78\ mg/100g\ \text{petals fresh weight}$$

Appendix 6: Linear HPLC gradient; solvent= % methanol in formic acid–water (5:95).



Appendix 7: Paper and thin–layer chromatography solvents.

Solvent	Preparation
Paper Chromatography:	
BAW	n–butanol: glacial acetic acid: water (4:1:5); mixed thoroughly in a separatory funnel, aged 24 hours prior to use
Bu–HCl	n–butanol: 2M hydrochloric acid (1:1); mixed thoroughly in a separatory funnel, aged 24 hours prior to use
1% HCl	concentrated hydrochloric acid in water (3:97)
HOAc–HCl	water: glacial acetic acid:12M HCl (82:15:3)
15% HOAc	glacial acetic acid: water (15:85)
Forestal	glacial acetic acid: concentrated hydrochloric acid: water (30:3:10)
Formic	formic acid: concentrated hydrochloric acid: water (5:2:3)
BBPW	n–butanol: benzene: pyridine: water (5:1:3:3)
Thin Layer Chromatography:	
EtOH–H_2O–NH_4OH	95% ethanol: water: concentrated ammonium hydroxide (16:3:1)
EtOAc–HOAc–H_2O	ethyl acetate: glacial acetic acid: water (3:1:1)

(adapted from Francis, 1982; Markham, 1982; and Takeda et al., 1986)

Appendix 8: Preparation of spectral shift reagents.

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

(adapted from Markham, 1982)

Appendix 9: Spray reagents used for detection of phenolic compounds in paper and thin–layer chromatography.

Spray reagent	Preparation	Compound Detected
Aniline Hydrogen phthalate	0.92ml aniline and 1.6g phthalic acid in 49ml n–butanol, 49ml ether, and 2ml water	Sugars
Glucose Aniline	1ml aniline and 1g glucose in 20ml 50% ethanol	Organic acids

(adapted from Markham, 1982 and Takeda et al., 1986)

Appendix 10: Spectral properties of various phenolic compounds (λ max)

Compound	Band II	Band I
Hydroxycinnamic acids	290–300	305–330
Anthocyanin pigments	270–280	315–325[a], 500–550
Flavonols	250–270	300sh, 350–380
Flavan–3–ols	270–280	
Flavones	250–270	330–350
Flavanones, Flavananols	270–295	300–330sh
Chalcones	220–270	300–320sh, 340–390
Aurones	240–270	370–430
Isoflavones	245–270	300–340

[a] In the case of acylation by hydroxycinnamic acids.

sh=shoulder

Appendix 11: Preparation of buffers for copigmentation study.

0.06M meta-Phosphoric acid buffer:

0.06 M/L X 79.99g/mol = 4.799g/L

0.2M Sodium acetate buffer:

0.2 M/L X 136.09g/mol = 27.22g/L

Preparation of 10M NaOH and 10N HCl for pH adjustment of buffer:

10M NaOH solution:

10 M/L X 40.01g/mol = 400.10g/L

10M HCl solution:

10 M/L X 36.47g/mol = 364.7 g/L

1.19 X 36.47g/mol = 43.40g/100ml

100ml X 36.47/43.40 g/100ml = 84.03ml/100ml distilled water

Appendix 12: **Sample calculation for preparation of anthocyanin stock solution for copigmentation study.**

Example: Malvin chloride, MW=691.04
Concentration of stock solution: 5.16×10^{-4}M

$$(5.16 \times 10^{-4}\text{M}) \times 691.04\text{g/mol} = 0.3566\text{g/L } 0.06\text{M } H_3PO_4$$

$$(0.3566\text{g/L } 0.06\text{M } H_3PO_4) \times 10^{-3} = 0.0003566\text{g/1ml } 0.06\text{M } H_3PO_4$$

Upon addition of an equal volume of 0.2M NaOAc buffer, the final concentration will be 2.58×10^{-4}M, on the basis of the following calculation:

$$\frac{0.0003566\text{g}}{2\text{ml buffer}} \times \frac{1\text{ mol}}{691.04} \times 10^3 = 2.58 \times 10^{-4}\text{M}$$

Similarly, the desired concentrations of pelargonidin 3–glucoside and monardaein were determined on the basis of the MW 468.85 and 881.23, respectively.

Appendix 13: Sample calculation for preparation of copigment solutions for copigmentation study.

Example: Chlorogenic acid, MW = 354.3

Concentration of pigment solution: 2.58×10^{-4}M

Copigment:Pigment molar ratio: 20

Volume of pigment solution = 2ml

Concentration of chlorogenic acid:

$$(2.58 \times 10^{-4}\text{M}) \times 20 = 5.16 \times 10^{-3}\text{M}$$

Amount of chlorogenic acid required to achieve 1:20 molar ratio:

$$(5.16 \times 10^{-3}\text{M}) \times 354.3\text{g/mol} \times 2\text{ml} = 0.00366\text{g}$$

Similarly, the required amounts of the copigments rutin and caffeic acid were determined based on the MW 610.51 and 180.15, respectively.

Appendix 14: Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of malvin chloride-chlorogenic acid solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λmax	Absorbance λmax	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	2.71	517	0.908	0.883	0	0.94
			5	2.69	519	1.010	0.998	0.130	
			10	2.71	522	1.137	1.134	0.284	
			20	2.71	526	1.596	1.593	0.804	
			40	2.71	534	2.079	2.014	1.281	
			80	2.71	535	2.315	2.296	1.600	
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	3.00	519	0.395	0.385	0	
			10	3.00	521	0.454	0.453	0.177	
			20	3.01	525	0.646	0.646	0.678	
			40	3.00	534	1.159	1.115	1.896	
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	3.25	517	0.218	0.213	0	0.86
			1	-	-	-	-	0.142*	
			2.5	3.24	519	0.253	0.252	0.183	
			5	3.26	521	0.280	0.279	0.310	
			7.5	3.25	522	0.310	0.307	0.441	
			10	3.25	527	0.329	0.329	0.545	
			20	3.26	530	0.477	0.468	1.197	
			40	3.24	535	0.872	0.837	2.930	
			80	3.25	541	1.410	1.288	5.047	
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	3.70	520	0.160	0.158	0	0.97
			1	3.72	520	0.168	0.165	0.044	
			5	3.72	521	0.267	0.266	0.684	
			10	3.71	-	-	-	0.694*	
			20	3.71	526	0.375	0.374	1.367	
			40	3.70	534	0.699	0.677	3.285	
			80	3.72	541	1.070	0.990	5.266	
			150	3.72	544	1.695	1.539	8.741	

Appendix 14 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	λ max	λ525	$\frac{A-A_0}{A_0}$	n*
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	4.00	523	0.072	0.071	0	
			10	4.00	525	0.111	0.111	0.563	
			20	4.00	532	0.148	0.147	1.070	
			40	4.01	534	0.255	0.244	2.437	
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	4.71	528	0.079	0.078	0	1.05
			5	4.70	533	0.104	0.104	0.333	
			10	4.70	533	0.112	0.111	0.423	
			20	4.71	533	0.151	0.148	0.897	
			40	4.69	535	0.249	0.240	2.077	
			80	4.70	542	0.485	0.451	4.782	
			150	4.70	547	0.920	0.826	9.590	
			200	4.71	549	1.151	1.022	12.103	
Malvin Chloride	2.58×10^{-4}	Chlorogenic Acid	0	5.71	535	0.061	0.059	0	1.17
			5	5.71	541	0.065	0.064	0.085	
			7.5	5.71	545	0.069	0.067	0.136	
			10	5.70	554	0.075	0.068	0.153	
			12.5	5.71	554	0.078	0.071	0.203	
			15	5.69	556	0.080	0.073	0.237	
			20	5.71	556	0.085	0.078	0.322	
			40	5.72	557	0.137	0.122	1.068	

Appendix 14 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_0}{A_0}$	n*
Malvin Chloride	7.73×10^{-4}	Chlorogenic Acid	0	2.70	514	2.802	2.740	0	0.28
			2.5	2.72	529	3.013	2.998	0.094	
			5	2.70	522	3.079	3.053	0.114	
			7.5	2.72	525	3.064	3.064	0.118	
			10	2.72	531	3.061	3.057	0.116	
			12.5	2.72	536	3.064	3.043	0.111	
			15	2.72	541	3.070	3.072	0.121	
			20	2.70	543	3.382	3.373	0.231	
Malvin Chloride	7.73×10^{-4}	Chlorogenic Acid	0	3.69	520	0.578	0.574	0	1.06
			2.5	3.69	525	0.740	0.740	0.289	
			5	3.69	525	0.938	0.938	0.634	
			7.5	3.69	529	1.108	1.102	0.920	
			10	3.70	530	1.282	1.268	1.209	
			15	3.73	533	1.708	1.663	1.897	
			20	3.70	537	2.208	2.153	2.751	

* extrapolated value

Appendix 15: Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λ max and at 525 nm of malvin chloride–caffeic acid solutions (solvent: aqueous H_3PO_4–NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	2.69	517	0.817	0.789	0	0.63
			2.5	2.70	518	1.106	1.083	0.373	
			5	2.69	525	1.106	1.106	0.402	
			7.5	2.71	523	1.212	1.210	0.534	
			10	2.69	525	1.522	1.522	0.929	
			15	2.70	528	1.538	1.532	0.941	
			20	2.69	529	1.786	1.775	1.250	
			40	2.69	534	2.286	2.239	1.838	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	3.00	518	0.615	0.602	0	
			10	3.00	524	0.972	0.970	0.611	
			20	3.00	531	1.523	1.502	1.495	
			40	3.00	537	2.044	1.941	2.224	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	3.25	521	0.381	0.376	0	
			10	3.24	525	0.677	0.677	0.801	
			20	3.25	531	1.035	1.018	1.707	
			40	3.24	537	1.468	1.395	2.710	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	3.70	521	0.158	0.157	0	0.86
			2.5	3.70	521	0.171	0.169	0.076	
			5	3.72	527	0.199	0.197	0.255	
			7.5	3.72	530	0.215	0.213	0.357	
			10	3.71	530	0.246	0.244	0.554	
			15	3.73	533	0.321	0.316	1.013	
			20	3.71	535	0.412	0.404	1.573	
			40	3.71	535	0.748	0.712	3.535	
			60	3.73	540	0.900	0.842	4.363	

Appendix 15 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	4.00	518	0.106	0.106	0	
			10	4.01	528	0.179	0.177	0.670	
			20	4.00	532	0.274	0.259	1.443	
			40	4.00	539	0.487	0.462	3.358	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	4.25	522	0.084	0.085	0	
			10	4.25	527	0.138	0.136	0.600	
			20	4.26	540	0.217	0.207	1.435	
			40	4.24	540	0.370	0.351	3.129	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	4.71	521	0.072	0.071	0	
			10	4.70	530	0.145	0.141	0.986	
			20	4.71	533	0.195	0.191	1.690	
			40	4.70	536	0.306	0.292	3.113	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	5.25	536	0.056	0.053	0	
			10	5.24	536	0.096	0.091	0.717	
			20	5.26	542	0.113	0.133	1.132	
			40	5.25	552	0.155	0.150	1.830	
Malvin Chloride	2.58×10^{-4}	Caffeic Acid	0	5.71	523	0.070	0.068	0	0.38
			1	5.70	531	0.088	0.086	0.265	
			2.5	5.70	531	0.093	0.092	0.353	
			5	5.69	533	0.103	0.102	0.500	
			10	5.69	533	0.105	0.102	0.500	
			20	5.70	540	0.136	0.134	0.971	
			40	5.69	553	0.142	0.136	1.000	

Appendix 15 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	λ max	λ 525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	7.73×10^{-4}	Caffeic Acid	0	2.71	514	2.802	2.740	0	0.53
			2.5	2.70	524	3.011	3.006	0.097	
			5	2.72	523	3.206	3.176	0.159	
			7.5	2.70	531	3.299	3.257	0.189	
			10	2.72	528	3.341	3.280	0.197	
Malvin Chloride	7.73×10^{-4}	Caffeic Acid	0	3.71	520	0.578	0.574	0	1.48
			2.5	3.69	525	0.715	0.715	0.246	
			5	3.70	529	1.011	1.004	0.749	
			7.5	3.70	533	1.418	1.447	1.521	
			10	3.69	532	1.736	1.690	1.944	
			12.5	3.69	535	2.154	2.072	2.610	

* extrapolated value

Appendix 16: Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of pelargonidin 3-glucoside-chlorogenic acid solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λmax	Absorbance λmax	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58×10^{-4}M	Chlorogenic acid	0	2.70	494	2.882	1.833	0	0.67
			1	2.70	494	2.814	1.870	0.020	
			2.5	2.70	497	2.765	1.874	0.020	
			5	2.70	496	2.701	1.917	0.046	
			7.5	2.70	497	2.593	1.932	0.054	
			10	2.70	499	2.559	1.970	0.075	
			15	2.70	500	2.468	2.078	0.134	
			20	2.70	504	2.367	2.099	0.145	
			40	2.70	508	2.227	2.113	0.153	
Pelargonidin 3-glucoside	2.58×10^{-4}M	Chlorogenic acid	0	3.24	496	2.516	1.577	0	0.63
			5	3.25	497	2.680	1.834	0.163	
			7.5	3.25	498	2.712	1.932	0.225	
			10	3.25	498	2.687	1.995	0.265	
			15	3.25	499	2.789	2.241	0.421	
			20	3.25	502	2.822	2.397	0.520	
			40	3.25	507	2.992	2.780	0.763	
			80	3.25	517	2.971	2.909	0.845	

Appendix 16 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Chlorogenic acid	0	3.72	496	0.996	0.636	0	1.14
			1	3.71	496	0.978	0.642	0.009	
			2.5	3.71	497	0.980	0.666	0.047	
			5	3.73	497	0.972	0.670	0.053	
			7.5	3.70	499	1.067	0.776	0.220	
			10	3.70	499	1.065	0.800	0.258	
			15	3.70	503	1.168	0.923	0.451	
			20	3.70	503	1.263	1.032	0.623	
			30	3.70	505	1.466	1.256	0.975	
			40	3.70	–	–	–	0.988*	
			80	3.70	512	1.981	1.845	1.901	
			150	3.71	516	2.299	2.221	2.492	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Chlorogenic acid	0	4.24	499	0.588	0.441	0	0.82*
			5	4.25	–	–	–	0.259*	
			10	4.26	502	0.692	0.674	0.528	
			20	4.25	506	0.839	0.722	0.637	
			40	4.24	510	1.200	1.101	1.497	
			80	4.26	514	1.666	1.604	2.637	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Chlorogenic acid	0	4.70	500	0.340	0.305	0	1.25
			5	4.70	509	0.329	0.293	0.069*	
			10	4.70	509	0.377	0.345	0.131	
			15	4.71	511	0.432	0.400	0.311	
			20	4.70	514	0.477	0.449	0.472	
			40	4.70	515	0.614	0.596	0.954	
			80	4.70	516	0.941	0.924	2.029	

Appendix 16 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance A_{max}	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Chlorogenic acid	0	5.25	511	0.292	0.281	0	1.26
			5	5.25	-	-	-	0.048*	
			10	5.25	-	-	-	0.116*	
			15	5.25	515	0.331	0.324	0.153	
			20	5.25	516	0.367	0.362	0.288	
			40	5.25	520	0.514	0.511	0.819	
			80	5.25	522	0.839	0.838	1.982	
			150	5.25	525	1.293	1.293	3.601	
			200	5.25	527	1.429	1.427	4.078	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Chlorogenic acid	0	5.70	517	0.278	0.273	0	1.22
			5	5.70	-	-	-	0.057	
			7.5	5.70	517	0.299	0.296	0.084	
			10	5.70	516	0.306	0.304	0.113	
			15	5.70	518	0.340	0.338	0.238	
			20	5.70	520	0.373	0.372	0.363	
			40	5.70	522	0.496	0.496	0.817	
			80	5.70	528	0.749	0.747	1.736	
			150	5.70	530	1.359	1.351	3.949	
			200	5.70	530	1.422	1.410	4.165	
Pelargonidin 3-glucoside	7.73 X 10^{-4}M	Chlorogenic acid	0	3.70	499	2.938	2.113	0	1.17
			1	3.70	497	2.945	2.177	0.030	
			2.5	3.70	501	2.910	2.379	0.126	
			5	3.70	501	3.215	2.807	0.328	
			7.5	3.70	506	3.317	3.085	0.460	
			12.5	3.70	519	3.248	3.180	0.505	

* extrapolated value

Appendix 17: Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of pelargonidin 3-glucoside–caffeic acid solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°$\pm$ 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λmax	Absorbance λmax	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Caffeic acid	0	2.70	492	2.715	1.702	0	0.46
			2.5	2.70	497	2.773	1.903	0.118	
			5	2.70	495	2.852	2.059	0.210	
			7.5	2.70	496	2.841	2.032	0.194	
			10	2.70	502	2.877	2.216	0.302	
			12.5	2.71	500	2.778	2.072	0.217	
			15	2.71	500	2.886	2.189	0.286	
			20	2.71	-	-	-	0.334*	
			40	2.71	504	3.145	2.499	0.468	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Caffeic acid	0	3.25	494	1.738	1.057	0	0.66
			2.5	3.25	495	1.878	1.185	0.121	
			5	3.25	495	2.047	1.356	0.283	
			7.5	3.25	495	2.005	1.367	0.293	
			10	3.25	-	-	-	0.357*	
			12.5	3.25	499	2.133	1.530	0.447	
			15	3.25	498	2.137	1.538	0.455	
			20	3.25	501	2.139	1.621	0.533	
			40	3.24	505	2.358	1.951	0.846	

Appendix 17 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Caffeic acid	0	3.71	497	0.861	0.565	0	0.66
			2.5	3.71	497	1.009	0.675	0.195	
			5	3.71	498	1.104	0.764	0.352	
			7.5	3.71	498	1.201	0.843	0.492	
			10	3.71	499	1.197	0.885	0.566	
			12.5	3.71	499	1.098	0.819	0.450	
			15	3.71	499	1.186	0.881	0.559	
			20	3.70	501	1.305	1.024	0.812	
			40	3.71	506	1.615	1.377	1.437	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Caffeic acid	0	4.24	499	0.388	0.301	0	0.69
			2.5	4.24	498	0.481	0.368	0.223	
			5	4.24	500	0.463	0.371	0.233	
			7.5	4.24	504	0.478	0.379	0.259	
			10	4.24	501	0.557	0.451	0.498	
			15	4.24	503	0.587	0.474	0.575	
			20	4.25	502	0.633	0.505	0.678	
			40	4.24	506	0.788	0.700	1.326	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Caffeic acid	0	4.71	508	0.242	0.216	0	0.67
			1	4.71	503	0.277	0.246	0.139	
			2.5	4.71	505	0.283	0.255	0.181	
			5	4.71	507	0.294	0.263	0.218	
			7.5	4.71	506	0.317	0.278	0.287	
			10	4.71	509	0.334	0.297	0.375	
			15	4.71	508	0.344	0.306	0.416	
			20	4.71	511	0.413	0.374	0.731	
			40	4.71	513	0.461	0.429	0.986	
			80	4.71	513	0.852	0.816	2.778	

Appendix 17 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58×10^{-4}M	Caffeic acid	0	5.68	516	0.200	0.196	0	0.53
			1	5.69	516	0.245	0.239	0.219	
			2.5	5.69	-	-	-	0.184*	
			5	5.68	516	0.240	0.240	0.224	
			10	5.70	515	0.252	0.247	0.260	
			15	5.71	518	0.253	0.249	0.270	
			20	5.71	518	0.295	0.292	0.490	
			40	5.71	518	0.317	0.313	0.597	
			80	5.71	521	0.537	0.533	1.719	
			150	5.71	523	0.705	0.701	2.576	

* extrapolated value

Appendix 18 Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of monardaein-chlorogenic acid solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λmax	Absorbance λmax	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Monardaein	2.58×10^{-4}	Chlorogenic acid	0	2.70	504	1.100	0.837	0	1.46
			5	2.69	507	1.066	0.857	0.024	
			10	2.70	507	1.041	0.863	0.031	
			15	2.69	509	1.101	0.929	0.110	
			20	2.69	509	1.169	1.003	0.198	
			40	2.70	512	1.369	1.254	0.498	
			80	2.70	516	1.691	1.620	0.935	
Monardaein	2.58×10^{-4}	Chlorogenic acid	0	3.70	503	0.219	0.179	0	0.99
			1	3.69	503	0.229	0.189	0.056	
			2.5	3.69	508	0.229	0.191	0.067	
			5	3.69	511	0.237	0.202	0.128	
			7.5	3.70	510	0.252	0.214	0.196	
			10	3.70	510	0.262	0.225	0.257	
			15	3.69	510	0.294	0.258	0.441	
			20	3.70	510	0.305	0.270	0.508	
			40	3.70	513	0.464	0.433	1.419	
			80	3.69	518	0.717	0.695	2.883	
			150	3.69	521	1.109	1.093	5.106	
			200	3.70	521	1.319	1.316	6.352	

Appendix 18 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Monardaein	2.58×10^{-4}	Chlorogenic acid	0	4.70	511	0.048	0.044	0	1.38
			5	4.70	518	0.050	0.046	0.045	
			7.5	4.70	519	0.053	0.049	0.114	
			10	4.69	516	0.057	0.055	0.250	
			15	4.69	517	0.063	0.060	0.364	
			20	4.70	519	0.072	0.069	0.568	
			40	4.69	519	0.104	0.101	1.295	
			80	4.70	519	0.190	0.187	3.250	
			150	4.70	523	0.359	0.358	7.136	
			200	4.69	523	0.467	0.466	9.590	
Monardaein	2.58×10^{-4}	Chlorogenic acid	0	5.70	513	0.038	0.037	0	0.90
			5	5.70	513	0.038	0.041	0.108	
			10	5.70	-	-	-	0.241*	
			15	5.70	516	0.040	0.042	0.135	
			20	5.70	522	0.045	0.044	0.189	
			40	5.70	525	0.063	0.063	0.703	
			80	5.70	524	0.101	0.101	1.730	
			150	5.70	525	0.196	0.196	4.298	
			200	5.70	525	0.284	0.284	6.676	

* extrapolated value

Appendix 19 Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of monardaein-caffeic acid solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Monardaein	2.58×10^{-4}	Caffeic acid	0	2.70	504	1.100	0.837	0	0.79
			5	2.69	506	1.270	1.021	0.219	
			10	2.69	509	1.473	1.242	0.484	
			20	2.69	511	1.497	1.325	0.583	
			40	2.70	512	2.061	1.911	1.283	
Monardaein	2.58×10^{-4}	Caffeic acid	0	3.70	503	0.219	0.179	0	0.79
			5	3.69	506	0.323	0.275	0.536	
			10	3.69	509	0.420	0.371	1.073	
			20	3.70	515	0.476	0.433	1.419	
			40	3.70	515	0.755	0.715	2.994	
Monardaein	2.58×10^{-4}	Caffeic acid	0	4.70	511	0.048	0.044	0	0.71
			5	4.70	509	0.074	0.067	0.523	
			10	4.70	515	0.084	0.079	0.795	
			20	4.70	516	0.088	0.083	0.886	
			40	4.70	515	0.170	0.159	2.614	
Monardaein	2.58×10^{-4}	Caffeic acid	0	5.70	513	0.038	0.037	0	1.23
			5	5.70	516	0.038	0.035	0.170*	
			10	5.70	515	0.047	0.045	0.216	
			20	5.70	515	0.050	0.045	0.216	
			40	5.70			0.081	1.189	

* extrapolated value

Appendix 20 Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of malvin chloride-rutin solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	2.58×10^{-4}	Rutin	0	2.71	515	1.103	1.060	0	1.10
			1	2.71	-	-	-	0.110*	
			2.5	2.71	534	1.369	1.342	0.266	
			5	2.72	531	1.908	1.891	0.784	
			7.5	2.72	548	2.565	2.257	1.129	
			10	2.72	538	2.437	2.272	1.143	
Malvin Chloride	2.58×10^{-4}	Rutin	0	3.80	522	0.130	0.153	0	1.14
			1	3.83	520	0.153	0.151	0.180	
			2.5	3.80	541	0.552	0.506	2.307	
			5	3.81	544	0.651	0.575	2.758	
			7.5	3.81	549	0.820	0.707	3.621	
			10	3.81	552	0.729	0.636	3.969	
			20	3.81	558	1.391	1.159	8.055	
Malvin Chloride	2.58×10^{-4}	Rutin	0	4.71	515	0.074	0.071	0	0.76
			1	4.71	527	0.150	0.149	1.099	
			2.5	4.70	534	0.241	0.240	2.380	
			5	4.71	540	0.369	0.348	3.901	
			7.5	4.71	564	0.498	0.433	5.099	

Appendix 20 continued

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λ max	Absorbance λ max	Absorbance λ 525	$\frac{A-A_o}{Ao}$	n*
Malvin Chloride	2.58×10^{-4}	Rutin	0	5.70	526	0.070	0.069	0	
			1	5.70	525	0.110	0.110	0.594	
			2.5	5.71	552	0.197	0.178	1.580	
Malvin Chloride	7.73×10^{-4}	Rutin	0	2.70	515	3.114	3.058	0	
			2.5	2.72	528	3.283	3.280	0.073	
			5	2.70	541	3.261	3.253	0.064	
Malvin Chloride	7.73×10^{-4}	Rutin	0	3.70	522	0.505	0.503	0	
			2.5	3.71	551	2.604	2.279	3.531	
			5	3.71	541	3.232	3.123	5.209	

* extrapolated value

Appendix 21 Effect of pH, anthocyanin and copigment concentrations on λmax and absorbance at visible λmax and at 525 nm of pelargonidin 3-glucoside-rutin solutions (solvent: aqueous H_3PO_4-NaOAc buffer; l=1cm; ionic strength=0.20M; T=20°± 1°C).

Pigment	Pigment Conc. (M)	Copigment	Copigment/pigment molar ratio	pH	Absorbance λmax	Absorbance λmax	Absorbance λ525	$\frac{A-A_o}{Ao}$	n*
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	2.70	492	2.715	1.702	0	0.143
			1	2.70	497	3.163	2.802	0.646	
			2.5	2.70	500	3.198	2.740	0.609	
			5	2.70	501	3.234	3.114	0.830	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	3.70	497	0.861	0.565	0	0.305
			1	3.70	500	1.538	1.166	1.064	
			2.5	3.70	-	-	-	1.407*	
			5	3.70	506	1.804	1.547	1.738	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	4.25	499	0.388	0.301	0	0.206
			1	4.25	503	0.669	0.568	0.887	
			2.5	4.25	502	0.713	0.585	0.937	
			5	4.25	505	0.815	0.678	1.252	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	4.70	508	0.242	0.216	0	
			1	4.71	508	0.389	0.362	0.676	
			2.5	4.71	505	0.400	0.365	0.690	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	5.25	514	0.209	0.199	0	0.286
			1	5.25	515	0.300	0.296	0.487	
			2.5	5.25	513	0.336	0.325	0.633	
Pelargonidin 3-glucoside	2.58 X 10^{-4}M	Rutin	0	5.70	516	0.200	0.196	0	0.258
			1	5.70	521	0.279	0.278	0.418	
			2.5	5.71	521	0.305	0.300	0.531	

* extrapolated value

Appendix 22 Equilibrium constants for the reaction of complexation between the anthocyanins malvin, pelargonidin 3-glucoside, and monardaein and chlorogenic acid and caffeic acid.

Pigment	Copigment	r_1 (A/A_o)	pH	K	$\overline{X}$
Malvin	Chlorogenic acid	1.054	2.7	30.1	
			3.7	216.9	
			4.7	250.7	
			5.7	321.6	204.8 ± 124
Malvin	Caffeic acid	1.121	2.7	29.2	
			3.7	1075.8	
			4.7	26.4	
			5.7	5.3	284.2 ± 528
Malvin	Rutin	1.072	2.7	905.3	
			3.7	3913.9	
			4.7	576.1	
			5.7	184.6	1395.0 ± 1705
Pelargonidin 3-glucoside	Chlorogenic acid	1.287	2.7	3.2	
			3.7	239.5	
			4.3	198.3	
			4.7	381.7	
			5.3	169.1	
			5.7	149.4	190.2 ± 123
Pelargonidin 3-glucoside	Caffeic acid	1.083	2.7	3.4	
			3.3	16.4	
			3.7	23.9	
			4.3	24.4	
			4.7	21.1	
			5.3	9.6	
			5.7	8.3	15.3 ± 8

Appendix 22 continued

Pigment	Copigment	r_1 (A/A_o)	pH	K	$\overline{X}$
Monardaein	Chlorogenic acid	1.143	2.7	304.4	
			3.7	99.0	
			4.7	581.9	
			5.7	44.1	257.4 ± 244
Monardaein	Caffeic acid	1.067	2.7	42.1	
			3.7	96.2	
			4.7	50.7	
			5.7	17.0	51.5 ± 33