A method for separation and characterization of individual anthocyanins was developed. High Performance Liquid Chromatography (HPLC) with a polymer based reversed-phase column was used to separate the pigments while on-line Photodiode Array Detection (PDA) was employed to record the UV and Visible spectrum of the individual peaks. Spectral information obtained from on-line PDA detection provided information about: 1) the nature of the aglycone, 2) the sugar substitution pattern and 3) the presence or absence of hydroxy aromatic organic acids. The nature of the glycosidation can be determined from the HPLC retention characteristics.

The HPLC/PDA methods were employed to characterize the anthocyanin profiles of the pigments in cranberry, roselle, cherry, bilberry, grape, red cabbage, black raspberry, blackberry, elderberry, plum, blackcurrant and strawberry. The anthocyanidin profiles were also determined for the samples for purposes of confirmation of the anthocyanin data.

In addition to the anthocyanin and anthocyanidin profiles, the general coloring properties for most of the samples were also determined. Included were Hunter L, a,
b values, total anthocyanin pigment concentration, wavelength maxima, percent tannin measurements, tinctoral strength, pH measurements and titratable acidity.
Characterization of Anthocyanins In Fruit Juices and Natural Colorants

by

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Typed by the author Victor Hong
I would like to express my gratitude to my major professor, Dr. Ronald E. Wrolstad for his advice, encouragement and patience in my education. He has opened many doors for me. I don’t know how to thank him enough.

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# Table of Contents

INTRODUCTION .................................................................................................................. 1

LITERATURE REVIEW ................................................................................................. 3

   The Chemistry of Anthocyanins................................................................. 3
   Anthocyanins As Chemical Markers.......................................................... 11
   Measurement of Color............................................................................... 14
   Analysis of Anthocyanins........................................................................ 20
   Anthocyanin Composition....................................................................... 35

USE OF HPLC SEPARATION/PHOTODIODE ARRAY DETECTION FOR
CHARACTERIZATION OF CRANBERRY, ROSELLE AND STRAWBERRY
ANTHOCYANINS......................................................................................................... 48

   Abstract........................................................................................................ 49
   Introduction................................................................................................ 50
   Experimental Section................................................................................ 52
   Results and Discussion............................................................................ 57
   Literature Cited........................................................................................ 88

CHARACTERIZATION OF ANTHOCYANIN CONTAINING COLORANTS AND
FRUIT JUICES BY HPLC/PHOTODIODE ARRAY DETECTION........................................... 90

   Abstract........................................................................................................ 91
   Introduction................................................................................................ 92
   Materials and Methods........................................................................... 94
   Results and Discussion............................................................................ 97
   Literature Cited........................................................................................ 144

BIBLIOGRAPHY............................................................................................................ 148
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Basic structure of the anthocyanidins</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Six principle resonance forms for the oxonium ion</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Structural transformations of anthocyanins with change in pH</td>
<td>7</td>
</tr>
<tr>
<td>2.1</td>
<td>HPLC chromatogram of blueberry anthocyanidins</td>
<td>74</td>
</tr>
<tr>
<td>2.2</td>
<td>HPLC chromatogram of cranberry anthocyanins, minimal sample cleanup (Cleanup Procedure I)</td>
<td>75</td>
</tr>
<tr>
<td>2.3</td>
<td>HPLC chromatogram of cranberry anthocyanins subjected to PVPP adsorption and precipitation by diethyl ether (Clean-up procedure II). 2.3A, detection at 520 nM. 2.3B, detection at 260 nM.</td>
<td>76</td>
</tr>
<tr>
<td>2.4</td>
<td>HPLC chromatogram of roselle anthocyanins. 2.4A, detection at 520 nM. 2.4B, detection at 260 nM.</td>
<td>77</td>
</tr>
<tr>
<td>2.5</td>
<td>HPLC chromatogram and spectral characteristics of bilberry and strawberry anthocyanidins chromatographed on the anthocyanin HPLC system</td>
<td>78</td>
</tr>
<tr>
<td>2.6</td>
<td>UV-visible spectra of cyanidin-3-galactoside (2.6A), peonidin-3-galactoside (2.6B) and delphinidin-3-sambioside (2.6C) determined by Photodiode Array Detection</td>
<td>79</td>
</tr>
<tr>
<td>2.7</td>
<td>HPLC chromatogram of commercial Malvin (malvidin-3,5-diglucoside)</td>
<td>81</td>
</tr>
<tr>
<td>2.8</td>
<td>UV-visible spectra of malvidin-3,5,-diglucoside determined by Photodiode Array Detection</td>
<td>82</td>
</tr>
<tr>
<td>2.9</td>
<td>UV-visible spectra of 2 unknown anthocyanins from red cabbage</td>
<td>83</td>
</tr>
</tbody>
</table>
List of Figures (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10</td>
<td>HPLC chromatogram of the fraction of cranberry anthocyanins remaining on a C18 Sep-Pak after elution with alkaline borate buffer</td>
<td>84</td>
</tr>
<tr>
<td>2.11</td>
<td>HPLC chromatograms of strawberry anthocyanins</td>
<td>85</td>
</tr>
<tr>
<td>2.12</td>
<td>UV-visible spectra of pelargonidin-3-glucoside from strawberry</td>
<td>87</td>
</tr>
<tr>
<td>3.1</td>
<td>HPLC chromatogram of black currant anthocyanins</td>
<td>130</td>
</tr>
<tr>
<td>3.2</td>
<td>HPLC chromatogram of blackberry anthocyanins</td>
<td>131</td>
</tr>
<tr>
<td>3.3</td>
<td>HPLC chromatogram of black raspberry anthocyanins</td>
<td>132</td>
</tr>
<tr>
<td>3.4</td>
<td>HPLC chromatogram of cyanidin-3-xylosylrutinoside</td>
<td>133</td>
</tr>
<tr>
<td>3.5</td>
<td>HPLC chromatogram of elderberry anthocyanins</td>
<td>134</td>
</tr>
<tr>
<td>3.6</td>
<td>HPLC chromatogram of plum anthocyanins</td>
<td>135</td>
</tr>
<tr>
<td>3.7</td>
<td>HPLC chromatogram of morello cherry anthocyanins</td>
<td>136</td>
</tr>
<tr>
<td>3.8</td>
<td>HPLC chromatogram of bilberry anthocyanins</td>
<td>137</td>
</tr>
<tr>
<td>3.9</td>
<td>HPLC chromatogram of Spreda Grape Skin Extract anthocyanins</td>
<td>138</td>
</tr>
<tr>
<td>3.10</td>
<td>HPLC chromatogram of the anthocyanins in Welches Grape Colorant</td>
<td>140</td>
</tr>
<tr>
<td>3.11</td>
<td>HPLC chromatogram of red cabbage anthocyanins</td>
<td>142</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Compilation of References on the Separation of Anthocyanidins and Anthocyanins by Reversed Phase HPLC</td>
<td>26</td>
</tr>
<tr>
<td>2.1</td>
<td>Percentages of Anthocyanidins in Cranberry and Roselle as determined by HPLC</td>
<td>71</td>
</tr>
<tr>
<td>2.2</td>
<td>Spectral Characteristics of Cranberry and Roselle anthocyanins determined by HPLC/Photodiode Array Detection along with Spectral Values for Selected Anthocyanins as Reported in the Literature</td>
<td>72</td>
</tr>
<tr>
<td>2.3</td>
<td>Spectral Characteristics of Strawberry anthocyanins as determined by HPLC/Photodiode Array Detection</td>
<td>73</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparison of General Colorant Properties for Commercial Colorants and Juice Concentrates</td>
<td>123</td>
</tr>
<tr>
<td>3.2</td>
<td>HPLC Anthocyanidin Profile of Commercial Colorants and Juice Concentrates</td>
<td>126</td>
</tr>
<tr>
<td>3.3</td>
<td>Spectral Characteristics of Commercial Colorants and Juice Concentrates as Determined by HPLC/Photodiode Array Detection</td>
<td>127</td>
</tr>
</tbody>
</table>
INTRODUCTION

Colorants, both natural and synthetic are an important part in the formulation of many food products. Without them, the visual appearance of many foods would greatly suffer. Recently, world wide restrictions have been applied to the legal use of synthetic food colorants. Red colorants have been particularly affected. The recent banning of Red No. 2 (Wallin and Smith, 1977) as a legal food colorant along with the questionable status of Red No. 40 in the United States has lead to increased interest in the use of natural red pigments as coloring agents.

There are principally 3 sources of allowed naturally occurring water soluble red colored pigments: 1) the anthocyanins, a large class of pigments derived from fruits and flowers; 2) betalains from red beet and 3) cochineal from the insect Coccus cacti (Wallin and Smith, 1977). This dissertation will be focused on the study and characterization of several economically important sources of anthocyanin containing colorants.

The emphasis of this work will be on the development of analytical methodology for characterization of the individual anthocyanin pigments from natural sources and the use of this methodology for analysis of commonly available anthocyanin containing colorants. Such data is useful from a regulatory standpoint. There are many different anthocyanins found in nature. The composition of anthocyanins in different plants is distinctive and has been useful as taxonomic
indicators. Development of analytical methods along with the compilation of a database for the individual anthocyanins from common commercial anthocyanin containing colorants can potentially serve as a systematic means for verification of the nature of added colorants and for checking the authenticity of an anthocyanin containing product.

This dissertation is divided into three sections. The first section, the Literature Review, is meant to give the reader 1) a general background on the basic chemistry of the anthocyanins, 2) a description of the analytical methods which have been applied to these pigments and 3) a review of the anthocyanin composition of the selected fruits and vegetables which will be studied in this research project. The second section is a paper entitled "Use of HPLC Separation/Photodiode Array Detection for Characterization of Individual Anthocyanins in Cranberry and Roselle". This paper describes the development and use of analytical methodology for separation and characterization of anthocyanins. As examples, the anthocyanins of both cranberry and roselle are characterized. The last section of this dissertation is a paper entitled "Determination of Anthocyanin Profiles in Commercial Fruit Colorants and Juices by HPLC/Photodiode Array Detection". In this paper, the methods developed in section 2 are used to characterize the anthocyanins in a number of commercial fruit juices and commercial colorants. The results from this last section not only serves as a database for the anthocyanin composition of the fruits and vegetables studied but is also a demonstration of the usefulness of the methods developed in this study.
LITERATURE REVIEW

The Chemistry of Anthocyanins

Introduction

The anthocyanins are of great interest to the Food Industry as they impart the pleasing red and blue colors associated with many fruits and vegetables. Often it is these colors or the lack of these colors which the consumer recognizes as a sign of good quality and freshness (Eskin, 1979). Anthocyanins are present in almost all higher plants and are found in all parts of the plant. Because man and animals have consumed these pigments from the beginning of time without any apparent ill effects, they are a highly desirable substitute for synthetic food colors which, obviously, cannot be subjected to such extensive testing (Brouillard, 1982).

The anthocyanins are phenolic substances belonging to the general group of C_6-C_3-C_6 secondary plant metabolites known as flavonoids. In highly acidic media, their basic structure is that of glycosidated derivatives of the 3,5,7,3' pentahydroxyflavylium cation. The non-glycosidated derivatives (the aglycones), are called the anthocyanidins. There are six commonly occurring anthocyanidins, their structures are listed in Fig. 1.1.
The occurrence of free anthocyanidins has been reported from time to time, however, it is unlikely that they occur in nature considering their instability and limited solubility in water (Shrikhande, 1976). The differences between the different anthocyanidins is due to the different hydroxylation and/or methoxylation patterns in the B-ring of the pentahydroxyflavylium molecule (Fig 1.1). Glycosidation of the anthocyanidin yields an "anthocyanin", it is this form that is almost always found in nature. Typically, glycosidation occurs at the 3,5 or 7 positions. The sugar moiety in the molecule serves to confer sap (water) solubility and stability to the molecule. The most common sugars found are the monosaccharides, such as glucose, galactose, arabinose and rhamnose. Di- and tri-saccharides also occur. The 3-hydroxyl position of the pentahydroxyflavylium molecule is almost always replaced by a sugar except in the case of 3-deoxyanthocyanins in which the sugar is in the 5 position. When a second sugar is present, it is generally at the C-5 position. Furthermore, the 5 or 7 position sugar is always glucose (Shrikhande, 1976). Glycosidation at the 3'
and 5' positions has also been demonstrated (Brouillard, 1982). In some cases, the sugar moieties are acylated by p-coumaric, caffeic, ferulic, or sinapic acids and sometimes by p-hydroxybenzoic, malonic or acetic acids. When present, these acyl substituents are usually bonded to the C-3 sugar (Harborne, 1964).

Structural Transformations in Water

As previously discussed, the anthocyanins are composed of as many as 3 chemical components: the 3,5,7,3' pentahydroxyflavylium cation derivative (anthocyanidin); one or more sugar moieties; and in some anthocyanins, one or more organic acid moieties. It is however, the flavylium cation that is responsible for most of the important chemical properties of the anthocyanins and therefore in this next section, the general chemistry of flavylium cations will be presented.

The flavylium cation is a heteroaromatic carboxonium cation. The positive charge is delocalized over the whole heteroaromatic system with the highest partial positive charges occurring at C-2 and C-4 (Bendz et al., 1967). Using the 5,7,4' tri-hydroxyflavalium cation as a simple example, 6 principal resonance structures can be drawn (Fig 1.2), consisting of an oxonium ion, two carbonium ions and three phenoxonium ions.

Figure 1.2 Principal Resonance Forms for the Oxonium Cation
These resonance structures help to illustrate the high reactivity of the flavylium cation. The partial positive charges at C2 and C4 make these carbon atoms potential sites for nucleophillic addition and/or substitution reactions, while the partial positive charges at the 5,7 and 4' hydroxyl groups suggest that the protons will be easily removed, even by weak acids (Brouillard, 1982).

One of the most noticeable and important reactions of the flavylum cation is its reactions with water. It is well known that anthocyanins act as acid-base indicators. In the low pH range, solutions of these pigments are red. As the pH of the solution is raised, the red color gradually fades away with the solution becoming colorless in the pH 4.0 - 4.5 range. Further increasing the pH produces a purple solution which eventually becomes deep blue at pH 7-8. Finally upon standing or with further increase in pH, the solution becomes yellow (Hrazdina, 1974). Kinetic, thermodynamic and spectroscopic methods have been employed (Brouillard, 1982) to show that in acidic solutions, 4 anthocyanin species exist (Figure 1.3), a quinonoidal base (A), the flavylium cation (AH+), the pseudobase or carbinol (B) and the chalcone (C).
Each of these different structures have very different spectral characteristics (and are consequently perceived as having different visual color). It should be noted that sugars, acylated sugars and methoxyl groups attached to the anthocyanidin have a marked effect upon the reactions leading to the formation of these different forms (Brouillard, 1982).

In solution, the four anthocyanidin species undergo the following series of equilibria (Brouillard and Delaporte, 1977; Brouillard and Dubois, 1977):
Equilibrium (1) is a fast diffusion-controlled acid-base reaction. Equilibrium (2) is much slower, the half-life being a few minutes. This reaction is a hydration reaction: nucleophillic attack on the pyrylium ring by water. Equilibrium (3) is a slow ring-chain tautomeric reaction leading to the formation of the chalcone.

In highly acidic solutions, it is the flavylium cation that predominates and is responsible for the characteristic red color. In slightly acidic media (pH 4-6), deprotonation of the hydroxylated flavylium cation occurs to produce the blue colored quinonidal species (eq 1). The flavylium cation can also be hydrated to form the colorless carbinol or pseudobase (eq. 2). The carbinol form can further undergo ring-chain prototropic tautomerism to form the yellow colored chalcone (eq 3). It should be noted that pH not only affects the structure of the flavylium cation but also its stability. At room temperatures, anthocyanins are stable only in acidic media (Brouillard, 1982). In alkaline media, destruction of the anthocyanin takes place rapidly (Brouillard, 1982).
A general point about nomenclature should be mentioned. Anthocyanins are assumed to occur in living cells associated with anions of organic acids. In text and formulae, the presence of the pigment is referred to by the name of the cation (Harborne, 1967).

Co-Pigmentation and Self-Association

It is well known that anthocyanins are responsible for the large variety of red to blue colors found in many fruits and flowers. It was however more difficult to explain how these pigments were able to produce such a wide variety of colors in nature. Differences in color due to pH effects and in different anthocyanin structure are not sufficient explanations. Most plant tissues are in the pH 4-6 range, which favors the colorless carbinol form of pure, isolated anthocyanins (Asen et al., 1972; Brouillard, 1982; Asen, 1976). Since the anthocyanins are highly colored at these pH levels in the plant, co-pigmentation was suggested as a possible mechanism.

Co-pigmentation is the interaction between anthocyanins and other molecules (referred to as the co-pigment), resulting in a change in color (usually a shift to a higher wavelength maxima), an increase in absorbance (when compared to the non co-pigmented anthocyanin at identical conditions) and an increase in stability (Osawa, 1982). The co-pigments by themselves do not significantly contribute to the color (Brouillard, 1982). It is thought that a complex is formed between the anthocyanin and the co-pigment resulting in altered spectral properties.

The most powerful co-pigments are flavonoids and other phenolic compounds such as cinnamic acids and benzoic acids (Brouillard, 1982; Osawa, 1982; Jurd and Asen, 1966; Asen et al., 1972). Other possible plant constituents have also been investigated for co-pigmentation effects. Asen et al. (1972) found that alkaloids such
as caffeine and brucine had an effect but that amino acids had little or no effect. Polyvalent cations such as aluminum ions are also known to act as co-pigments. These ions form chelation type complexes with those anthocyanins containing o-dihydroxyl systems, eg. cyanidin, petunidin and delphinidin. The effect of metal ions on anthocyanin color is well studied (Asen et al., 1969), however, there is still doubt as to whether this mechanism is significant in plant tissues (Asen, 1976).

Anthocyanins containing at least 2 acyl groups have been found to be extremely stable throughout the entire pH range (Brouillard, 1982). Anthocyanins with less than 2 acyl groups do not exhibit this property. It was also found that for the diacylated caffeoylferuloyl cyanidin 3,7,3'-triglucoside, the chalcone and carbinol forms did not exist. These facts have led Brouillard to infer that one acyl group is situated above the pyrylium ring and the other beneath it, protecting the pyrylium ring from attack from water (Brouillard, 1981). This is another example of how co-pigmentation can change the chemistry of the anthocyanins, in this case, at the intramolecular level.

It has been observed that solutions of pure anthocyanins do not follow Beer's law (Timberlake and Bridle, 1983). That is, linearly increasing the anthocyanin concentration does not produce a linear increase in absorbance values. For example, Asen et al. (1972) at pH 3.16 found that increasing the concentration of cyanidin-3,5-diglucoside from 0.0001 M to 0.01M (100 fold) produced a 300 fold increase in absorbance. This effect, which still awaits an adequate explanation is known as self-association (Timberlake and Bridle, 1983). Hoshino et al. (1981) studying anthocyanins in neutral solutions by circular dichroism, suggest that self association
at pH 7 is due in part to hydrophobic interactions between aromatic nuclei and hydrophilic interactions between sugar moieties.

Much of the work in the area of co-pigmentation and self-association has been in an attempt to explain the mechanisms behind color variation in fruits and flowers. It should be realized however that this affect is also important in anthocyanin containing beverages. Timberlake and Bridle, (1983) studying fruit juices and wines found that both self-association and co-pigmentation occur in the same solution, the relative rates being pH dependent. Self-association predominates at lower pH's whereas co-pigmentation is the major effect at higher pH's. The authors emphasized that because of these effects, color in anthocyanin containing beverages is arbitrary in a sense that so many variables exist which contribute to color. For example, losses in anthocyanin due either to dilution or degradation will produce proportionally greater losses in visual color. It is important that these effects are understood and taken into consideration when studying color in anthocyanin containing juices and wines.

Anthocyanins As Chemical Markers

Chemical analysis of plant constituents (chemotaxonomy) is a excellent objective method for positive identification and classification of plants. Asen (1982) gives an excellent summary of the usefulness of flavonoids as chemical markers:

"All inherent morphological manifestations of cultivar (and species) differences must ultimately have a biochemical difference, but not all biochemical differences are expressed morphologically."
Thus, biochemical differences should be more numerous than morphological differences. The loss or modification of a single step in a biochemical pathway might occur without any significant morphological changes, and plants which are judged similar, on the basis of their morphological characteristics, might differ widely in their chemical constitution.

Among all plant chemical constituents, flavonoids (anthocyanins are included in this large class of phenolic secondary plant metabolites) have been more widely used as chemical markers than any other group of plant substances. The advantages, over most other low molecular weight constituents, are their universal distribution in vascular plants, considerable structural diversity, chemical stability, and the ease and rapidity of identification. The exceptional usefulness of flavonoids is that they are not actively concerned in cellular metabolic processes. Although the concentration of flavonoids can be influenced by the tissue sampled and changes in environment, any particular flavonoid constituent can be relied on to be present in more or less constant amounts when uniformly sampled from plants grown under the same environment."

Analysis of anthocyanin and/or flavonoid composition has been very useful in distinguishing between species and higher levels. This is especially true if there are qualitative differences. With closely related taxa (eg. at the cultivar or variety
level) however, the use of chemotaxonomy has been limited as most of the differences are quantitative and not qualitative (Stewart et al., 1979).

Much work has been focused on the use of flavonoid profiles to distinguish between closely related taxa. Asen (1977) found only quantitative differences in the petal flavonoid profile between two indistinguishable seedlings of sister New Guinea White Impatiens seedlings. Similar results were obtained for roses and poinsettias (Asen, 1982; Stewart et al., 1979). Flavonoid profiles between different cultivars or varieties of fruits appear to show greater differences, possibly due to greater genetic diversity. Both quantitative and qualitative differences have been shown for the anthocyanin profiles in red raspberries (Torre and Barritt, 1977; Spanos and Wrolstad, 1987), blackberries (Torre and Barritt, 1977; Sapers et al., 1986) and blueberries (Sapers et al., 1984). Only quantitative differences however have been reported for cranberry anthocyanins (Sapers and Hargrave, 1987; Durst and Wrolstad, 1988).

For the purposes of verification of authenticity in fruit juice products, flavonoid profiles have become a powerful tool. Paper chromatography of anthocyanins have been used to detect adulteration between different grape varieties (Fitelson, 1967; Mattick, 1967) and adulteration in blackberry juice (Fitelson, 1968). More recently, detection of adulteration of cranberry juice drinks with grape skin extract was made possible by HPLC analysis of both the anthocyanins (Hale et al., 1986) and of the anthocyanidins (Hong and Wrolstad, 1986b). Wald et al. (1986) used quercetin-3-O-β-glucuronide as a chemical marker to detect adulteration of blackcurrant products with blackberries.
Measurement of Color

Color is the major quality that anthocyanins impart to food and drink (Van Buren et al., 1974). Consequently, measurement of color, from both the standpoint of measuring the quantity of anthocyanins and describing perceived color are important. This section of the Literature Review will focus on both of these important areas: quantitation of anthocyanins and measurement of red color in anthocyanin containing foods.

Quantitation of Anthocyanins

The beautiful red to purple hues that anthocyanins exhibit are due to their intense absorption of visible light in the 510-540 nM range. Absorbance measurements in this range would therefore be a simple method of measuring anthocyanins. Direct determination of anthocyanins has been applied to analysis of cranberry anthocyanins (Lees and Francis, 1972) and to the estimation of grape anthocyanins (Lin and Hilton, 1980). Unfortunately, direct determination of anthocyanins by absorbance measurements in the visible spectrum is not the most accurate method due to the presence of interfering compounds. This interference is due to absorbance from chlorophyll compounds, degradation products of anthocyanins, sugar-amino acid Maillard-type reactions and probably many others (Francis, 1982). This has lead to the development of indirect methods for measurement of anthocyanins.

As mentioned above, the anthocyanins are acid base indicators; they exhibit a change in absorbance when the pH is changed. This property has been widely used
to quantitate anthocyanins. Sondheimer and Kertesz (1948) quantitated strawberry anthocyanins by measuring the change in absorbance of the anthocyanins at 2 different pH's, pH 2.0 and 3.4. The difference in the absorbance values being proportional to the total monomeric anthocyanin content. Swain and Hillis (1959) used pH values of 0.5 and 1.0. Later work by Fuleki and Francis (1968a; 1968b) employed pH values of 1.0 and 4.5. These "pH differential" methods have been the most successful for quantitation of anthocyanin pigments (Francis, 1982).

Subtractive methods for measurement of anthocyanins have also been developed. These methods employ reagents which either destroy the pigments or destroy the interfering compounds (Francis, 1982). Dickinson and Gawler (1956) used sulfite to bleach the anthocyanins while Swain and Hillis (1959) used hydrogen peroxide. Subtractive methods have not been well accepted because they introduce other errors (Francis, 1982). Bisulfite bleaching however, has found uses in measurement of polymeric pigments as the polymeric pigments are resistant to bisulfite bleaching (Somers, 1971).

Color Measurement

The color of an object is difficult to measure in that it is not a physical characteristic such as melting point, particle size, concentration and so on, but rather, it is the human perception of appearance (Francis, 1983). This is particularly important in food products. Consumers first judge the quality of a food product by its color. Experience has conditioned us to associate color with quality and sensory properties (Expert Panel on Food Safety and Nutrition, 1986). It is not
surprising therefore that much work has been done on the measurement of color in foods. In this section, the discussion will be focused on the measurement of color in anthocyanin containing beverages as it is in beverages that anthocyanins would most likely be used.

**Tinctoral Strength**

In order to compare the coloring power of different colorants, the concept of tinctoral strength has been developed. This is an important specification, especially with natural colorants such as anthocyanins, as natural colorants possess a great deal of variation due to their biological origin (Clydesdale, 1978).

In the food industry, tinctoral strength is often specified in terms of optical density (absorbance) at the maximum wavelength (Clydesdale, 1978). Unfortunately, this is not the best method for measuring color. While some dyes give good correlation between optical density measurements and visual rank, others show poor correlation between optical density measurements and visual rank. Much of this error is due to the psychophysics of the human eye. Visual perception takes into consideration the entire visual spectrum, this is analogous to the total area encompassed by the spectral absorption curves. Optical density measurement is not an adequate measurement of tinctoral strength in that it considers only the absorbance at a single wavelength and ignores the importance of band width (Clydesdale, 1978).
Tristimulus Colorimetry

The importance of color measurement has led to the development of many different types of colorimeters which attempt to describe color as perceived by the human eye. These systems operate on the entire visible spectral range taking into consideration the response characteristics of the human eye. The results are expressed as coordinates in a 3-dimensional color space. A common description of these three coordinates are: hue, which can be thought of as the color; value, which can be described as lightness or darkness; and chroma, which is vividness. The theory and mathematics behind color theory are beyond the scope of this literature review and can be found elsewhere (Francis, 1983; Clydesdale, 1984; Francis and Clydesdale, 1975; Hunter, 1975; Judd and Wyszecki, 1963).

The standard system for expression of colorimetric data is the CIE system, developed by the Commission Internationale de l'Eclairage in the 1930's (Clydesdale, 1984). This system is based on the three mathematical primaries X, Y and Z. Another system commonly used is the Hunter L, a and b coordinates. L representing value or lightness and darkness, +a is redness, -a is greenness, +b is yellowness and -b is blueness (Francis, 1983). CIE and Hunter values are interconvertible by mathematical transformation.

It is important to point out that any kind of instrumentation used in color measurement has a serious defect in that different readings are obtained from instrument to instrument even using the same make and model and measuring the same sample. These differences are due to problems associated with energy (light) sources, filters and detectors and the instrument's inability to produce exact
responses. Because of this, color-measuring equipment is not normally used to specify the absolute color of a sample, but rather to measure small color differences between samples. When used in such a manner, tristimulus colorimeters are highly reproducible (Francis and Clydesdale, 1975).

Tristimulus data can further be manipulated. The total color difference ($\Delta E$), using the Hunter system, between two samples is often expressed as:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (i)$$

Data can also be expressed as a single color function called Hue and can be calculated as:

$$\text{Hue angle} = \tan^{-1} \left( \frac{a}{b} \right) \quad (ii)$$

Finally, the saturation index, which is closely related to the chroma (intensity of color) of the sample can be expressed as:

$$\text{Saturation Index} = \sqrt{\Delta a^2 + \Delta b^2} \quad (iii)$$

Tristimulus colorimetry has been widely used for measurement of color in foods, the literature is replete with references employing tristimulus colorimetric methods for both specifying color or for measuring color changes. Some of the more fundamental work with anthocyanin containing beverages has been in the area of comparison of tristimulus measurements to that of visual response. In general,
anthocyanin containing beverages are transparent and therefore transmission measurements are typically made. For light colored solutions, Johnson et al. (1976) found that all tristimulus parameters except for hue and "b" correlated highly with visual assessment. Dark colored solutions measured by transmission colorimetry exhibit an "area of confusion" where changes in pigment concentration do not correlate with changes in colorimetric measurements. Photocells do not adequately measure visual chroma or hue at low luminosities, the resulting measurement may be more of a lightness or darkness color assessment (Eagerman et al. 1973). Transmission colorimetry of dark colored beverages has been successful by use of modified color scales (Eagerman et al. 1973). Van Buren et al., (1974) using purified anthocyanins found that anthocyanins give a range of colors depending on concentration and pH. However in concentrated solutions, the anthocyanins all exhibited a similar dark red color. In terms of tristimulus values, they found that a decrease in the concentration of the anthocyanins increases L values. Also, the hue angle and the saturation index were both dependent on the L value and on pH.
Analysis of Anthocyanins

In this next section of the literature review, methods for the analysis of anthocyanins will be discussed. The interest in anthocyanins from both the standpoint of color quality and stability in foods and as taxonomic indicators has resulted in a considerable body of work in the area of analytical methodology for anthocyanins. A brief review of these methods will be given. Most of the emphasis however, will be on the discussion of the use of High Performance Liquid Chromatography (HPLC) for separation of individual anthocyanin pigments.

Quantitation of Anthocyanins

A review of the methods for quantitation of total anthocyanins is found in the "Color Measurement" section of this Literature Review.

Paper and Thin-Layer Chromatography of Anthocyanins

Paper Chromatography (PC) is the established method for separation of anthocyanins. PC R_f values in a number of "standard" solvents are available for most of the known anthocyanins. A description of the most widely used PC methods along with the R_f values of most of the common anthocyanins are given by Harborne (1967).

As a natural extension of PC, Thin-Layer Chromatographic methods (TLC) have also been employed for the analysis of anthocyanins. The advantages of TLC over PC is that analysis time is much shorter and sensitivity and resolution are higher. Unfortunately, R_f values are not as reliable as with PC because of the
differences in layer thickness from plate to plate. In most cases, reference compounds are needed to confirm identification (Hrazdina, 1979). A comprehensive review of previous work with TLC separation of anthocyanins is given by Hrazdina (1979) and Francis (1982). Two recent papers illustrate the usefulness of TLC for resolution of complex mixtures of anthocyanins. Hrazdina et al. (1977), using cellulose TLC plates and 5 different solvent systems were able to resolve 8 anthocyanins from red cabbage. This work is significant in that the anthocyanins of red cabbage is highly acylated with a mixture of both aromatic and non-aromatic organic acids (Timberlake and Bridle, 1982) representing a considerable chromatographic challenge. Ohta et al. (1979) developed a 2 dimensional cellulose TLC system for analysis of grape and grape products. These workers were able to separate 20 anthocyanins from Concord Grape in a single run.

Electrophoresis

As previously discussed, the anthocyanins are ionic in nature (depending on pH) and would therefore be expected to be mobile in an electric field. Electrophoresis however, has found little use in the field of anthocyanin separations as it offers little or no advantage over paper chromatography. In acetate buffer, the anthocyanins do not migrate far. Ionization of the anthocyanins by alkali causes oxidative decomposition with air (Hrazdina, 1979). Successful results have been reported by von Elbe et al., (1969) with paper electrophoresis using 0.1 M citric acid at pH 2.00 as an electrolyte. Four anthocyanins from sour cherries were separated by these workers. Osawa et al., (1971) using cellulose acetate film
electrophoresis has also obtained excellent results. A review of previous work in separation of anthocyanins by electrophoresis is given by Hrazdina (1979).

**Column Chromatography**

In search of methods for larger scale separation and quantitation of individual anthocyanins, column chromatographic methods have been developed. A number of column support materials have been tried without success, including aluminum oxide, cellulose powders, ion exchange resins and Sephadex gel. In most cases, sample matrix components were found to interfere with the separation. Polyamide powders showed good retention of the anthocyanins but chromatographic resolution was poor (Hrazdina, 1979).

The most success has been obtained with insoluble polyvinylpyrrolidone (PVP), a polymer which is quite specific for phenolic substances (Loomis and Battaile, 1966). The mechanism of binding is by hydrogen bond formation (Andersen and Sowers, 1968). The anthocyanidins elute from PVP columns in the order corresponding to the hydroxylation pattern in the B ring. As the number of free hydroxyl groups increase, retention on PVP also increases. The elution order is as follows (listed from the earliest eluting or least retained to the latest eluting, or most retained): malvidin, peonidin, pelargonidin, petunidin, cyanidin, delphinidin (Van Teeling et al., 1971). Glycosidation of the anthocyanidins decreases retention. Thus, trisaccharides elute before disaccharides which in turn elute before monosaccharides (Van Teeling et al., 1971).

A review of all of the aforementioned column chromatographic methods is given by Hrazdina (1979). For a discussion of the general characteristics of the
different adsorbents commonly used for separation of anthocyanins and other flavonoids, the reader is referred to Markham (1982).

**Gas-Liquid Chromatography**

Gas-Liquid Chromatography (GC)/Mass Spectrometry (MS) is a powerful technique for the separation and identification of natural products. The individual components are separated by GC while MS yields information useful for identification of the individual components. Unfortunately, the anthocyanins are of limited volatility and therefore, more volatile derivatives must be made. The most success has been achieved with reaction of the anthocyanins with trimethylchlorosilane and hexamethyldisilazane (Bombardelli et al., 1976). The result is a nitrogen containing derivative which after injection into the gas chromatograph, further transforms (this transformation is due to the high temperatures required for GC analysis) into a quinoline derivative. The quinoline derivatives of the anthocyanins and anthocyanidins when chromatographed, give rise to sharp peaks and when subjected to MS, show fragmentation patterns useful for the identification of the original anthocyanin or anthocyanidin (Baj et al., 1983). This method has been successfully used to separate four of the six common anthocyanidins (Bombardelli et al., 1976) and 15 anthocyanins from bilberry (Baj et al., 1983). In the latter study, the usefulness of MS was well illustrated in that the MS data not only yielded information about the nature of the aglycone but was also useful for determination of the nature of the attached sugar.

Despite the excellent results obtained by previous workers using GC/MS, derivatization in general introduces problems of stability in the anthocyanins and
thus, future development is likely to be in the direction of High Performance Liquid Chromatography (HPLC) rather than GC (Francis, 1982).

High Performance Liquid Chromatography (HPLC)

Recent developments in area of higher performance solvent delivery systems and microparticulate column packing materials have made HPLC the method of choice for the separation of low molecular weight non-volatile compounds. These developments have opened up new horizons in the field of analytical separation and identification of plant phenolic compounds (Camire and Clydesdale, 1979).

The most success for separation of both anthocyanins and anthocyanidins has been with Reversed Phase HPLC (RP-HPLC). This method employs a non-polar or hydrophobic stationary phase with a polar eluting solvent (mobile phase). The most popular RP-HPLC stationary phases are octylsilane (C₈) or octyldecylsilane (C₁₈). These stationary phases are usually chemically bonded onto silica column support material (Joseph, 1986). The mobile phases in RP-HPLC are based on water to which a water-miscible organic solvent (organic modifier) is added to modify the elution characteristics of the sample. Compounds elute more rapidly when the proportion of the organic modifier in the mobile phase is increased. Typical mixtures are water-methanol or water-acetonitrile (Parris, 1976).

Table 1.1 gives a summary of recent work with RP-HPLC analysis of anthocyanidins and anthocyanins. The reader is referred to the individual publications for specific details. The remainder of this section on HPLC analysis of anthocyanidins and anthocyanins will be a discussion of some of the important chromatographic factors which affect analytical separation and detection of these
pigments. While the information presented will be focused on analytical separation of anthocyanins, the general principles are applicable to a wide range of related phenolic compounds.
Table 1.1 Compilation of References on the Separation of Anthocyanidins and Anthocyanins by Reversed Phase HPLC

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Solvent</th>
<th>Anthocyanin Source</th>
<th>Mixes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanidins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db,gal,ga,pa,rv</td>
<td>w/a/m</td>
<td>grape, rose</td>
<td></td>
<td>Wilkinson, 1977</td>
</tr>
<tr>
<td>db,gal,ga,pa,rv</td>
<td>w/a/m</td>
<td>citrus fruits</td>
<td>1</td>
<td>Atlanta and S restr, 1980</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv</td>
<td>w/a/m</td>
<td>cranberry</td>
<td></td>
<td>Hong and Westest, 1986</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>bilberry</td>
<td></td>
<td>Be et al., 1985</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>blackberry</td>
<td></td>
<td>Sepers et al., 1986</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>blueberry</td>
<td></td>
<td>Sepers et al., 1984</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>bag wth he berry</td>
<td></td>
<td>Anderson, 1987</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>cranberry</td>
<td></td>
<td>Currv and Currvval, 1979</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>cranberry</td>
<td></td>
<td>Meas et al., 1985</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>cranberry</td>
<td></td>
<td>Meas et al., 1986</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>cranberry</td>
<td></td>
<td>Sepers and Mengr, 1987</td>
</tr>
<tr>
<td>db,gal,pa,pg,rv,3-glu,aran,gal</td>
<td>w/l/m</td>
<td>cranberry</td>
<td></td>
<td>Currv and Currvval, 1988</td>
</tr>
</tbody>
</table>
Table 1.1 (Continued)

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Solvent</th>
<th>Anthocyanin Source</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>dp, as, pi, ga, av = -3-glu, ara, gel</td>
<td>w/o/m</td>
<td>strawberry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu, ar, gel</td>
<td>p/h</td>
<td>elderberry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu, -3,3-diglu and acylated arylamines</td>
<td>w/o/m</td>
<td>grapes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu and acylated arylamines</td>
<td>w/t/acetone</td>
<td>grapes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu and acylated arylamines</td>
<td>w/t/m</td>
<td>grapes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu and acylated arylamines</td>
<td>w/t/m</td>
<td>grapes</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu, ara, gel</td>
<td>w/o/m</td>
<td>lingonberry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, ap, 3-rot, glu, gel</td>
<td>w/o/acetone</td>
<td>petunia bracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, ap, 3-rot, glu, gel</td>
<td>w/o/acetone</td>
<td>petunia bracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, ap, 3-rot, glu, gel</td>
<td>w/o/acetone</td>
<td>petunia bracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp, as, pi, ga, av = -3-glu and acylated arylamines</td>
<td>w/HCl04/m</td>
<td>port wine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, ap, 3-rot, glu, ara, gel</td>
<td>w/o/m</td>
<td>red raspberry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, ap, 3-rot, glu, ara, gel</td>
<td>w/o/m</td>
<td>red raspberry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as, pi, ga, av = -3-glu, ara, gel</td>
<td>w/o/m</td>
<td>Syringa berries</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.1 (continued)

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Solvent</th>
<th>Anthocyanin Source</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>lttlut*</td>
<td>water</td>
<td>Misc. sources</td>
<td></td>
<td>Castelli et al., 1983</td>
</tr>
</tbody>
</table>

* Cyg (ODS) columns employed unless otherwise stated.
** This listing of pigments is to serve as a guide. For specific details, the original reference should be consulted.

**Key to Pigments:**
- G = cyanidin
- d = delphinidin
- b = pelargonidin
- g = peonidin
- p = petunidin
- m = malvidin
- v = peonidin

**Key to Solvent Systems:**
- a = acetic acid
- f = formic acid
- v = a variety of organic modifiers were reported: acetonitrile, 1-propanol, acetonitrile and ethanol
- m = methanol
- a = acetonitrile
- p = phosphate or potassium phosphate buffer
- s = trifluoroacetic acid
- w = water

**Key to Notes:**
- 1 = C18 column also used
- 2 = preparative HPLC
- 3 = polyether reversed phase column employed
- 4 = rapid compression system employed
The Nature of the Stationary Phase

ODS bonded phase columns are by far the most widely used columns for RP-HPLC analysis of anthocyanidins and anthocyanins. All but one of the workers listed in Table 1.1 employed this stationary phase. In an ideal system, relative retention would be based on the relative polarity of the analytes. The most polar analytes would elute first and the least polar analytes eluting last. For the anthocyanins, the key factors which determine retention are: 1) the overall polarity of the molecule; 2) the substitution of the B-ring; 3) for glycosides, the nature of the attached sugar (Casteele et al., 1983; Strack et al., 1980). Thus, for identical glycosides, the anthocyanins elute in the following order (listed from earliest eluting to latest eluting) delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin (Hrazdina, 1979). The nature of the attached sugar group(s) also play an important role in the retention characteristics of the anthocyanin. In general, for the same aglycone, diglycosides are eluted more rapidly than monoglycosides which in turn are eluted more rapidly than the aglycone (Casteele et al., 1983). The type of sugar also greatly affects retention. A number of workers studying the anthocyanins in several species of *Vaccinium* showed that for the same aglycone, the 3-galactoside elutes before the 3-glucoside which in turn elutes before the 3-arabinoside (Durst and Wrolstad, 1988; Sapers and Hargrave, 1987; Andersen, 1985; Hicks et al., 1985; Hale et al., 1986; Andersen, 1987). The position of glycosidation is also a factor, for example, cyanidin-3-glucoside elutes before cyanidin-5-glucoside (Strack et al., 1980). Finally, acylation with either acetic
acid or a hydroxy aromatic acid increases retention time (Hrazdina, 1979; Williams, 1978; Wulf and Nagel, 1978).

Selection of the correct HPLC column is important for optimal results. Chromatographic materials classified under the general label of C\textsubscript{8} or C\textsubscript{18} can behave in very different ways, providing a wide range of unique selectivities. A particular separation cannot always be reproduced on another column of the same type or from the same manufacturer (Wouters et al., 1986). These problems are inherently associated with silica-based support materials (Joseph, 1986). Most commercially available bonded phases are chemically bonded to the silica support by a siloxane (Si-O-Si-R) bond, where R = the alkyl group (Abbott and Simpson, 1986). Due to steric hindrance, the bonding reaction is always incomplete, leaving unreacted silanol groups (Si-OH) on the silica surface. These residual silanol groups can potentially interact with analyte molecules producing secondary retention effects. The most important interactions are those of hydrogen bonding and of ion exchange (Dolan, 1986). These interactions are especially significant with basic compounds (Joseph, 1986). For example, a recent study showed large differences in the retention order of cephalosporins (a group of nitrogen and sulfur containing heterocyclic compounds) when separated on eight brands of C\textsubscript{8} and C\textsubscript{18} packing materials by RP-HPLC (Wouters et al., 1986).

Fortunately, such differences in retention order for anthocyanins has not been demonstrated. The single case of a conflicting retention order has been by Casteele et al. (1983) who reported that cyanidin-3-glucoside eluted after cyanidin-3-arabinoside. As mentioned above, the recent literature reports the opposite elution order. Since the solvent system used by Casteele et al.
(water/formic acid/methanol) was quite similar to that used by Andersen (1985, 1987), the differences are most likely to be due to stationary phase effects.

Another disadvantage of silica based columns is their instability at pH extremes. Under these conditions, degradation of the stationary phase occurs (Joseph, 1986). In general, most manufacturers do not recommend the use of mobile phases outside the range of pH 2-8. This presents a problem for analysis of anthocyanins as a low pH is essential to keep the anthocyanins in the red flavylium cation form, which results in increased peak sharpness. Early workers did not employ mobile phases below pH 2.0 in fear of damage to their silica based columns (Hale, et al., 1986). Several workers however, have reported that there is no loss in column performance of silica columns when used with mobile phases below pH 2, (Spanos and Wrolstad, 1988; Wulf and Nagel, 1978; Bronnum-Hansen and Hansen, 1983).

Recently, macroporous poly(styrene-divinylbenzene) copolymer reversed phase adsorbents, such as the PRP-1 (Hamilton), have become available. The advantage of these materials is that they do not exhibit any of the previously mentioned shortcomings associated with silica based packings (Joseph, 1986). Hence, eluants of very low pH can be used and the possibility of secondary retention effects due to silanol interactions are eliminated.

The chromatographic characteristics of these columns for phenolic substances appears to be quite similar to that of silica based reversed phase adsorbents. Buta (1984) showed that plant phenolics and flavonoids could be effectively separated on polymer based reversed phase columns and that retention order is identical to that of a silica based ODS column. Similar results have been
obtained by Hale et al., (1986) working with cranberry anthocyanins. It has been observed however, that with the polymer columns, there is somewhat less resolution with methoxylated phenolic acids than with the reversed phase ODS systems (Buta, 1986).

The Nature of the Solvent

The most common solvents for RP-HPLC are mixtures of either methanol or acetonitrile with water (Parris, 1976). For analysis of anthocyanidins and anthocyanins, pH must also be controlled to insure that all of the pigments are in the same colored form. As discussed above, a low pH is essential for optimum peak sharpness. Acetic acid, formic acid or phosphate buffers have been the most commonly used agents for controlling pH (Table 1.1).

The nature of the organic modifier is also important in determining the success of a chromatographic separation. Different organic solvents may offer different chromatographic selectivities. Table 1.1 shows that both methanol and acetonitrile have been the most commonly employed organic modifiers for analysis of anthocyanins. Akavia and Strack (1980) found that acetonitrile was superior to methanol, ethanol, isopropanol and acetone for RP-HPLC separation of anthocyanidins while Bronnum-Hansen and Hansen (1983) reported that tetrahydrofuran (THF) gave better selectivity and shorter retention times than either methanol or acetonitrile.

Detection

One of the major characteristics of the anthocyanins is their color. This is due to their intense absorption in the visible spectrum (465-560 nM). A second
absorption maxima occurs in the UV region from 270-280 nM (Markham, 1982). For detection of anthocyanin and anthocyanidins, most workers have employed detection in the visible region. Typically at ca. 525 nM (Casteele et al., 1983). UV detection of anthocyanins is also possible and its use has been reported by several workers (Casteele et al., 1983; Mazza, 1986). The advantage of detection in the visible range is that only the anthocyanins are detected whereas when detection in the UV is employed, all the UV absorbing compounds are also detected, which with typically "dirty" natural products samples, results in complex chromatograms.

Recently, several new modes of detection have become available which not only serve as a means of detection but can also yield information as to the structure of the individual compounds. The first of these is the Photodiode Array Detector (PDA). This detector allows UV-Visible spectra to be taken of individual compounds as they elute from the column. For flavonoids in general, spectral characteristics give valuable information as to the nature of the flavonoid, its oxygenation pattern, its sugar substitution pattern and acylation if present (Markham, 1982). Andersen (1985) used RP-HPLC/PDA to characterize the anthocyanins in lingonberry (Vaccinium vitis-idaea) and later used the same method to characterize 15 anthocyanins from bog whortleberries (Vaccinium uliginosum L.), (Andersen, 1987). Mazza (1986) used similar methods to separate and characterize anthocyanins and other phenolic compounds from saskatoon berries (Amelanchier alnifolia Nutt.). Unfortunately, the latter study reports only the spectral characteristics of the anthocyanins in the UV region.

The second type of detection that has been used for both detection and structure determination has been electrochemical detection (EC). When two
electrodes are used in series, the ratio of the response of the downstream electrode to that of the upstream electrode (Collection Efficiency, N₀) is dependant on the electrochemical properties of the analyte (Rosten and Kissinger, 1982). The best illustration of the usefulness of EC is reported by Lunte (1987). Using both PDA and EC detection, unknown flavonoids from grape juice and wine were characterized without isolation of the pure compound. PDA detection was used to classify the flavonoid while EC detection was used to determine the hydroxylation and conjugation pattern of the unknown flavonoids.

In the field of plant phenolic chemistry, where thousands of different flavonoids are known to exist in nature and where standards are generally not available (Lunte, 1987), the advent of both these types of detection methods will undoubtly lead to advances in phytochemical studies.
Anthocyanin Composition

Grapes

Much work has been done in the area of characterization of anthocyanin pigments in grapes. Much of this has been prompted by the interest in the color of red wines during the winemaking process. Ribereau-Gayon (1982) has written an excellent review on the anthocyanin composition of wine grapes. There are numerous species of grapes, each with a characteristic anthocyanin profile. Commercially, the two most readily available sources of grape pigments are Enociannin and Concord Grape Colorant. In terms of color, both Enociannin and Concord Grape Colorant exhibit a typically reddish-blue hue (Francis, 1975). In a survey of 17 grape skin powders and extracts, Riboh (1977) reported color descriptions ranging from "light grayish pink" to "purplish red".

Enociannin

One of the more readily available commercial grape colorants is, enociannin or Grape Skin Extract (GSE), a grape skin colorant made usually as a by-product of winemaking. It is therefore likely that the major variety of grape present is that of wine grapes, *Vitis vinifera* (Wallin, 1977). A variety of processes are used to produce these colorants. Commonly used methods range from simple alcohol extraction of wine grape skins after racking to fermentation of the pressed grape skins (Francis, 1975). A comprehensive survey of potential methods for purification of grape pigments is given by Lin and Hilton (1980).
A survey of the anthocyanins of *Vitis vinifera* shows the major pigments to be malvidin-3-glucoside with p-coumaroyl-malvidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, delphinidin-3-glucoside and cyanidin-3-glucoside as minor pigments (Wrolstad, 1976; Singleton and Esau, 1969; Koppen and Basson, 1966; Van Buren et al., 1970; Rankine et al., 1958). Even the anthocyanin composition of different varieties within this species has been found to be quite varied. Using High Performance Liquid Chromatographic (HPLC) techniques, Wulf and Nagel (1978) found 21 different anthocyanins in Cabernet Sauvignon grapes and only 5 different anthocyanins in Pinot Noir grapes. The additional pigments found in Carbernet Sauvignon and not in Pinot Noir grapes were acylated acetic acid or coumaric acid derivatives of malvidin, peonidin, delphinidin, cyanidin or petunidin-3-glucosides. Caffeic acid derivatives have also been reported for vinifera grapes (Sakellariades and Luh, 1974).

Early work by Bockian et al. (1955) reported the presence of delphinidin, petunidin and malvidin-3,5-diglucosides in *Vitis vinifera var Cabernet sauvignon*. However, these findings have been much debated, and their conclusions have been eventually proved to be false, on the basis of ineffective chromatographic resolution in the techniques used, Ribereau-Gayon (1982). It is now established that *Vitis vinifera* grapes do not contain anthocyanin diglucosides.

An excellent summary of the general anthocyanin content of *Vitis vinifera* grapes are as follows (Ribereau-Gayon, 1982):

1) Malvidin-3-glucoside is the major pigment in *Vitis vinifera* but does not represent the majority of the total anthocyanin
pigments. It has been found to be present as approximately 36% of the total pigments.

2) Free anthocyanidins are not found.

3) Anthocyanidin diglucosides do not occur in *Vitis vinifera* but do occur in hybrids and in other species.

4) Cyanidin and peonidin derivatives are systematically found in all species but are abundant in only a few.

5) The absence of acylated anthocyanins is characteristic of the Pinot variety of *Vitis vinifera*.

**Concord Grape Colorant**

The other commercially available source of grape pigments is Concord Grape Colorant, an extract made from the grapes of *Vitis labrusca var. Concord*. The pigments from the lees of Concord grape juice are easy to recover, requiring only an acid-alcohol extraction (Francis, 1975).

A survey of anthocyanins from this species show a very complex pigment profile with delphinidin-3-glucoside, cyanidin-3-glucoside and malvidin-3,5-diglucoside as the major pigments with peonidin-3-glucoside, peonidin-3,5-diglucoside, peonidin-3-glucoside-p-coumaric acid, peonidin-3,5-diglucoside-p-coumaric acid, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, cyanidin-3,5-diglucoside-p-coumaric acid, malvidin-3-glucoside-p-coumaric acid, malvidin-3,5-diglucoside-p-coumaric acid, petunidin-3-glucoside, petunidin,3,5-diglucoside, petunidin-3,5-diglucoside-p-coumaric acid, petunidin-3-glucoside-p-coumaric acid, delphinidin-3,5-diglucoside, delphinidin-3-glucoside-p-coumaric acid and
delphinidin-3,5-diglucoside-p-coumaric acid present as minor pigments (Wrolstad, 1976; Ohta et al., 1979).

The differences in the pigment profiles between vinifera and labrusca are large. The labrusca species contains 3,5 diglucosides which are not found in the vinifera species. The use of anthocyanin pigment profiles should therefore be useful for differentiating between the two species.

Roselle

The calyces of roselle (Hibiscus sabdariffa L.) have been used to produce red jams, jellies, preserves, extracts, sauces, chutneys, beverages and wine (Clydesdale et al., 1979). A red colored, pleasantly flavored extract has also been produced for use in a number of tropical countries (Du and Francis, 1973). There is considerable interest in the use of Roselle pigment isolates as a potential natural red colorant. The plant is high in pigment, Du and Francis (1973) reported the calyces to contain as much as 1.5% anthocyanin pigment on a dry weight basis. Pigment extraction is easily accomplished with water (Francis, 1975). In terms of color, roselle pigments exhibit similar spectral characteristics to that of the now banned Red No. 2 (Clydesdale et al., 1979) and has been described to have an overall red shade associated with that of raspberry color (Francis, 1975). Riboh described the color of several roselle powders as being "medium brown, red-orange". Several workers have investigated the use of roselle colorant in jellies, drinks, carbonated beverages, roselle concentrates and gelatin desserts (Clydesdale et al., 1979; Esselen and Sammy, 1975). A potential problem with roselle extracts is that of the flavor of the extract. Sensory analyses conducted
roselle colored gelatin and beverages by Clydesdale et al. (1979) concluded that the flavor imparted by the roselle colorant were found to be detectable and disliked by the panelists.

The anthocyanin profile of roselle is simple, consisting of only 4 pigments. The 2 major pigments are cyanidin-3-sambubioside (xylose-glucose) and delphinidin-3-sambubioside. The 2 minor pigments are delphinidin-3-glucoside and cyanidin-3-glucoside (Du and Francis, 1973).

**Red Cabbage Colorant**

Red cabbage (*Brassica oleracea*) leaves are an inexpensive and abundant source of anthocyanins (Timberlake and Bridle, 1982; Shewfelt and Ahmed, 1977). Shewfelt and Ahmed (1977), felt that colorants derived from red cabbage offered superior color to that of Red No. 40 and suggested that it could be used to replace Red No. 2. Visually, red cabbage colorant has been described to be predominantly red with some blue hue, the color is close to that of Red No. 2 (Shewfelt and Ahmed, 1977) and to that of beet colorant (Sapers et al., 1981). An overall description of the functional properties of red cabbage colorant is given by Sapers et al. (1981).

The anthocyanin pigments of red cabbage are well characterized (Timberlake and Bridle, 1982). As with many other cruciferous plants, the anthocyanin pattern of red cabbage is complex, containing a sophisticated pattern of acylated anthocyanins (Timberlake and Bridle, 1982). Early work reported the presence of cyanidin-3-sophoroside-5-glucoside acylated with p-coumaric acid and ferulic acid (Tanchev and Timberlake, 1969). Later work by Hrazdina et al. (1977) using
column, paper and thin layer chromatography resolved eight pigments. They were all identified as derivatives of cyanidin-3-sophoroside-5-glucoside. In addition to cyanidin-3-sophoroside-5-glucoside, acylation was found at the position 3 sugars by the following groups: malonic acid, p-coumaric acid in both 1 and 2 mol ratios, ferulic acid in both 1 and 2 mol ratios and sinapic acid in both 1 and 2 mol ratios. In the case of acylation with more than 1 acid, mixed acylation (where 2 different acylating acids occur in the same anthocyanin) does not occur (Hrazdina, et al., 1977). Recently, new acylated forms of the red cabbage pigments have been characterized, including the discovery of acylation with glucose derivatives of ferulic and p-coumaric acid to the sophoroside sugar moiety (Ideka, et al., 1987; Itaka, 1986).

Bilberry

The bilberry or whortleberry (*Vaccinium myrtillus*) is a native of parts of Europe and northern Asia (Timberlake and Bridle, 1982). Suomalainen and Keranen (1961) reported that this species contains cyanidin, delphinidin, petunidin and malvidin glucosides and arabinosides. Later work by Baj, et al. (1983) using both HPLC and Gas Liquid Chromatography (GLC) found all possible combinations of cyanidin, delphinidin, petunidin, peonidin and malvidin 3-galactosides, 3-glucosides and 3-arabinosides in bilberry. Qualitatively, the delphinidin glycosides were present in the largest quantities and the peonidin glycosides were the least abundant. Since the bilberry is closely related to the lowbush blueberry (*Vaccinium angustifolium*), it is likely that their anthocyanin patterns are similar (Timberlake and Bridle, 1982). It is appropriate then to include a brief discussion of the
anthocyanins of the various blueberry species. Francis, et al. (1966), using paper chromatographic techniques and a wide variety of solvent systems found that the ripe fruits of the lowbush blueberry contained all possible combinations of delphinidin, malvidin, petunidin, peonidin and cyanidin 3-galactosides, 3-glucosides and 3-arabinosides. The glucosides were found to be present in much greater quantity than the galactosides. Only trace quantities of the arabinosides were found. Diglycosides were also detected but not identified. A review of the anthocyanins of another related species, the highbush blueberry (Vaccinium corymbosum) shows that this species contains the same anthocyanins as the lowbush blueberry but in different proportions (Timberlake and Bridle, 1982).

Recently, Sapers, et al. (1984) in a study of lowbush blueberry, separated as many as 16 anthocyanins by HPLC. Three distinct anthocyanin patterns were found for the 11 different cultivars studied. The anthocyanins of a related species, the Bog Whortleberry (Vaccinium uliginosum L.) was also found to contain all possible combinations of the previously mentioned pigments, with malvidin-3-glucoside as the predominant pigment (Andersen, 1987).

Black Currants

The very high pigment content of black currants (Ribes nigrum L.) make this a potential source of anthocyanin pigments despite their high cost. Early work by Chandler and Harper (1961) identified cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside and delphinidin-3-glucoside. Later, Morton (1968) using preparative thin layer chromatographic methods quantified the anthocyanins in blackcurrant juice. The amounts and relative percentage of total pigments (in
parenthesis) of the anthocyanins and anthocyanidins reported were as follows:
cyanidin and delphinidin, 17 mg/100ml (11.7%); cyanidin-3-glucoside, 12
mg/100ml (8.3%); cyanidin-3-rutinoside, 33 mg/100ml (22.8%); delphinidin-3-
rutinoside, 61.8 mg/100ml (42.6%); and delphinidin-3-glucoside, 21.3 mg/100ml
(14.7%). Recent work by Francis and Andersen (1984) using droplet countercurrent chromatography (DCCC) resolved four pigments from blackcurrant juice.
Anthocyanidins were not found. Although no quantitation was attempted, from the
peak areas of the separated anthocyanins, the relative amounts of the individual
pigments appears as follows: delphinidin-3-rutinoside > cyanidin-3-rutinoside >
delphinidin-3-glucoside > cyanidin-3-glucoside. These results are in excellent
agreement to those of Morton (1968).

**Blackberry**

Blackberries, *(Rubus ursinus, Rubus laciniatus, Rubus procerus* and Marion),
another expensive commodity, are sufficiently high in pigment such that it would be
possible to utilize a blackberry juice or juice concentrate as a colorant.

The anthocyanin profiles of blackberries is simple, as sophorose or xylose
pigments are not synthesized (Timberlake and Bridle, 1982). The major pigment
has been identified as cyanidin-3-glucoside with lesser amounts or even traces of
cyanidin-3-rutinoside depending on the species analyzed (Barritt and Torre, 1973).
**Black Raspberry**

Like the blackberry, the Black Raspberry (*Rubus leucodermis, Rubus occidentalis*) is sufficiently high in pigment such that it could conceivably be used as a colorant despite its very high cost.

European species of black raspberries are characterized by xylose containing derivatives of cyanidin, the major pigments being cyanidin-3-xylosylrutinoside, cyanidin-3-sambubioside, cyanidin-3-rutinoside and cyanidin-3-glucoside (Harborne and Hall, 1964; Nybom, 1968; Timberlake and Bridle, 1982). American varieties of black raspberries are reported to show different anthocyanin patterns, a major difference being the lack of xylose containing pigments (Timberlake and Bridle, 1982). Daravingas and Cain (1966, 1968) reported cyanidin-3-rutinoside-5-glucoside, cyanidin-3-diglucoside, cyanidin-3,5-diglucoside and cyanidin-3-glucoside in the North American "Monger" variety. In a more recent study, Torre and Barritt (1977) examined 4 American varieties of black raspberries and found various distributions of cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-sambubioside and cyanidin-3-xylosylrutinoside. These findings are in contrast to those of Daravingas and Cain (1966) and tends to support earlier findings (Harborne and Hall, 1964; Nybom, 1968; Timberlake and Bridle, 1982) that xylose containing derivatives of cyanidin are characteristic of black raspberries.

**Elderberry**

Another source of anthocyanins for use as food colorants are elderberries (*Sambucus nigra*), a highly pigmented fruit grown freely in the northern hemisphere.
Elderberry extracts are now commercially available (Riboh, 1977). Visually, the color has been described to range from light grayish pink to medium light brown-red.

The anthocyanin composition of elderberries is simple and contain few other phenolic substances (Hansen and Hansen, 1983). Recent work using High Performance Liquid Chromatographic (HPLC) techniques identified and quantitated the following 4 pigments (Percentage of total anthocyanins given in parenthesis): cyanidin-3-sambubioside-5-glucoside, (1.1%); cyanidin-3-glucoside-5-glucoside (0.8%); cyanidin-3-sambubioside, (32.4%); and cyanidin-3-glucoside, (65.7%); (Hansen and Hansen, 1983).

Cherry

Cherries are a popular and highly colored fruit. There are two major species of cherries, the sweet (Prunus avium) and the sour cherry, (Prunus cerasus). Like that of blackberry and black raspberries, cherry products are expensive, but due to their very high pigment content, the use of cherry juices or concentrates as colorants may be economically feasible.

The pigment profiles of the sweet cherry is relatively simple. Using various paper chromatographic systems, Lynn and Luh (1964) reported the pigments (estimates of percentages of total anthocyanin in parenthesis) to be: cyanidin-3-rutinoside, (75%); cyanidin-3-glucoside, (15%); peonidin-3-rutinoside, (1-2%); and peonidin-3-glucoside (1%).

The anthocyanin pigments of sour cherries is more complex (Timberlake and Bridle, 1982). Harborne and Hall (1964) reported cyanidin-3-glucosylrutinoside,
cyanidin-3-sophoroside, cyanidin-3-rutinoside and cyanidin-3-glucoside in 6
different varieties of sour cherries. The "Morello A" variety was found to contain
only cyanidin-3-glucoside and cyanidin-3-glucosylrutinoside. The "Montmorency"
variety was found to contain in addition the following minor pigments: cyanidin-3-
xylosylrutinoside (Shrikhande and Francis, 1973), peonidin-3-glucoside and
peonidin-3-rutinoside (Dekazos, 1970). Previous reports of cyanidin-3-
gentiobioside in sour cherries are incorrect (Du et al., 1975).

**Plum**

This popular, readily available fruit is potentially useful as a colorant
source. While not as highly colored as some of the other sources, by-products of
plum (*Prunus domestica*) processing may make pigment production from this source
economically feasible.

Harborne and Hall (1964) in a survey of 15 common plum varieties reported
the major pigments to be cyanidin-3-glucoside and cyanidin-3-rutinoside with
traces of peonidin-3-glucoside and peonidin-3-rutinoside.

**Vaccinium vitis-ideae and Vaccinium oxyccoccus**

The lingonberry, cowberry or mountain cranberry (*Vaccinium vitis-ideae*) is
a small (usually less than 1/2 inch in diameter) red berry native to the British Isles
and cooler parts of the Northern Hemisphere (Timberlake and Bridle, 1982). The
European Cranberry (*Vaccinium oxyccoccus*) is a smaller relative to the American
Cranberry (*Vaccinium macrocarpon*), (Timberlake and Bridle, 1982). Specific
details regarding the coloring power or potential use of either the *vitis-ideae* or
*oxycoccus* species as colorants is not known. Concern however regarding these two species has been in the area of cranberry juice adulteration.

Because of its importance, much work has been done in the characterization of the pigments in the American Cranberry, *Vaccinium macrocarpon* (Sakamura and Francis, 1961; Zapsalis and Francis, 1965). The pigment profile is simple, consisting of only 4 major pigments. Fuleki and Francis (1968c), using Thin Layer Chromatography/Scanning Densitometry reported the following pigment profile: cyanidin-3-galactoside, (36%); peonidin-3-galactoside, (36%); cyanidin-3-arabinoside, (19%); and peonidin-3-arabinoside (9%). Trace quantities of cyanidin and peonidin 3-glucosides have also been detected (Fuleki and Francis, 1967; Fuleki and Francis, 1968c).

Extensive studies have recently been performed on the study of the anthocyanin composition of cranberries. In general, these results show that peonidin-3-galactoside is present in the largest quantities followed by cyanidin-3-galactoside. Lesser quantities of cyanidin-3-arabinoside and peonidin-3-arabinoside are present and as previously reported, the glucosides of cyanidin and peonidin are present in trace quantities (Durst and Wrolstad, 1988; Sapers and Hargrave, 1987).

The pigment profiles of Lingonberries (*Vaccinium vitis-ideae*) is simpler than that of the American Cranberry. Recently, Andersen (1985) identified and quantititated the following pigments from this species: cyanidin-3-galactoside, (88%); cyanidin-3-arabinoside, (10.6%); and cyanidin-3-glucoside, (1.4%).

Little is known about the anthocyanins of the European Cranberry, *Vaccinium oxycoccus*. Until a complete identification is reported, it would be reasonable to
expect that this berry follows the basic anthocyanin patterns which are characteristic of the *Vaccinium* genus: Mono glycosidic combinations of cyanidin, delphinidin, petunidin, peonidin and malvidin with galactose, arabinose or glucose (Timberlake and Bridle, 1982).

**Strawberries**

A survey of the anthocyanins of strawberry (*Fragaria anannassa*) show that the major pigment is pelargonidin-3-glucoside with smaller amounts of cyanidin-3-glucoside (Wrolstad, et al., 1970; Timberlake and Bridle, 1982). A third pigment, composed of pelargonidin and glucose in a 1:1 molar ratio and chromatographically different (by paper and thin layer chromatography) from pelargonidin-3-glucoside was also identified. No acylation was detected. It was suggested that this third pigment was an isomer of pelargonidin-3-glucoside. (Wrolstad, et al., 1970).
Title: USE OF HPLC SEPARATION/PHOTODIODE ARRAY DETECTION FOR CHARACTERIZATION OF CRANBERRY, ROSELLE AND STRAWBERRY ANTHOCYANINS

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Technical Paper No. _________ From the Oregon State Agricultural Experiment Station
A systematic procedure for separation and characterization of cranberry (Vaccinium macrocarpon), roselle (Hibiscus sabdariffa, L.) and strawberry (Fragaria anannassa) anthocyanins is described. Separation of pigments was achieved by High Performance Liquid Chromatography (HPLC) on a polymer reversed phase column. Methods for preparation of an anthocyanin isolate free of other interfering phenolics was developed. Photodiode array detection was employed to determine the UV-visible spectral characteristics of the pigments. Derivatives of delphinidin (delphinidin, petunidin and malvidin) can be distinguished from derivatives of cyanidin (cyanidin and peonidin) which in turn can be distinguished from pelargonidin derivatives on the basis of their different UV-visible spectra. Acylation with cinnamic acids and differentiation between 3 and 3,5 glycosidation patterns can also be determined from the UV-visible spectrum. In addition to these methods, sample pre injection treatment techniques were also useful for characterization of the pigments. Alkaline hydrolysis of the anthocyanins is useful for determination of acylation while anthocyanins which do not contain an o-diphenolic system can be enriched on a C18 reversed phase cartridge by elution with alkaline borate buffer. Using a combination of these techniques, peak assignments for the anthocyanins from sources whose anthocyanin composition is known can be readily made.
The anthocyanins are the natural pigments responsible for the red, blue and purple colors of many plants. There are many different anthocyanins found in nature. The individual anthocyanin composition for any given plant is distinctive, and the analysis of anthocyanin and/or other flavonoids has therefore been very useful in distinguishing between species. This is especially true if there are qualitative differences (Stewart, et al., 1979). With more closely related plants, such as those which differ only at the cultivar level, the differences in the chemical profiles are mostly quantitative (Asen, 1982).

Classically, anthocyanins have been separated by a number of techniques including paper, thin-layer and column chromatographic methods. Of these methods, paper chromatography, has been the most widely used method for separation of the individual anthocyanins (Hrazdina, 1979). The mobilities (Rf values) of most of the naturally occurring anthocyanins in a number of different solvent systems have been reported (Harborne, 1967). Unfortunately, paper chromatographic methods are time consuming and quantitation is difficult. A number of workers have reported High Performance Liquid Chromatographic (HPLC) methods for separation of both the anthocyanins (Wulf and Nagel, 1978; Casteele, et al., 1983; Strack, et al, 1980) and their aglycones (Wilkinson, et al., 1977; Akavia and Strack, 1980). The advantages of HPLC is that it is fast, sensitive and quantitative. One of the problems with the use of HPLC for analysis of anthocyanins is that absolute peak retention times can vary from worker to worker even when
using similar analytical conditions. This is made more complex by the fact that there is a general lack availability of pure anthocyanin standards. The combination of a lack of standards and the problems with specification of absolute peak retention times are two factors which contribute to the difficulty of using HPLC for analysis of anthocyanins. There are however, a number of commonly available materials for which the anthocyanins have been thoroughly characterized. These materials can often be used as references.

Recently, the availability of photodiode array detection has allowed the chromatographer to determine the spectra of compounds as they elute from the HPLC column. Andersen (1985, 1987) has characterized a number of anthocyanins using this technique. This investigation, describes a systematic approach for use of HPLC for the separation of anthocyanins and the use of on-line photodiode array detection for characterization of the spectral properties of the anthocyanins. The analysis of the anthocyanins in cranberry (Vaccinium macrocarpon), roselle (Hibiscus sabdariffa) and strawberry (Fragaria anannassa) will be described.
Experimental Section

Samples.

Single strength cranberry juice pressed from frozen cranberries was available from another study (Durst and Wrolstad., 1988). Dried roselle was obtained from General Foods Inc. (New York). Strawberry and blueberry juice concentrates were supplied by Kerr Concentrates (Salem, Oregon). Commercial Technical grade Malvin was obtained from the Aldrich Chemical Company (Milwaukee, Wisconsin). Bilberry powder was supplied by Spreda (Burgdoff, Switzerland). Red cabbage extract was supplied by G. Shimizu and Co., LTD. (Osaka, Japan). Tamarillo fruit was purchased from a local supermarket.

Extraction of Pigments

For dried samples: 10 g of dried material was crushed and extracted with 100 mL of 1% methanolic HCl (1 mL concentrated HCl in 99 mL Methanol) overnight at 0°C. The slurry was filtered and the solids washed with an additional 100 mL of 1% methanolic HCl. The methanol extracts were combined and concentrated to ca. 10 mL in a rotary evaporator (30°C). An aqueous pigment extract was prepared by evaporating the methanol extract to dryness and redissolving in an equal volume of 0.01% HCl (0.1 mL conc. HCl in 1 L of distilled water). Juice samples: Juice samples were diluted ca. 10 fold and used as is. Sample size: For injection onto
HPLC, sufficient sample was used such that the final aqueous pigment extract had an absorbance of ca. 4 (1 cm pathlength) at 520 nM.

**Paper Chromatography**

Descending paper chromatography was performed using the methods described by Harborne, (1967) and Du and Francis, (1973). For isolation, cleaned-up samples (see below) were streaked on Whatman 3MM and chromatographed in either BAW (n-butanol/acetic acid/water, 4/1/5, top layer), 1% HCl (water/conc. HCl, 97/3) or AHW (acetic acid/HCl/water, 15/3/82) in the descending mode. Bands were cut, eluted with methanol containing 0.01% conc. HCl and concentrated in a rotary evaporator. When necessary, pigments were rechromatographed in one of the other different solvent systems.

**Sample Preparation for HPLC Analysis of Anthocyanidins**

The hydrolysis procedure previously described by Hong and Wrolstad (1986) was used.

**Sample Preparation for HPLC Analysis of Anthocyanins.**

Two different sample preparation methods were used. For quantitation (Cleanup Procedure I), the pigments were adsorbed onto an activated C$_{18}$ Sep-Pak cartridge (Waters Associates, Milford, MA). To activate, pass 5 mL of methanol through the cartridge followed by 5 mL of distilled water. All Sep-Pak procedures were performed with a 25 mL syringe. The anthocyanins were adsorbed onto the reversed phase material by passing an aqueous solution of the pigments into the
cartridge. The cartridge was washed with 0.01% HCl and the pigments eluted with 0.01% Methanolic HCl. The methanolic extract was then evaporated to ca. 1 mL. For HPLC, a fraction of the methanolic extract was redissolved to an appropriate concentration with 4% phosphoric acid (concentrated phosphoric acid/ water, 4/96, w/w) and filtered with a 0.45 μm Type HA Millipore filter (Millipore Corp., Bedford, MA).

For HPLC separation and Photodiode Array analysis (Cleanup Procedure II). The pigments (in aqueous solution) were adsorbed onto hydrated polyvinylpolypyrrolidone (PVPP; GAF Corp., New York, NY), washed with 0.01% HCl until the eluate was almost colorless and eluted with 0.01% methanolic HCl. The eluate was concentrated in a rotary evaporator to dryness, re-dissolved in ca. 2 mL methanolic HCl and the pigments precipitated with ca. 20 mL cold (0 °C) diethyl ether. The pigments were filtered and redissolved in methanolic HCl. The precipitation procedure was repeated. The final methanolic pigment extract was evaporated to ca. 1 mL. For HPLC, the pigments were redissolved to an appropriate concentration with 4% phosphoric acid and filtered with a 0.45 μm Type HA Millipore filter.

**HPLC separation of Anthocyanidins**

Liquid Chromatograph: Perkin Elmer Series 400 equipped with a Varian UV-50 variable wavelength detector and a Perkin Elmer LCl-100 integrator. Column: Suplecosil ODS (5 mm x 25 cm ) 5 micron particle size with Bio-Rad ODS guard column. Solvent: Solvent A:15% Acetic Acid (conc. acetic acid/water, 15/85, v/v). Solvent B: Acetonitrile. Conditions: isocratic elution at 1.5 mL/min with
85% A and 15% B at room temperature. Detection at 520 nM. Injection volume 25 μl.

Selective Elution of Anthocyanins with Alkaline Borate Buffer

Anthocyanins were adsorbed onto an activated C₁₈ Sep-Pak. Alkaline borate buffer (0.1N Sodium Borate, ca. pH 9) was passed through the column until the eluant was colorless (ca. 50 mL). The anthocyanins were reconverted into their red oxonium salt form by passing ca. 5 mL of 2N HCl through the column. The procedure with Borate buffer and HCl was repeated one additional time. The anthocyanins remaining on the cartridge were subsequently eluted with 0.01% methanolic HCl, concentrated to ca. 1 mL in a rotary evaporator. For HPLC, the pigments were redissolved to an appropriate concentration with 4% phosphoric acid and filtered with a 0.45 μm Type HA Millipore filter.

Alkaline Hydrolysis of Anthocyanins

Approximately 5 mg of "cleaned-up" pigment was added to 10 ml of 10% aqueous of KOH in a screw cap test tube. The tube was flushed with nitrogen and capped. The pigments were hydrolyzed for 8 minutes at room temperature in the dark. The pigments were reconverted to their red oxonium salt form by addition of 2N HCl. The pigments were adsorbed onto a C₁₈ Sep-Pak, washed with excess 0.01% HCl and the pigments eluted with 0.01% methanolic HCl. The pigments were concentrated to ca. 1 mL. For HPLC, the pigments were redissolved to an appropriate concentration with 4% phosphoric acid and filtered with a 0.45 μm Type HA Millipore filter.
HPLC separation of Anthocyanins

Liquid Chromatograph: Perkin Elmer Series 400 equipped with a Hewlett-Packard 1040A Photodiode Array Detector and a Hewlett-Packard 9000 computer. Column: Polymer Labs PLRP-S 5 micron (4.6 X 25 cm) with Polymer Labs guard column. Solvent: Solvent A, 4% Phosphoric Acid (conc. phosphoric acid/water, 4/96, v/v). Solvent B, 100% Acetonitrile. Program: isocratic elution with 6% B from 0-10 min, linear gradient to 20% B from 10-55 min, isocratic elution at 20%B from 50-65 min. Room Temperature operation. Primary detection at 520 and 260 nM (20 nM bandwidth) Injection Volume: 20 μl. All spectral data were smoothed with the HP system smoothing function (filter length = 15).
Results and Discussion

**HPLC Separation of Anthocyanidins**

Shown in Figure 2.1 is a typical HPLC chromatogram of blueberry anthocyanidins. Blueberry (and bilberry, *Vaccinium myrtillus*) anthocyanins were used as a standard source of pigments as it contains all of the common anthocyanidins (Francis, et al., 1966) except for pelargonidin which can be found in Strawberries (Hong and Wrolstad, 1986). Pelargonidin elutes between petunidin and peonidin. Retention on reversed phase (RP) systems is based on the polarity of the anthocyanidin, the most hydrophillic anthocyanidin (delphinidin) eluting first and the least hydrophillic anthocyanidin (malvidin) eluting last (Wilkinson, et al., 1977).

Analysis of anthocyanidins is a good initial method in that it yields information as to the nature of the aglycones. Since there are only 6 common anthocyanidins, identification is easily done. Although pure standards are difficult to obtain, suitable natural sources are readily available as discussed above. Table 2.1 lists the results of anthocyanidin analysis of cranberry, roselle and strawberry. Cranberry was found to contain only cyanidin and peonidin. These results are in agreement to those previously reported (Hong and Wrolstad, 1986). Roselle was found to contain only delphinidin and cyanidin which is in agreement with the results of Wilkinson et. al. (1977). Strawberry was found to contain pelargonidin as the major pigment with lesser amounts of cyanidin.
HPLC Separation of Anthocyanins

The anthocyanin composition of cranberry is well characterized, both by classical paper chromatographic methods and by more recent HPLC methods. Shown in Figure 2.2A is a HPLC chromatogram of cranberry anthocyanins and their relative peak areas. Peak assignments were made by comparison with results reported by Durst and Wrolstad, (1988, in press), Sapers and Hargrave (1987); Hicks, et al. (1985); Hale, et al. (1986). Again, as discussed above, retention is based on the relative hydrophobicity of the molecule. With the anthocyanins, the degree of glycosidation and the nature of the sugar moiety becomes an important factor, (Strack, et al., 1980; Casteele, et al., 1983). From these results and those of previous workers, for a given anthocyanidin, the analogous glycosides will elute in the following order (in terms of chromatographic retention in RP HPLC systems): galactoside faster than the glucoside which is in turn faster than the arabinoside. The results from the anthocyanidin analysis of cranberry showing the presence of only cyanidin and peonidin glycosides is additional support in confirming the results of the anthocyanin analysis.

In developing an analytical method for the separation of the anthocyanins, several factors were taken into consideration. Most workers have obtained excellent results using silica based ODS HPLC columns with mobile phases consisting of acetic acid/water with either acetonitrile or methanol as the organic modifier. Acetic acid plays an important role in the separation by acting both as an organic modifier and by lowering the pH of the mobile phase. A low pH is essential to keep the anthocyanins in the red flavylum cation form, resulting in increased peak
peak sharpness (Hale, et al., 1986). Early workers did not employ mobile phases below pH 2.0 due to the possibility of damage to silica based columns (Hale, et al., 1986). There are reports however, that there is no loss in column performance of silica columns when used with mobile phases below pH 2, (Spanos and Wrolstad, 1988; Wulf and Nagel, 1978; Bronnum-Hansen and Hansen, 1983). In this study, a polymer reversed phase column was employed so that mobile phases at very low pH's could be used without fear of column degradation. A phosphoric acid/water and acetonitrile solvent system was chosen over acetic acid/methanol as the former possesses desirable spectral properties in the low UV. Transparency in the low UV is important for taking diode array spectra as compounds are eluted from the column.

Two different sample preparation methods were employed in this study. For quantitation of the relative amounts of the anthocyanins, samples were subjected to minimal clean-up (clean-up procedure I) to minimize changes in pigment composition. When spectra were to be taken, samples were subjected to a more vigorous cleanup procedure (cleanup procedure II) which included steps involving adsorption of pigments onto PVPP and precipitation of pigments by diethyl ether. Such a procedure is necessary to remove the non-anthocyanin, UV-visible absorbing compounds from the sample matrix. Through the use of a photodiode array detector, spectra will be taken directly from the HPLC runs. Therefore, any compounds which elute with the anthocyanins and absorb in the UV-Visible range will contribute to the apparent spectra of the anthocyanins, thereby resulting in inaccuracies.
Shown in Figure 2.2A is a chromatogram of cranberry anthocyanins in which the sample was subjected to minimal cleanup (cleanup procedure I). When monitored at 520 nM where only the anthocyanins are detected, the chromatogram appears quite "clean". When monitored at 260 nM however (Figure 2.2B), it becomes apparent that there are other compounds eluting near or co-eluting with the anthocyanins. Figure 2.3A shows a chromatogram of cranberry anthocyanins when cleaned-up with the more vigorous clean-up procedure (cleanup procedure II). It can be seen that this clean-up procedure is effective in reducing the amount of interference by UV absorbing compounds (Figure 2.3B). Unfortunately, this clean-up procedure also changes the relative proportions of the anthocyanins (compare Figure 2.2A with Figure 2.3A). This demonstrates the necessity of using a minimal cleanup procedure (cleanup procedure I) when quantitation of the relative peak areas is desired.

Shown in Figure 2.4 is a chromatogram of roselle anthocyanins and their relative peak areas. As expected, the delphinidin glycosides eluted before the cyanidin glycosides and the diglycosides eluted before the corresponding monoglycosides. A late eluting peak (Peak 4-e) remains unidentified. Identities of the roselle anthocyanins was established by separation with paper chromatography and identification by comparison with the Rf values reported by Du and Francis (1973).

Comparison of the relative percentages of the anthocyanidins (Table 2.1) to the sum of relative percentages of the corresponding anthocyanins (Figure 2.2A for cranberry; Figure 2.4 for roselle) in each of the samples show that both methods give comparable results.
Figure 2.5 shows an HPLC chromatogram for a mixture of bilberry (Vaccinium myrtillus) and strawberry anthocyanidins chromatographed on the anthocyanin HPLC system. The occurrence of free anthocyanidins has been reported from time to time, however, it is unlikely that they occur in nature (Shrikhande, 1976). Retention times and spectral characteristics were determined for identification purposes. All of the common anthocyanidins were well resolved except for peonidin and malvidin. Delphinidin elutes near peonidin-3-galactoside. The remaining anthocyanidins elute well after malvidin-3-glucoside.

Spectral Characterization of Anthocyanins

UV-visible absorption spectroscopy has been a valuable method for characterization of anthocyanins. Important structural properties about the anthocyanin can be obtained from spectral information (Harborne, 1963) including the nature of the aglycone (anthocyanidin), the position of attachment of the sugar molecule (Harborne, 1958) and information regarding acylation by aromatic organic acids. Typically the spectra of anthocyanins as well as other flavonoids are measured in methanol containing 0.01% HCl. Most of the reports in the literature employ this solvent. In measurement of spectra directly from the HPLC column, the choice of solvent to be used is limited to the mobile phase of the HPLC system. Since the spectral characteristics of the anthocyanins is dependent on both pH and on the nature of the solvent, direct comparison of spectral characteristics with those published in the literature may be inappropriate. Harborne (1958) reports that in general, there is a 15 nM shift towards shorter wavelengths when water is substituted for methanol.
Shown in Figure 2.6 is the spectra of cyanidin-3-galactoside (from cranberry), delphinidin-3-sambubioside (from roselle) and peonidin-3-galactoside (from cranberry). Table 2.2 lists the visible $\lambda_{\text{max}}$ and the $E_{440}/E_{\lambda \text{ vis max}}$ wavelength ratio of the anthocyanins of both cranberry and roselle as determined in this study along with values as reported in the literature. The wavelength maxima in the visible range is closely related to the hydroxylation pattern of the anthocyanin. In acidic methanol solutions, pelargonidin 3-glycosides exhibit a visible $\lambda_{\text{max}}$ at about 505 nM, cyanidin and peonidin 3-glycosides have a visible $\lambda_{\text{max}}$ at 520-526 nM and delphinidin derivatives (delphinidin, malvidin and petunidin) at 532-537 nM (Table 2.2). The nature of the sugar substitution has no effect on spectra, (Harborne, 1958). The visible wavelength maximum for the cyanidin and peonidin 3-glycosides from both cranberry and roselle (Table 2.2) were similar, the values being lower than previously reported for the same pigments in acidified methanolic solvents. The two delphinidin 3-glycosides from roselle exhibited a higher wavelength maxima than for the cyanidin and peonidin 3-glycosides. These values are lower than those previously reported for the same pigments in acidified methanolic solvents. The HPLC/photodiode array results of Andersen (1985, 1987), also using a part aqueous, part organic HPLC solvent (formic acid/methanol/water), showed similar trends.

Information regarding the glycosidation substitution pattern of the anthocyanins can also be obtained from spectra. The 3- and 3,5- diglycosides have similar spectral maxima but show differences in the 400-460 nM region (Harborne, 1967). These differences have been traditionally expressed in terms of the $E_{440}/E_{\lambda \text{ vis max}}$ ratio, the 3-glycosides exhibiting ratios that are about twice as
large as for the 3,5-diglycosides. The $E_{440}/E_{\lambda \text{vis max}}$ values obtained for all of the 3-glycosides in both cranberry and roselle (Table 2.2) are similar, the values ranging from 29-35%. These values are higher than those previously reported for spectra in methanolic solvents. The results of Andersen (1985, 1987) are also higher than for those values reported for methanolic solvents. It appears that decreasing solvent polarity influences the spectral characteristics of the anthocyanins by increasing the $E_{440}/E_{\lambda \text{vis max}}$ ratio and decreasing the visible $\lambda_{\text{max}}$. Figure 2.7 shows an HPLC chromatogram of commercial technical grade malvin (malvidin-3,5-diglucoside). Several unidentified pigments are also present. Figure 2.8 shows the UV-visible spectra of malvin. The visible wavelength maxima of malvin was 523 nM which is indicative of a delphinidin derivative. The $E_{440}/E_{\lambda \text{vis max}}$ ratio of malvin was 16%, a value about half of that for the 3-glycosides. This value is higher than for those values reported for this pigment in methanolic solvents (Table 2.2).

Information regarding the presence of acylation by hydroxylated aromatic organic acids can also be obtained from spectra. This is detected by the presence of a peak in the 310 nM range. The ratio of the absorbance at the acyl $\lambda_{\text{max}}$ to the absorbance at the visible $\lambda_{\text{max}}$(E_{\lambda \text{acyl max}}/E_{\lambda \text{vis max}} ratio) is a measure of the molar ratio of the cinnamic acid to the anthocyanin in the pigment. In acidified methanolic solutions, a E_{\lambda \text{acyl max}}/E_{\lambda \text{vis max}} ratio of 48-71% is indicative of a 1:1 molar ratio of the cinnamic acid to the anthocyanin while E_{\lambda \text{acyl max}}/E_{\lambda \text{vis max}} ratio of 83-107% is characteristic of a 2:1 molar ratio of the cinnamic acid to the anthocyanin (Harborne, 1958). Both the acyl $\lambda_{\text{max}}$ and the E_{\lambda \text{acyl max}}/E_{\lambda \text{vis max}} ratio is characteristic of the nature of the acylating acid (Harborne, 1958).
None of the anthocyanins analyzed in this work exhibited a peak in this area, indicating no acylation with hydroxylated aromatic organic acids. Figures 2.9A and 2.9B show the UV-Visible spectra of 2 unidentified acylated anthocyanins from red cabbage (Brassica oleracea). The absorbance peak in the 310 nM range indicates acylation with a cinnamic acid. The $E_{\lambda,\text{acyl max}}/E_{\lambda,\text{vis max}}$ ratio shows that the anthocyanin in Figure 2.9A is acylated with a 1:1 molar ratio of the cinnamic acid to the anthocyanin. The $E_{\lambda,\text{acyl max}}/E_{\lambda,\text{vis max}}$ ratio for the anthocyanin shown in Figure 2.9B shows that it is acylated with a 2:1 molar ratio of the cinnamic acid to the anthocyanin.

In addition to acylation with cinnamic acid derivatives, anthocyanins have also been found to be acylated with either acetic acid or with malonic acid. Detection of acylation of anthocyanins with either of these acids is not possible by spectral means as they do not have a characteristic absorbance spectra in the UV. It is possible however, to determine the presence of acylation with these acids by their HPLC elution characteristics. Acylation with acetic acid results in an increase in retention time (Wulf and Nagel, 1978). While there is little information on the HPLC elution characteristics of malonic acid acylated pigments, the polar nature of a malonic acid ester would most likely result in decreased retention when compared to the non-acylated anthocyanin. Another means of detecting any of acylated anthocyanins is that the ester bond is susceptible to alkaline hydrolysis. Hence, the disappearance of a peak after mild alkaline hydrolysis would be indicative of either an acylated anthocyanin or an alkaline labile anthocyanin.

The spectral characteristics of the anthocyanidins are given in Figure 2.5. These results are similar to that for the anthocyanins in that the same basic
spectral patterns are followed: 1) Pelargonidin derivatives have a lower visible \( \lambda_{\text{max}} \) than cyanidin derivatives which in turn have a lower visible \( \lambda_{\text{max}} \) than the delphinidin derivatives; 2) Pelargonidin exhibits the lowest visible wavelength maxima and the highest \( E_{440}/E_{\lambda_{\text{vis max}}} \) ratio (Table 2.2). A pronounced shoulder in the 410-460 nM region is evident. 3) Anthocyanidins within each class have similar spectral characteristics (eg. cyanidin and peonidin have similar spectral characteristics and delphinidin, petunidin and malvidin have similar spectral characteristics). Comparison of the \( \lambda_{\text{vis max}} \) of the anthocyanidins (Figure 2.5) to that of the anthocyanins (Table 2.2) show that in general, the anthocyanidins exhibit a 6-10 nM higher visible \( \lambda_{\text{max}} \) than for the corresponding anthocyanins. These results are consistent with those of Harborne (1958) who reported a 6-12 nM larger visible \( \lambda_{\text{max}} \) for the anthocyanidins in 0.01% methanolic HCl. On the basis of this information and of their elution profile (discussed above), anthocyanidins can be readily differentiated from anthocyanins.

Class Separation of Anthocyanins with Borate Ion.

We have observed that when anthocyanins are adsorbed onto a C18 Sep-Pak cartridge and eluted with an alkaline borate solution that non-acylated anthocyanins containing o-dihydroxyl systems (cyanidin, delphinidin and petunidin) are preferentially eluted while those anthocyanins which do not contain o-dihydroxy groups (pelargonidin, peonidin and malvidin) tend to be enriched on the cartridge. Figure 2.10 compares the HPLC chromatogram of the fraction of cranberry anthocyanins which remains on the Sep-Pak after elution with an alkaline borate solution with an untreated cranberry chromatogram. It can be seen that the
peonidin glycosides are highly enriched. This technique provides an additional method for characterization of the anthocyanins. Spectral analysis can yield useful information as to the nature of the anthocyanin, the visible \( \lambda_{\text{max}} \) differentiating cyanidin derivatives, delphinidin derivatives and pelargonidin. However, anthocyanins within each class cannot be differentiated (e.g. similarly glycosidated cyanidin and peonidin glycosides cannot be differentiated from each other; and similarly glycosidated delphinidin, petunidin and malvidin glycosides cannot be differentiated from each other) by spectral characteristics alone. The use of this \( C_{18} \) Sep-Pak/Borate Buffer method allows differentiation of cyanidin from peonidin glycosides and differentiation of delphinidin and petunidin from malvidin glycosides.

Alkaline borate solutions have been used as buffers in the electrophoretic separation of phenols. The primary function of borate has been to enhance electrophoretic mobility through the formation of negatively charged complexes which result from the reaction of borate ion with phenols possessing o-dihydroxyl groups.\((Pridham, 1964)\). In our borate sample treatment scheme, it appears that those anthocyanins which possess the o-dihydroxyl groups form a charged borate complex which results in a more hydrophillic species. The complex is preferentially eluted from the reversed phase cartridge. Those anthocyanins which do not complex with the borate ion remain in the cartridge. In the analysis of grape anthocyanins, we have observed that highly retained species (long retention times) such as acylated cyanidin and delphinidin glycosides, are also not eluted from the cartridge. Assuming that borate complexes form, the hydrophobicity of these anthocyanins are such that any complex formed is not sufficiently hydrophillic enough to be eluted from the column. The anthocyanidins behave differently in that
they are completely eliminated by the borate/Sep-Pak treatment. We believe that the very unstable anthocyanidins are completely destroyed under the alkaline conditions employed. This behavior could be applied to distinguish anthocyanidins from anthocyanins with similar retention times.

**Characterization of Strawberry Anthocyanins**

The potential applications of these methods can be illustrated in characterizing the anthocyanins in strawberries (*Fragaria anannassa*). Our interest in strawberries extends from the repeated occurrence of unidentified minor peaks in the strawberry HPLC chromatogram.

A survey of the anthocyanins in strawberry show that the major pigment is pelargonidin-3-glucoside with smaller amounts of cyanidin-3-glucoside (Timberlake and Bridle, 1982). A third pigment, possibly a isomer of pelargonidin-3-glucoside was reported by Wrolstad, et al., (1970). The anthocyanidin profile (Table 2.1) show that the major anthocyanidin is pelargonidin with lesser amounts of cyanidin. Figure 2.11A shows the HPLC chromatogram for strawberry anthocyanins. The spectral characteristics of the individual peaks are included in Table 2.3. Figure 2.11B shows a typical HPLC chromatogram of the strawberry anthocyanins remaining on a C18 Sep-Pak after elution with alkaline borate buffer. This treatment had little effect on the major strawberry anthocyanins (peaks 11-b,c,d,e), the conclusion therefore is that they are most likely to be pelargonidin derivatives and not cyanidin derivatives. Peak 11-a which has the same retention time as cyanidin-3-glucoside is reduced in its relative peak area but is not completely eliminated. The major peak (peak 11-b) is identified as pelargonidin-3-
glucoside. Its UV-visible spectra is included in Figure 2.12. Its spectral characteristics are similar to that of its aglycone, in that a pronounced shoulder in the 410-450 nM region is present. This results in a higher $E_{440}/E_{\text{vis}}$ ratio than for the other common anthocyanidin glycosides (Table 2.2). These results are consistent with those of Harborne (1958). The visible wavelength maxima (Table 2.3) is lower than for cyanidin glycosides.

The $\lambda_{\text{vis}} \text{max}, E_{440}/E_{\text{vis}}$ ratio (Table 2.3) and the appearance of a shoulder in the 400-460 nM region for peaks 11-c and 11-e indicate that these unknown pigments are pelargonidin-3-glycosides. Both peaks elute later than pelargonidin-3-glucoside, indicating that the sugar substituent of unidentified compounds exhibit greater hydrophobicity than for glucose. Some possibilities are 1) glycosidation with a more hydrophobic sugar group, eg. rutinose, arabinose, xylose; 2) acylation of the sugar moiety; 3) formation of polymerized pigments. No UV absorbance maxima was found in the 310 nM range for any of the strawberry anthocyanins, indicating no acylation with hydroxy-aromatic organic acids.

Peak 11-c is tentatively identified as pelargonidin-3-rutinoside on the following basis: 1) Its spectral characteristics show that it is a pelargonidin derivative; 2) This peak co-elutes with pelargonidin-3-rutinoside from Tamarillo (Cyphomandra betacea) fruit (Wrolstad and Heatherbell, 1974). Figure 2.11C shows an HPLC chromatogram of strawberry anthocyanins after mild alkaline hydrolysis. The disappearance of peak 11-e indicates that it is acylated. The UV spectrum eliminates the possibility of acylation with an aromatic acid. The late retention time of peak 11-e is consistent with the possibility of acylation with acetic acid. From this information, we conclude that peak 11-e is an acetic acid
acylated pelargonidin derivative. For definitive identification, further studies will need to be performed on purified fractions of these anthocyanins. Peak 11-d remains unidentified. The $\lambda_{vis\text{ max}}$ is higher than that for a pelargonidin derivative and lower than that of a cyanidin derivative. A shoulder in the 400-460 nM region is evidence that it may be a pelargonidin derivative. It is not hydrolyzed under alkaline conditions indicating that it is not acylated, it does not exhibit any characteristics to indicate that it is pelargonidin (aglycone) and it is not eluted from a C$_{18}$ Sep-Pak with borate buffer supporting the possibility that it is a glycosidated pelargonidin derivative.

Conclusion

In addition to HPLC retention order, the use of on-line photodiode array detection is a powerful tool to aid in the characterization of anthocyanins. Removal of interfering phenolic compounds by sample preparation procedures results in a sufficiently clean anthocyanin extract such that spectra obtained by photodiode array detection can be used for identification. Derivatives of delphinidin, cyanidin and pelargonidin can be distinguished from each other by their visible $\lambda_{max}$ spectral means while anthocyanins acylated with hydroxy-aromatic organic acids can be identified by their characteristic spectral absorption maxima in the 310 nM range. The presence of different glycosidic substituents can be differentiated due to different retention characteristics by reversed phased HPLC. Non-acylated anthocyanins without o-dihydroxyl groups (pelargonidin, malvidin and peonidin) can be selectively concentrated on a C$_{18}$ Sep-Pak using an alkaline borate buffer as an elutant, thus serving as a means of distinguishing those anthocyanins from cyanidin.
petunidin and delphinidin derivatives. Acylated anthocyanins can be eliminated from a chromatogram by mild alkaline hydrolysis.
Table 2.1  Percentages of Anthocyanidins in Cranberry, Roselle and Strawberry as determined by HPLC

Relative Percentages of Anthocyanidins based on total peak area

<table>
<thead>
<tr>
<th>Sample</th>
<th>Delphinidin</th>
<th>Cyanidin</th>
<th>Petunidin</th>
<th>Petargonidin</th>
<th>Peonidin</th>
<th>Malvidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranberry</td>
<td>NF</td>
<td>46%</td>
<td>NF</td>
<td>NF</td>
<td>54%</td>
<td>NF</td>
</tr>
<tr>
<td>Roselle</td>
<td>59%</td>
<td>41%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Strawberry</td>
<td>NF</td>
<td>24%</td>
<td>NF</td>
<td>75%</td>
<td>NF</td>
<td>NF</td>
</tr>
</tbody>
</table>

NF = Not found
Table 2.2  Spectral Characteristics of Cranberry and Roselle anthocyanins determined by HPLC/Photodiode Array Detection along with Spectral Values for Selected Anthocyanins as Reported in the Literature

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Visible Wavelength (nm)</th>
<th>(K40/E) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cranberry Anthocyanins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyanasin-3-glucoside</td>
<td>M</td>
<td>515</td>
</tr>
<tr>
<td>cyanisin-3-glucoside</td>
<td>M</td>
<td>515</td>
</tr>
<tr>
<td>cyanasin-3-arabinoside</td>
<td>M</td>
<td>515</td>
</tr>
<tr>
<td>pelargonin-3-glucoside</td>
<td>M</td>
<td>515</td>
</tr>
<tr>
<td>pelargonin-3-glucoside</td>
<td>M</td>
<td>515</td>
</tr>
<tr>
<td>pelargonin-3-arabinoside</td>
<td>M</td>
<td>517</td>
</tr>
</tbody>
</table>

| **Roselle Anthocyanins**     |                         |                |
| cyanasin-3-sambubioside      | M                       | 523            |
| cyanasin-3-glucoside         | M                       | 20             |
| cyanasin-3-sambubioside      | M                       | 517            |
| cyanasin-3-glucoside         | M                       | 515            |

<table>
<thead>
<tr>
<th><strong>LITERATURE VALUES</strong></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanasin-3-glucoside</td>
<td>b 528  23 Zappulli and Frendo, 1965</td>
</tr>
<tr>
<td>cyanasin-3-arabinoside</td>
<td>c 520  31 Anderan, 1985</td>
</tr>
<tr>
<td>cyanasin-3-glucoside</td>
<td>b 528  23 Zappulli and Frendo, 1965</td>
</tr>
<tr>
<td>cyanasin-3-arabinoside</td>
<td>c 520  30 Anderan, 1985</td>
</tr>
<tr>
<td>cyanasin-3-glucoside</td>
<td>a 525  22 Harborne, 1950</td>
</tr>
<tr>
<td>cyanasin-3-arabinoside</td>
<td>c 520  22 Anderan, 1985</td>
</tr>
<tr>
<td>cyanasin-3-sambubioside</td>
<td>a 526  24  Duv and Freenco, 1973</td>
</tr>
<tr>
<td>cyanasin-3-glucoside and gluicoside</td>
<td>c 516  20-31 Anderan, 1985</td>
</tr>
<tr>
<td>pelargonin-3-glucoside</td>
<td>b 527  29 Zappulli and Frendo, 1965</td>
</tr>
<tr>
<td>pelargonin-3-sambubioside</td>
<td>b 524  20 Zappulli and Frendo, 1965</td>
</tr>
<tr>
<td>pelargonin-3-glucoside</td>
<td>a 523  26 Harborne, 1950</td>
</tr>
<tr>
<td>pelargonin-3-arabinoside</td>
<td>a 523  25 Sambubioside and gluicoside, 1974</td>
</tr>
<tr>
<td>pelargonin-3-glucoside</td>
<td>c 518  30 Anderan, 1987</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Dietricins, Papilins and Highery Glucosides</strong></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpinolin-1-glucoside</td>
<td>a 535  18 Harborne, 1950</td>
</tr>
<tr>
<td>alpinolin-1-arabinoside</td>
<td>a 541  22 Duv and Freenco, 1973</td>
</tr>
<tr>
<td>alpinolin-3-glucoside</td>
<td>b 528  25 Anderan, 1985</td>
</tr>
<tr>
<td>alpinolin-3-arabinoside</td>
<td>a 542  20 Duv and Freenco, 1973</td>
</tr>
<tr>
<td>alpinolin-3-arabinoside,</td>
<td>c 524  27 Anderan, 1985</td>
</tr>
<tr>
<td>alpinolin-3-glucoside,</td>
<td>a 535  16 Harborne, 1950</td>
</tr>
<tr>
<td>alpinolin-3-glucoside, and gluicoside</td>
<td>c 524-528  24-25 Anderan, 1987</td>
</tr>
<tr>
<td>alpinolin-3-glucoside</td>
<td>a 535  18 Harborne, 1950</td>
</tr>
<tr>
<td>alpinolin-3,5-glucoside</td>
<td>a 533  12 Harborne, 1950</td>
</tr>
<tr>
<td>alpinolin-3-glucoside,</td>
<td>c 526  28-37 Anderan, 1987</td>
</tr>
<tr>
<td>alpinolin-3-glucoside, gluicoside and arabinoside</td>
<td>a 518  26 Harborne, 1950</td>
</tr>
</tbody>
</table>

* = 0.01% HCl in buffer
+ = 0.1% HCl in buffer
M = HPLC solvent, formic acid/water/methanol
P = methanol used in this study, phosphoric acid/water/methanol

Pipe: [Image]
<table>
<thead>
<tr>
<th>Peak #</th>
<th>Visible Wavelength Maxima (nM)</th>
<th>E440/Evis Ratio (%)</th>
<th>Acyl Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-a</td>
<td>ND</td>
<td>ND</td>
<td>N</td>
</tr>
<tr>
<td>11-b</td>
<td>501</td>
<td>45</td>
<td>N</td>
</tr>
<tr>
<td>11-c</td>
<td>501</td>
<td>50</td>
<td>N</td>
</tr>
<tr>
<td>11-d</td>
<td>509</td>
<td>61</td>
<td>N</td>
</tr>
<tr>
<td>11-e</td>
<td>501</td>
<td>51</td>
<td>N</td>
</tr>
</tbody>
</table>

ND = not determined
Y = Acylation with hydroxy aromatic acid detected
N = No Acylation with hydroxy aromatic acid detected

Table 2.3 Spectral Characteristics of Strawberry anthocyanins as determined by HPLC/Photodiode Array Detection
Figure 2.1    HPLC Chromatogram of Blueberry Anthocyanidins. Detection at 520 nM. Peak identification: 1-a, delphinidin; 1-b, cyanidin; 1-c, petunidin; 1-d, peonidin; 1-e, malvidin
Figure 2.2  HPLC Chromatogram of Cranberry Anthocyanins, minimal sample cleanup (Cleanup procedure I). 2.2A, detection at 520 nM. 2.2B, detection at 260 nM. Peak Identification. Relative peak areas calculated with detection at 520 nM in parentheses. 2-a. cyanidin-3-galactoside (33%); 2-b. cyanidin-3-glucoside (2%); 2-c. cyanidin-3-arabinoside (14%); 2-d. peonidin-3-galactoside (38%); 2-e. peonidin-3-glucoside; (4%) 2-f. peonidin-3-arabinoside (9%). Proportion of cyanidin glycosides: 49% Proportion of peonidin glycosides: 51%
Figure 2.3  HPLC Chromatogram of Cranberry Anthocyanins subjected to PVPP adsorption and precipitation by diethyl ether (Clean-up procedure II).  2.3A, detection at 520 nM.  2.3B, detection at 260 nM  Peak Identification.  3-a. cyanidin-3-galactoside ; 3-b. cyanidin-3-glucoside; 3-c. cyanidin-3-arabinoside ; 3-d. peonidin-3-galactoside ; 3-e. peonidin-3-glucoside; 3-f. peonidin-3-arabinoside
Figure 2.4  HPLC Chromatogram of Roselle Anthocyanins. Detection at 520 nM. Peak Identification. Relative peak areas calculated with detection at 520 nM in parentheses. 4-a. delphinidin-3-sambioside (56%); 4-b. delphinidin-3-glucoside (4%); 4-c. cyanidin-3-sambioside (33%); 4-d. cyanidin-3-glucoside (3%); 4-e. unknown (4%). Proportion of delphinidin glycosides: 60%. Proportion of cyanidin glycosides 36%
Figure 2.5  HPLC Chromatogram and Spectral Characteristics of Bilberry and Strawberry Anthocyanidins Chromatographed on the Anthocyanin HPLC system. Peak identification with spectral information in parentheses, (\(\lambda_{\text{vis max}}\) in nM, \(E_{440}/E_{\lambda_{\text{vis max}}}\) ratio expressed as %): 5-a. delphinidin (529 nM, 25%); 5-b. cyanidin (523 nM, 27%); 5-c. petunidin (531 nM, 26%); 5-d pelargonidin (512 nM, 34%); 5-e. peonidin (525 nM, 27%)+ malvidin (533 nM, 28%).
Figure 2.6 UV-Visible Spectra of cyanidin-3-galactoside (2.6A), peonidin-3-galactoside (2.6B) and delphinidin-3-sambioside (2.6C) determined by Photodiode Array Detection.
Figure 2.7  HPLC Chromatogram of Commercial Malvin (malvidin-3,5-diglucoside). Peak identification: 7-a, 7-c, 7-d unknown; 7-b.malvidin-3,5-diglucoside. Spectral characteristics, visible wavelength maxima = 523 nM; $E_{440}/E_{\lambda \text{vis max}}$ ratio = 16%,
Figure 2.8 UV-Visible spectra of A, malvidin-3,5-diglucoside and B, malvidin-3-glucoside as determined by Photodiode Array Detection
Figure 2.9  UV-Visible spectra of 2 unknown anthocyanins from red cabbage. Spectral information in parentheses, ($\lambda_{\text{vis}}$ max in nM, $\lambda_{\text{acyl}}$ max in nM, $E_{440}/E_{\lambda}$ vis max ratio expressed as %, $E_{\lambda}$ acyl max/$E_{\lambda}$ vis max ratio expressed as %). Anthocyanin 2.9A, (529, 333, 21%, 58%). Anthocyanin 2.9B, (537, 320, 18%, 100%)
Figure 2.10  A, HPLC Chromatogram of the fraction of cranberry anthocyanins remaining on a C18 Sep-Pak after elution with alkaline borate buffer. B, untreated cranberry anthocyanin chromatogram.
Peak identification: 10-a cyanidin-3-galactoside; 10-b cyanidin-3-glucoside; 10-c cyanidin-3-arabinoside; 10-d peonidin-3-galactoside; 10-e peonidin-3-glucoside; 10-f peonidin-3-arabinoside
Figure 2.11  HPLC Chromatograms of Strawberry Anthocyanins
2.11A: Untreated strawberry sample
2.11B: Anthocyanin fraction remaining on a C_{18} Sep-Pak after elution with alkaline borate buffer.
2.11C: Strawberry anthocyanins after alkaline hydrolysis
Peak identification: 11-a cyanidin-3-glucoside; 11-b pelargonidin-3-glucoside; 11-c pelargonidin-3-rutinoside (tentative); 11-d pelargonidin derivative (tentative); 11-e pelargonidin-3-glycoside acylated with acetic acid (tentative)
Figure 2.11 (Continued)
Figure 2.12  UV-Visible spectra of pelargonidin-3-glucoside from strawberry
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Title: CHARACTERIZATION OF ANTHOCYANIN CONTAINING COLORANTS AND FRUIT JUICES BY HPLC/PHOTODIODE ARRAY DETECTION

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Abstract

The anthocyanin pigment profiles of commercial black currant, blackberry, black raspberry, elderberry, cherry, plum, grape, bilberry and red cabbage products were characterized by High Performance Liquid Chromatography (HPLC)/Photodiode Array Detection. Removal of acylated anthocyanins by alkaline hydrolysis and selective removal of anthocyanins on a reversed phase cartridge with borate buffer were auxiliary techniques which proved helpful in making peak assignments. Both the retention properties on reversed phase HPLC and the spectral properties by photodiode array detection were used to characterize the anthocyanins. Other properties including tinctoral strength, total anthocyanin concentration, browning, titratable acidity and Hunter tristimulus values were also determined for these colorants.
Introduction

The recent banning of Red No. 2 in the United States along with the questionable status of Red No. 40 has led to the increased use of natural red pigments as coloring agents (Wallin and Smith, 1977). The anthocyanins are one such class of natural red pigments which have found use as a suitable alternative for synthetic colorants in many applications.

The anthocyanins are widely distributed in nature, occurring in most higher plants. They are found in all parts of the plant but are most obvious in fruits and flowers (Brouillard, 1982). Pigment extracts are commercially available, grape skin extract (GSE) being the most common. In the United States only 2 sources of anthocyanin extracts are allowed to be used in foods. GSE is restricted for use in coloring beverages while grape color extract (GCE), an extract of concord grapes, can be used to color non-beverage foodstuffs (CFR, 1986). In the European Economic Community (EEC) countries, anthocyanin extracts from food sources are generally allowed (for specific information this class of colorants is listed under EEC #E163).

In addition to extracts, the concentrated juice of red fruits such as cranberries, raspberries, elderberries, etc., can also be used in food products that are compatible with the acidity and flavor of the fruit juice concentrate involved (Riboh, 1977). In the United States and for most EEC countries, fruit juice and concentrates can be used without restriction.

The demand for anthocyanin containing colorants is increasing. Fruit juice concentrates have become an important ingredient in the manufacture of many foods
and beverages. Production of fruit juice concentrates has become highly competitive in both domestic and foreign markets. Analytical methodology able to identify the anthocyanins and to determine their source are needed for quality control, for determination of the authenticity of fruit juice concentrates and color extracts as well as for regulatory activities (Wrolstad, et al., 1981).

Chemical analysis of plant constituents (chemotaxonomy) is an excellent objective method for identification and classification of plants. The anthocyanin composition of many fruits is quite distinctive (Wrolstad, et al., 1981) and analysis of anthocyanins has been successfully used to detect adulteration of Concord Grape Juice with Vinifera or hybrid varieties (Mattick, et al., 1967; Fitelson, 1967), adulteration of blackberry and cherry juice with elderberry or grape skin extract (Fitelson, 1968) and adulteration of cranberry juice cocktail with grape skin extract (Hale, et al., 1986; Hong and Wrolstad, 1986).

The objectives of this work are twofold. First, to compare the general coloring properties of several commercially available berry colorants and berry juice concentrates. This information is useful in product development and in quality assurance. The data presented may be helpful in selecting a colorant with the appropriate hue and/or tinctoral strength. The second objective is to use HPLC coupled with photodiode array detection to make peak assignments and characterize anthocyanin profiles. This information is useful from a regulatory standpoint such as identification of an added food colorants or detection of adulteration in anthocyanin containing products.
Materials and Methods

Samples

Colorants: San Ei "San Red ELF" (elderberry colorant) and Red Cabbage colorants: San Red RCF, San Red RC liquid and San Red RC powder was supplied by G. Shimizu and Co., LTD (Osaka, Japan), Obi-Pektin "Black currant Powder (200)"; Obi-Pektin "Elderberry (200)"; Spreda Enocianine Powder, Type 501 (This sample will be designated as "Spreda #501"); Obi-Pektin Bilberry Powder (250); Obi-Pektin Morello Cherry Powder (348); Obi-Pektin Morello Cherry Powder (240); Obi-Pektin Morello Cherry Powder (240 D) was supplied by Spreda (Burgdoff, Switzerland). Welch's Grape Colorant, Type 250 was supplied by Welch Foods (Westfield, NY). An additional sample of Spreda Grape Skin Extract #501 (This sample will be designated as "Spreda GSE") was supplied by General Foods, Inc. (New York)

Berry Juice Concentrates: Plum, black raspberry and blackberry juice concentrate was supplied by Kerr Concentrates (Salem, OR).

Red FDC #40 was obtained from International Flavors and Fragrances (New York, NY)

Red Raspberry juice pressed in our laboratory was available from a previous study (Spanos and Wrolstad, 1987)

Spectral and Acidity Analyses:

Wavelength maxima, Anthocyanin concentration as cyanidin-3-glucoside (ε = 29,600), Degradation Index, Color Density, Polymeric Color,
Percent Tannins and Browning Index was measured by the procedure described by Wrolstad (1976).

Tinctoral strength was expressed as the absorbance at the visible wavelength maxima of a 1% solution of the pigment in the appropriate solvent, either distilled water or pH 1 buffer as described by Wrolstad (1976). A 1% stock solution of the pigment in the appropriate solvent was made and diluted such that the absorbance at the visible maxima was less than 1. Absorbance readings were taken and the tinctoral strength calculated by multiplication with the dilution factor.

Hunter Tristimulus Values (Hunter DP-25P-2, Hunter Instruments, Reston, VA) were determined both on 1% solutions (in distilled water) of the colorants, and at constant absorbance where the pigment solutions (in pH 3.5 buffer) were diluted such that the absorbance at the visible wavelength maxima = 1. The procedure is described in detail by Sapers et al. (1981). All Hunter Colorimeter measurements were made with 1 cm (i.d.) cells with the instrument in the transmittance mode (specular component included).

Titratable Acidity was determined using the Glass Electrode Method, AOAC 22.059 (Official Methods of Analysis, AOAC, 1984).

Chromatographic Methods

HPLC methodology for analysis of Anthocyanidins and Anthocyanins are described by Hong and Wrolstad, (1988).

Photodiode Array measurement of spectral properties for the individual peaks were determined at the apex of the peak except in the case
of closely eluting peaks. For closely eluting peaks, spectral measurement were determined at the front of the peak for the first peak and at the rear of the peak for the second peak.
Results and Discussion

Sample Information

A variety of different anthocyanin containing colorants were examined, including fruit juice concentrates which are readily available and may be used without restriction in the United States. The "Obi-Pektin" samples all come as powders. The manufacturer has stated the following specifications for these products: Morello Cherry 240-D, 40% dry fruit material, 60% maltodextrin added, vacuum dried. Morello Cherry 240, 40% dry fruit material, 60% sucrose added, vacuum dried. Morello Cherry 348, 48% dry fruit material, 12% sucrose, 37% starch, 3% citric acid, drum dried. Bilberry 250, 50% dry fruit material, 50% sucrose added, vacuum dried. Blackcurrant 200, 100% dry fruit powder, vacuum dried. Elderberry 200, not specified. All of the above products are essentially dried fruit juices. From a regulatory standpoint, these products may be legally used as food ingredients in the United States.

The remaining samples are extracts of anthocyanins from natural sources. Enocianin Powder A (Spreda GSE Type 501) is a spray dried product specified by the manufacturer to contain 40-70% dry fruit material with 30-60% maltodextrin added. San Red RC, RC powder and RCF are anthocyanin extracts derived from red cabbage (Brassica oleracia var rubla). The manufacturer states the following specifications for their colorants: San Red RC, 15% propylene glycol, 1% citric acid. San Red RC powder, 36% red cabbage color, 2% citric acid, 62% starch syrup powder. San Red RCF, 20% ethyl alcohol, 2% citric acid. San Red ELF is an
extract of elderberry anthocyanins. The extract is specified to contain 20% ethyl alcohol and 5% citric acid. The Welch's Grape Color Extract is declared to be an extract prepared from citric acid extraction of Concord Grapes. Of the colorants in this group, only the grape samples are permitted to be used in the United States. As previously discussed, Enociannin A is allowed to be used only in beverages while Welch's Grape Color Extract is allowed to be used only in non-beverage applications (CFR, 1986).

**General Colorant Properties**

Listed in Table 3.1 are the general properties of 13 colorants and 2 juice concentrates (blackberry and plum). The concentration of monomeric anthocyanins was measured by both the pH differential method and by the single pH method (Wrolstad, 1976). All values are expressed as cyanidin-3-glucoside even if the sample did not contain major quantities of this pigment, allowing for easy comparison in total anthocyanin concentrations between different commodities. Both of the Spreda GSE samples, the "San Ei" elderberry colorant and all of the cabbage colorants were high in total anthocyanin. This is not unexpected as they are all concentrated pigment extracts. In addition to the contribution of color by the monomeric anthocyanins, there can be contribution of color from polymerized anthocyanins and brown pigments arising from enzymic and non-enzymic browning (Wrolstad, 1976). A useful index for relative amount of polymeric color in these pigments can be determined from their resistance to bleaching by bisulfite. This is expressed as "% polymeric color". In general, the % polymeric color in all of the samples analyzed were close to 50%, indicating that the samples had undergone
considerable polymerization. The exceptions are Welch's Grape Color Extract and all of the cabbage colorants which are very low in % polymeric color. It has been suggested that acylated anthocyanins are more stable during storage than non-acylated anthocyanins (Sapers, et al., 1981). The data in Table 3.1 tends to support this hypothesis as grape and cabbage are the only samples analyzed that contain acylated anthocyanins.

Tinctoral Strength is commonly used to express coloring power and is often defined as the Absorbance (Optical Density) at the visible wavelength of maximum absorbance (Clydesdale, 1978). The color of anthocyanin solutions is pH dependent, the pigments exhibiting their maximum color intensity at about pH 1 and being nearly colorless at pH values of about 4.5, (Skrede, 1985). Listed in Table 3.1 are the tinctoral strength values for the samples in both water and in pH 1 buffer. In every case, the tinctoral strength is higher in pH 1 buffer than in distilled water. Comparison of the tinctoral strength data with the monomeric anthocyanin data shows that there is a direct relationship between monomeric anthocyanin and tinctoral strength at pH 1. Samples with high pigment concentrations exhibiting high tinctoral strength values. Such a relationship does not hold for the monomeric anthocyanin data when it is compared with the tinctoral strength values for the pigments in distilled water. This is not surprising, as the range in pH for the samples varied from 3.18 to 4.35 (Table 3.1). All of the samples showed at least a 2 fold increase in tinctoral strength when the pH 1 buffer was substituted for water except for the "Obi-Pektin" elderberry sample which exhibited only a small increase. This is most likely due to the presence of large quantities of non-anthocyanin pigments present. The low degree of spectral
responsiveness to changes in pH in this sample would also indicate that the anthocyanins may have undergone significant alteration during processing or storage (Wallin and Smith, 1977). This is also reflected in the high % polymeric color value measured for the elderberry sample.

It should be emphasized that although tinctoral strength is commonly used to specify coloring power, it is not necessarily the most accurate measurement of coloring power. It has been noted that there can be a poor correlation between optical density and visual rank. This is especially true when comparisons are being made between different pigments (Clydesdale, 1978). The major reason is that Optical Density (Absorbance) measurements at a single wavelength does not consider band width, as visual perception is analogous to the total area encompassed by the spectral curve (Clydesdale, 1978); hence absorbance at a single wavelength will not necessarily reflect color intensity as visually observed.

Tristimulus colorimetry is perhaps the best method for estimating visual color. For cranberry juice cocktail, Hunter "L" and "a" values are highly correlated to anthocyanin concentration while Hunter "b" and Hunter "hue" show poor correlation to anthocyanin concentration as these functions are affected by browning components (Johnson, et al., 1976). Two sets of Hunter tristimulus transmittance data are included in Table 3.1. The first set shows the values for a 1% solution of the pigments in distilled water. At these concentrations, the Hunter data is of limited usefulness as the luminosity (L) values fall within the "area of confusion", a region where changes in pigment concentration fail to correlate with changes in instrumental readings (Eagerman, et al., 1973; Clydesdale, 1978).
The second set of Hunter data compares the tristimulus values for the pigment solutions at constant absorbance (A visible max = 1) at pH 3.5 (Sapers, et al., 1981). The values for Red FD&C #40 are included as a point of reference for comparison. All the samples exhibited lower L values than for Red FD&C #40, indicating that all of the colorants were darker than Red FD&C #40 (Skrede, et al., 1983; Sapers, et al., 1981). There was a large variation in the Hue angle between the samples. When compared to Red FDC #40, the "Obi-Pektin" elderberry, blackcurrant, two of the cherry and the plum sample showed higher Hue angles indicating that these samples produced a more orange shade of red. The remaining samples had lower hue angles, especially the cabbage samples, indicating a less orange (more purple) shade of red. In terms of total color difference (ΔE), the blackberry concentrate, the plum concentrate and the cherry samples were the closest in color to Red FD&C #40 while the cabbage colorant samples were the most dissimilar in color to Red FD&C #40.

Titratable acidity (TA) and pH are also listed as they are both influential factors in determining the color of anthocyanin solutions. Titratable acidity and pH values were similar for all of the samples except for black currant, plum, morello cherry 348 and the Spreda Grape Skin Extract (GSE) samples which were all high in titratable acidity, exceeding 7.0 g of anhydrous citric acid per 100 g.
The anthocyanins of the blackcurrant (*Ribes nigrum* L.) have been well characterized. Early work by Chandler and Harper (1962) identified the pigments of blackcurrant to be cyanidin and delphinidin 3-glucosides and 3-rutinosides. Later workers using thin-layer chromatography (Morton, 1968) and droplet counter-current chromatography (DCCC), (Francis and Andersen, 1984) reported that the anthocyanins are present in the following relative amounts, delphinidin-3-rutinoside > cyanidin-3-rutinoside > delphinidin-3-glucoside > cyanidin-3-glucoside. Table 3.2 shows that cyanidin and delphinidin are the only anthocyanidins present in the blackcurrant sample analyzed. Figure 3.1 shows the HPLC chromatogram of the blackcurrant sample along with the relative peak areas. Spectral data of the individual anthocyanins are included in Table 3.3. Both the anthocyanidin and the anthocyanin profile is consistent with the results of other workers as discussed above.

Peak identification of the anthocyanins was achieved as follows: Cyanidin-3-glucoside and delphinidin-3-glucoside were identified by comparison of retention times and spectral data (Table 3.3) with cyanidin and delphinidin 3-glucoside from roselle (Hong and Wrolstad, 1988). Peak identification for cyanidin-3-rutinoside was assigned on the basis of 1) comparison with cyanidin-3-rutinoside isolated by preparative paper chromatography from cherry, and 2) comparison with cyanidin-3-rutinoside from red raspberry (Spanos and Wrolstad, 1987). The major anthocyanin in blackcurrant is delphinidin-3-rutinoside (Morton, 1968; Francis and
Andersen, 1984). This anthocyanin was assigned to the peak with the largest area, (peak 1-b). Additional support for this assignment is that 1) the visible $\lambda_{\text{max}}$ and $E_{440}/E_{\lambda_{\text{vis}} \text{max}}$ ratio (Table 3.3) for this peak is consistent for a delphinidin-3-glycoside, (Hong and Wrolstad, 1988) and 2) its retention order is consistent for that of a rutinoside; cyanidin-3-rutinoside elutes later than cyanidin-3-glucoside, similarly, the retention time for this peak is later than for delphinidin-3-glucoside. This elution order has been previously reported in reversed phase HPLC systems by Spanos and Wrolstad (1987). and Sapers, et al. (1986).

**Blackberry**

The anthocyanins of blackberries along with other members (red and black raspberries, boysenberries and loganberries) of the *Rubus* species has been well studied (Jennings and Carmichael, 1980; Torre and Barritt, 1977; Barritt and Torre, 1973; Harborne and Hall, 1964). The anthocyanins in this family are characterized by cyanidin glucosides with pelargonidin glucosides present in some species. Additional glucose, xylose or rhamnose residues may be present in various combinations to give di or triglycosides (Jennings and Carmichael, 1980; Torre and Barritt, 1977). The anthocyanin profile of blackberries is characterized by cyanidin-3-glucoside and in some cases lesser quantities of cyanidin-3-rutinoside (Torre and Barritt, 1977; Barritt and Torre, 1973; Harborne and Hall, 1964). Pelargonidin-3-glucoside has been reported in one species (Sapers et al., 1986). Marion blackberries are unusual in that the major pigment is cyanidin-3-rutinoside (Barritt and Torre, 1973). Recently, Sapers et al., (1986) using HPLC, showed that in addition to cyanidin-3-rutinoside and cyanidin-3-glucoside,
blackberries contained cyanidin-3-xyloside along with several acylated cyanidin derivatives which were tentatively identified as being acylated with a dicarboxylic acid. The anthocyanidin profile (Table 3.2) for blackberry show that cyanidin is the only aglycone present. A HPLC chromatogram along with peak assignments and relative peak areas is given in Figure 3.2. Peak identification was achieved as follows: Peaks 2-c and 2-d correspond to cyanidin-3-glucoside and cyanidin-3-rutinoside respectively on the basis of retention time (compare with blackcurrant) and spectra (Table 3.2). Peak 2-a, matches retention time and spectral characteristics with cyanidin-3-sophoroside, previously identified and characterized from red raspberry and peak 2-b matches the retention time and spectral characteristics of cyanidin-3-glucosylrutinoside also from red raspberry (Spanos and Wrolstad, 1987). Both cyanidin-3-sophoroside and cyanidin-3-glucosylrutinoside have not been previously reported in blackberries. These pigments are however, associated with red raspberries (Harborne and Hall, 1964).

There are several possible explanations for the presence of these unexpected pigments. The first is that the sample analyzed was a contaminated sample, possibly containing fruit of another species of *Rubus* (red raspberry or its clones). Alternatively, it is possible that these pigments are actually present in blackberries but have been previously unidentified. This is less likely as there has been a considerable amount of work in the characterization of blackberry anthocyanins. Further studies with authentic fruit are needed to determine if these pigments are actually present in blackberries.
**Black Raspberry**

Unlike the blackberry, black raspberries (*Rubus occidentalis*, *Rubus leucodermis*) are characterized by the presence of xylose containing pigments. The four anthocyanins of black raspberry are cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-sambubioside and cyanidin-3-xylosylrutinoside (Harborne and Hall, 1964; Nybom, 1968; Barritt and Torre, 1973; Torre and Barritt, 1977). A previous report indicates that the 3-xylosylrutinoside and the 3-rutinoside are generally present in the largest quantities (Torre and Barritt, 1977). Analysis of anthocyanidins in black raspberry (Table 3.2) show that cyanidin is the only aglycone present. The HPLC anthocyanin profile is given in Figure 3.3. Spectral information of the individual peaks is given in Table 3.3. Peak identification was achieved as follows: In order to establish the retention time for cyanidin-3-xylosylrutinoside, black raspberry anthocyanins were separated by preparative paper chromatography in AHW (acetic acid/HCl/water, 15/3/82). Four bands of pigments were found, corresponding to the four anthocyanins previously reported for black raspberry, the darkest bands corresponding to the 3-rutinoside and to the 3-xylosylrutinoside. The band (R_f=0.73) corresponding to cyanidin-3-xylosylrutinoside (Harborne, 1967) was isolated and then chromatographed by HPLC. The resulting chromatogram is shown in Figure 3.4. The retention time is similar to that of cyanidin-3-glucoside, which is later than one might anticipate for a triglycoside. One would normally expect triglycosides to elute sooner than diglycosides which in turn be eluted before monoglycosides. The late elution of cyanidin-3-rutinoside suggests that the hydrophobic methyl group of rhamnose causes increased retention (Spanos and Wrolstad, 1987). Sapers et al. (1986)
suggested that cyanidin-3-xyloside would be more hydrophobic than either cyanidin-3-glucoside or cyanidin-3-rutinoside. Thus, the hydrophobicity of both rhamnose and xylose may account for the late retention of cyanidin-3-xylosylrutinoside.

From a chromatographic standpoint, samples suspected of containing both cyanidin-3-glucoside and cyanidin-3-xylosylrutinoside will need to be separated on an alternate chromatographic system capable of resolving this pair.

The anthocyanin profile for black raspberry is consistent with previous reports for this species. Determination of the proportion of the major pigment was not possible due to co-elution of cyanidin-3-glucoside with cyanidin-3-xylosylrutinoside.

**Elderberry**

The anthocyanin profile of elderberry (Sambucus nigra L.) has been previously characterized by HPLC. The major anthocyanins along with their relative percentages (based on HPLC peak area) are cyanidin-3-glucoside (65.7%) and cyanidin-3-sambubioside (32.4%). The two minor pigments are cyanidin-3-sambubioside-5-glucoside (1.1%) and cyanidin-3-glucoside-5-glucoside (0.8%), (Bronnum-Hansen and Hansen, 1983). Table 3.2 shows that cyanidin is the only aglycone present in both samples of elderberry analyzed. Figure 3.5 shows the anthocyanin profile for elderberry along with the peak area percentage for both of the elderberry samples analyzed. Spectral information of the individual peaks is given in Table 3.3. Peak identification was achieved as follows: Peaks 5-c and 5-d correspond to cyanidin-3-sambubioside and cyanidin-3-glucoside respectively on
the basis of both retention time and spectral characteristics. Peaks 5-a and 5-b elute very closely together. Both the anthocyanidin data (Table 3.2) and the visible wavelength maxima (Table 3.3) indicate that peaks 5-a and 5-b are cyanidin glycosides. Early elution relative to cyanidin-3-glucoside indicates that these peaks are either di or tri glycosides. The $E_{440}/E_{vis}$ ratio of peaks 5-a and 5-b is approximately 40% lower than for any of the other cyanidin-3-glycosides, indicating substitution at the 5 position (Harborne, 1958). From these data and the HPLC retention data of Bronnum-Hansen and Hansen (1983), peaks 5-a and 5-b are tentatively assigned as cyanidin-3-sambubioside-5-glucoside and cyanidin-3-glucoside-5-glucoside respectively.

**Plum**

In a survey of 15 common plum (*Prunus domestica*) varieties, Harborne and Hall (1964) reported the major anthocyanins to be cyanidin-3-glucoside and cyanidin-3-rutinoside accompanied by traces of the related peonidin derivatives. Table 3.2 shows the results from HPLC analysis of the anthocyanidins (aglycones) for plum. The major aglycone is cyanidin with trace amounts of peonidin. Figure 3.6A shows the HPLC chromatogram of the plum anthocyanins while Figure 3.6B shows the HPLC chromatogram of the plum anthocyanins remaining on a reversed phase cartridge after elution with alkaline borate buffer. This treatment tends to concentrate non-acylated anthocyanins without o-diphenolic groups (pelargonidin, peonidin and malvidin) on the cartridge (Hong and Wrolstad, 1988). Table 3.3 lists the spectral characteristics for each of the peaks. Peaks 6-b and 6-c correspond to cyanidin-3-glucoside and cyanidin-3-rutinoside respectively based on previously
established retention time and spectral data (Hong and Wrolstad, 1988). Peak 6-a is present in insufficient quantities for spectral determination. Based on the anthocyanidin data (Table 3.2) and on its retention characteristics (more polar than cyanidin-3-glucoside), it is likely to be either cyanidin-3-galactoside or a cyanidin di- or tri-glycoside. Peak 6-f shows spectral properties indicative of a peonidin derivative and is identical in retention time to that of peonidin-3-glucoside from cranberries (Hong and Wrolstad, 1988). The fact that it is concentrated in the Sep-Pak after treatment with alkaline borate is additional evidence that this is a peonidin glycoside. Peaks 6-d,e,g have spectral characteristics which do not correspond with any of the previously identified anthocyanins. They all are selectively concentrated on the Sep-Pak after elution with alkaline borate buffer. An unknown pigment with similar properties was observed for an anthocyanin from strawberry (Hong and Wrolstad, 1988). Further studies are needed to characterize these pigments.

**Sour Cherry**

The anthocyanin profile of sour cherries (*Prunus cerasus*) is complex (Timberlake and Bridle, 1982). Harborne and Hall (1964) reported cyanidin-3-glucosylrutinoside, cyanidin-3-sophoroside, cyanidin-3-rutinoside and cyanidin-3-glucoside in 6 different varieties of sour cherries; the "Morello A" variety was reported to contain only cyanidin-3-glucoside and cyanidin-3-glucosylrutinoside. Peonidin-3-glucoside and peonidin-3-rutinoside was reported as minor pigments in this same variety (Dekazos, 1970). Later work by Shrikhande and Francis (1973) showed that cyanidin-3-xylosylrutinoside was a minor component in the
Montmorency variety of sour cherries. Previous reports of cyanidin-3-gentiobioside in sour cherries are incorrect (Du et al., 1975).

Figure 3.7 shows the anthocyanin profile of Morello cherry, all three of the samples showed similar profiles. The results of anthocyanidin analysis for Morello cherry (Table 3.2) show that cyanidin is the only aglycone present. The spectral data for the anthocyanins in Figure 3.7 are shown in Table 3.3. Peak identification was achieved as follows: Peaks 7-c and 7-d correspond to cyanidin-3-glucoside and cyanidin-3-rutinoside, respectively, matching previously established retention time and spectral characteristics. Peak 7-a is identified as cyanidin-3-sophoroside and peak 7-b is identified as cyanidin-3-glucosylrutinoside, these assignments based on comparison with previously established retention times and spectral characteristics (Hong and Wrolstad, 1988) and production of a single symmetrical peak when spiked with the same pigment from red raspberries. A number of trace pigments are present in the chromatogram but were present in such small quantities that spectra could not be taken.

**Bilberry**

The bilberry or whortleberry (Vaccinium myrtillus) is a native of parts of Europe and northern Asia (Timberlake and Bridle, 1982). Suomalainen and Keranen (1961) reported that this species contains cyanidin, delphinidin, petunidin and malvidin glucosides and arabinosides. Later work by Baj, et al. (1983) using both HPLC and Gas Liquid Chromatography (GLC) found all possible combinations of cyanidin, delphinidin, petunidin, peonidin and malvidin 3-galactosides, 3-glucosides and 3-arabinosides in bilberry. Quantitatively, the delphinidin glycosides were
present in the largest quantities and the peonidin glycosides were the least abundant. Since the bilberry is closely related to the lowbush blueberry (*Vaccinium angustifolium*), it is likely that their anthocyanin patterns are similar (Timberlake and Bridle, 1982). It is appropriate then to include a brief discussion of the anthocyanins of the various blueberry species. Francis, et al. (1966), found that the ripe fruits of the lowbush blueberry contained all possible combinations of delphinidin, malvidin, petunidin, peonidin and cyanidin 3-galactosides, 3-glucosides and 3-arabinosides. The glucosides were found to be present in much greater quantity than the galactosides. Only trace quantities of the arabinosides were found. Diglycosides were also detected but not identified. A review of the anthocyanins of another related species, the highbush blueberry (*Vaccinium corymbosum*) shows that this species contains the same anthocyanins as the lowbush blueberry but in different proportions (Timberlake and Bridle, 1982). Recently, Sapers, et al. (1984) in a study of lowbush blueberry, separated as many as 16 anthocyanins by HPLC. Three distinct anthocyanin patterns were found for the 11 different cultivars studied. The anthocyanins of a related species, the Bog Whortleberry (*Vaccinium uliginosum L.*) was also found to contain all possible combinations of the previously mentioned pigments, with malvidin-3-glucoside as the predominant pigment (Andersen, 1987).

Figure 3.8A shows the anthocyanin profile for the bilberry colorant sample while Figure 3.8B shows the fraction of bilberry anthocyanins that remain on the Sep-Pak after elution with alkaline borate buffer. Table 3.3 lists the spectral characteristics for the individual peaks. Anthocyanidin analysis (Table 3.2) shows that all of the common aglycones except for pelargonidin are present in this sample.
Previous work with cranberries show that the 3-galactoside elutes earlier than the 3-glucoside which in turn elutes earlier than the 3-arabinoside when attached to the same aglycone (Sapers and Hargrave, 1987; Hicks, et al., 1985; Hale, et al., 1986; Andersen, 1987; Durst and Wrolstad, 1988; Hong and Wrolstad, 1988).

Referring to the spectral data for bilberry (Table 3.3), peaks 8-d, 8-f and 8-j exhibit spectral characteristics typical of cyanidin or peonidin 3-glycosides. The retention time for peak 8-d matches that of cyanidin-3-galactoside from cranberry and peak 9f for cyanidin-3-glucoside. Peak 9-j has a retention time which corresponds to peonidin-3-glucoside, also from cranberry (Hong and Wrolstad, 1988). Of these peaks, only 8-j remains in the cartridge after treatment with borate buffer, confirming that it is a peonidin glycoside. The remaining bilberry peaks listed in Table 3.3 all show spectra characteristic of delphinidin, petunidin and malvidin 3-glycosides. Peak 8-c corresponds in retention time to delphinidin-3-glucoside (see blackcurrant). Since the galactoside elutes earlier than the glucoside, peak 8-b is likely to be delphinidin-3-galactoside. The earliest eluting peak (peak 8-a) was present in quantities too low for spectral determination. It does appear to remain on the Sep-Pak after elution with borate buffer, hence it may not be a delphinidin or cyanidin derivative as its early retention time indicates. Peak 8-e elutes just before cyanidin-3-glucoside and is identified as delphinidin-3-arabinoside as previous workers have shown that delphinidin-3-arabinoside elutes before cyanidin-3-glucoside (Baj, et al., 1983; Andersen, 1987). Peak 8-h elutes after cyanidin-3-glucoside (8-f) and before peonidin-3-glucoside (8-j) and is therefore most likely to be petunidin-3-glucoside. Peak 8-I has the same retention time as malvidin-3-glucoside from grapes. Relative to peak 8-I, the later eluting
peak 8-m is therefore assigned as malvidin-3-arabinoside. Both peaks 8-l and 8-m
are concentrated on the Sep-Pak after elution with borate buffer which supports
their identity as malvidin derivatives. The remaining peaks are present in
quantities too low for spectral characterization.

Grape

Pigment from wine grape skins (Vitis vinifera) has been available
commercially for some time (Francis, 1975). The anthocyanin composition of
grapes have been well studied. So specific are the pigment profiles of grapes that
differentiation of the various species of Vitis are possible through the study of the
anthocyanin composition (Ribereau-Gayon, 1982). The anthocyanins of the vinifera
species are based largely on malvidin, to lesser extents on delphinidin, petunidin and
peonidin and only to a small extent on cyanidin. They are found not only as the 3-
glucosides, but also as 3-glucosides acylated with acetic acid, p-coumaric acid and
caffeic acid (Bakker and Timberlake, 1985a; Wulf and Nagel, 1978). Numerous
works have been published involving the characterization of grape anthocyanins and
the specific results of those studies have been summarized by Ribereau-Gayon
(1982). A brief summary of the conclusions by the same author follows:

1) Malvidin-3-glucoside is the major pigment in Vitis vinifera.

2) Anthocyanidin diglucosides do not occur in Vitis vinifera but do occur in
hybrids and in other species.

3) Free Anthocyanidins are not found.

4) Cyanidin and peonidin derivatives are systematically found in all species
but are abundant in only a few.
5) The absence of acylated anthocyanins is characteristic of Pinot variety of *Vitis vinifera*.

Figure 3.9A shows the HPLC chromatogram for Spreda GSE. Figure 3.9B shows the HPLC chromatogram of the fraction of GSE pigments that remain on a Sep-Pak after elution with borate buffer while Figure 3.9C shows the chromatogram of GSE after mild alkaline hydrolysis. Spectral data for the major peaks are given in Table 3.3. The anthocyanidin analysis (Table 3.2) shows that this sample contains all of the common anthocyanidin aglycones except for pelargonidin. Malvidin and delphinidin are the major anthocyanidins present. Peak identification was achieved as follows: The spectral data for peaks 9-c, 9-f and 9-i (Table 3.3) show spectral properties indicative of cyanidin or peonidin 3-glycosides. Peak 9-c corresponds in retention time to cyanidin-3-glucoside and peak 9-f corresponds in retention time to peonidin-3-glucoside. The fact that 9-f remains on the Sep-Pak after elution with borate buffer confirms that it is a peonidin glycoside. The remaining peaks that were spectrally characterized show spectral properties typical of a delphinidin derivative. Peak 9-a corresponds in retention time to delphinidin-3-glucoside, peak 9-d to petunidin-3-glucoside and peak 9-g to malvidin-3-glucoside. Peaks 9-b and 9-e were present in quantities too low for spectra to be taken and remain unidentified. Peak 9-k exhibits a spectral profile typical of a 3-glycoside of a delphinidin derivative. It is removed with alkaline hydrolysis (Figure 3.9C), and is therefore an acylated pigment. Its late retention time (after malvidin-3-glucoside) is consistent with acylation with either a cinnamic acid or acetic acid. Since no absorbance maxima in the 310 nm range was found for this peak, acylation with acetic acid is probable. Peak 9-k is
therefore assigned the tentative assignment of malvidin-3-glucoside acetate as previous workers have reported that this anthocyanin is a predominant pigment in Vitis vinifera (Wulf and Nagel, 1978; Bakker and Timberlake, 1985b). Peaks 9-h, 9-j and 9-I were present in quantities too low for spectral characterization. However, since they do elute after malvidin-3-glucoside and before malvidin-3-glucoside acetate, it is likely that these peaks correspond to the 3-glucoside acetates of delphinidin, cyanidin, petunidin and peonidin, respectively. Peak 9-o exhibits a wavelength maxima higher than observed for any of the anthocyanins analyzed with this system. The presence of an absorbance maxima in the 310 nM region is indicative of acylation with a hydroxy aromatic moiety. The Eacyl/Evis ratio of 68 shows that acylation is in a 1:1 molar ratio. Since peak 9-o is present in relatively large amounts, it is tentatively identified as malvidin-3-p-coumarylglucoside as previous workers have reported this as a major pigment in Vitis vinifera (Bakker and Timberlake, 1985a,b; Wulf and Nagel, 1978). Peaks 9-I, 9-m and 9-n are present in insufficient quantities for determination of spectral properties. From their position in the chromatogram, it is likely that they are the 3-p-coumarylglucosides of delphinidin, cyanidin, petunidin and/or peonidin.

Figure 3.9B shows a chromatogram of those anthocyanins that remain on the Sep-Pak after elution with alkaline borate buffer. Traces of peak 9-d (petunidin-3-glucoside) remain. When compared to the untreated chromatogram (Figure 3.9A) however, it can be seen that the amount of this pigment has been greatly reduced, thus characterizing this as an o-diphenolic anthocyanin. Peak 9-g is also highly concentrated which is expected for malvidin-3-glucoside. All of the peaks eluting later than peak 9-g are also concentrated on the Sep-Pak after elution with borate
and support the evidence that they may be acylated. The disappearance of all of these peaks from the chromatogram except for peak 9-i after alkaline hydrolysis (Figure 3.9C) confirms that these peaks are acylated.

Peak 9-i is unusual in that exhibits spectral properties indicative of a cyanidin derivative but it elutes very late. It is not affected by alkaline hydrolysis which indicates that it is not acylated.

The other sample of grape colorant, "Welch's Grape Color Extract" is specified by the manufacturer to be "A natural color pigment extracted from Concord Grape (Vitis labrusca var. Concord) using citric acid". A review specific for the anthocyanins for the Concord variety of Vitis labrusca show that the major pigment is delphinidin-3-glucoside followed by (listed in descending order by relative quantity) the 3-glucosides of cyanidin, petunidin, malvidin and peonidin. Acylation with p-coumaric acid and 3,5-diglucosides with cyanidin and delphinidin have also been reported (Wrolstad, 1976; Ingalsbe, et al., 1963; Ohta, et al., 1979). Figure 3.10A shows a HPLC chromatogram of Welch's Grape Color Extract while Table 3.3 lists the spectral data for the individual peaks. The anthocyanidin profile (Table 3.2) for this sample show that delphinidin and cyanidin are the major aglycones. Peaks 10-c,10-d,10e,10-f and 10-g correspond to the 3-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, respectively. The spectral data for peak 10-a show that it is 1) a delphinidin derivative and 2) that it contains a 3,5-glycosidic substitution pattern evident from the low E440/Evis ratio. Similarly, peak 10-b is a 3,5 substituted cyanidin derivative. Taking into consideration the early retention of these peaks and the results of previous workers (see above), peaks 10-a and 10-b are likely to be delphinidin-3,5,-
diglucoside and cyanidin-3,5-diglucoside respectively. Peaks 10-h, 10-i and 10-j all exhibit E440/Evis ratios indicative of 3-glycosides. They disappear after alkaline hydrolysis (Figure 3.10C) indicating that they are acylated. They exhibit no absorption maxima in the 310 nM range indicating that acylation is not with a cinnamic acid. Their retention characteristics is similar to that of the acetic acylated anthocyanins for GSE. Based on their \( \lambda_{\text{vis}} \) max, peak 10-h and 10-j are likely to be acetic acid acylated delphinidin derivatives whereas peak 10-i is an acetic acid acylated cyanidin derivative. The remaining peaks (10-k to 10-r) are all acylated in a 1:1 molar ratio with a hydroxy aromatic acid (Eacyl/Evis ratio = 50-70%), most likely being p-coumaric acid, the commonly reported acylating acid for Concord Grape. For most of the peaks, the acyl absorption band was wide and exhibited more of a shoulder in the 300-315 nM region rather than a distinct wavelength maxima. Similar observations for the spectra of anthocyanidin-3-glucoside-p-coumarates has been made by Wulf and Nagel, (1978). The E440/Evis ratio for peaks 10-k, 10-l, 10-m and 10-o show that in addition to acylation, they all are 3,5-substituted. The visible wavelength maxima for these acylated pigments is higher than for the non-acylated counterparts. However, if we assume that the delphinidin derivatives still exhibit a higher wavelength maxima than for the cyanidin derivatives, then the spectra for peaks 10-k, 10-m and 10-o indicate that they are delphinidin derivatives whereas peak 10-l is a cyanidin derivative. Taking into consideration the spectral data for these peaks and their HPLC retention order, it is likely that peaks 10-k, 10-l, 10-m and 10-o are the p-coumaric acid acylated derivatives of the 3,5-diglucosides of delphinidin, cyanidin, petunidin and malvidin, respectively. The presence of anthocyanin-3,5-diglucosides acylated
with p-coumaric acid has been reported by Ingalsbe, et al., (1963) and Ohta, et al., (1979).

Spectral data for the remaining peaks (10-o, 10-p, 10-q and 10-r) show that they are most likely to be the p-coumaric acid acylated derivatives of anthocyanin 3-glycosides. In a similar fashion, taking into consideration the visible wavelength maxima, previously discussed spectral data and the HPLC retention order, peaks 10-n, 10-p, 10-q and 10-r are tentatively identified as the p-coumaric acid acylated derivatives of the 3-glucosides of delphinidin, cyanidin, petunidin and malvidin, respectively. Again, the presence of anthocyanidin-3-glucosides acylated with p-coumaric acid has been reported (Ingalsbe, et al., 1963; Ohta, et al., 1979).

Figure 3.10B shows the HPLC chromatogram of the fraction of Concord anthocyanins that remain on the Sep-Pak after elution with alkaline borate buffer. Peaks 10-b to 10-e are significantly reduced indicating cyanidin, delphinidin or petunidin glycosides. The disappearance of all of the peaks eluting later than peak 10-g after alkaline hydrolysis (Figure 3.10C) confirms that these pigments are acylated.

**Red Cabbage**

The usefulness of anthocyanin pigment preparations from red cabbage (*Brassica oleracea*) has been discussed in detail (Sapers, et al., 1981; Shewfelt and Ahmed, 1977, 1978). Red cabbage, in common with other Cruciferous plants, has a very sophisticated pattern of acylated anthocyanins. This complexity is reflected in the many differing reports regarding the exact composition of the red cabbage
anthocyanins (Timberlake and Bridle, 1982). The definitive work for red cabbage anthocyanin composition is by Hrazdina, et al. (1977). What follows is a summary of their findings: Eight anthocyanins were isolated and characterized. They were all identified as derivatives of cyanidin-3-sophoroside-5-glucoside. In addition to cyanidin-3-sophoroside-5-glucoside, acylation was found at the position 3 sugars by the following groups: malonic acid, p-coumaric acid in both 1 and 2 mol ratios, ferulic acid in both 1 and 2 mol ratios and sinapic acid in both 1 and 2 mol ratios. In the case of acylation with more than 1 acid, mixed acylation (where 2 different acylating acids occur in the same anthocyanin) does not occur (Hrazdina, et al., 1977). Recently, new acylated forms of the red cabbage pigments have been characterized, including the discovery of acylation with glucose derivatives of ferulic and p-coumaric acid to the sophoroside sugar moiety (Ideka, et al., 1987; Itaka, 1986).

Shown in Figure 3.11A is a typical chromatogram of the anthocyanins in either the San Red RCF or San Red RC powder red cabbage samples. The spectral properties of the individual peaks are listed in Table 3.3. The adsorption of the pigments onto PVPP was omitted in the sample preparation steps (Hong and Wrolstad, 1988) as these pigments did not adsorb well to the PVPP, resulting in a severe loss of pigments. The anthocyanidin profile (Table 3.2) for red cabbage shows that cyanidin is the only aglycone present. Several early eluting peaks were present in the anthocyanidin chromatogram, most probably the result of incomplete acid hydrolysis. The early eluting peaks remained in the chromatogram even after the red cabbage anthocyanins were subjected to acid hydrolysis for twice the
normal hydrolysis time. This attests to the high stability of acylated anthocyanins (Sapers, et al., 1981).

Peaks 11-a to 11-m exhibit E440/Evis ratios which are indicative of 3,5 glycosidation. Peak 11-a is the only peak that shows no acylation by an aromatic organic acid. Its very early elution time indicates that it may be either cyanidin-3-sophoroside-5-glucoside or cyanidin-3-malonylsophoroside-5-glucoside as reported by Hrazdina, et al., (1977). Peaks 11-b, 11-c, 11-d, 11-g and 11-h show Eacyl/Evis ratios in the 50-70% range, indicating acylation with 1 mol of the acylating acid. The nature of the acylating hydroxy aromatic acid can be determined by the absorption spectra in the 300-340 nM range. Ayclated anthocyanins containing sinapic and ferulic acid both have UV acyl absorption maxima (in acidified methanol) in the 330 nM range whereas anthocyanins acylated with p-coumaric acid exhibit a lower UV maxima at 310 nM. (Hrazdina, 1977). All of the spectral information indicate that peaks 11-b, 11-d, and 11-h are derivatives of cyanidin-3-sophoroside-5-glucoside monacylated with either sinapic or ferulic acid. Similarly, peaks 11-c and 11-g are derivatives of cyanidin-3-sophoroside-5-glucoside monacylated with p-coumaric acid.

Peaks 11-e, 11-f, 11-i, 11-j, 11-k, 11-l and 11-m have Eacyl/Evis ratios of 90-120% indicating acylation with 2 mol of the acylating acid. The acyl UV maxima for peak 11-e shows that it is likely to be a di(p-coumaric acid) acylated derivative of cyanidin-3-sophoroside-5-glucoside. Similarly, higher acyl wavelength maxima for peaks 11-f, 11-i, 11-j, 11-k, 11-l and 11-m shows that these peaks are likely to be di(sinapic acid) or di(ferulic acid) derivatives of cyanidin-3-sophoroside-5-glucoside.
The latest eluting peak (11-n) exhibit E440/Evis ratios indicative of a 3-glycoside. The $E_{acyl}/E_{vis}$ ratio show that the peak is di-acylated. The acyl wavelength maxima indicates that acylation is likely to be with either sinapic acid or ferulic acid. From this information, the late retention time for this peak is not unexpected. The presence of acylated derivatives of cyanidin-3-sophoroside-5-glucoside is well established in red cabbage (Timberlake and Bridle, 1982) and there are no reports of acylated 3-glycosides. One possibility is that this peak is an acylated derivative of cyanidin-3-sophoroside arising from hydrolysis of the cabbage anthocyanins.

All of the anthocyanins in red cabbage were found to be cyanidin (Table 3.2) which agrees with previous findings. Inspection of the visible wavelength maxima of the red cabbage anthocyanins show that the visible wavelength maxima of cyanidin-3-sophoroside-5-glucoside is 515 nM, a figure consistent with our previous observations of cyanidin glycosides. The acylated pigments however, tended to have a much higher wavelength maxima, even higher than for the delphinidin derivatives. Previous studies with anthocyanins in acidified methanol showed only small (1-5 nM) differences in the visible wavelength maxima between acylated and non-acylated pigments for the same aglycone (Hrazdina, et al., 1977; Wulf and Nagel, 1978). These results show that in a predominantly aqueous HPLC solvent, the visible wavelength maxima for acylated cyanidin glycosides is very different from that of non-acylated cyanidin glycosides. The difference in some cases by as much as 20 nM. For example, peak 11-b, identified as a mono-acylated derivative of cyanidin-3-sophoroside-5-glucoside, has a similar retention time to that of cyanidin-3-glucoside, yet the wavelength maxima for this peak is 529 nM,
14 nM higher than for cyanidin-3-glucoside. From these observations, it appears that acylation (with hydroxy aromatic acids) of the anthocyanins produces a shift to a higher visible wavelength maxima when spectra are measured in the aqueous HPLC solvent used. Additional support for this hypothesis is that anthocyanins which are diacylated have a higher visible wavelength maxima than for the monacylated anthocyanins. This could account for the large difference in Hunter values for the red cabbage samples when compared to the other colorants examined. In light of these findings, when acylation with cinnamic acids is suspected, the $\lambda_{\text{vis max}}$ will not be an accurate indicator of the nature of the aglycone. Further studies are needed to establish more precisely the effect of acylation with cinnamic acids on the spectra of anthocyanins in aqueous solvent systems.

Figure 3.11B shows the HPLC anthocyanin profile of red cabbage anthocyanins after alkaline hydrolysis. The disappearance of all of the peaks except for 11-a and 11-z confirms that those peaks are acylated.

In regard to the anthocyanin profile of the 3 different samples of red cabbage colorants, the San Red RC powder and the San Red RCF samples were found to have very similar pigment profiles. The San Red RC liquid sample (Figure 3.11C) was different from the other two samples in that an extra early eluting peak (11-z) was present and that the major pigment was 11-1. Peak 11-z exhibits spectral characteristics corresponding to that of a cyanidin derivative.

Conclusions

In this paper, we demonstrate the utility of HPLC/photodiode array detection as a valuable tool for characterization of anthocyanins. The spectral
characteristics of the anthocyanins yield useful information in regard to the nature of the aglycone and the sugar substitution pattern while the retention characteristics on reversed phase HPLC yields information as to the nature of the sugar moieties. The behavior of the anthocyanins after alkaline hydrolysis and after selective elution from a reversed phase cartridge with borate buffer are additional indices useful for characterizing these pigments. These methods are particularly useful for assignment of peak identities in materials for which the anthocyanins have been previously identified. The information presented provides color indices for a variety of potentially useful colorants along with analytical information which can be used to identify the presence of these colorants.
Table 3.1  Comparison of General Colorant Properties for Commercial Colorants and Juice Concentrates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wavelength Maxima (nm) (In pH 1 buffer)</th>
<th>pH of 1% Solution</th>
<th>Titratable Acidity (a)</th>
<th>Anthocyanin Conc. (b) (mg/l for a 1% solution)</th>
<th>Degradation Index</th>
<th>Color Density</th>
<th>Polym eric Color</th>
<th>% Tannins</th>
<th>Browning Index</th>
<th>Tinctoral Strength (Abs. of 1% solution)</th>
<th>Tinctoral Strength at pH 1 (Abs. of 1% solution)</th>
<th>Hunter Parameters (1% solution)</th>
<th>Hunter Parameters (Constant Abs) (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Red FDC #40</td>
<td>San-El</td>
<td>Elderberry</td>
<td>Obi-Pektin</td>
<td>Elderberry</td>
<td>Obi-Pektin</td>
<td>Blackcurrent</td>
<td>Blackberry</td>
<td>Cone.</td>
<td>Spreda</td>
<td>OSE</td>
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<tr>
<td>Wave length Maxima (nm) (In pH 1 buffer)</td>
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<td>518</td>
<td>514</td>
<td>517</td>
<td>513</td>
<td>523</td>
<td></td>
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<tr>
<td>pH of 1% Solution</td>
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<td>4.4</td>
<td>3.2</td>
<td>3.3</td>
<td>3.0</td>
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<td>Titratable Acidity (a)</td>
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<td>6.3</td>
<td>20.4</td>
<td>10.6</td>
<td>11.8</td>
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<tr>
<td>Anthocyanin Conc. (b) (mg/l for a 1% solution)</td>
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<td>29.5</td>
<td>27.7</td>
<td>37.7</td>
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<td>Polym eric Color</td>
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<td>0.9</td>
<td>0.7</td>
<td>3.2</td>
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<td>35%</td>
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<tr>
<td>Browning Index</td>
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<td>3.1</td>
<td>0.6</td>
<td>0.5</td>
<td>1.5</td>
<td></td>
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<tr>
<td>Tinctoral Strength (Abs. of 1% solution)</td>
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<td>2.7</td>
<td>1.0</td>
<td>0.6</td>
<td>5.8</td>
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<tr>
<td>Hunter Parameters (1% solution)</td>
<td>L</td>
<td>x</td>
<td>18</td>
<td>29</td>
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<td>40</td>
<td>x</td>
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<tr>
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<td>Hue (degrees)</td>
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<td>Sat'n Index</td>
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<td>Hue (degrees)</td>
<td>22</td>
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<tr>
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<td>Total Color Difference (e)</td>
<td>-</td>
<td>22</td>
<td>26</td>
<td>19</td>
<td>15</td>
<td>26</td>
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</tr>
</tbody>
</table>

**Notes:**

- *a* = calculated as anhydrous citric acid per 100 g of sample
- *b* = anthocyanin concentration calculated as cyanidin-3-glucoside, pH differential method used
- *c* = anthocyanin concentration calculation as cyanidin-3-glucoside, single pH method used
- *d* = Hunter values of the sample at A vis max = 1, pH 3.5
- *e* = Total Color Difference when compared with Red FD&C #40
- *x* = Not determined, 1% solution is opaque
Table 3.1 (continued)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spada GSE</th>
<th>Welch's Grape</th>
<th>Obl-Pektin</th>
<th>Obl-Pektin</th>
<th>Obl-Pektin</th>
<th>Obl-Pektin</th>
<th>Bilberry</th>
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<tbody>
<tr>
<td></td>
<td>nM (in pH 1 buffer)</td>
<td></td>
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<td>Wavelength Maxima</td>
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<td>515</td>
<td>516</td>
<td>521</td>
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<td>3.6</td>
<td>3.3</td>
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<tr>
<td>Titratable Acidity (a)</td>
<td>7.1</td>
<td>4.7</td>
<td>7.1</td>
<td>3.8</td>
<td>4.4</td>
<td>4</td>
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<tr>
<td>Monomeric Anthocyanin Conc. (b) (mg/l for a 1% solution)</td>
<td>226.4</td>
<td>48.7</td>
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<td>2.9</td>
<td>4.8</td>
<td>18.3</td>
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<td>6.9</td>
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<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
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<td>16%</td>
<td>45%</td>
<td>50%</td>
<td>35%</td>
<td>41%</td>
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<tr>
<td>Browning Index</td>
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<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
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<td>3.3</td>
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<td>Tinctorial Strength at pH 1 (Abs. of 1% solution)</td>
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<td>0.5</td>
<td>0.4</td>
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</tr>
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<td>8</td>
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<td>Sat'n Index x</td>
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<td>Hunter Parameters (Constant Abs) (d)</td>
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<td>18</td>
<td>19</td>
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<td>6</td>
<td></td>
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<tr>
<td>Hue (degrees)</td>
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<td>6</td>
<td>24</td>
<td>26</td>
<td>18</td>
<td>8</td>
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<tr>
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<td>45</td>
<td>43</td>
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<td>45</td>
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<tr>
<td>Total Color Difference (e)</td>
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<td>16</td>
<td>20</td>
<td>14</td>
<td>23</td>
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</table>
Table 3.1 (continued)

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<thead>
<tr>
<th>Sample</th>
<th>Plum Concentrate</th>
<th>San Red</th>
<th>San Red RC IIq</th>
<th>San Red RC powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength Maxima (nm)</td>
<td>512</td>
<td>528</td>
<td>531</td>
<td>529</td>
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<tr>
<td>(in pH 1 buffer)</td>
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<tr>
<td>pH of 1% Solution</td>
<td>3.5</td>
<td>3.2</td>
<td>3.2</td>
<td>3.3</td>
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<td>Titratable Acidity (a)</td>
<td>5.6</td>
<td>2.7</td>
<td>2.9</td>
<td>3.8</td>
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<tr>
<td>Monomeric Anthocyanin Conc. (b)</td>
<td>3.7</td>
<td>100.7</td>
<td>95.5</td>
<td>244.9</td>
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<td>(mg/l for a 1% solution)</td>
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<td>136.7</td>
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<td>366.9</td>
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<td>(mg/l for a 1% solution)</td>
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<td>Degradation Index</td>
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<td>1.7</td>
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<td>Color Density</td>
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<td>Polymeric Color</td>
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<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>% Tannins</td>
<td>48%</td>
<td>13%</td>
<td>12%</td>
<td>9%</td>
</tr>
<tr>
<td>Browning Index</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
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<tr>
<td>Tinctorial Strength (Abs. of 1% solution)</td>
<td>0.1</td>
<td>2.4</td>
<td>4.2</td>
<td>6.8</td>
</tr>
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<td>Tinctorial Strength at pH 1 (Abs. of 1% solution)</td>
<td>0.4</td>
<td>9.1</td>
<td>10.6</td>
<td>24.4</td>
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<td>Hunter Parameters (1% solution)</td>
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<td>41</td>
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<td>b</td>
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<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Hue (degrees)</td>
<td>34</td>
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<td>15</td>
<td>16</td>
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<tr>
<td>Sat'n Index</td>
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<td>61</td>
<td>49</td>
<td>43</td>
</tr>
<tr>
<td>Hunter Parameters (Constant Abs.) (d)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>62</td>
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<td>-16</td>
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<td>Hue (degrees)</td>
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<td>64</td>
<td>66</td>
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<td>Total Color Difference (e)</td>
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<td>41</td>
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</table>
Table 3.2 HPLC Anthocyanidin Profile of Commercial Colorants and Juice Concentrates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Delphinidin</th>
<th>Cyanidin</th>
<th>Petunidin</th>
<th>Pelargonidin</th>
<th>Peonidin</th>
<th>Malvidin</th>
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</thead>
<tbody>
<tr>
<td>Elderberry *</td>
<td>NF</td>
<td>100%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Blackcurrant</td>
<td>47%</td>
<td>53%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Blackberry</td>
<td>NF</td>
<td>100%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Blackraspberry</td>
<td>NF</td>
<td>100%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Obi-Pektln Morello Cherry *</td>
<td>NF</td>
<td>100%</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Plum Concentrate</td>
<td>NF</td>
<td>96%</td>
<td>NF</td>
<td>NF</td>
<td>4%</td>
<td>NF</td>
</tr>
<tr>
<td>Obi-Pektln Bilberry</td>
<td>31%</td>
<td>24%</td>
<td>19%</td>
<td>NF</td>
<td>8%</td>
<td>18%</td>
</tr>
<tr>
<td>Spreda GSE *</td>
<td>32%</td>
<td>15%</td>
<td>18%</td>
<td>NF</td>
<td>5%</td>
<td>30%</td>
</tr>
<tr>
<td>Welch's Grape Color Extract</td>
<td>49%</td>
<td>28%</td>
<td>12%</td>
<td>NF</td>
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<td>7%</td>
</tr>
<tr>
<td>San Red Cabbage Colorants *</td>
<td>NF</td>
<td>100%</td>
<td>NF</td>
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NF = not found

* Summary of results for all similar samples

(a) = Incomplete hydrolysis
Table 3.3 Spectral Characteristics of Commercial Colorants and Juice Concentrates as Determined by HPLC/Photodiode Array Detection

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<th>Peak #</th>
<th>VISIDE Wavelength Maxima (nm)</th>
<th>E440/Evils Ratio (%)</th>
<th>Acyl Wavelength Maxima (nm)</th>
<th>Eacly/Evils Ratio (%)</th>
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<tbody>
<tr>
<td>1-a</td>
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<td>29</td>
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<td>1-b</td>
<td>525</td>
<td>31</td>
<td>31</td>
<td>31</td>
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<tr>
<td>1-c</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>1-d</td>
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<td>2-b</td>
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<td>31</td>
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<td>2-c</td>
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<td>3-b</td>
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**Bilberry Anthocyanins**

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HD = not determined
Sh = shoulder
Y = Acylation with hydroxy aromatic acid detected
N = No Acylation with hydroxy aromatic acid detected
Figure 3.1  HPLC chromatogram of black currant anthocyanins. Peak identification and peak area percentages: 1-a delphinidin-3-glucoside (9%); 1-b delphinidin-3-rutinoside (44 %); 1-c cyanidin-3-glucoside (5%); 1-d cyanidin-3-rutinoside (42%)
Figure 3.2  HPLC chromatogram of blackberry anthocyanins. Peak identification and peak area percentages: 2-a cyanidin-3-sophoroside (9%); 2-b cyanidin-3-glucosylrutinoside (4%); 2-c cyanidin-3-glucoside (57%); 2-d cyanidin-3-rutinoside (30%)
Figure 3.3  HPLC chromatogram of blackraspberry anthocyanins. Peak identification and peak area percentages: 3-a cyanidin-3-sambubioside (3%); 3-b cyanidin-3-glucoside + cyanidin-3-xylosylrutinoside (33%); 3-c cyanidin-3-rutinoside (64%); 3-d unidentified
Figure 3.4  HPLC chromatogram of cyanidin-3-xylosylrutinoside
Figure 3.5  HPLC chromatogram of elderberry anthocyanins. Peak identification: 5-a + 5-b unidentified, see text for tentative identification; 5-c cyanidin-3-sambubioside; 5-d cyanidin-3-glucoside; 5-e unidentified.

Peak area percentages.
San Red ELF: 5-a + 5-b (31%); 5-c (42%); 5-d (27%); 5-e trace.
Elderberry 200: 5-a + 5-b (37%); 5-c (37%); 5-d (27%); 5-e not found.
Figure 3.6  HPLC chromatogram of plum anthocyanins. 3.6A, untreated. 3.6B, fraction of anthocyanins remaining on a Sep-Pak after elution with alkaline borate buffer.  
Peak identification and peak area percentages: 6-a unknown (less than 1%); 6-b cyanidin-3-glucoside (37%); 6-c cyanidin-3-rutinoside (45%); 6d unknown (3%); 6-e unknown (3%); 6-f unknown (12%); 6-g unknown (trace).
Figure 3.7  HPLC chromatogram of Morello cherry anthocyanins. Peak identification: 7-a cyanidin-3-sophoroside; 7-b cyanidin-3-glucosylrutinoside; 7-c cyanidin-3-glucoside; 7-d cyanidin-3-rutinoside.

Peak area percentages:
Morello Cherry 240, 7-a (less than 1%); 7-b (65%); 7-c (14%); 7-d (14%); 7-e (7%).
Morello Cherry 240D, 7-a (3%); 7-b (74%); 7-c (3%); 7-d (16%); 7-e (2%).
Morello Cherry 348, 7-a (3%); 7-b (72%); 7-c (4%); 7-d (15%); 7-e (3%).
Figure 3.8 HPLC chromatogram of Bilberry anthocyanins. 3.8A, untreated. 3.8B, fraction of anthocyanins remaining on a Sep-Pak after elution with alkaline borate buffer. Peak identification and peak area percentages: 8-a unknown (trace, less than 1%); 8-b delphinidin-3-galactoside (19%); 8-c delphinidin-3-glucoside (9%); 8-d cyanidin-3-galactoside (15%); 8-e petunidin-3-galactoside (14%); 8-f cyanidin-3-glucoside (9%); 8-g unknown (trace); 8-h unknown (2%); 8-i unknown + 8-j unknown (9%); 8-k unknown (trace); 8-l malvidin-3-glucoside (22%); 8-m malvidin-3-arabinoside (1%).
Figure 3.9  HPLC chromatogram of Spreda GSE anthocyanins. 3.9A, untreated. 3.9B, fraction of anthocyanins remaining on a Sep-Pak after elution with alkaline borate buffer. 3.9C, anthocyanin profile after alkaline hydrolysis.

Peak identification: 9-a delphinidin-3-glucoside; 9-b unidentified; 9-c cyanidin-3-glucoside; 9-d petunidin-3-glucoside; 9-e unknown; 9-f peonidin-3-glucoside; 9-g malvidin-3-glucoside; 9-h to 9-o unidentified.

Peak area percentages:

Spreda GSE, 9-a (17%); 9-c (3%); 9-d (21%); 9-f (7%); 9-g (43%); 9-k (4%); 9-o (2%); the remaining peaks were present in quantities less than 1%.

Spreda GSE #501, 9-a (15%); 9-c (3%); 9-d (17%); 9-f (6%); 9-g (45%); 9-k (3%); 9-o (7%); the remaining peaks were present in quantities less than 1%.
Figure 3.9 (continued)
Figure 3.10  HPLC chromatogram of the anthocyanins in Welches Grape Colorant.  3.10A, untreated.  3.10B, fraction of anthocyanins remaining on a Sep-Pak after elution with alkaline borate buffer.  3.10C, anthocyanin profile after alkaline hydrolysis.

Peak identification:  10-a unknown ; 10-b unknown ; 10-c delphinidin-3-glucoside ; 10-d cyanidin-3-glucoside ; 10-e petunidin-3-glucoside; 10-f peonidin-3-glucoside ; 10-g malvidin-3-glucoside ; The remaining peaks are unknown.  See text for tentative identification.

Peak area percentages:  10-a (2%); 10-b (1%); 10-c (31%); 10-d (16%); 10-e (10%); 10-f (3%); 10-g (5%); 10-h (3%); 10-i (1%); 10-j (trace, less than 1%); 10-k (5%); 10-l (5%); 10-m (9%); 10-n (3%); 10-o (2%)
Figure 3.10 (continued)
**Figure 3.11**  HPLC chromatogram of red cabbage anthocyanins. 3.11A, San Red RC powder, untreated. 3.11B, San Red RC liquid after alkaline hydrolysis. 3.11C, San Red RC liquid, untreated.

Peak identification incomplete. Characterization of the individual pigments given in text.

Peak area percentages:

**San Red RC powder:** 11-a (3%); 11-z (not found); 11-b (7%); 11-c + 11-d (4%); 11-e (3%); 11-f (5%); 11-g + 11-h (36%); 11-i (trace, less than 1%); 11-j (trace); 11-k (8%); 11-l (31%); 11-m (trace); 11-n (trace)

**San Red RCF:** 11-a (3%); 11-z (not found); 11-b (9%); 11-c + 11-d (7%); 11-e (3%); 11-f (5%); 11-g + 11-h (42%); 11-i (trace, less than 1%); 11-j (trace); 11-k (8%); 11-l (trace); 11-m (trace); 11-n (trace)

**San Red RC liquid:** 11-a (trace); 11-z (6%); 11-b (10%); 11-c + 11-d (2%); 11-e (2%); 11-f (7%); 11-g + 11-h (16%); 11-i (trace); 11-j (trace); 11-k (10%); 11-l (43%); 11-m (trace); 11-n (trace)
Figure 3.11 (continued)

[Graph B]

[Graph C]
AOAC Official Methods of Analysis, 14th Ed., AOAC, Arlington, VA.


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