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# Chemical Characterization of Wild Maine Cranberries

Dolly Jean Watson

# CHEMICAL CHARACTERIZATION OF WILD MAINE CRANBERRIES

By

Dolly Jean Watson

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Advisory Committee:

Alfred A. Bushway, Professor of Food Science, Co-Advisor

Rodney J. Bushway, Professor of Food Science, Co-Advisor

Denise Skonberg, Assistant Professor of Food Science

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# CHEMICAL CHARACTERIZATION OF WILD MAINE CRANBERRIES

By Dolly Jean Watson

Thesis Co-Advisors: Dr. Alfred A. Bushway
Dr. Rodney J. Bushway

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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This study was conducted to analyze wild Maine cranberries for anthocyanin (ACY), anthocyanidin, organic acid, L-ascorbic acid, and moisture content. Cranberries were analyzed in both the fresh and frozen states. Analysis for ACY and anthocyanidin content was performed by High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE); organic acids by HPLC; and L-ascorbic acid (L-AA) and moisture by AOAC methods for titration and drying, respectively.

ACY results showed similar qualitative profiles for all cultivars except one where no detectable ACY were present. Four major peaks and two minor peaks were detected, which have been tentatively identified as the galactosides (Gal), arabinosides (Arab), and glucosides (Glu) of cyanidin (Cy) and peonidin (Pn). Quantitatively, significant differences ($p<0.05$) existed among the different cultivars for total and individual ACY content, with totals ranging from 9.64 to 126.05 mg/100g and 13.11 to 94.48 mg/100g for fresh and frozen samples, respectively (results reported as Cy-3-Gal). The proportion of

individual ACY content varied notably with Cy-3-Gal comprising 20-35% of the total, Cy-3-Arab 14-22%, Pn-3-Gal 30-45%, and Pn-3-Arab 13-18%.

Aglycones obtained through acid hydrolysis of the ACY and analyzed by HPLC resulted in 55% Cy and 35% Pn. The CE method developed in this study employed an acidic run buffer, which kept the aglycones in their flavylium cation form. Thus, the same concentrations of pigments used in HPLC were applied to CE. For comparison of methods, HPLC and CE analyses were performed on commercial cranberries. Results showed that the methods were comparable despite the noisy baseline of the CE online detection. The correlation coefficient was 0.967.

The primary organic acids in cranberries are citric, malic, quinic, and L-ascorbic. Mean organic acid content of fresh and frozen berries varied slightly (2041 mg/100g and 2035 mg/100g, respectively) despite the large differences in individual acids. Malic was the primary acid in fresh berries at 64%. Citric and quinic made up 25% and 11%, respectively. In the frozen berry, citric and malic acids were evenly matched at 42% and 41%, respectively. Quinic remained low at 17%.

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early Sunday mornings to say hello and ask how my week went. And even though she was miles away she always asked if there was anything she could do or anything that I needed. She is the most generous woman I know, and has given me the greatest gift there is to give. Thank you, Mom. I love you.

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## INTRODUCTION

The American Cranberry, *Vaccinium macrocarpon*, like its relative, the Small Cranberry *(Vaccinium oxycoccus)* is a native berry to North America. It is a low-growing, woody perennial plant with thick elliptical leaves that is capable of producing fruit for decades. It thrives in the cool, damp climates of the north Atlantic and Pacific Coast lines of North America (Eck, 1990). Early American Natives used the berry not only for food, but also for dyes, medicinal purposes, and symbols of peace.

The American Cranberry is a popular fruit and a multi-million dollar industry with a large majority of the cranberry harvest going into the manufacture of juice and juice products. The perfect formula for breeding, growing, and harvesting cranberries for maximum fruit production as well as maximum anthocyanin production is proving to be an endless search. This is complicated by the fact that cranberries require unique growing conditions, including an acidic soil, an adequate water supply, sand, and a growing season stretching over the months of April to November (CCCGA, 1999). Thus, their production is limited to certain climates and areas in the United States. The leading cranberry producers in the U.S. are Massachusetts, Wisconsin, New Jersey, Oregon, and Washington. The state of Maine has recently initiated an interest in the cranberry industry. In 1998, 176 acres of cranberries were planted.

The purpose of this study was to evaluate the wild cranberries grown in Maine primarily for their anthocyanin content and secondly for their organic acid and vitamin C

content. The results determined in this study would be useful information for breeding wild Maine cranberries with known cultivars. The slightly colder climate in Maine, compared to the other cranberry producing states previously mentioned, may influence the metabolism and production of ACY or other nutrients in the berry, which would improve the quality of the fruit in manufacturers' perspective.

# COMPOSITION

Fresh cranberries primarily consist of fruit sugars, organic acids, and pectin. Their water content averages 88% (Table 1). Hong and Wrolstad (1986) reported the sugar content of whole cranberries ranged from 5.25 to 7.13 g/100g, of which glucose comprised approximately 80% and fructose 20%.

The high content of organic acids is responsible for the tartness and low pH of cranberries, with the primary acids being citric, malic, quinic, and benzoic (Table 1). L-ascorbic acid is also an organic acid, but is more often categorized as a vitamin, vitamin C. It is the acidic nature of cranberries that decreases the occurrence of urinary tract infections for people who regularly consume cranberry juice and juice products. This is achieved via the conversion of quinic and benzoic acids to hippuric acid in the human body, which is then excreted in the urine (Eck, 1990; CCCGA, 1999). Unlike quinic and benzoic acids, citric and malic acids are metabolized and used for energy; and therefore, do not contribute pH lowering effects in urine. Organic acids are discussed later in the literature review.

As with the sugar content of cranberries, the ash content is also low, but comparable to that of the closely related lowbush blueberry. Potassium and calcium are the predominant minerals, and despite the high acidity of fresh cranberries, the ash of the fruit is slightly alkaline (Eck, 1990).

The high pectin content of cranberries influences the quantity of sugar needed during jelly processing with as little as 40% sugar being added, unlike other fruits that require 65% added sugar. The amount of pectin can vary between cultivars, from bog to bog, and from one growing season to the next, producing a range of 0.40 to 1.36% (Eck, 1990).

**TABLE 1.**

**COMPOSITION OF CRANBERRY FRUIT***

| CHEMICAL COMPOSITION | % |
|---|---|
| Moisture | 88.00 |
| Reducing sugars | 4.20 |
| Acids (as citric) | 2.40 |
| Pectin | 1.20 |
| Fat (ether extract) | 0.40 |
| Protein | 0.20 |
| Ash | 0.25 |
| Fiber | 1.60 |

| VITAMIN CONTENT | per 100g |
|---|---|
| Vitamin A | 40 IU |
| Vitamin C | 7.5-10.5 mg |
| Thiamine | 13.5 mg |
| Riboflavin | 3.0 mg |
| Nicotinic Acid | 33.0 mg |
| Pantothenic Acid | 25.0 mg |
| Pyridoxine | 10.0 mg |
| Biotin | trace |

| MINERAL CONTENT | mg/100g |
|---|---|
| Potassium | 53 |
| Sodium | 2 |
| Calcium | 13 |
| Phosphorus | 8 |
| Magnesium | 5.5 |
| Iodine | 0.005 |
| Sulfur | 5 |
| Chlorine | 4 |
| Iron | 0.4 |
| Manganese | 0.6 |
| Copper | 0.4 |

| ACID CONTENT | % |
|---|---|
| Citric Acid | 1.10 |
| Malic Acid | 0.26 |
| Quinic Acid | 0.5-1.0 |
| Benzoic Acid | 0.065 |

| ENERGY | kcal/100g |
|---|---|
| Fresh berries | 26 |
| Sauce | 125 |

*Adapted from Eck, 1990.

# CRANBERRY GROWTH & HARVESTING

Cranberries grow wildly along the Atlantic Coast and some other areas of the United States. They prefer conditions offering sandy soil, an abundant supply of fresh water, and a growing season that lasts for at least 150 frost free days (CCCGA, 1999).

Whereas most crops require a neutral pH of the soil, cranberries thrive in acidic conditions; typically in the range of 4.0 to 5.5. Eck (1990) reported that the preferred substrate for cranberry growth was that of an acid peat soil, which provided a significant source of nitrogen and boron when the organic matter decomposed. The acidic nature of the soil also facilitated the suppression of weeds.

The growth of the cranberry involves two primary stages, an active stage and a dormant or resting stage. During the dormant stage in winter, the cranberry bogs are flooded to prevent damage to the vines from severe drops in temperature and early spring frosts. Some farmers choose to apply a layer of sand every couple of years over this protective ice sheet to stimulate growth, improve drainage, and control weeds and insects (CCCGA, 1999). Others may "dry sand" on the vines directly during the summer months.

With the arrival of spring and warmer temperatures, the winter floodwater is drained. Farmers must be extremely careful of spring frosts since the cranberry is in a stage of rapid growth and is very susceptible to less than optimal growing conditions. Optimal growth temperatures range between 15.6°C and 26.7°C (60°F and 80°F), with temperatures below -0.6°C (31°F) causing significant damage to crop yield once the bud is exposed (Eck, 1990).

During the summer months, rainfall and irrigation are critical for the success of the crop. Cranberries require up to one inch of water per week during the growing season (CCCGA, 1999). In the first months of summer, flowering of the cranberry bud occurs, and bees take on the responsibility of pollination. Typically, flowering takes place on old uprights rather than new ones, and it is the old flowering uprights that bear fruit. Eck

(1990) reported that berry yield was associated with the percentage of uprights with flowering buds from the previous fall season. Theoretical yields have been estimated at 450 barrels of cranberries from a single acre, but this is unlikely since only one-third of potential blossoms in a cranberry bog will set fruit (Eck, 1990).

Once pollination has taken place, fruit growth and development begin. The cranberry growth curve follows that of an initial burst of growth that is maintained until maturity of the fruit. In the early stages of fruit development the berry is small and green owing to the presence of chlorophyll pigments. Only as the berry matures and the red anthocyanins and other pigments predominate does the cranberry inherit its characteristic red hue. It is the development of these anthocyanins that is crucial to the commercialization of the cranberry and cause for extensive research for their enhancement.

Harvesting of the fruit begins in early September and lasts until November. Harvest time is dependent on fruit maturity, cultivar, and weather conditions, since frost damage is still an offender of berry quality during the harvest season. There are two kinds of harvesting for cranberries depending on the final use of the fruit: dry and wet harvesting. Cranberries that are destined to be frozen or processed into juice, jellies, and sauces are usually wet harvested. This accounts for more than 85% of the total cranberry harvest (CCCGA, 1999). Wet harvesting involves flooding of the bog with approximately a foot of water, agitating the water to free the berries from the vine, and corralling the fruit for transportation. Due to the increase in rate of deterioration of wet harvested fruit, berries are subjected to a drying step prior to further processing (Eck, 1990).

The other 15% of the cranberry harvest is dry harvested and enters the grocery store as fresh fruit. Originally accomplished by handpicking, dry harvesting has since progressed from scoops to picking machines. These berries are delivered to receiving stations, graded, and screened based on their color and ability to bounce (Ocean Spray, 1999).

## CRANBERRY QUALITY

The quality of cranberry fruit is a complex term to define since there are many factors affecting it -- weather and other environmental conditions, cultivar, method of harvesting, fruit handling, berry size, pesticide use -- with wide variations in fruit composition occurring from one growing season to the next. Several researchers (Sapers et al., 1986; Sapers et al., 1983b; Andersen, 1989) reported that color, or anthocyanin content, was the most heavily weighed factor when determining the value and quality of cranberries. Sapers et al. (1983a) further defined quality in terms of juice yield, sugar content, and acidity.

Breeding programs continually experiment with different growing conditions and cultivars to achieve high pigment expression yet maintaining the hardiness of the berry. Mazza and Miniati (1993) described the optimal ACY content in quality cranberries was higher than 67 mg/100 g.

# ANTHOCYANIN CONTENT

Anthocyanins (ACY) are the compounds responsible for the vibrant reds, blues, and violets of flowers, fruits, and vegetables. They are water-soluble glycosides and acylglycosides of anthocyanidins and number more than 240 naturally occurring compounds (Strack & Wray, 1989). ACY are located in the outer layer of plant tissue called the exocarp, and may be used to characterize a specific variety of cranberry (or other plant) since different cultivars may have different ACY profiles.

The demand for natural food colorants by the consumer has stimulated the food industry to use such pigments as anthocyanins. Unfortunately, ACY are difficult to isolate and very unstable in aqueous and slightly acidic or neutral solutions, which encompasses the majority of beverages and food products of desired application. An explosion of research has been conducted to address these problems of pigment extraction, isolation, and stabilization, which will be discussed later in this review.

Recently, another area of research has focused on the potential health benefits of ACY as antioxidants and free radical scavengers. It is believed that ACY may play an important role in preventing the oxidation of lipids; thus, reducing the risk of certain forms of heart disease (Frankel et al., 1993; Fuhrman et al., 1995; Hertog et al., 1993; Satué-Gracia et al., 1997; Tamura & Yamagami, 1994; Teissedre et al., 1996). The benefits of ACY's antioxidant activity has also been associated with aiding in several other disease states afflicting human health: diabetic retinopathy (Wang et al., 1997), certain cancers, and neurodegeneration (Prior et al., 1996).

## Structure

Collectively, anthocyanins belong to the flavonoid family of compounds and consist of a basic flavan nucleus (Figure 1), made up of two benzene rings and a γ-pyran ring, and an attached sugar moiety (Figure 2). When one sugar molecule is attached (mainly glucose, but also rhamnose, galactose, xylose, arabinose, and fructose), it is almost always at position 3. Other sugars may be linked at positions 5, 7, 3', 4', and 5' with usually no more than three sugar moieties attached at different positions due to steric hindrance (Britton, 1983; Jackman et al., 1987; Francis & Markakis, 1989). Glycosylation occurs as mono-, di-, and trisaccharides of the same or combinations of different sugar molecules. Many ACY also contain acyl acid linkages (acylation) in their structure, which are attached to the sugar moiety. In order of occurrence, the acyl acids are coumaric, caffeic, ferulic, p-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic, and malic acids (Francis, 1989). As with glycosylation, acylation is believed to play an important role in the stabilization of ACY structure and color, not just the presence of acyl acids, but also the type of the acyl group. An example of this was reported by Baublis et al. (1994). It was determined that decreases in degradation by hydration were a result of intramolecular copigmentation due to the high degree of acylation and substitution in the B ring of the chromaphore. Research has not reported any acyl acids associated with the American Cranberry.

Francis and Markakis (1989) listed 17 naturally occurring aglycones with only six of them having importance to the food industry as food colorants. These aglycones and their substitution patterns on the flavan nucleus are listed in Table 2. The aglycones of

importance to the food industry are pelargonidin (Pel), cyanidin (Cy), peonidin (Pn), delphinidin (Del), petunidin (Pet), and malvidin (Mal), which are most likely due to their abundance in comparison to the other aglycones.

**FIGURE 1.**

**FLAVAN NUCLEUS**

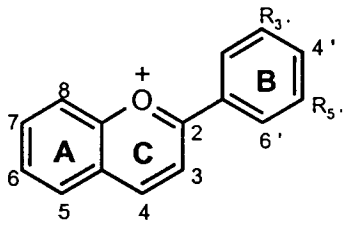



**TABLE 2.**

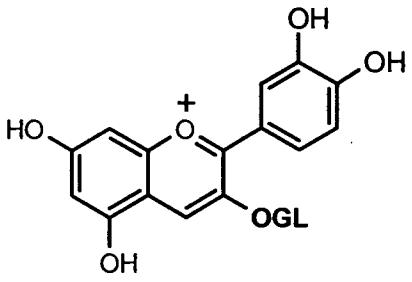
**STRUCTURES OF NATURALLY OCCURRING ANTHOCYANIDINS***

| | SUBSTITUTION PATTERN | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3 | 5 | 6 | 7 | 3' | 4' | 5' |
| Pelargonidin | OH | OH | H | OH | H | OH | H |
| Cyanidin | OH | OH | H | OH | OH | OH | H |
| Peonidin | OH | OH | H | OH | $OCH_3$ | OH | H |
| Delphinidin | OH | OH | H | OH | OH | OH | OH |
| Petunidin | OH | OH | H | OH | $OCH_3$ | OH | OH |
| Malvidin | OH | OH | H | OH | $OCH_3$ | OH | $OCH_3$ |
| Apigenin | H | OH | H | OH | H | OH | H |
| Luteolidin | H | OH | H | OH | OH | OH | H |
| Triacetidin | H | OH | H | OH | OH | OH | OH |
| Aurantidin | OH | OH | OH | OH | H | OH | H |
| 5-MethylCy | OH | $OCH_3$ | H | OH | OH | OH | H |
| Rosinidin | OH | OH | H | OH | $OCH_3$ | OH | H |
| 6-HydroxyCy | OH | OH | OH | OH | OH | OH | H |
| Pulchellidin | OH | $OCH_3$ | H | OH | OH | OH | OH |
| Euopinidin | OH | $OCH_3$ | H | OH | $OCH_3$ | OH | OH |
| Capensinidin | OH | $OCH_3$ | H | OH | $OCH_3$ | OH | $OCH_3$ |
| Hirsutidin | OH | OH | H | $OCH_3$ | $OCH_3$ | OH | $OCH_3$ |

*Francis & Markakis, 1989.

**FIGURE 2.**

**ANTHOCYANIN STRUCTURE**



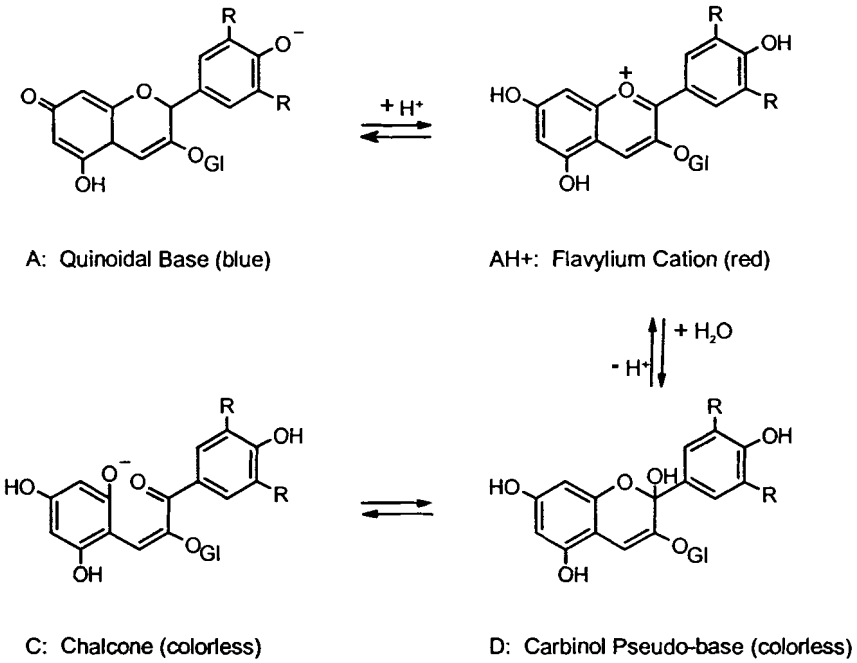

CYANIDIN-3-GLYCOSIDE

As evident from Table 2, some substitutions on the flavan nucleus are hydroxyl groups, while others are methoxyl groups. Methylation may occur before or after glycosylation (Strack & Wray, 1994) and was reported to decrease the stability of ACY; whereas, hydroxylation increased their stability via hydrogen bonding (H-bonding) with other phenolic compounds. The number of hydroxyl and methoxyl groups also played a significant role in the color shifts of ACY. With increased hydroxyl substitutions on the B ring, the color changed from orange to blue. Replacing the hydroxyls with methoxyl groups reversed this trend. For example, cyanidin glycosides, which have two hydroxyl groups on the B ring are red; whereas, peonidin glycosides, which have only one hydroxyl group on the B ring are orange.

In acidic or neutral solution, it has been demonstrated that four ACY structures existed in equilibrium: the flavylium cation (AH+), the quinonoidal base (A), the carbinol pseudobase (B), and the chalcone (C) (Figure 3).

## FIGURE 3.

## STRUCTURAL TRANSFORMATIONS OF ANTHOCYANINS IN AQUEOUS MEDIA*




*Redrawn from Francis & Markakis, 1989.

The mechanism driving these reactions in Figure 3 involved the deprotonation of the flavylium cation in slightly acidic media (pH 4-6) to the anhydro base (A). This in turn, through hydration, gave rise to the carbinol pseudobase (B) and chalcone (C) (Chen & Hrazdina, 1982; Strack & Wray, 1989). Two of these structures are colorless, the pseudobase and chalcone, explaining the loss of color at higher pH values where they are the dominant species. This is further explained in the next section under pH.

## Color Stability

### pH

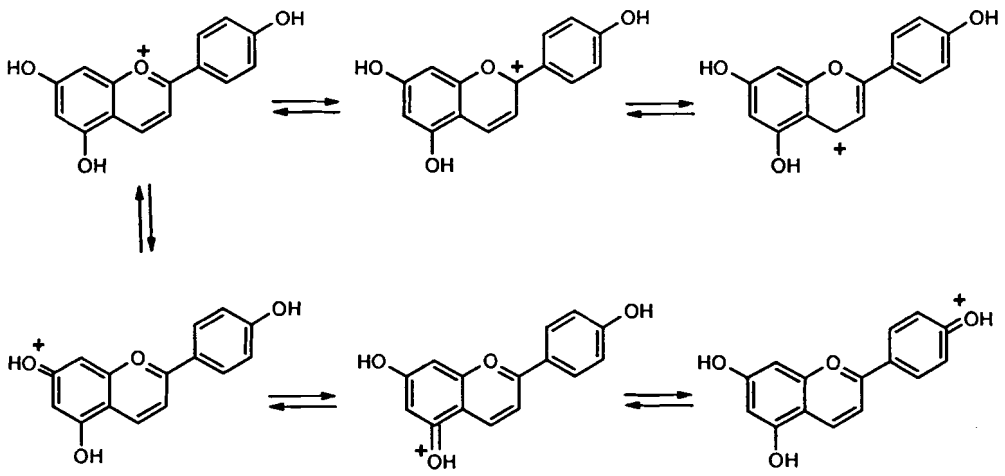
In aqueous solutions, ACY behave like pH indicators. They are red at low pH, blue at intermediate pH values, and colorless at high pH. This change in and loss of color is due to the change in equilibrium between the four anthocyanin structures (Figure 3). The quinoidal base is blue; whereas, the flavylium cation is red. As previously mentioned, the pseudobase and chalcone are colorless. Britton et al. (1983) reported the different spectra of each structural compound, with the flavylium salt (pH 1.0) having a maximum absorption near 510 nm; the pseudobase (pH 4.0) maximum near 380 nm; the anhydro base (pH 7.5) maximum near 535 nm; and the chalcone (in ethanol) maximum near 380 nm. Research by Mazza and Brouillard (1987) discovered six structural species of cyanidin at varying pH values by spectrophotometric data. The two additional structures were the anionic forms of the quinonoidal base and the chalcone.

Further explanation of these structural changes centers on the flavylium cation. It is relatively stabile under acidic conditions and exists as six resonance forms (Figure 4), with the highest partial positive charges occurring at positions 2 and 4 (Jackman et al., 1987; Strack & Wray, 1989). With even slight increases in pH, hydroxyl protons dissociate leaving the flavylium cation more susceptible to nucleophilic attack by water, forming the carbinol pseudobase. The pseudobase then equilibrates with the colorless chalcone. Figure 5 depicts the distribution of these four structures under equilibrium conditions with pH for cyanidin-3,5-glucoside. In their research, Mazza and Brouillard

(1987), also showed a diagram of the spectra of cyanidin-3,5-diglucoside at varying pH, which allowed the reader to follow the disappearance of the flavylium cation with increases in pH while the other structural species arose (Figure 6). Consistent with this diagram, Fuleki and Francis (1968b) described the highest absorbency for cranberry juice occurred at a pH around 1.0, with increases in pH correlating to sharp decreases in optical density.

**FIGURE 4.**

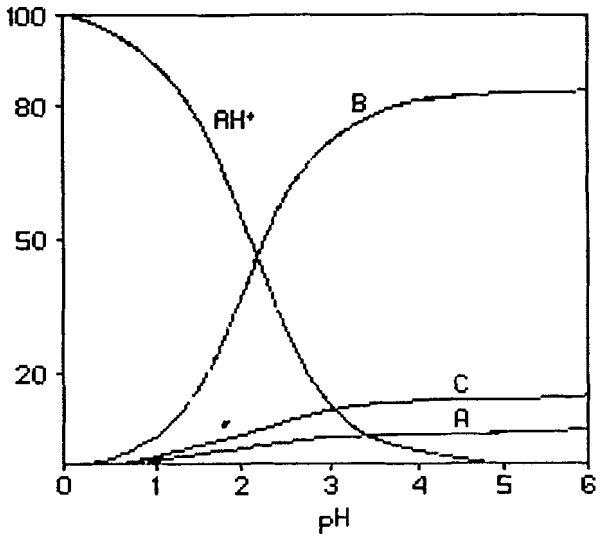
**RESONANCE FORMS OF THE FLAVYLIUM CATION***



*Redrawn from Jackman et al., 1987.

## FIGURE 5.

## DISTRIBUTION OF THE FOUR ANTHOCYANIN STRUCTURES WITH pH UNDER EQUILIBRIUM CONDITIONS*



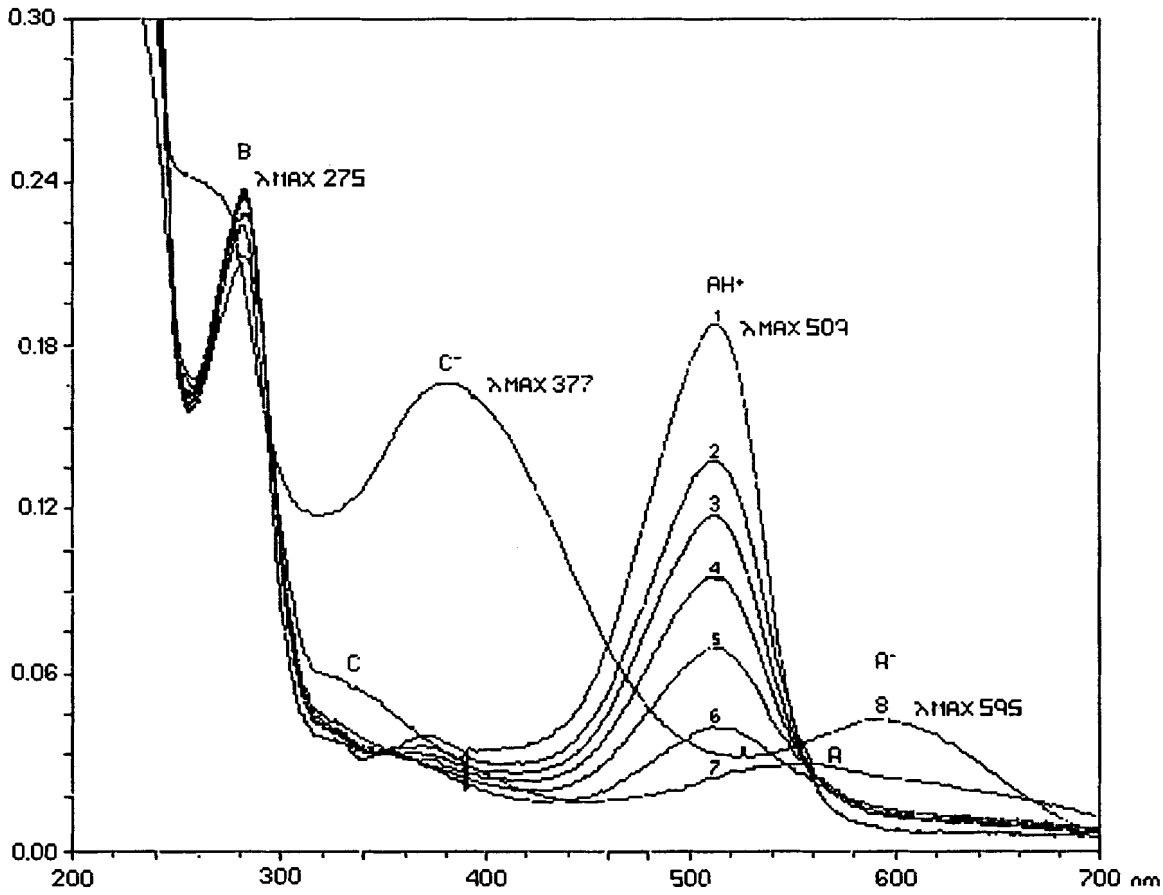

*Scanned image from Mazza & Brouillard, 1987.

| | | | |
|---|---|---|---|
| AH+ | Flavylium Cation | A | Quinonoidal Base |
| B | Carbinol Pseudobase | C | Chalcone |

Chen and Hrazdina (1982) reported that the complete ionization of the anhydro bases of malvidin-3-glucoside and malvidin-3,5-diglucoside occurred at pH 8.5. Reversal of equilibrium to the flavylium cation may be achieved when acidic conditions are reapplied and if the alkali treatment is not too severe.

## FIGURE 6.

## SPECTRA OF CYANIDIN-3,5-DIGLUCOSIDE AT VARYING pH VALUES*




*Scanned from Mazza & Brouillard, 1987.
pH values: 1=2.21, 2=2.49, 3=2.58, 4=2.72, 5=2.91, 6=3.77, 7=4.81, 8=10.66.

## Temperature & Light

Temperature greatly affects the stability and integrity of ACY. When the temperature is increased, the rate of pigment degradation is also increased. Exposure to light also has been shown to have adverse effects on ACY. These factors have been investigated (Withy et al., 1993; Dao et al., 1998; Inami et al., 1996; Bakker et al., 1992). Both processing and storage temperatures and light affect the stability of anthocyanins; thus, making the job of the food processor even more challenging.

Beverages, especially fruit juices, and fruit products are normally held at room temperature and packaged in clear containers on the grocer's shelves and in the consumer's home where exposure to light is almost unavoidable, providing less than optimal conditions for color retention. A study conducted by Bakker et al. (1992) showed that two fruit puree samples and one clarified juice sample lost almost 50% of ACY concentration after three weeks at 20°C (68°F). The same samples held at -20°C (-4°F) showed little or no change in pigment concentration after eight weeks. Dao et al. (1998) conducted a similar study on black bean ACY and reported the disappearance of pigments (aglycones) after 48 hours when held at 25°C (77°F) in acidified methanol (MeOH). Color retention improved slightly when samples were held at 2°C (35.6°F) for which total pigment loss occurred after day six. The proposed mechanism of degradation was the favored formation of colorless chalcones at high temperatures.

Another study by Inami et al. (1996) suggested that ACY composition played a role in color stability. The researchers compared the effects of temperature and other processing conditions on two species of elderberry with different ACY profiles. Their

results showed that the pigments of *Sambucus canadensis* were superior to pigments of *S. nigra* for color retention under high temperatures. Specifically, the primary ACY in *S. canadensis*, Cy-3-*O*(6-*O*-*E*-*p*-coumaroyl-2-*O*-α-D-xylopyranosyl)-α-D-glucopyranoside-5-*O*-α-D-glucopyranoside, expressed an 80% pigment retention in comparison to 50% retention by the primary ACY in *S. nigra*, cyanidin-3-sambubioside.

Inami et al. (1996) also subjected elderberry samples to solar energy to determine the effects of sunlight on ACY. Again, it was determined that *S. canadensis* retained more pigment color than *S. nigra* when exposed to light, 50% and 20%, respectively. This is more evidence in support of the stabilizing effects of acyl acids on ACY and the importance of structure, in general, on ACY stability. These are only a few cases, but from these experiments, it can be seen the effect structure has on the stability of ACY color, with aglycones being less stable than their glycosides, which in turn are less stable than acylglycosides.

## Copigmentation, Condensation, & Metal Reactions

Copigmentation and condensation reactions play significant roles in the color stability of ACY. Color is intensified in these reactions through the association of ACY with other flavonoids, alkaloids, amino acids, benzoic acids, and other ACY compounds through the formation of H-bonds (Francis & Markakis, 1989). There are two types of copigmentation reactions: intramolecular and intermolecular copigmentation.

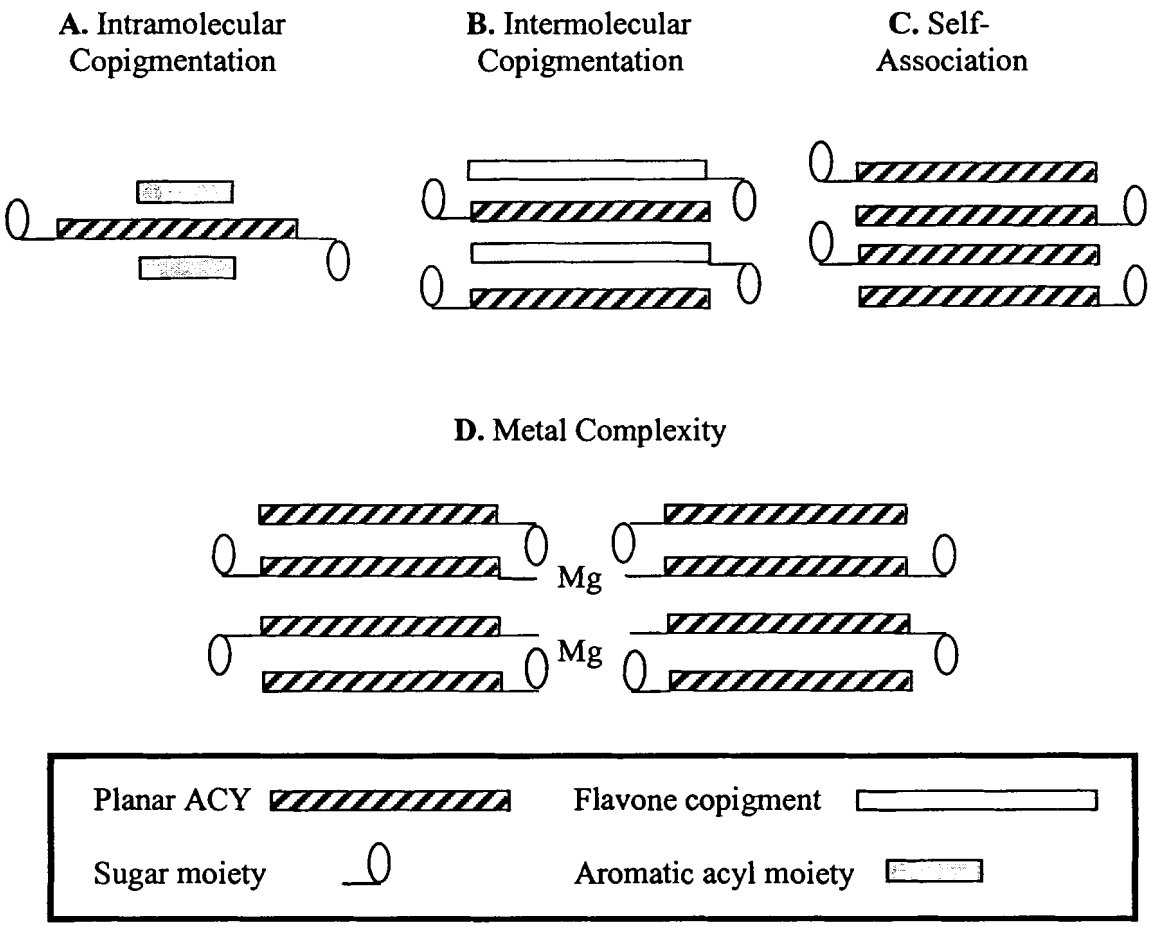
Intramolecular copigmentation is more efficient in its stabilization capability (Jackman et al., 1987; Francis & Markakis, 1989), but both types cause a bathochromic

shift in color from red to blue. Intramolecular copigmentation involves the reaction between ACY and its aromatic acyl substituents. The ACY is protected from degradative hydration reactions by the sandwich-like configuration of the aromatic acyl groups around the pyrylium ring of the ACY (Figure 7a) (Harborne, 1988; Strack & Wray, 1989). A prime example of intramolecular copigmentation is the heavenly blue ACY from *Ipomoea tricolor* flower. Stability is increased with increased content of aromatic acyl groups and increased substitution of the aglycone.

Intermolecular copigmentation (Figure 7b) is the association of ACY with colorless flavonoids and other related compounds that act as copigments. It is affected more by pH than is intramolecular pigmentation (Harborne, 1988), but offers protection from hydration of the ACY through the same mechanism. The degree of copigmentation is also dependent on ACY concentration and type and copigment concentration and type. A study conducted by Baranac et al. (1996) looked at the spectrophotometric data of copigmentation reactions between Mal-3,5-diglucoside and quercetin-3-rutinoside to identify the factors influencing the reaction as well as characteristics of the copigment formed. Their findings showed that the reaction was under the influence of pH, mole ratio, and temperature as previously mentioned. Francis and Markakis (1989) reported that copigmentation was more pronounced with 3,5-diglucosides than with 3-glucosides, again suggesting the importance of ACY structure in stabilizing reactions.

**FIGURE 7.**

**MECHANISMS OF ANTHOCYANIN STABILIZATION *IN VIVO**




*Redrawn from Harborne, 1988.

When the concentration of ACY is relatively high, they are more likely to associate with other ACY than with copigments. These reactions are termed self-association, or condensation reactions, and offer explanation as to why ACY veer from the linearity of the Beer-Lambert Law. Color may increase more than proportionally to pigment concentration (Hoshino, 1992; Jackman et al., 1987), creating the need for care when making dilutions for analysis. Hoshino (1992) demonstrated that during self-association, anthocyanidin nuclei stacked vertically, which is illustrated in Figure 7c. It

was the vertical stacking that offered protection and color intensification of the pigments. Self-association differs from copigmentation reactions in that they are more prominent at lower pH values (Jackman et al., 1987) and result more from hydrophobic interactions than H-bonding.

Stabilization of ACY and their associated color may also be a result of complexes with metals (Figure 7d). This may occur along with copigmentation and condensation reactions. In order for metal complexes to occur an orthodiphenolic group in the B-ring must be present; thereby, making it possible to differentiate cyanidin-, delphinidin-, and petunidin-glycosides from pelargonidin-, peonidin-, and malvidin-glycosides. The latter three do not have adjacent hydroxyl groups (Jackman et al., 1987). Another application for metal-ACY complexes is for the determination of food adulteration for those foods having simple ACY profiles.

Different metals cause different degrees of stability and bathochromic shifts. Bivalent metal ions are not able to form colored complexes, but chelates will form with tin, titanium, chromium, iron, aluminum, and others (Francis & Markakis, 1989). Experiments conducted by Takeda et al. (1985) showed marked increases in absorbency and bathochromic shifts when aluminum chloride ($AlCl_3$) was added to solutions of ACY (delphinidin-3-glucoside). It was also shown that above molar ratios of 5:1 ($AlCl_3$:ACY) there were no additional increases in absorbency or bathochromic shift.

Metal complexes may have important applications in the food industry since they occur naturally in foods and are sources of contamination during processing. Consideration must be given, though, to the undesirable formation of brown pigments due to metal complexes with tannins.

## Vitamin C & Oxygen

The water-soluble vitamin C is well known for its health benefits as an antioxidant, but when associated with ACY, the combination of the two compounds has not been favored. When pigment and vitamin come together, the result is mutual destruction involving a condensation mechanism (Francis & Markakis, 1989; Jackman et al., 1987). This is an important issue of concern for the food industry since product quality and nutrition are both compromised.

It has also been shown that oxygen acts synergistically with vitamin C in the degradation of ACY (Francis & Markakis, 1989; Jackman et al., 1987). The proposed mechanism is the formation of an intermediate molecule of hydrogen peroxide, which is known to bleach ACY. These reactions can be inhibited under low temperatures. A study by Bakker et al. (1992) on the effects of some processing variables on strawberry pigments showed that ACY degradation was greatly inhibited when samples were held under air at lower temperatures (-20°C, -4°F) than higher ones (20°C, 68°F). When comparing samples stored under nitrogen, there were no significant differences in the degree of ACY degradation for cold or room temperature storage conditions.

Because vitamin C and ACY coexist in so many fruits without complexing, it has been suggested that the antioxidant activity of flavonols offers protection to both compounds (Francis et al., 1989). At the same time flavonols stabilized ACY color by acting as copigments, they prevented the reaction between vitamin C and pigment, sparing both compounds. This may prove beneficial for the food scientist in achieving maximum protection of ACY in food products. Another study carried out by Sarma et al.

(1997) showed that the oxidation of ascorbic acid by metal ions was prevented in the presence of ACY (cyanidin derivative) via metal chelation. The researchers offered a second explanation for the synergistic effect between vitamin C and ACY: ascorbic acid acted as a copigment and directly interacted with the metal-chelated ACY, forming a table ACY-metal-copigment coordinate complex (Sarma et al., 1997).

## Analytical Methods for Anthocyanins

The extraction and isolation of pigments are procedures that have been in practice for quite some time. The documentation on ACY research is extensive. Table 3 is a list of research literature reviewed for this study, including the source of ACY, method of extraction, and chromatographic information. Other methods of separation and quantification have been employed besides that of HPLC. These include Thin-Layer Chromatography (TLC) (Goiffon et al., 1991; Frøytlog et al., 1998), Paper Chromatography (Hong & Wrolstad, 1990), Spectrophotometry (Toldam-Andersen & Hansen, 1997; Sapers et al., 1986; Sapers et al., 1983b; Fuleki & Francis, 1968a & 1968b), and more recently Capillary Electrophoresis (Bicard et al., 1999; da Costa et al., 1998; Bridle & García-Vigueraí, 1997; Bridle et al., 1996).

## TABLE 3.

## SUMMARY OF ANTHOCYANIN RESEARCH BY HPLC

| ACY SOURCE | SOLVENT | MOBILE PHASE | COLUMN | AUTHOR |
|---|---|---|---|---|
| *Vaccinium vites-idaea* | Acidified EtOH (1% HCl) | Gradient FA[1]:$H_2O$ (1:9) & FA: $H_2O$: MeOH (1:4:5) | ODS-Hypersil | Andersen, 1985 |
| *Vaccinium Myrtillus* | MeOH | Gradient FA: $H_2O$ (1:9) & MeOH:FA: ACN[2]: $H_2O$ (22.5:22.5:45:10) | Aquapore RE-300 | Baj et al., 1983 |
| *Vitis vinifera* | EtOH: $H_2O$ (8:2) Acidified w/0.1% HCl | Gradient $H_2O$:AC (93:7) & $H_2O$:AC[3]: ACN:MeOH (47:23:23:7) | Aquapore | Baldi et al., 1995 |
| *Vaccinium* Species | 10% FA | Gradient ACN & 10% FA | HS-5-C18 | Ballington et al.,1988, 1987 |
| Raspberry Juice | Diluted d $H_2O$ | Gradient 15% AC & 100% ACN | Supelcosil LC-18 | Boyles & Wrolstad, 1993 |
| Black Beans | Acidified MeOH (0.5% HCl) | | | Doa et al., 1998 |
| *Ribes nigrum* | MeOH w/0.1% TFA[4] | Gradient $H_2O$:AC: (9:1) & $H_2O$:AC: MeOH (4:1:5) | ODS Hypersil | Frøylog et al., 1998 |
| *Vaccinium Anustifolium* | MeOH:FA: $H_2O$ (70:1:29) | Gradient 5% FA MeOH | SuperPac Pep-S | Gao & Mazza, 1995 |
| ACY Stds | Dissolved in Acidified MeOH (2N HCl) | Gradient $H_2O$:FA (99:1) & MeOH | SuperPac Pep-S | Gao & Mazza, 1994 |
| *Vaccinium Anustifolium* | MeOH:FA: $H_2O$ (70:2:28) | Gradient 5% FA & MeOH | SuperPac Pep-S | Gao & Mazza, 1994 |
| Fruit Jams | MeOH:AC: $H_2O$ (25:1:24) | Gradient 5% FA & MeOH | Lichrochart RP-18 | García-Viguera et al.,1997 |
| Red Fruit | None | Isocratic $H_2O$:FA: ACN (81:10:9) | RP-18 LiChrospher | Goiffon et al., 1991 |
| Cranberry Juice & Fruit | Acidified MeOH (0.1N HCl) | Gradient 10% AC & MeOH:AC: $H_2O$ (6:1:3) | PLRP-S | Hale et al., 1986 |

| | | | | |
|---|---|---|---|---|
| Cranberry Juice & Fruit | Juice: diluted 10x Others: acidified MeOH (1% HCl) | Gradient 4% PA[5] & ACN | PLRP-S | Hong &, Wrolstad 1990 |
| Cranberry Juice | Diluted to 50 Brix° | Isocratic $H_2O$: AC:MeOH:ACN (7:1:1:1) | MCH-10 C18 (Micropak) | Hong &, Wrolstad 1986 |
| *Sambucus Canadensis & S. nigra* | Acidified MeOH (0.1% HCl) | Gradient 0.5% PA in 60% THF[6] & 0.5% PA in $H_2O$ | Capcell Pack C18 | Inami et al., 1996 |
| *Ocimum Basilicum* | Acidified MeOH (0.1% HCl) | Gradient 1.4% Perchloric Acid & MeOH | SynChropak RP-100 | Phippen &, Simon 1998 |
| *Vaccinium Macrocarpon* | 95% EtOH:1.5M HCl (85:15) | Gradient 0.1M Phosphate (pH 1.5) & ACN | µBondapak C18 | Sapers et al., 1984 |
| *Vaccinium Macrocarpon* | 95% EtOH:1.5M HCl (85:15) | Isocratic $H_2O$: ACN:AC:PA (81.7:8.4:8.4:1.5) | µBondapak C18 | Sapers et al., 1983a |

[1] FA = Formic Acid
[2] ACN = Acetonitrile
[3] AC = Acetic Acid
[4] TFA = Trifluoroacetic Acid
[5] PA = Phosphoric Acid
[6] THF = Tetrahydrofuran

## Extraction

The general rule for the extraction of ACY applies an acidified organic solvent with repeated maceration. Four of the papers listed in Table 3 used a mixture of acidified ethanol (EtOH); whereas, eleven others use acidified methanol (MeOH). An organic solvent was preferred over that of an aqueous one due to the degradation of ACY by hydration as previously explained. Hydrochloric acid (HCl) (~0.1%) was the predominant acid used to obtain acidic conditions. Recent studies have leaned toward the use of EtOH and less harsh acids such as formic acid and acetic acid, due to the toxicity of MeOH and the degradation of solvents containing HCl (Strack & Wray, 1989). As

mentioned earlier, an acidic medium is required to keep the ACY in the flavylium cation form where it is most stable and offers the greatest UV-Vis absorbency (510-530 nm) for detection.

The extraction process was further characterized by repeated maceration of the plant in solvent. This usually occurred under low temperatures and subdued light. Prior to chromatographic analysis, many methods subjected samples to clean-up procedures, such as ion-exchange and preparative chromatography (Frøylog et al., 1998), or solid-phase extraction (SPE) (Dao et al., 1998; Kraemer-Schafhalter et al., 1998).

The variation in solvent type and concentration as applied to the extraction of plant ACY is evident from Table 3. Revilla et al. (1998) investigated several different methods of extraction administered on Cabernet Sauvignon Grape ACY. Their purpose was to compare the efficiency of pigment extraction by different solvents, temperatures, and lengths of extraction. The extraction solvents varied in their concentrations of MeOH (50-100%), acetone (75%), and acid (1-2% 12N HCl). Two methods used solely MeOH as the extraction solvent. The duration of extraction (9-120 hours) and temperature (-25°C to room temperature) also varied between methods.

The results from their study showed that acidity and length of extraction played a role in the extraction of ACY from grapes. The method obtaining the greatest total ACY extraction and recovery employed the highest concentration of acid, the longest duration, and coldest temperature: acidified MeOH with 2% 12N HCl for five-24 hour extraction periods at 4°C. The method of least recovery used neutral solvents of 100% MeOH and 80% MeOH, which spanned over 20 hours. The length of extraction in these procedures

created an obvious problem and called for improvement to make analysis more time efficient.

It was also shown that different solvents varied in the recovery of individual ACY. Acidic solvents proved more efficient than neutral solvents in the extraction of non-acylated ACY. Revilla et al. (1998) concluded that the use of solvents containing up to 1% 12N HCl produced the partial hydrolysis of some acetylated ACY during extraction and should not be applied to samples known to have these substituents. Cranberries have not been reported to have attached acyl groups. Thus, it has been safe to use these strong acids in the extraction solvent for cranberry pigments.

Because of the complexity of plant tissue matrices and presence of polar, non-phenolic compounds, metals, and other degradative compounds, it has sometimes been necessary to subject the ACY extract to clean-up procedures. This has been accomplished through solid-phase extraction (SPE), with carbon-18 (C18) cartridges being the most popular adsorbent. Several articles addressed the application of SPE for the purification of ACY. Kraemer-Schafhalter et al. (1998) showed that SPE was a quick and efficient means for the elimination of impurities in the sample matrix, while at the same time offering increased ACY stability due to self-association on the cartridge. They also reported the efficiency of different adsorbent materials, with reversed-phase silica gels achieving the highest color retention. Loss of color was less than five percent.

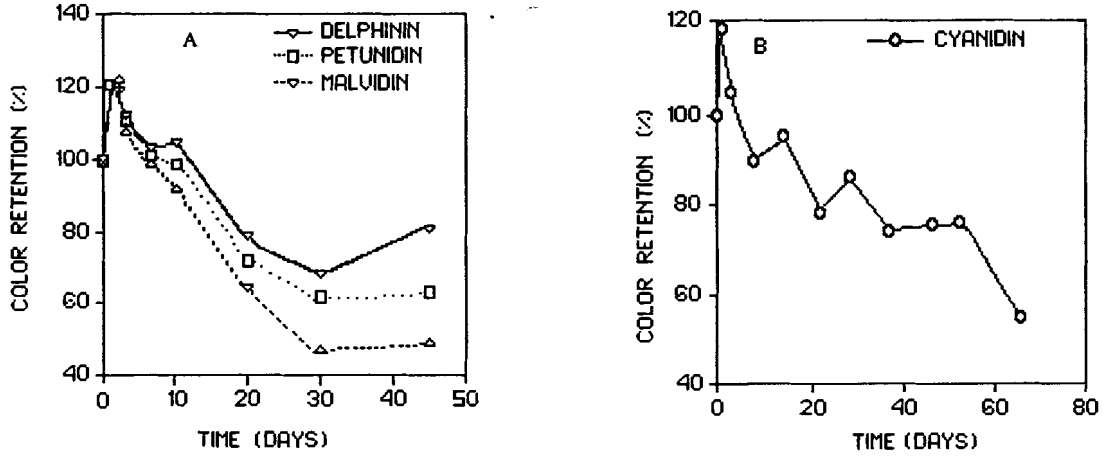
Another study investigating the short- and long-term storage effects of SPE on anthocyanidin stability was conducted by Dao et al. (1998). Variables in the study included type of SPE cartridge, temperature and atmosphere of storage, and type of anthocyanidin. It was found that temperature played a critical role in anthocyanidin

stability in the adsorbed form as well as in solution, as previously noted. Aglycones from black beans stored in acidic MeOH (0.01% HCl) at 25°C were not detectable after 48 hours. When the same pigments were stored at 2°C, anthocyanidins were not detectable after six days, and when stored on a C18 SPE cartridge at 2°C, the same compounds were still present after 45 days, with 82% Del, 63% Pet, and 49% Mal still remaining of the original concentration. When subjected to all variables (25°C, 2°C, SPS cartridge) Del showed the highest stability, followed by Pet and Mal.

All of the aglycones from black beans and Cy from evergreen blackberry puree, expressed a similar pattern of color retention on SPE cartridges (Figure 8a & 8b). It can be seen that all four compounds showed an initial increase in color retention. For Del, Pet, and Mal loss of color occurred up to day 30 where they experience slight increases in color retention until the end of the study period. This was not the pattern followed by Cy, which showed a steady decrease in color retention throughout the entire study.

**FIGURE 8.**

**STABILITY OF ANTHOCYANIDINS STORED ON C18 SPE CARTRIDGES AT 2°C***




*Scanned from Doa et al. (1998).

Reasons for the increased stability of anthocyanic compounds on C18 SPE cartridges are not known, but Doa and colleagues (1998) offered some possibilities. Firstly, they proposed that anthocyanidins formed salts on the adsorbent after rinses of 0.01% HCl. Secondly, the compounds may have formed H-bonds with the reactive silanol groups (SiOH) on the surface of the cartridge. And thirdly, the flavylium cations may have expressed self-preservation through hydrophobic stacking known as self-association. Self-association has been discussed previously under the section 'ACY Stability.'

Hong and Wrolstad (1990) showed the importance of SPE clean-up in quantitative analysis as applied to pigments of cranberry, strawberry, and blueberry juices, roselle, bilberry powder, red cabbage extract, and tamarillo fruit. When samples were subjected to a minimal clean-up step on a C18 cartridge and monitored at 520 nm,

the chromatogram appeared quite "clean." When the absorbance was changed to 260 nm, the resulting chromatogram showed the presence of interfering eluting peaks. More intense clean-up procedures did not prove better since proportions of ACY were affected. Several other studies (Hong & Wrolstad, 1986; García-Viguera et al., 1997; and Phippen & Simon, 1998) included sample clean-up with C18 SPE cartridges in their methods of ACY analysis. It is now considered common practice to use SPE cartridges for the clean-up of ACY, as evidenced by the abundance of SPE cartridges used in the analysis of ACY as reported in the literature.

## High Performance Liquid Chromatography (HPLC)

With improved technology, reversed-phase high performance liquid chromatography (RP-HPLC) has become the favored analytical technique for the separation and quantitation of ACY. Complex plant extracts have been easily resolved on the non-polar, reversed-phase column support material with several examples already mentioned. Some problems have occurred with the use of high acid concentrations in the mobile phase and the degradation of the support material, but this has been chiefly overcome with sturdier columns. As Baj et al. (1983) explained, the low pH was necessary to avoid peak broadening, which arose from the inter-conversion of the structural forms of ACY. Through small changes in mobile phase concentration, the retention time of individual ACY can be manipulated. Increased organic solvent decreases retention times, but may adversely affect resolution. With complex ACY profiles, acid hydrolysis has been useful in breaking down co-eluting pigments into their

aglycones, sugars, and acyl groups, which have then been separated and looked at more easily to determine the sample matrix.

Research conducted by Baldi et al. (1995) employed HPLC coupled with mass spectroscopy (MS) for the qualitative evaluation of ACY in *Vitis vinifera*. With HPLC/MS, researchers have been able to compile more information on a molecular level about the individual compounds being separated. Baldi et al. (1995) found that it was possible to obtain the mass spectra of all ACY compounds, which are characteristic of each compound, even if they were co-eluting or in trace amounts. Through this method, the researchers confirmed the presence of two caffeoyl derivatives and discovered the presence of diglucosidic ACY in the species *V. vinifera*.

Many HPLC methods applied to the separation and quantification of ACY employed the use of a PhotoDiode Array Detector (DAD) for spectral analysis. Data generated from DAD analysis allows for the determination of the complete UV-Vis spectra of all types of compounds. In regards to ACY, important structural properties have been obtained, such as the nature of the aglycone, the position of the glycoside, and information of any attached aromatic acyl groups (Hong & Wrolstad, 1990). Research by Baranac et al. (1996) and Mazza and Brouillard (1987) investigated the structural transformations and reactions of anthocyanic compounds in aqueous solutions by studying spectrophotometric data. The ACY solvent has been a very important parameter in spectrophotometry and has required special consideration for reproducible results since ACY are dependent on pH for structural conformation and unstable in aqueous solutions. Most spectral data for ACY has been measured in a methanol solvent acidified with 0.01% HCl (Hong & Wrolstad, 1990).

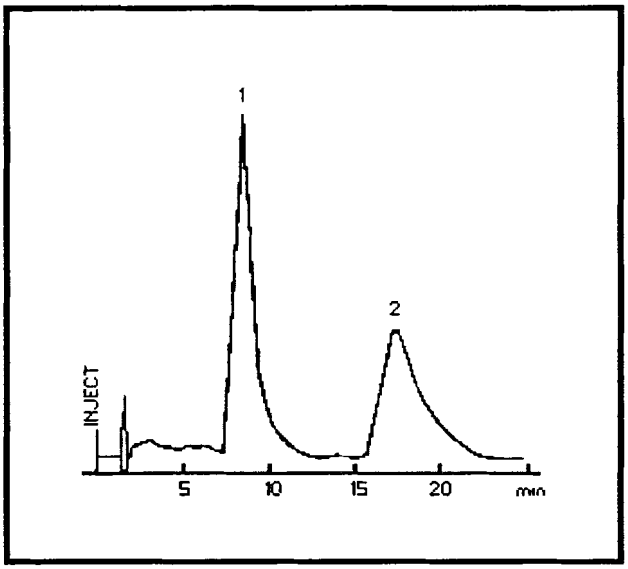
Qualitatively, the American Cranberry appears to have a much simpler ACY profile than other fruits in the *Vaccinium* genus, even the closely related Small Cranberry (*V. oxycoccus*). Only four major ACY with two aglycones are represented in the American Cranberry; whereas, studies conducted by Ballington et al. (1988 & 1987) on *V. crassifolium*, *V. sempervirens*, *V. ovatum*, *V. stamineum*, *V. erythrocarpum*, and seven species of blueberries reported complex profiles of up to 15 known ACY, with all of the six common aglycones except pelargonidin being present. The glycosides reported were the *O*-3-glucosides, -galactosides, and -arabinosides. Although acyl acid substituents were not mentioned in these papers, studies conducted by Gao and Mazza (1995 & 1994) on blueberries identified acetylated ACY in some high-bush and low-bush berries by means of acid hydrolysis and spectral analysis.

Several research articles have been dedicated to the cranberry – evaluating berry quality, breeding programs (Sapers et al., 1983a), juice recovery (Sapers et al., 1983b), individual and total ACY content (Sapers et al., 1987 & 1986, Hong & Wrolstad, 1990, Fuleki & Francis, 1968a & 1968b, and Andersen, 1989), fruit composition (Hong & Wrolstad, 1986), and processing effects (Sapers et al., 1983b).

Hong and Wrolstad (1986) conducted a thorough investigation on the composition of cranberry fruit, from titratable acidity and brix to spectral analysis and Hunter parameters to organic acid, sugar, and anthocyanidin content. Consistent with other researchers' results, Hong and Wrolstad (1986) reported the presence of two anthocyanidins, cyanidin (Cy) and peonidin (Pn). The chromatogram on the following page shows the peaks eluting at 8-9 minutes for Cy and 16-17 minutes for Pn (Figure 9). The peaks are broad, which may be a result of inadequate pH for the flavylium cation.

## FIGURE 9.

## LC CHROMATOGRAM OF CRANBERRY ANTHOCYANIDINS[a]




[a]Scanned from Hong & Wrolstad, 1986.
Peak Assignment: 1 – Cyanidin, 2 – Peonidin

Calculated as Cy-3-Glu, the content of Cy and Pn from eight popular cranberry varieties ranged from 7.6 to 20.5 mg/100g and 6.2 to 12.1 mg/100g, respectively. The average content of Cy and Pn was 12.3 and 8.9 mg/100g with percent coefficients of variance (%CV) equal to 38% and 25%, respectively. The average total anthocyanidin content was 21.2 mg/100g with a %CV of 31%. The percentages of Cy and Pn varied less than their individual contents among cultivars. Cy contributed 57% (9%CV), while Pn contributed 43% (12%). These percentages were inconsistent with later work conducted by the same researchers (Hong & Wrolstad, 1990) when they report 46% Cy and 54% Pn based on total peak area. Discrepancies were most likely due to differences in cranberry cultivar, extraction, available standards, and quantification methods.

Later work conducted by Hong and Wrolstad (1990) further analyzed cranberry pigments by looking at the individual ACY. In agreement with other research (Sapers et al., 1987 & 1983a), they found the cranberry contained the 3-galactosides and 3-arabinosides of Cy and Pn. Two minor peaks also present were believed to be the 3-glucosides of Cy and Pn. Values of individual and total ACY content varied in the literature, most likely due to differences in experimental design. Experiments led by Sapers et al. (1987 & 1986) took into account the variation in berry surface color, and they subdivided samples into light, medium, and dark berries since the highest concentration of pigment is located in the berry skin. Total ACY values reported ranged from 25.3-34.4 mg/100g for light subsamples, 38.1-62.7 mg/100g for medium subsamples, and 61.3-100.6 mg/100g for dark subsamples in the 1987 published article and 7-12 mg/100g for light subsamples, 15-37 mg/100g for medium subsamples, and 34-72 mg/100g for the dark subsamples as published in the 1986 article. Average total ACY contents from these two articles are listed in Table 4. Earlier work by Sapers et al. (1983a) obtained higher total ACY contents of 13 cranberry cultivars when analyzed by a tristimulus reflectance method. The range and average of total ACY content was 45.9-171.9 mg/100g and 86.3 mg/100g, respectively.

It was concluded that berry total ACY content decreased in proportion to the berry surface-to-volume ratio; and thus, was affected more by berry size than berry surface color (Sapers et al., 1986).

**TABLE 4.**

**AVERAGE ANTHOCYANIN CONTENT IN CRANBERRIES SORTED BY SURFACE COLOR**

| Color | 1987[a] | 1986[b] |
|---|---|---|
| Dark | 80.60[c] | 46.13 |
| Medium | 48.03 | 24.33 |
| Light | 28.40 | 8.50 |

[a] Average total ACY content within subsamples for 'Franklin,' 'McFarlin,' and 'Searles' cultivars in Sapers et al., 1987.
[b] Average total ACY content within subsamples for 'McFarlin,' 'Stevens,' and 'Early Black' cultivar in Sapers et al., 1986.
[c] Reported in mg/100g

Two articles published by Sapers et al. (1987 & 1983a) also looked at the individual proportions of ACY based on peak area. The results took into account a fifth unknown peak assumed to be Pn-3-Glu. The results are listed in Table 5.

**TABLE 5.**

**PROPORTIONS OF INDIVIDUAL ANTHOCYANINS IN CRANBERRIES**

| YEAR | Cy-3-Gal | Cy-3-Arab | Pn-3-Gal | Unknown | Pn-3-Arab |
|---|---|---|---|---|---|
| 1987[a] | 24.6 | 14.5 | 36.9 | 4.7 | 13.1 |
| 1983[b] | 25.8 | 15.6 | 40.1 | 3.1 | 15.2 |

[a] Individual proportions based on peak area percent as published in Sapers et al., 1987.
[b] Individual proportions based on peak area percent as published in Sapers et al., 1983a.

From Table 4 it can be seen that the total ACY content varied significantly for different subsamples of cranberries according to surface color. Data not shown but discussed by the researchers also showed that total ACY content varied greatly among

cultivars (Sapers et al., 1987, 1983a, & 1986). On the other hand, the proportions of individual ACY varied little among different species, which has importance for taxonomic purposes and cross-breeding studies, as well as determination of product adulteration.

García-Viguera et al. (1997), Hale and Fagerson (1986), and Goiffon et al. (1991) addressed the issue of adulteration in fruit products. García-Viguera et al. (1997) developed a technique to determine jam authenticity via ACY profiles, where previously other flavonoids had been used. Some manufactures have tried keeping the cost of cranberry juice down by adding less expensive fruit juices without affecting the quality of the final product. Hale et al. (1986) were able to separate a mixture of cranberry juice cocktail adulterated with grape enocyanin by HPLC. They were able to detect adulteration of the cranberry juice when just 5% of the enocyanin solution was added. Goiffon et al. (1991) performed HPLC analysis on the ACY of several red fruits to determine their profiles and the retention characteristics of different substituents in different mobile phases in order to establish references for manufactures. It was determined for analysis of ACY by RP-HPLC with weakly acidic media that the elution order of the aglycones was Del, Cy, Pet, Pel, Pn, and Mal. With identical aglycone moieties, the elution order of the 3-glycosides was -galactoside (Gal), -glucoside (Glu), -rutinoside, and -arabinoside (Arab) with some exceptions (Goiffon et al., 1991).

## Capillary Electrophoresis (CE)

The literature on the analysis of ACY by capillary electrophoresis (CE) is in its infancy with only a handful of articles in existence. The list is much longer for research conducted on flavonoids (Delgado et al., 1994; Seitz et al., 1992; Fernández de Simón et al., 1995; Morin et al., 1997; Gil et al., 1995; Ferreres et al., 1994; Ng et al., 1992; Morin & Dreux, 1993; and Boyce & Bennett, 1996), encompassing flavonols, flavones, flavanones and other plant phenolics. All of these flavonoid experiments employed a borate buffer at pH 7.5 or higher; hence, the initial research on ACY (Bridle & García-Viguera, 1997; Bridle et al., 1996) also applied a borate buffer with a pH near 8.0. As seen with HPLC applications, solvents and mobile phases were of acidic nature, as low as pH 1.8. The necessity for very low pH values when analyzing ACY has been explained: the flavylium cation is the predominant structure in acidic solution; it is the most stable structure; and it has the highest chromaphore absorbency. It was determined by Bridle and García-Viguera (1997) that a much larger sample concentration (87 times) was needed for an equivalent CE response at pH 8.0 compared to HPLC at pH 1.8. Figure 10 illustrates the differences in DAD spectra of pelargonidin-3-glucoside as determined in the study by Bridle and García-Viguera (1997).

**FIGURE 10.**

**SPECTRA OF PELARGONIDIN-3-GLUCOSIDE AT pH 1.8 AND pH 8.0[a]**


[a] Scanned from Bridle and García-Viguera, 1997.

Two of the CE articles reviewed followed the same analysis conditions, which are outlined in Table 6. At pH 8.0, the dominant colored species was the blue quinonoidal base. In borate buffer, it has a characteristic absorbency maximum in the range of 560-580 nm. The DAD was set to monitor at 560 nm and 580 nm for studies by Bridle and Gracía-Viguera (1997) and Bridle et al. (1996), respectively.

## TABLE 6.

## ANTHOCYANIN METHOD BY CAPILLARY ZONE ELECTROPHORESIS IN BASIC MEDIA*

| CAPILLARY | BUFFER | INJECTION | ELECTRIC FIELD |
|---|---|---|---|
| L=57 cm<br>l=50 cm<br>id.=75 μm | 150 mM NaBorate<br>pH 8.0 | Hydrodynamic<br>2 seconds<br>9 nL | 25 kV<br>~30 μA |
| **TEMPERATURE** | **CONDITIONING** | **BTWN RUNS** | **SAMPLE SOLVENT** |
| 25° C<br><br>**WAVELENGTH**<br><br>580 nm | MeOH, 5 min<br>1M NaOH, 5 min<br>0.1M NaOH, 5 min<br>$dH_2O$, 3 min<br>Buffer, 3 min | 0.1M NaOH, 3 min<br>$dH_2O$, 2 min<br>MeOH, 3:1 | 25 mM Phosphate<br>pH 2.5 |

* Adapted from Bridle and García-Viguera, 1997 and Bridle et al., 1996.

At pH 8.0, the silanol groups on the capillary wall were deprotonated and possessed a negative charge. The ACY were also carrying a negative charge; and therefore, did not interact with or adsorb on the capillary wall. The driving force behind the migration and resolution of the pigments was three-fold: first, and foremost, was the electro-osmotic flow (EOF), which has been the basis for almost all CE operations; second was the charge/mass ratio; and third was the selectivity obtained from borate complexation with ACY (Bridle et al., 1996). Despite the attraction of the negatively charged quinonoidal base for the anode (inlet end of the capillary), the net movement was toward the cathode and detector via the forces of the EOF. The charge/mass ratio for negative analytes correlated to larger massed species being detected before smaller-in-mass species with the same charge since they had less electrophoretic mobility toward the

anode (inlet end). ACY that complexed with borate in the buffer also experienced longer migration times since the negative charge was increased more than molecular mass of the complex. Borate complexation required a free ortho-dihydroxyl group on the ACY. Pigments with this characteristic are Cy, Del, and Pet. This has importance when analyzing cranberry pigments since they contain the glycosides of Cy, which has a free ortho-dihydroxyl group, and Pn, which does not.

Migration time was also affected by glycosylation (Bridle & Gracía-Viguera, 1997; Bridle et al., 1996). With increased glycosylation, there was a decrease in migration time, which followed the charge/mass ratio mechanism previously described. In the first study by Bridle et al. (1996) on work with ACY standards, the migration of analytes was Mal-3,5-diglucoside, Pel-3-Glu, Mal-3-Glu, Cy-3-Rutinoside, Cy-3-Glu, and Del-3-Glu. All peaks were baseline separated except for Pel-3-Glu and Mal-3-Glu, and the run time was just short of eight minutes.

In the second study by Bridle and García-Viguera (1997), the ACY in strawberries and elderberries were analyzed. Four major ACY in both fruits were separated. The order of detection was different than that by HPLC for the strawberry pigments, but the same for the elderberry pigments. For both analyses by CE, the run time was under six minutes. This CE method proved much more time efficient than the corresponding HPLC analyses, which took up to 25 minutes for the strawberry pigments.

## TABLE 7.

## ANTHOCYANIN METHOD BY CAPILLARY ZONE ELECTROPHORESIS IN ACIDIC MEDIA

| CAPILLARY[a] | BUFFER | INJECTION | ELECTRIC FIELD |
|---|---|---|---|
| L=75 cm<br>l=70.4 cm<br>id.=50 μm | 50 mM NaPhosphate<br>apparent pH 1.5<br>10-30% ACN<br>MeOH | Hydrostatic pressure<br>4 seconds | 25 kV<br><br>positive polarity |
| **TEMPERATURE**<br><br>20° C<br><br>**WAVELENGTH**<br><br>520 nm | **CONDITIONING**<br><br>not described | **BTWN RUNS**<br><br>Buffer, 1 min<br>at high pressure | **SAMPLE SOLVENT**<br><br>1g Blackcurrant powder + 25 mL water |

| CAPILLARY[b] | BUFFER | INJECTION | ELECTRIC FIELD |
|---|---|---|---|
| L=72 cm<br>id.=50 μm | 0.25 mM CTAB<br>160 mM NaPhosphate<br>pH 2.1 | Hydrodynamic<br>8 seconds<br>50 mbar | -25 kV<br>~-70 μA<br>negative polarity |
| **TEMPERATURE**<br><br>25° C<br><br>**WAVELENGTH**<br><br>520 nm | **CONDITIONING**<br><br>1 M NaOH, 10 min<br>0.1 M NaOH, 10 min<br>$H_2O$, 10 min<br>Buffer, 15 min | **BTWN RUNS**<br><br>0.1M NaOH, 3 min<br>$H_2O$, 3 min<br>Buffer, 5 min | **SAMPLE SOLVENT**<br><br>100 mM Citric Acid<br>25 mM NaPhosphate<br>pH 2.1<br>MeOH, 30% |

[a] CE conditions for ACY analysis from da Costa et al., 1998
[b] CE conditions for ACY analysis from Bicard et al., 1999

Two more recent studies analyzed ACY by CE under acidic conditions and are outlined in Table 7. The advantage of greater sensitivity at low pH values has been mentioned, but da Costa et al. (1998) also discussed the fact that at higher pH values (≥7) ACY rapidly degrade, making accurate quantitation difficult. Other advantages noted by the researchers were increased peak sharpness and improved resolution of the pigments

with decreasing buffer pH. This was at the cost of increased migration time. The migration time was significantly longer (>20 minutes) than that found under basic conditions. The explanation for this goes back to the forces of the EOF: as the pH of the buffer was decreased, the silanol groups on the capillary wall were progressively protonated, decreasing the wall charge and drag of the EOF. Under acidic conditions, the analytes migrated almost entirely under their own electrophoretic mobility. This was another important point of consideration when analyzing ACY by CE because, as previously mentioned, ACY possess different charges at different pH values. Under slightly acidic conditions, the predominant ACY forms are the neutral quinoidal bases, which do not migrate or resolve in the absence of the EOF.

In order to resolve the four ACY compounds in blackcurrants, an organic modifier was added to the running buffer. Trials of 10-30% acetonitrile (ACN) and MeOH were conducted. The best results were obtained with 30% ACN. The improvement in peak resolution and shape with the addition of an organic modifier was attributed to changes in the EOF and the analyte electrophoretic mobility, which in turn were due to changes in buffer pH (da Costa et al., 1998). Even the slightest change in buffer pH resulted in changing the apparent $pK_a$ of the analyte, which caused subsequent changes in solute-analyte interactions (da Costa et al., 1998).

The other study employing acidic conditions for the analysis of ACY used a quaternary amine, cetyltrimethylammoniumbromide (CTAB). CTAB is a long chain alkylammonium salt that has the ability of reversing the direction of the EOF when added to the running buffer. At levels well below its critical micellar concentration, CTAB coats the capillary wall and creates an overall positive charge. Reversing the EOF has

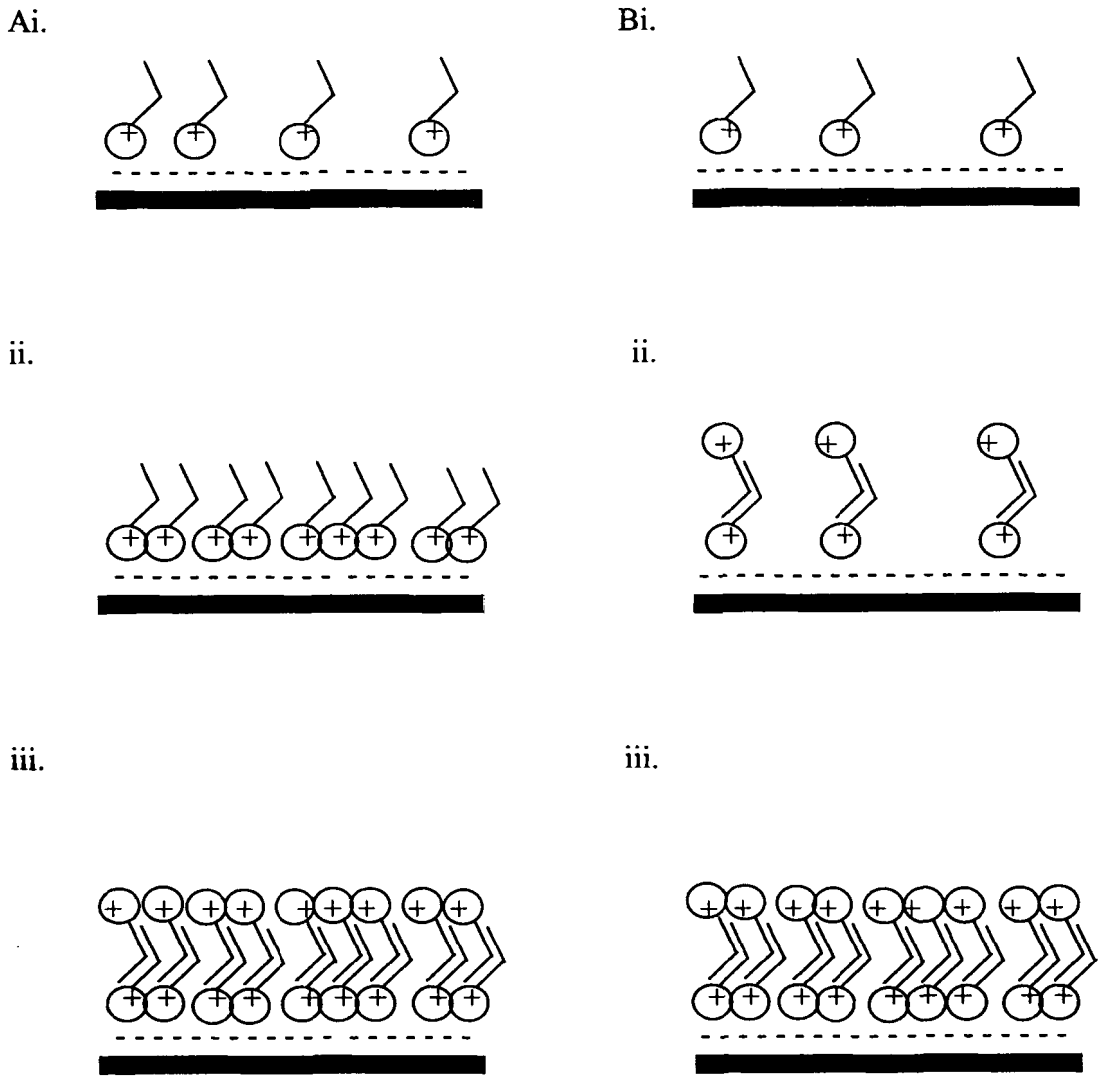
offered advantages in detecting negative analytes, which have shown the longest migration times under normal CE conditions. Lucy and Underhill (1996) investigated the mechanism behind CTAB's adsorption on to the capillary wall. They discussed two possible methods of how CTAB formed its bilayer on the capillary wall. Figure 11 shows a schematic of the proposed mechanisms, which are explained in the following paragraphs.

The first mechanism involved small concentrations of the surfactant that, because their positive heads were more attracted to the negative silanol groups of the capillary wall, they individually formed a single layer on the capillary (Figure 11 Ai-ii). As more surfactant was introduced in to the capillary, the long hydrophobic tails of the surfactant quickly lined up with those adsorbed to the capillary wall to form a second layer (Figure 11 Aiii). The positively charged heads of this second layer were then exposed on the capillary wall and gave it an overall positive charge.

The second mechanism proposed by Lucy and Underhill (1996) involved larger initial concentrations of the surfactant. In this case, the hydrophobic interactions of the long alkylammonium chains were stronger than the electrostatic attraction between the positive charge on the CTAB head and the negative charge on the capillary wall. Therefore, the surfactant existed in solution as hemimicelles. Initially, a few single molecules adhered to the wall as before through electrostatic attraction (Figure 11 Bi), but then the hemimicelles adsorbed on to the capillary wall solely through hydrophobic attraction (Figure 11 Bii-iii). The authors also described factors that affected these two processes: ionic strength, pH, and temperature.

## FIGURE 11.

## SCHEMATIC REPRESENTATION OF THE ADSORPTION OF CTAB ON THE CAPILLARY WALL*




* Redrawn from Lucy & Underhill (1996).

Bicard et al. (1999) explained that it was the hydrophobic interactions between the chromaphore and the alkyl chain of the CTAB and influence of the positive charge of the CTAB on the capillary wall that were responsible for the resolution of the colored flavylium cation in acidic media. The researchers experimented with slightly acidic run buffers (pH 3-4) without CTAB present, but detection of the flavylium cation was not found at any wavelength. They offered the explanation that the cation was adsorbed on the capillary wall via ionic interactions with the negative silanoate groups. Thus, the cationic surfactant was employed to eliminate wall-analyte interactions. Under these conditions, three Cy- and one Mal- glycosides from different natural sources were able to be analyzed.

## ORGANIC ACID CONTENT

Organic acids, such as citric, malic, quinic, benzoic, and ascorbic acid, which are the predominant acids in the American Cranberry, play an important role in maintaining the quality of a wide variety of fruits and vegetables. The presence and quantity of acids can indicate the quality and properties of a product and can reflect contamination or adulteration. Organic acids also offer nutritional value (L-ascorbic acid), flavor, color, and other functions to food and food components. In general, organic acids are weak, non-oxidizing compounds. Citric and malic are the most abundant organic acids in plants, making up to 3% of tissue on a fresh weight basis.

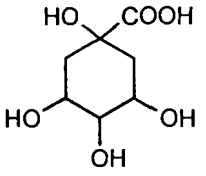
## Structure

The structures of the five major acids present in cranberries are shown in Figure 12. It can be seen that these compounds are small and relatively simple in configuration, but as with ACY these acids owe their different functions to their structure. Ascorbic acid possesses antioxidant activity making it an important health contributor. It also spares the degradation of other food components. Benzoic acid has shown antifungal properties; thus, offering natural protection for food safety and keeping quality. It offers maximum activity at pH 2.5-4.0. Kraemer-Schafhalter et al. (1998) described citric acid as a chelator of metal ions, which may have application in the protection of ACY during processing. Malic acid offers several flavoring characteristics to food. It has taste-blending characteristics, aftertaste masking abilities, as well as flavor-fixing qualities, which hold onto essential essences (Malic Attributes, 1999). There are probably other benefits and functions of organic acids that remain undiscovered.

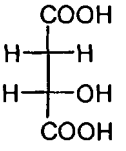
All of these acids contribute tart flavors to fruits and some vegetables. The $pK_a$ values of individual acids vary, depending on the number of carboxylic acid and hydroxyl groups present. Table 8 lists the $pK_a'$ values for the acids found in cranberries.

## FIGURE 12.

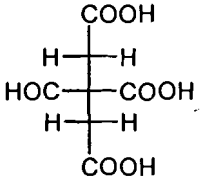
## STURCTURE OF THE FIVE MAJOR ORGANIC ACIDS IN CRANBERRIES



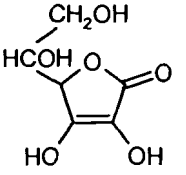

QUINIC ACID



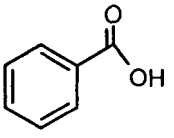

MALIC ACID




CITRIC ACID




L-ASCORBIC ACID




BENZOIC ACID

## TABLE 8.

## $pK_a$ VALUES FOR THE MAJOR ORGANIC ACIDS IN CRANBERRIES

| ACID | $pK_a$ VALUE | | |
|---|---|---|---|
| | $pK_{a1}$ | $pK_{a2}$ | $pK_{a3}$ |
| L-ascorbic | 4.2 | 11.6 | |
| Benzoic | 4.2 | | |
| Citric | 3.1 | 4.8 | 6.4 |
| Malic | 3.4 | 5.2 | |
| Quinic | 4.9 | | |

## Analytical Methods for Organic Acids

Organic acids have been analyzed by a variety of methods: ion-exchange column chromatography, thin-layer and paper chromatography, spectrophotometry, gas-liquid chromatography, RP-HPLC and CE, and on a variety of foods: grapes (López & Gómez, 1996; Hunter et al., 1991), pineapples (Bartolomé et al., 1996), raspberries (Riaz & Bushway, 1994), fruit juices (Coppola et al., 1978; Blanco et al., 1996), potatoes (Bushway et al., 1984), and other fruits or vegetables (Nisperos-Carriedo et al., 1992; Kovács & Djedjro, 1994). The articles I reviewed come solely from the RP-HPLC category. Improvements in column technology have been made for the analysis of organic acids since the acidic content of the mobile phase has resulted in column degradation. There are now columns that are able to withstand mobile phases with pH values less than 2.0. This is important when considering the range of p$K_a$ values of different acids and stabilizing individual acids in either their protonated or deprotonated state..

The extraction procedure for organic acids can be extremely easy or very difficult, depending on the food matrix. For solid foods, either a dilute acid (phosphoric or sulfuric) or an organic solvent (EtOH or MeOH) or both have been used. The complexity of the extraction exists when samples are subjected to ion-exchange columns and/or Sep-Paks® for clean up. The ion-exchange columns are laborious and time consuming since they are not reusable and must be made fresh for each sample analyzed.

The mobile phase plays an important role in keeping each of the different acids in a stable ionized state, usually the protonated state to minimize analyte-column

interactions. Therefore, many solvents have consisted of concentrated acids; namely phosphate and sulfate. Some methods have been developed to isocratically analyze organic acids as well as other food components (López & Gómez. 1996). This has been made possible through the involvement of both UV and refractive index detection. Most other methods employed UV detection only, monitoring at 214 nm or 245 nm for vitamin C.

Three of the articles reviewed specifically addressed the organic acid content in cranberries. Coppola et al. (1978) determined the mean content of individual acids in cranberry juice was 1.32%, 0.92%, and 1.08% for quinic, malic, and citric, respectively. The analysis time for all three acids was under 12 minutes. Acids eluted according to increasing polarity with quinic acid (less polar) eluting first and citric acid (more polar) eluting last (Coppola et al., 1978). These results were in close agreement with later work by Hong and Wrolstad (1986), who also reported mean percent of total acids: quinic 39.2%, malic 27.2%, citric 32.4%, and shikimic 1.2%. Shikimic is an acid metabolically related to quinic acid.

Later work conducted by Coppola (1984) exploited chromatographic analysis of acid content in juices for the purpose of monitoring authenticity. Cranberry juice adulteration can be monitored easily by looking at the ratio of citric to quinic. Although these acids are present in other fruits, quinic acid is characteristically higher in cranberries and their juice than other fruits. Other methods exist for monitoring adulteration, such as sugar or anthocyanin analysis. Sometimes these methods are used in conjunction with one another because of varietal differences within classes of fruits and vegetables.

# MATERIALS & METHODS

The cranberries used in this study were generously given to the Department of Food Science & Human Nutrition at the University of Maine by John Harker, an independent grower in Southern Maine. The berries were received cleaned and individually packaged in 16 slotted plastic containers. Sample sizes of the 16 cultivars were small and varied, requiring the entire sample to be macerated for homogeneity. Sample remaining after the analysis on fresh cranberries was packaged and sealed in Ziploc freezer-storage bags and stored in the freezer until ready for further analysis. When sample size allowed, samples were analyzed in triplicate. Within a week of delivery, entire samples of the berries were macerated in a Cuisinart (Cuisinarts Corp.; East Windsor, NJ) and immediately examined for moisture and L-AA content. Samples for ACY and organic acid content were prepared in their respective extraction solvents and refrigerated until analysis was completed for moisture and L-AA.

Anthocyanidin analysis was carried out on frozen cranberries only for the 1998 cranberry cultivars. Cranberries from the 1999 harvest were also supplied and analyzed for ACY content only. The berries were fresh and extracted within two days. Unlike the 1998 cranberries, the 1999 berries were not held in solvent for any longer than the extraction process. CE analysis of anthocyanidins was carried out on commercial cranberries. The extracted pigments were then injected into the HPLC for comparison and correlation between the developed CE method and the established HPLC method for the analysis of anthocyanidins.

The following chemicals were supplied from EM Science (Gibbstown, NJ): sulfuric acid, HPLC grade water, HPLC grade MeOH, HPLC grade ACN, hydrochloric acid, ammonium hydroxide, and glacial acetic acid. Chemicals from Fisher Scientific Co. (Fair Lawn, NJ) included 2-propanol, n-propanol, potassium phosphate, and phosphoric acid. Chemicals from J.T. Baker Chemical Co. (Phillipsburg, NJ) included meta-phosphoric acid and sodium bicarbonate. L-ascorbic acid and 2,6-dichloroindophenol sodium salt were supplied from Sigma Chemicals (St. Louis, MO), and ACY standards were from Indofine Chemical Co. (Somerville, NJ). The C18 Sep-Pak® Plus cartridges came from Waters (Milford, MA), and the deionized water ($dH_2O$) was made by glass distillation in the Food Science Analytical Chemistry Lab (Orono, ME).

## ANALYSES PERFORMED

The fresh and frozen harvested cranberries were analyzed for the following: anthocyanin content, anthocyanidin content, organic acid content (citric, malic, quinic, and ascorbic acids), and moisture content. The 1999 harvested berries were analyzed for ACY content only.

## Anthocyanin Extraction

The extraction procedure for ACY was adapted from Sapers et al. (1983a). Fresh cranberries were macerated in a Cuisinart. In triplicate, 5-gram samples were weighed out into cylindrical tubes and wrapped with aluminum foil to block light. Approximately 20g of extraction solvent (95% EtOH:1.5 M HCl 85:15) was added to the berries and then polytroned for three minutes at 45 x 100 rpm. The slurry was then centrifuged for 10-12 minutes at 15,000 x g. The supernatant was collected into an amber beaker. The residue was extracted two more times with approximately 20g of solvent. All three supernatants were combined and a 10:1 dilution was made with $dH_2O$. This mixture was then subjected to a clean up step by loading it onto a C18 Sep-Pak®, activated with 5ml of 2-propanol and 10ml of $dH_2O$. The pigments were then eluted with 2ml of 1% HCl in MeOH. Samples were not filtered prior to injection into the High Performance Liquid Chromatograph (HPLC) due to loss of pigment on the filter paper.

## HPLC Conditions for Anthocyanin Analysis

Samples were analyzed on an HP 1050 HPLC system equipped with an HP 1050 series isocratic pump and an HP 1040A PhotoDiode Array Detector (Hewlett-Packard Co.; Avondale, PA). The column used was a Columbus C18; 150x4.6mm; 5μm (Phenomenex,; Torrance, CA). The mobile phase consisted of HPLC grade water, acetonitrile, acetic acid, and phosphoric acid in the ratio 81.7:8.4:8.4:1.5. The volume of the sample injected was 10μl, and flow rate was 1.0 ml/minute. The DAD was set to

monitor at a wavelength of 520 nm. Each sample was injected once with a standard injected after every third sample. The temperature of the room reflected the temperature of the HPLC system; and therefore, it was difficult to control. One standard (Cy-3-Gal) was made up in 1% HCl in MeOH at a concentration of approximately 0.01mg/mL. Sample calculations were based on peak areas.

## Acid Hydrolysis of Anthocyanins

The extraction procedure for isolating the two anthocyanidins in cranberries followed that for anthocyanins up to and including the step combining the three successive extraction supernatants. After this step, 3mL of supernatant were combined with 15mL of 1% HCl in MeOH in an amber, flat-bottomed round flask. The flask was weighted down and placed in a hot water bath (~100°C) for 30 minutes. The flask was then put immediately into a cold water bath until cool enough to be handled. The dark liquid was then loaded onto an activated C18 Sep-Pak® (5ml 2-propanol, 10ml $dH_2O$), eluted with 1% HCl in MeOH, and injected onto the HPLC.

## HPLC Conditions for Anthocyanidin Analysis

HPLC analysis for anthocyanidins was similar to that of the anthocyanins except for the mobile phase. More organic solvent was needed to resolve the peaks and maintain efficient retention times. The ratio of the mobile phase used was 70:20:7.5:1.5 (HPLC grade water:acetonitrile:acetic acid:phosphoric acid). The two standards were made up in

1% HCl in MeOH at a concentration of approximately 0.01mg/mL. Calculations were based on peak areas.

## CE Conditions for Anthocyanidin Analysis

Anthocyanidin content for cranberries was analyzed on an HP 3D Capillary Electrophoresis System with an HP ChemStation and an on-line DAD detector (Hewlett-Packard Co.; Waldbronn, Germany). The capillary dimensions were 48.5cm with an effective length of 48.0cm, an inner diameter of 75μm, a bubble factor of 2.7, and an optical path length of 200μm. The system was conditioned each day with 0.1M phosphoric acid for 15 minutes at 40°C, HPLC grade water for 15 minutes at 40°C, and running buffer for 15 minutes at 27°C. The composition of the running buffer was 150mM phosphoric acid, 50mM β-cyclodextrin, and 3M urea with a pH of ~2.11. Hydrodynamic injection involved applying 50mBar of pressure for five seconds. The system was then run at a constant voltage of 20kV, which resulted in a current of approximately 110μA. The temperature remained steady at the specified 27°C. The DAD was set to monitor a wavelength of 525nm. Impurities were expelled from the capillary during the post-conditioning, which involved flushing the capillary with 0.1M phosphoric acid for five minutes, HPLC grade water for five minutes, and then the run buffer for five minutes. All post-conditioning rinses were at 27°C. The buffer vials, as well as the post-conditioning vials, were replenished after every fourth run to maintain a consistent pH.

## Organic Acid Extraction

The extraction procedure for organic acids was adapted from that of Bushway et al. (1984) and Riaz and Bushway (1994). Fresh cranberries were again macerated in a Cuisinart, and three 12.5g samples were put in a conical centrifuge tube along with approximately 25g of solvent (95% EtOH:$H_2O$:$H_2SO_4$ 60:40:0.2). This mixture was polytroned for four minutes at 55 x 100 rpm. The supernatant was collected by vacuum filtration. A 2mL aliquot was taken, dried under nitrogen, and reconstituted in 3mL of $dH_2O$. The pH was adjusted to 6-7 with 5% $NH_4OH$ and then put on an ion-exchange column. The column was prepared by packing it with a 2mL slurry of a Bio-Rad #5 Anion Exchange Resin (100-200 mesh) (Bio-Rad Laboratories; Richmond, CA) and capping it with a frit. The resin was good for only one application.

Before applying the sample, the resin was rinsed with 3mL of $dH_2O$. The sample was applied and then washed with 3mL of $dH_2O$. The acids were eluted with 3.5mL of 10% $H_2SO_4$ and 3.5mL of $dH_2O$. This approximate 7mL volume was then passed through an activated C18 Sep-Pak® for further clean up. It was activated with 5mL 95% EtOH and 5mL $dH_2O$. Samples were filtered prior to injection on the HPLC.

## HPLC Conditions for Organic Acid Analysis

Samples were analyzed on the same HPLC system as the ACY. The RP column was a BetaBasic 18; 250x4.6 mm; 5mm (Keystone Scientific, Inc.; Bellefonte, PA). The mobile phase was adapted from an article in BASELINE Chromatography Newsletter by

Keystone Scientific, Inc. (1998) and consisted of 0.05M $KH_2PO_4$, 0.03M $H_3PO_4$, and 0.1% n-Propanol. The flow rate was 1.0 mL/min; injection volume was 10.0μL; and the DAD was set to monitor at 214nm. Standards were prepared according to the method outlined by Bushway et al. (1984).

## L-Ascorbic Acid Extraction

L-Ascorbic acid was extracted from fresh cranberries by polytroning a 10g sample and approximately 20g of 3% meta-phosphoric acid for three minutes at 55 x 100 rpm in a conical centrifuge cone wrapped in aluminum foil. The supernatant was collected by vacuum filtration. A 5mL aliquot of the supernatant was diluted with 10mL of 3% meta-phosphoric acid. This was then passed through an activated C18 Sep-Pak® (4mL MeOH, 10mL $dH_2O$, and 10mL sample). A 5mL aliquot was collected after the initial 2mL were allowed to pass through the Sep-Pak®.

## Quantification of L-Ascorbic Acid

The AOAC method of titration was used to determine the quantity of L-ascorbic acid in the sample. The amount of titrant needed for the sample analysis was compared to a standard curve, which was also described in the AOAC method for vitamin C determination.

## Moisture Content Determination

The moisture content of cranberries was determined by weighing a 2g sample into a pre-weighed crucible and drying it for 14 hours in a forced air oven (Fisher Isotemp® Oven Model 350) at 105°C. The sample and container were allowed to cool in a desiccator before reweighing.

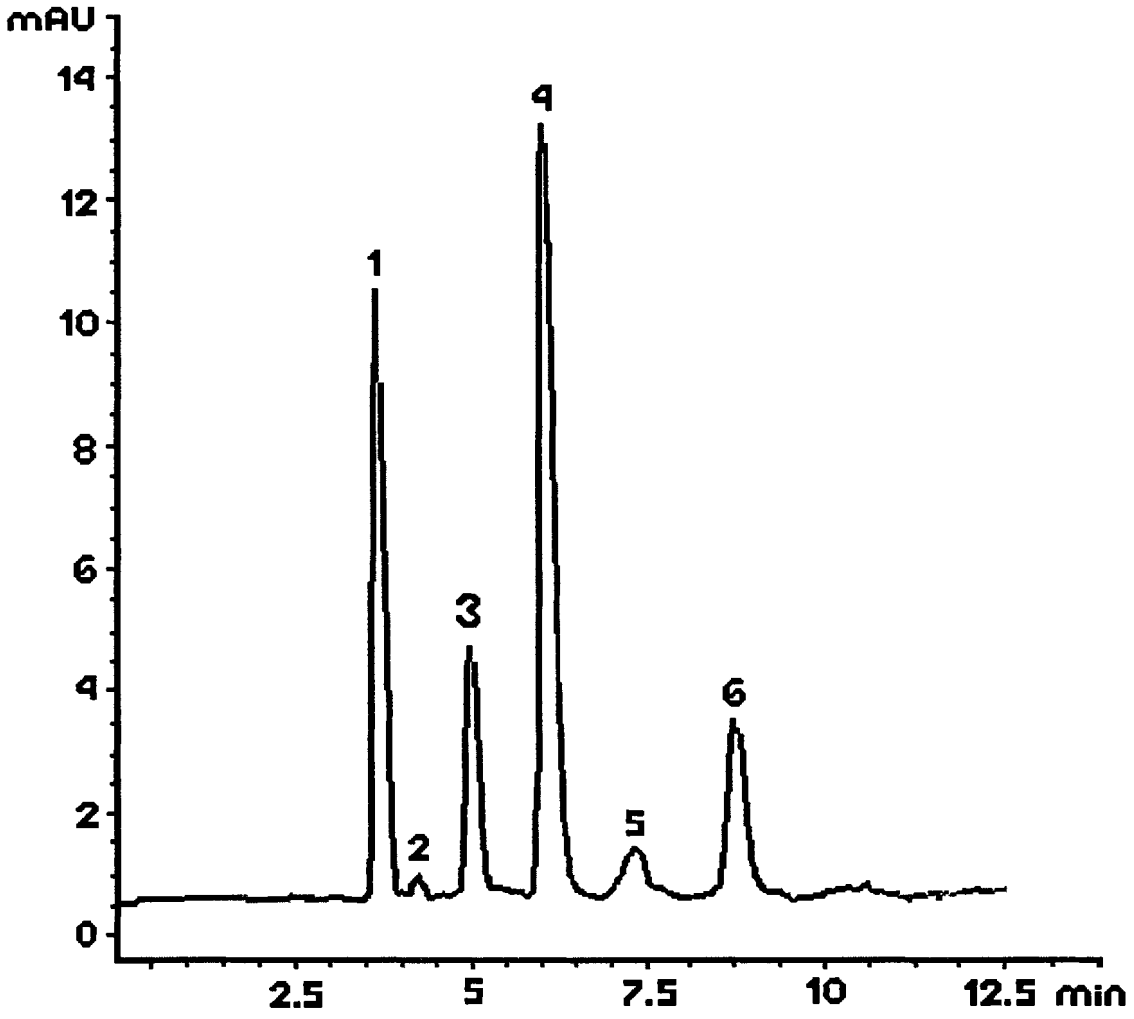
# RESULTS & DISCUSSION

## ANTHOCYANIN & ANTHOCYANIDIN CONTENT

In this study, cranberry pigments were analyzed in both the fresh and frozen state by HPLC. Of the 16 original cultivars from the 1998 harvest, 14 were analyzed in the fresh state and 13 in the frozen due to small sample size and sample depletion for other analytical testing. All cultivars showed similar ACY profiles, but varied in content. The ACY profiles of the cranberries were consistent with those reported in the literature, which have been discussed earlier in this paper. A typical chromatogram of the purified pigment extract is illustrated in Figure 13. Unlike its close relative, the Small Cranberry, which has the *O*-3-glucosides of cyanidin (Cy), peonidin (Pn), delphinidin (Del), petunidin (Pet), and malvidin (Mal) (Andersen, 1989), the American Cranberry has only the *O*-3-glucosides of Cy and Pn.

FIGURE 13.

HPLC CHROMATOGRAM OF CRANBERRY ANTHOCYANINS




Peak assignment: 1 – Cy-3-Gal, 2 – Unknown, 3 – Cy-3-Arab,
4 – Pn-3-Gal, 5 – Unknown, 6 – Pn-3-Arab.

The chromatogram above shows the detection of four major peaks (1,3,4,6) and two minor peaks (2, 5). The analysis time was just under 12 minutes. The average retention times for individual ACY, along with %CV values are listed in Table 9. The small %CV values for individual retention times indicated the reproducibility of this

method from run to run and day to day (Table 9). Retention times for the individual ACY on any of the dates shown in Table 9 varied less than 20%.

Listed in Table 10 are the absorbency maxima, collected by DAD. The absorbency spectra provided insurance that the compounds detected were indeed anthocyanins, based on the characteristic spectra of anthocyanic compounds reported in the literature. ACY with attached glycosides in the 3-position followed the typical

**TABLE 9.**

**AVERAGE RETENTION TIMES (IN MINUTES) FOR CRANBERRY ANTHOCYANINS BY HPLC**

| Date | Cy-3-Gal | Cy-3-Arab | Pn-3-Gal | Pn-3-Arab | Stats |
|---|---|---|---|---|---|
| All Dates | 105 | 105 | 105 | 105 | # of cases |
| | 3.864 | 5.200 | 6.355 | 9.393 | Mean |
| | 15.0 | 12.6 | 14.9 | 12.3 | % CV |
| 11/17/98 | 38 | 38 | 38 | 38 | # of cases |
| | 3.622 | 4.901 | 5.862 | 8.907 | Mean |
| | 7.2 | 7.1 | 13.0 | 7.4 | % CV |
| 1/23/99 | 32 | 32 | 32 | 32 | # of cases |
| | 3.773 | 4.886 | 5.963 | 8.631 | Mean |
| | 17.6 | 8.7 | 7.5 | 5.9 | % CV |
| 7/17/99 | 11 | 11 | 11 | 11 | # of cases |
| | 3.989 | 5.874 | 7.649 | 11.258 | Mean |
| | 9.9 | 5.7 | 9.4 | 7.4 | % CV |
| 11/11/99 | 14 | 14 | 14 | 14 | # of cases |
| | 4.557 | 6.145 | 7.467 | 10.832 | Mean |
| | 14.8 | 11.8 | 10.0 | 6.8 | % CV |
| 11/13/99 | 10 | 10 | 10 | 10 | # of cases |
| | 3.966 | 5.278 | 6.498 | 9.615 | Mean |
| | 11.7 | 9.3 | 9.6 | 8.4 | % CV |

**TABLE 10.**

**UV-Vis MAXIMA OF CRANBERRY ANTHOCYANINS & AGLYCONES**

| ANTHOCYANIN | UV-Vis MAXIMA (nm) |
|---|---|
| Cy-3-Gal | 280, 520[a] |
| Cy-3-Arab | 285, 518 |
| Pn-3-Gal | 280, 520 |
| Pn-3-Arab | 285, 518 |
| Cyanidin | 275, 525[b] |
| Peonidin | 275, 525 |

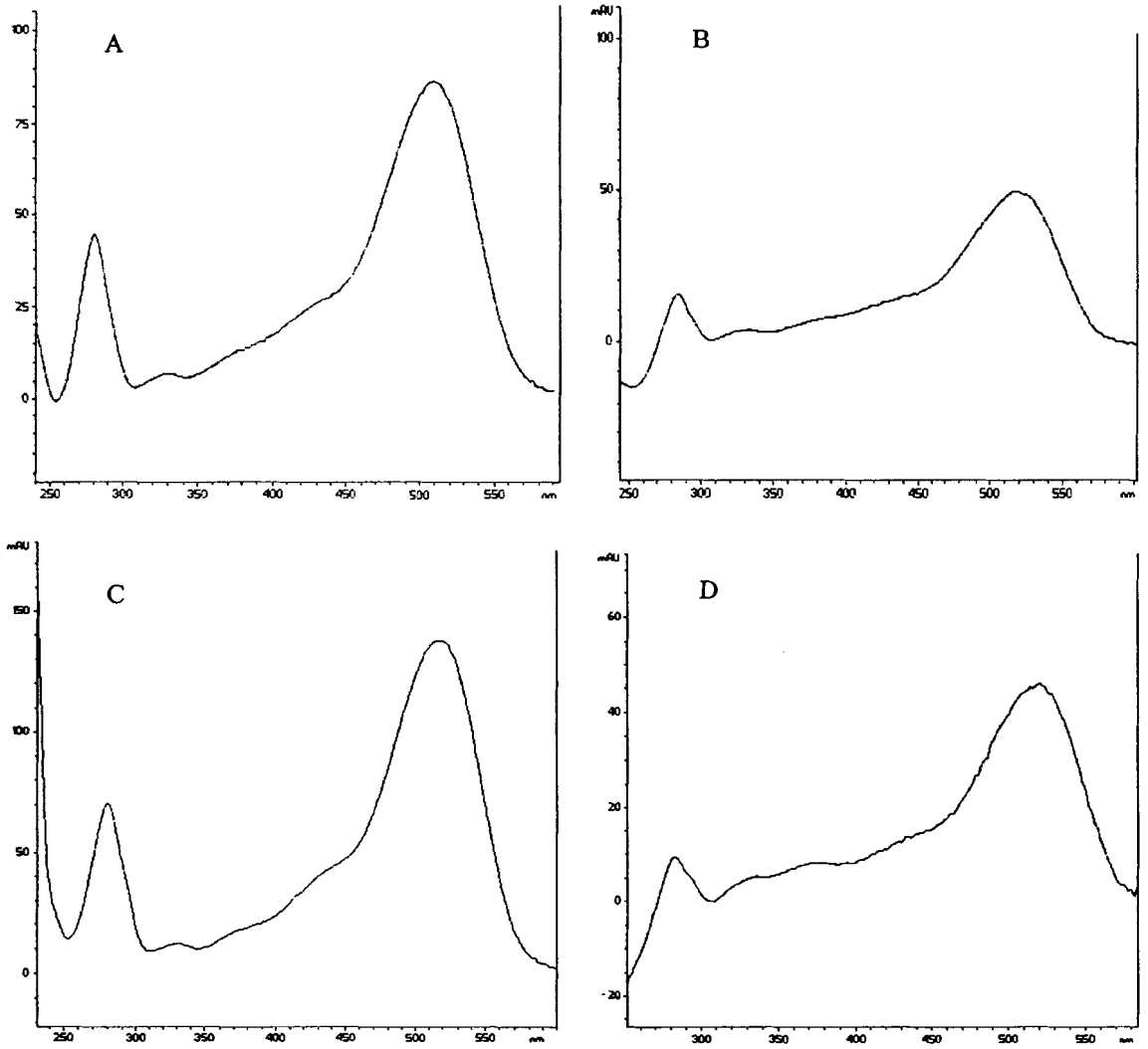
[a] In $H_2O$:ACN:Acetic Acid:Phosphoric Acid (81.7:8.4:8.4:1.5) pH<2.0.
[b] In $H_2O$:ACN:Acetic Acid:Phosphoric Acid (70:20:8.5:1.5) pH<2.0.

spectra shown in Figure 14a-d. Slight differences in the absorbency maxima existed with the attachment of different sugar moieties. The galactosides of both Cy and Pn expressed maxima at 280 and 520 nm; whereas, the arabinosides absorbed at 285 and 518 nm. These values differed even further from the individual aglycones, which showed maxima at 275 and 525 nm.

Besides spectral analysis, peaks were identified by comparing retention times of peaks from previous studies (Sapers et al., 1983a; Sapers & Hargrave, 1987) and one available standard, Cy-3-Gal. The four major peaks were identified as Cy-3-Gal, Cy-3-Arab, Pn-3-Gal, and Pn-3-Arab. Due to the lack of standards, results were reported in Cy-3-Gal equivalents. One cultivar, which was yellow, showed no ACY detection. The results from this yellow cultivar were not included in any of the calculations for total ACY content or determination of significant differences between cultivars.

## FIGURE 14.

## SPECTRA OF THE FOUR MAJOR ANTHOCYANINS IN CRANBERRIES*

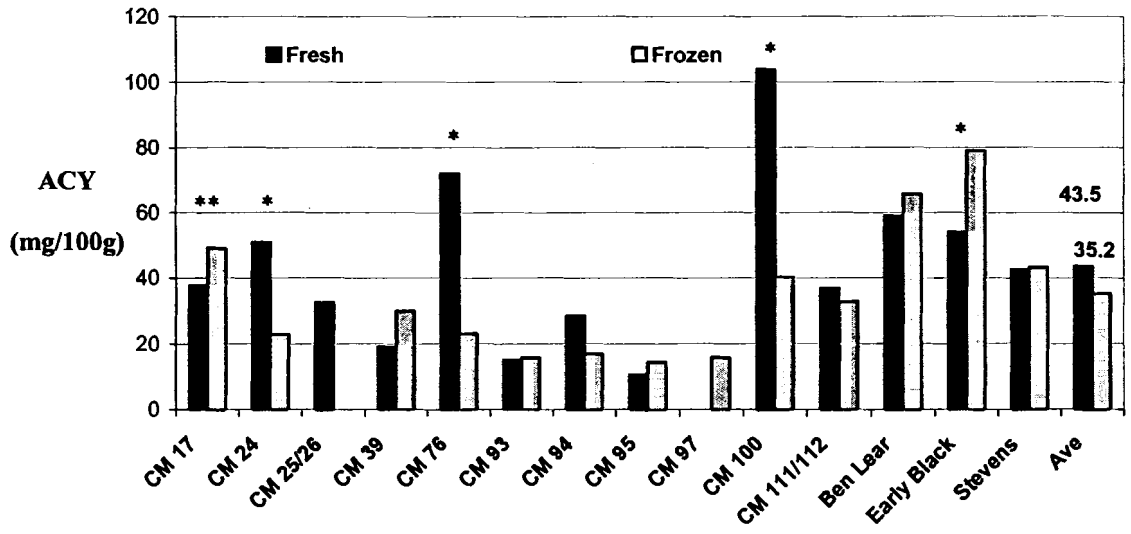



* In $H_2O$:ACN:Acetic Acid:Phosphoric Acid (81.7:8.4:8.4:1.5) pH < 2.0.
[A] Cy-3-Gal
[B] Cy-3-Arab
[C] Pn-3-Gal
[D] Pn-3-Arab

As previously mentioned, the content of ACY varied greatly among different cultivars. This was evident by the large %CV values obtained when all data entries were compiled. The %CV was 59.8% (bases on 38 samples) for fresh total ACY and 64.4% (32 samples) for frozen total ACY. The content of individual ACY also varied greatly among cultivars with a range of %CVs from 56.2-104.9% (both fresh and frozen samples considered). The total ACY content of the four major peaks ranged from 9.64 to 126.05 mg/100g for fresh samples and 13.11 to 94.48 mg/100g for frozen samples (Figure 15). The mean content of the four major peaks was 43.49 and 35.17 mg/100g for fresh and frozen samples, respectively. The cultivar labeled 'CM 100' expressed the highest average ACY content for fresh samples with 103.85 mg/100g; whereas, 'Early Black' had the highest content for the frozen samples with 79.12 mg/100g. Cultivar 'CM 95' showed the lowest average ACY content for both fresh and frozen samples with 10.76 and 14.47 mg/100g, respectively. These values fell within the ranges reported in other studies (Sapers & Hargrave, 1987; Sapers et al., 1986; Sapers et al., 1983a; Fuleki & Francis, 1968b).

FIGURE 15.

TOTAL ACY CONTENT
IN FRESH & FROZEN WILD MAINE CRANBERRIES[a]




[a] Total content reflects the content of the four major ACY. 1998 harvest.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test for MC 17, CM 25/26, CM 95, CM 97, CM 111/112.

I would like to point out four of the cultivars in Figure 15: 'CM 24', 'CM 76', 'CM 94', and 'CM 100.' These cultivars showed large differences in their total ACY content between the fresh and frozen states. Consequently, they were the first samples extracted and followed the original dilution factors of 1:50 (sample:water) ('CM 76') and 1:25 ('CM 24', 'CM 94', 'CM 100'). These proved to be too dilute and resulted in a very weakly colored solution. The rest of the fresh samples and all of the frozen samples were diluted 1:10. The calculations for total ACY content accounted for the dilution factor, but not the large difference between the fresh and frozen samples for these four cultivars. It has been well documented that ACY do not follow the Beer-Lambert law where

absorbency increases linearly with concentration. But this does not explain why the more dilute samples were higher in content then the less dilute samples. If anything, it should be the reverse for a couple of reasons: 1) as pigment concentration increases the color may increase more than proportionally (Jackman et al.; 1987), and 2) in weakly acidic solutions of water, ACY are unstable and will degrade under nucleophilic attack; thus, decreasing their color intensity. This would be an interesting issue to look into further. It was also noteworthy to point out that if these four pairs of means were set aside, there would be only one significant difference in mean total ACY content from fresh to frozen berries. The frozen berries for 'Early Black' showed higher total ACY content than fresh 'Early Black' berries.

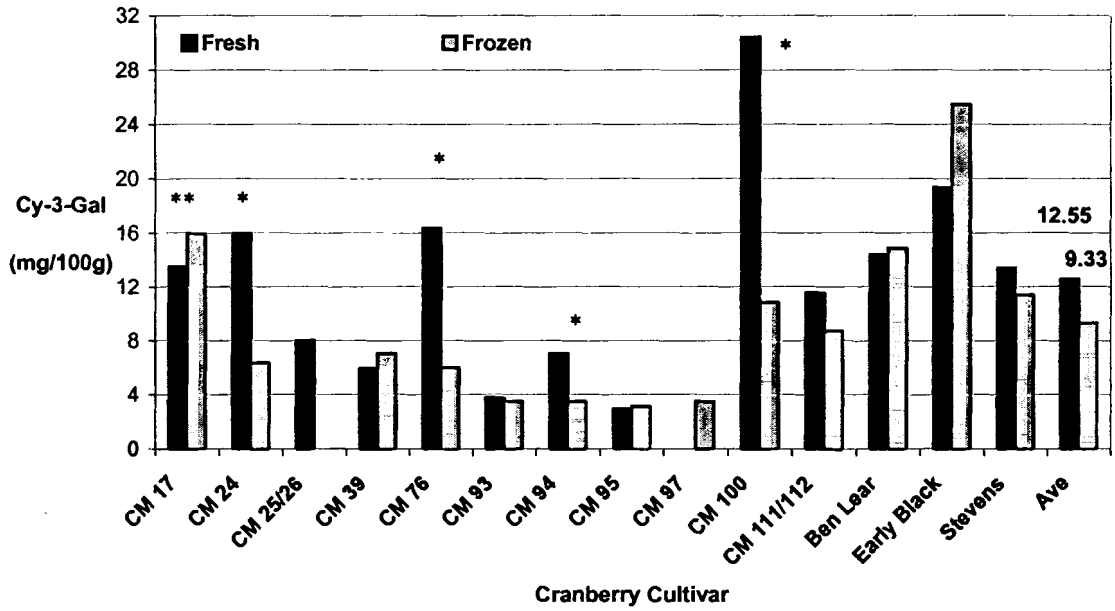
An increase in ACY content was expected from the fresh to frozen samples due to an increase in extraction caused by cell wall rupture and continued ACY production in the frozen state. This was not the case in this study, as evident from Figure 15. Possible reasons may be that the storage time and/or temperature were not adequate for further ACY development, or because of the presence and activity of degradative enzymes in macerated berries, allowing enzyme and ACY to come in contact with each other.

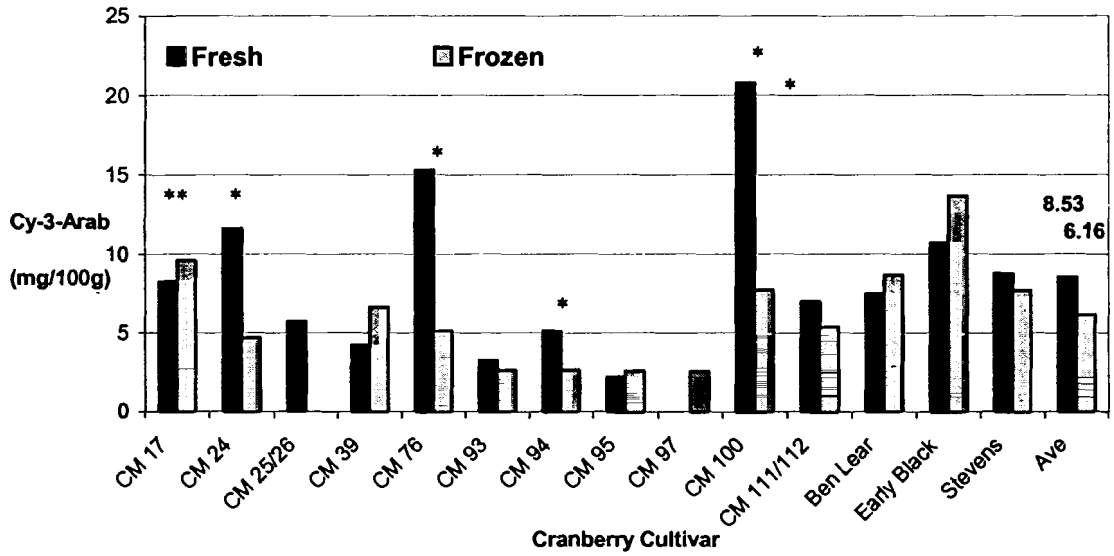
There were slight increases in total ACY content for five of the sixteen frozen cultivars, but they were not significant ($p<0.05$). The average content of individual ACY followed a similar pattern as the average total content in Figure 15. Graphs for individual ACY contents are shown in Figure 16. Significant differences existed ($p<0.05$). Pairs of bars with asterisks (*) were significantly different as computed with the two-tailed t-test. As with the average total content, the individual ACY content of the berries also varied greatly between cultivars. Figure 17 represents the sorted data of individual ACY to

facilitate distinguishing significant differences (data for frozen berries not shown). Bars with different letters were significantly different, determined by one-way ANOVA and Tukey's Multiple Comparison Test ($p<0.05$).

## FIGURE 16.

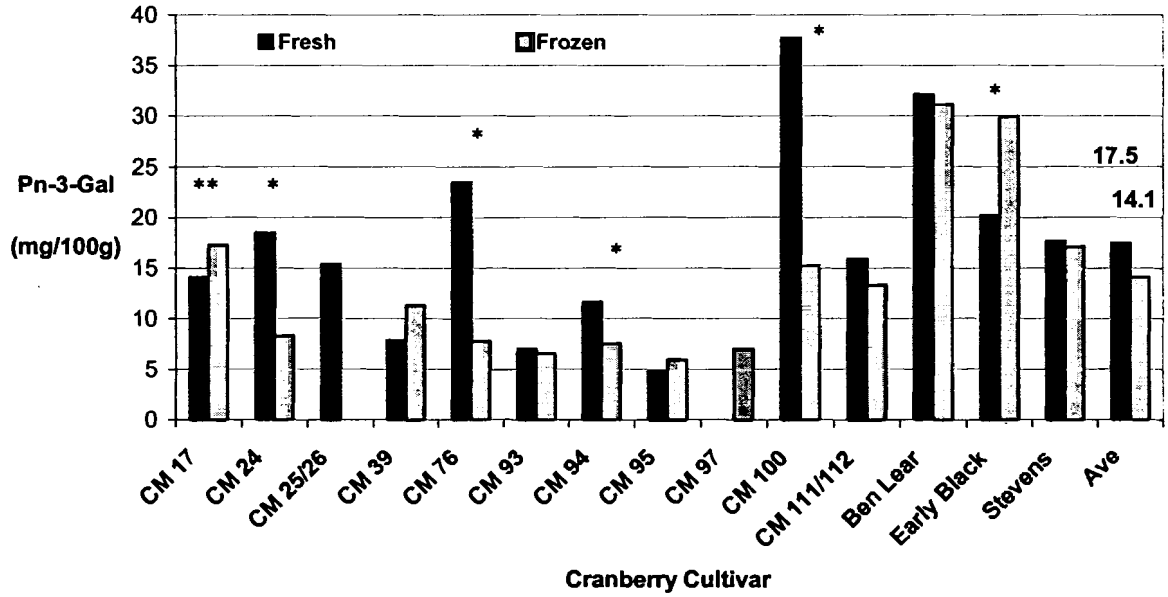
## INDIVIDUAL ACY CONTENTS IN FRESH & FROZEN WILD MAINE CRANBERRIES[a]

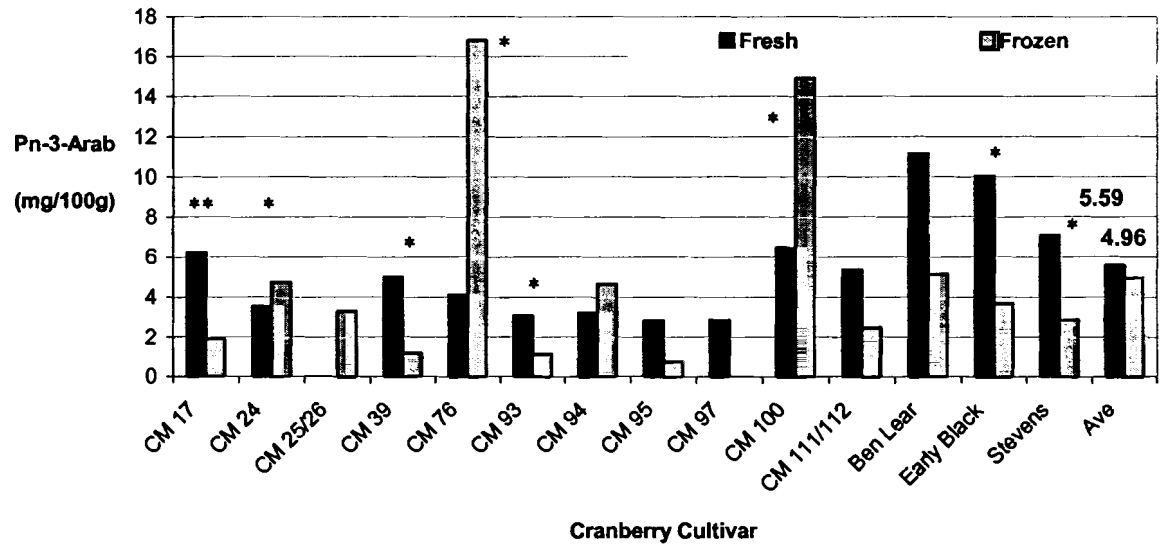



[a] 1998 Harvest.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test for CM17, CM25/26, CM95, CM97, CM111/112.

## FIGURE 16. CONTINUED

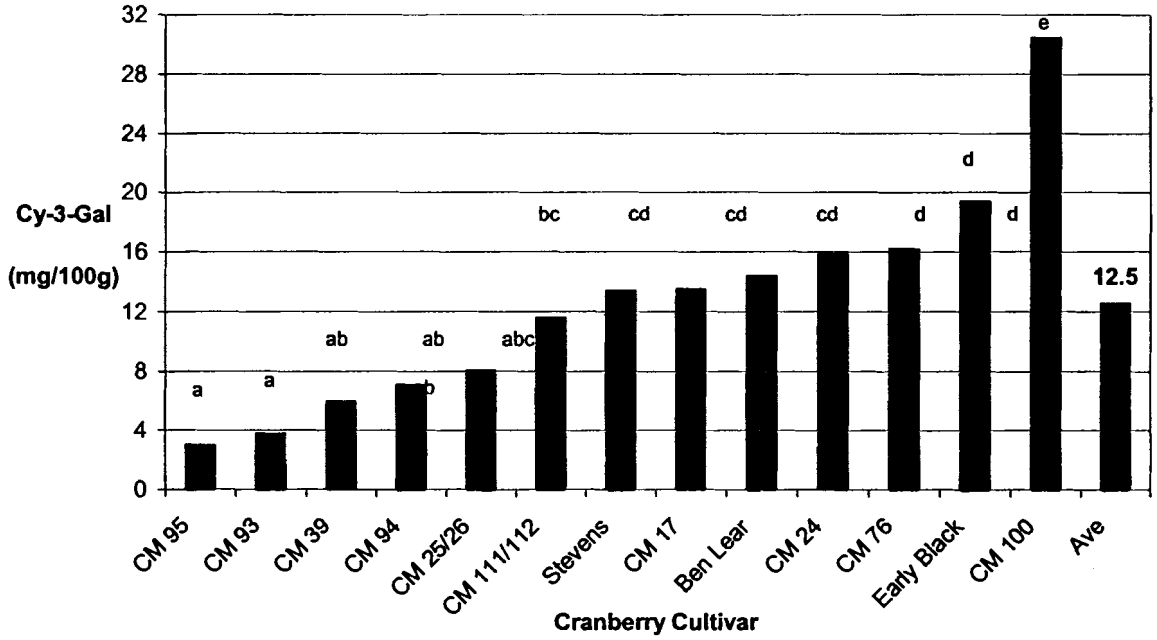


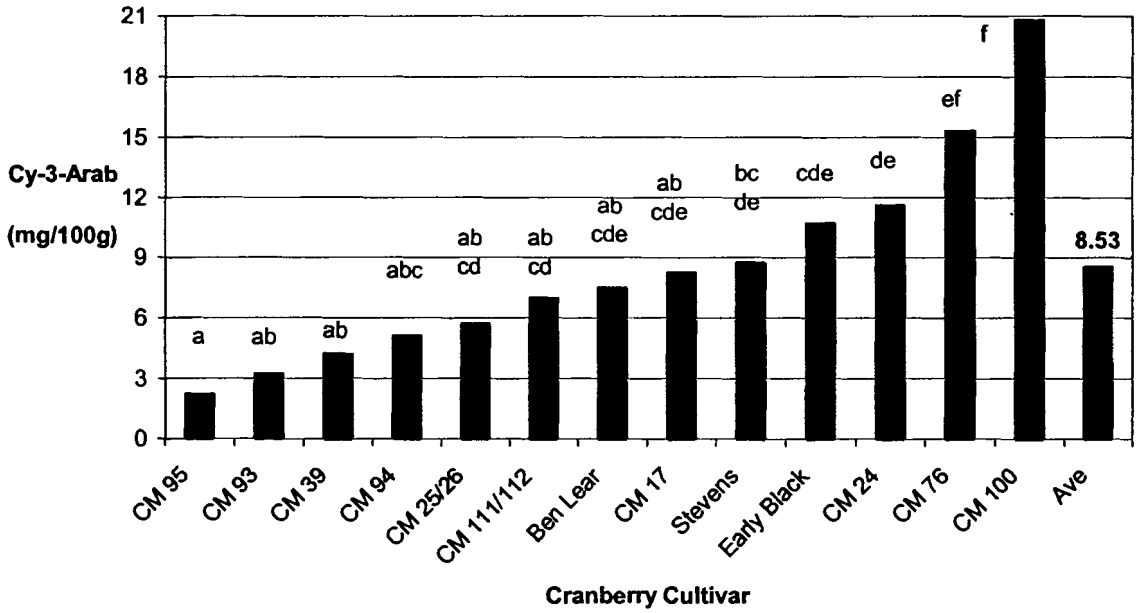




[a] 1998 Harvest.
*Significantly different, $p<0.05$.
** Not enough data to conduct t-test for CM17, CM25/26, CM95, CM97, CM111/112.

## FIGURE 17.

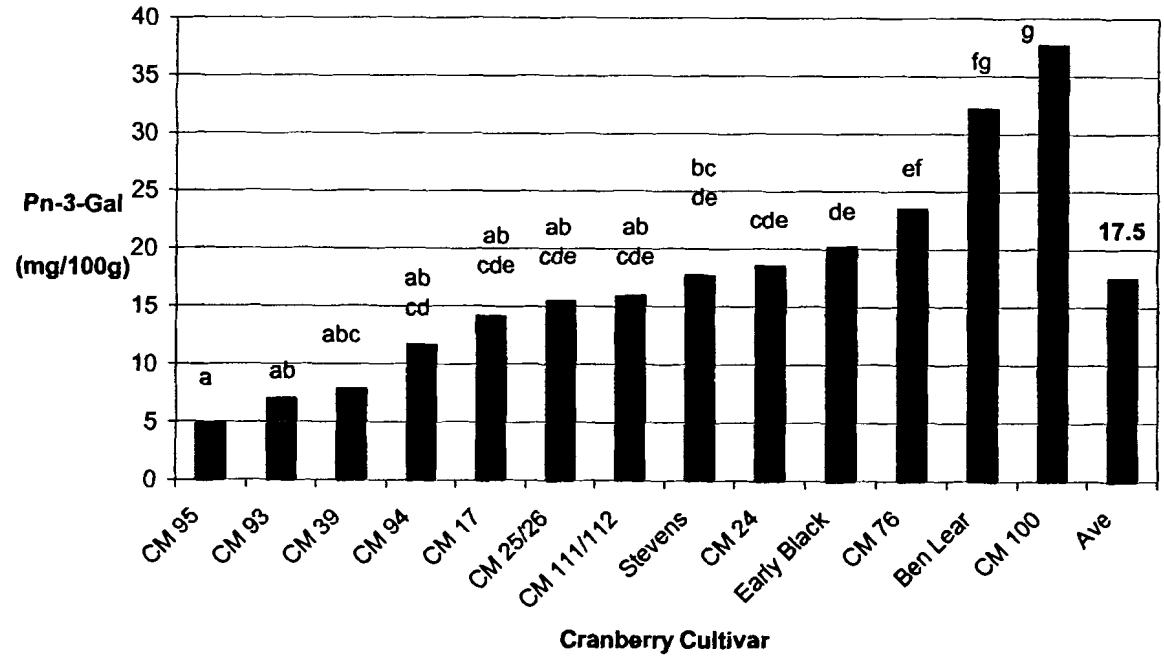
## SIGNIFICANT DIFFERENCES IN INDIVIDUAL & TOTAL ACY CONTENTS IN FRESH WILD MAINE CRANBERRIES*

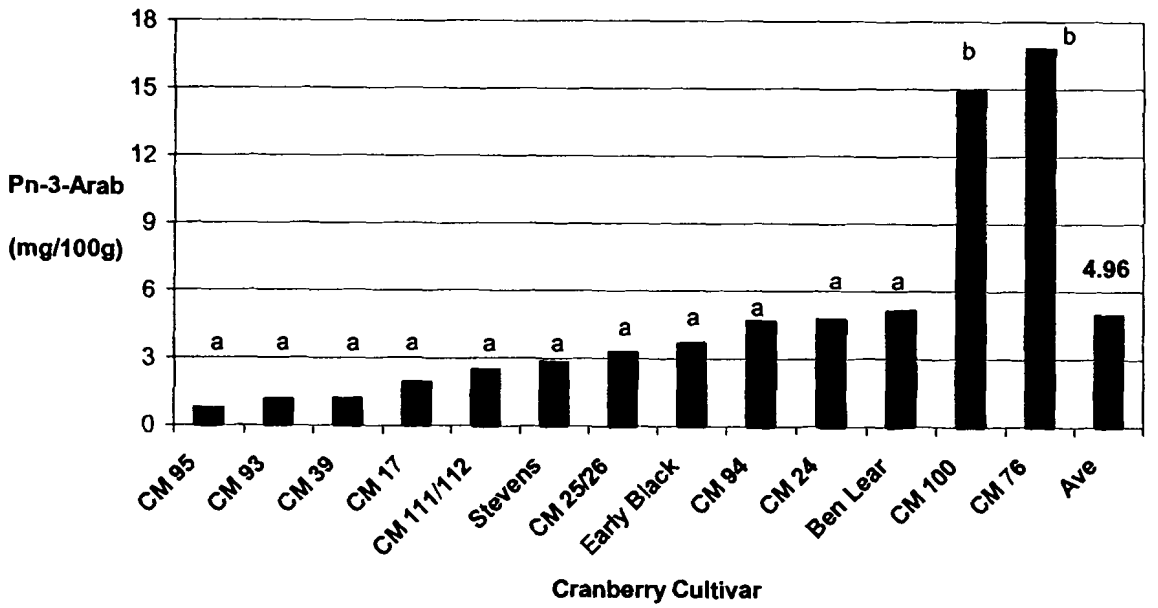



* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

## FIGURE 17. CONTINUED

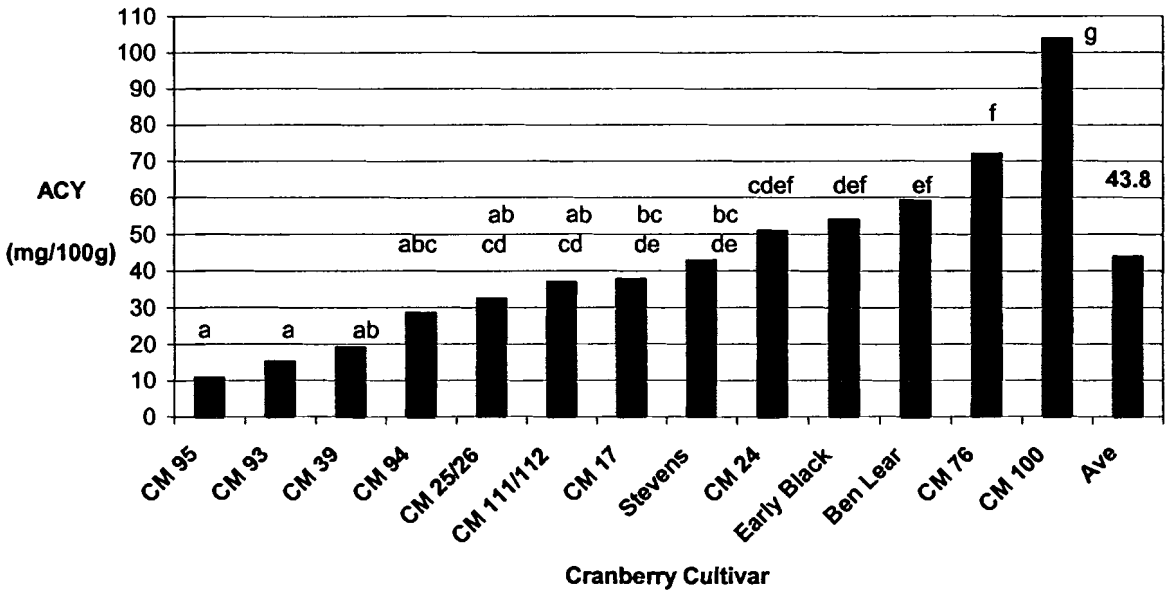



* 1998 Harvest.

Bars with different letters are significantly different, $p<0.05$.

## FIGURE 17. CONTINUED



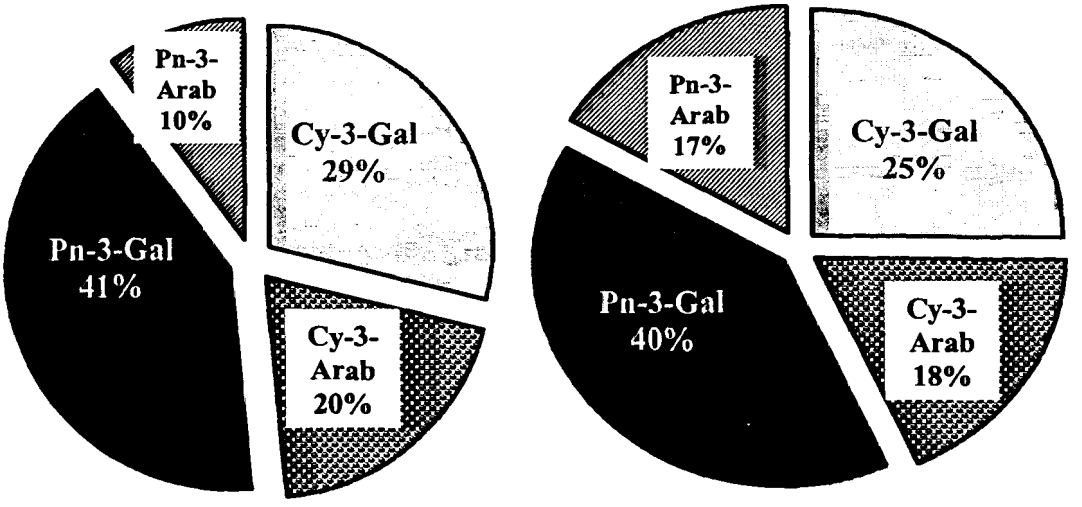

* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

Unlike the findings of Sapers and Hargrave (1987) who reported that the relative proportions of individual ACY for most clones varied within narrow limits, the proportion of individual ACY in this study varied significantly among cultivars and between fresh and frozen samples (Figure 18). In this study, Cy-3-Gal made up an average of 29% (21-37%) and 25% (17-33%) for fresh and frozen samples, respectively; Cy-3-Arab 20% (11-26%) and 18% (13-25%); Pn-3-Gal 41% (31-59%) and 40% (33-51%); and Pn-3-Arab 10% (5-25%) and 17% (12-20%). On the other hand, Sapers et al. (1983a) reported average percentages similar to results obtained in this study with 25% for Cy-3-Gal; 15% Cy-3-Arab; 40% Pn-3-Gal; and 16% Pn-3-Arab. Where other researchers reported little variation in proportions of individual ACY and suggested that the rate of pigment development was rather constant throughout the growth and

development of the berry, my data did not support this reasoning. In order to draw conclusions, more data should be generated from subsequent growing seasons on the same cultivars.

**FIGURE 18.**

**AVERAGE % OF TOTAL ACY IN FRESH & FROZEN WILD MAINE CRANBERRIES***




* 1998 Harvest.

Near the end of this project, cranberry samples from the next growing season (1999) were sent to the Food Science Department and extracted for ACY content only. Table 11 compares the ACY content between the two growing seasons. It is of interest to evaluate successive growing seasons for variations in pigment content as well as other berry characteristics. Comparing berries of different seasons may tell how hardy the berry is or if it is greatly affected by environmental and/or growth conditions.

**TABLE 11.**

**COMPARISON OF INDIVIDUAL & TOTAL ACY CONTENTS IN CRANBERRIES FROM TWO CONSECUTIVE SEASONS**

| YEAR | Cy-3-Gal | Cy3-Arab | Pn-3-Gal | Pn-3-Arab | Total |
|---|---|---|---|---|---|
| 1998[a] | 13.00[c] | 8.78 | 18.78 | 5.69 | 46.26 |
| 1999[b] | 14.24 | 11.50 | 33.64 | 13.26 | 72.65 |

[a] Based on 29 samples from 10 cultivars.
[b] Based on 20 samples from the same 10 cultivars analyzed in the 1998 harvest.
[c] Reported in mg/100g.

It is evident that ACY content was higher in the 1999 berries. There are several possibilities for difference in ACY content from one season to the next, as already explained. It may also have been due to the long holding period of the 1998 berries in their extraction solvent while performing vitamin C and moisture content analyses. The berries from the 1998 harvest were held for a couple of weeks in solvent in the refrigerator before they were extracted; whereas, the 1999 berries were extracted directly after maceration in the solvent. Despite the all organic and acidic solvent, the ACY in the 1998 harvested berries most likely underwent some degradation during that holding time. Therefore, the 1999 berries were probably more accurate in measuring the ACY content in these cultivars.

Seven of the twelve cultivars for the 1999 season averaged over 67mg/100g of ACY; whereas, only two of the fourteen cultivars for the 1998 season averaged ACY contents over 67mg/100g. (Recall that Mazza and Miniati (1993) reported that the optimum ACY content in cranberries was 67mg/100g or higher.) Because of time

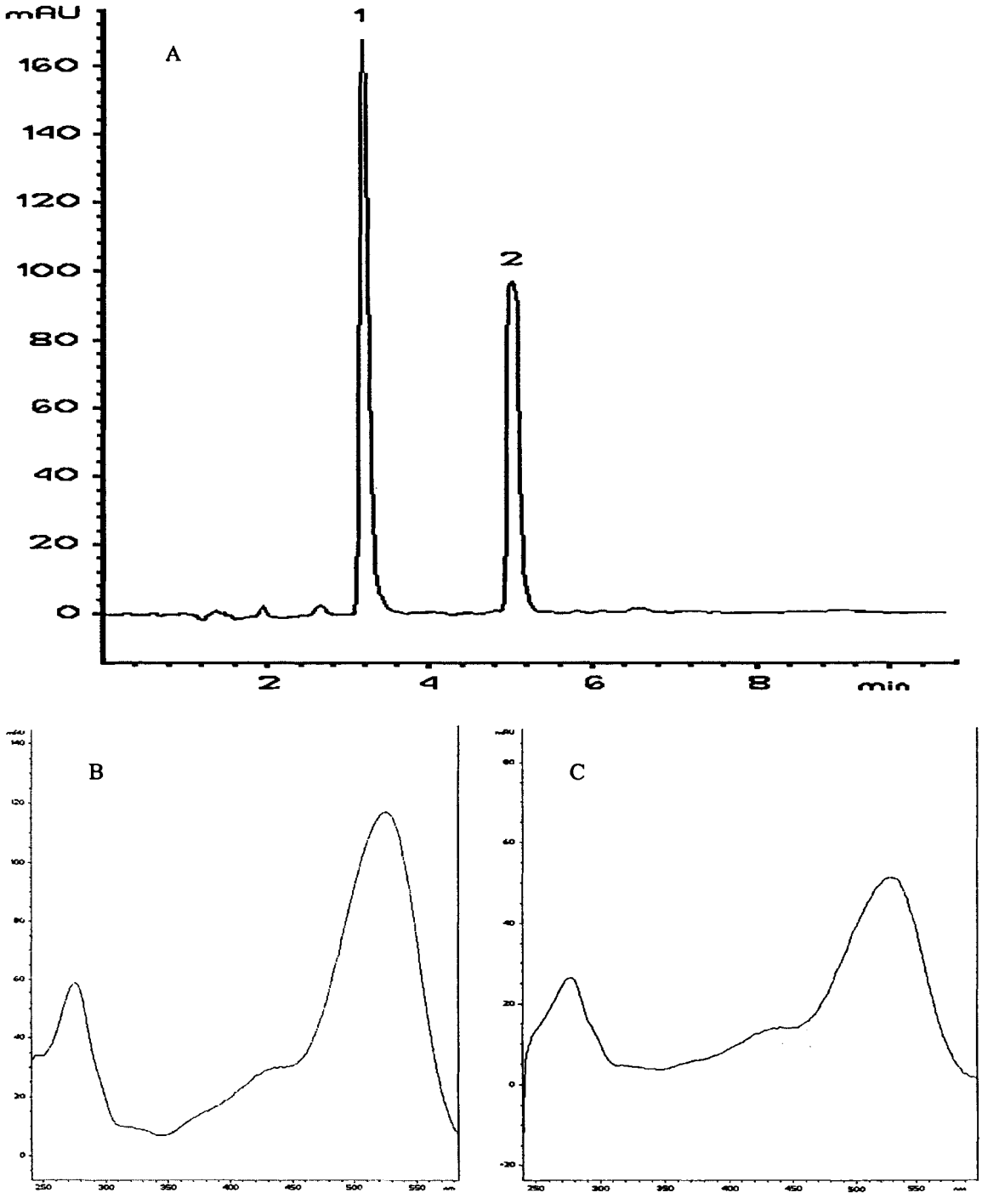
restraints, the 1999 berries were not analyzed for their aglycone content, which is discussed below for the 1998 berries.

Fortunately, standards were available for the aglycones (Cy and Pn); and thus, the ACY were subjected to an acid hydrolysis treatment (outlined in the 'Methods and Materials' section). Only eight of the frozen samples remained of the original samples to be analyzed for their anthocyanidin content by HPLC. An HPLC chromatogram of the aglycones is represented in Figure 19 along with the spectra of each component. The analysis time was under five minutes, which offset the long extraction and acid hydrolysis procedures. The absorbency maxima were previously listed in Table 10 (pp. 61), but from Figure 19 it is evident that the maxima are 275 nm and 525 nm for each compound.

Table 12 lists the average retention times and %CV's for the HPLC analysis of cranberry anthocyanidins. The coefficients of variance were small for this HPLC method, but they varied more than those for the ACY HPLC method. Nonetheless, the %CV values hovered around 8-10% for individual dates of analysis.

**FIGURE 19.**

**HPLC CHROMATOGRAM**
**& SPECTRA OF CRANBERRY ANTHOCYANIDINS**




[A] HPLC Chromatogram. Peak assignment: 1 – Cyanidin, 2 – Peonidin.
[B] Cyanidin spectra.
[C] Peonidin spectra.

TABLE 12.

AVERAGE RETENTION TIMES
(IN MINUTES) FOR CRANBERRY ANTHOCYANIDINS BY HPLC

| Date | Cyanidin | Peonidin | Stats |
|---|---|---|---|
| All Dates | 111 | 111 | # of cases |
| | 3.186 | 4.829 | Mean |
| | 15.0 | 15.2 | % CV |
| 5/24/99 | 21 | 21 | # of cases |
| | 3.063 | 4.596 | Mean |
| | 4.5 | 4.6 | % CV |
| 7/31/99 | 26 | 26 | # of cases |
| | 2.900 | 4.38 | Mean |
| | 8.7 | 5.6 | % CV |
| 11/5/99 | 13 | 13 | # of cases |
| | 3.899 | 6.070 | Mean |
| | 19.9 | 12.9 | % CV |
| 11/6/99 | 6 | 6 | # of cases |
| | 3.408 | 5.803 | Mean |
| | 8.8 | 21.6 | % CV |
| 11/7/99 | 16 | 16 | # of cases |
| | 3.039 | 4.464 | Mean |
| | 11.8 | 8.7 | % CV |
| 11/19/99 | 29 | 29 | # of cases |
| | 3.248 | 4.844 | Mean |
| | 11.3 | 9.1 | % CV |

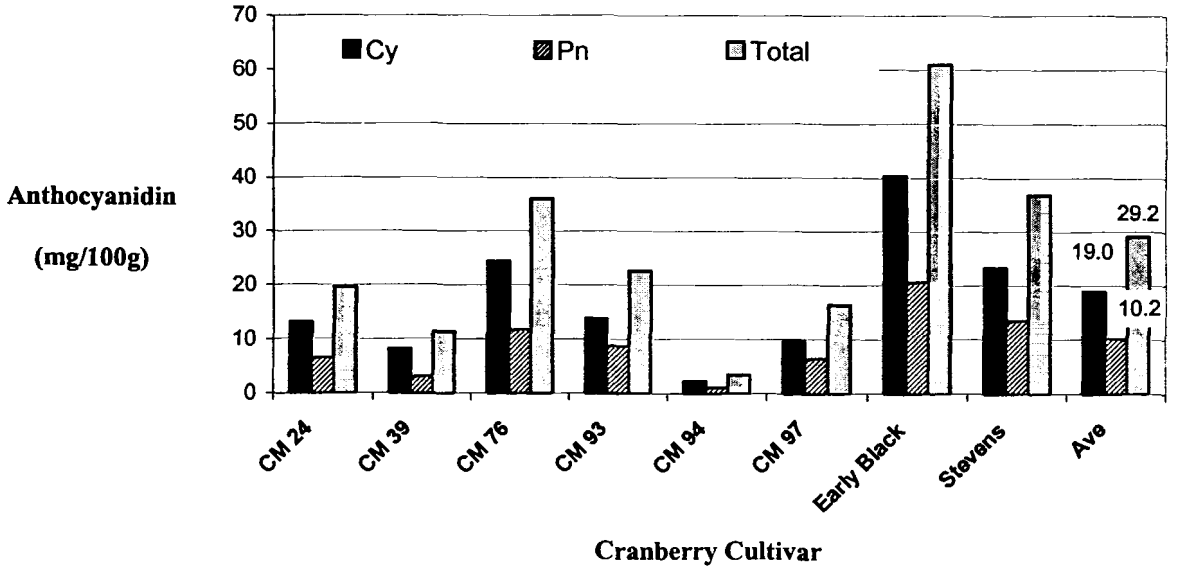
The total content for anthocyanidins ranged from 3.44 to 77.46 mg/100g and averaged 29.18 mg/100g (Figure 20). Individual mean values for Cy and Pn were 18.99 and 10.19 mg/100g, respectively (Figure 20). On the average, Cy contributed 65%, while Pn contributed 35%. It was interesting to note that the peonidin glycosides appeared to

contribute more to the total ACY content than did the cyaniding glycosides (Figure 18. pp. 72) on the same berries. As previously mentioned, peonidin appeared very sensitive to processing conditions, and possibly degraded under the intense boiling temperatures of the acid hydrolysis; thus, explaining the differences in results between aglycone contents.

The acid hydrolysis and HPLC separation performed by Hong and Wrolstad (1986) on single strength cranberry juice pigments resulted in much smaller values for aglycone content then reported in this study. (Keep in mind that the procedure I followed used three sequential extractions to collect the cranberry pigments.) They reported average anthocyanidin contents of 12.3 and 8.9 mg/100g (as Cy-3-Glu) for Cy and Pn, respectively. Average total content was 21.2 mg/100g. Their LC chromatogram, which is represented in Figure 9 (pp. 33) showed wide, tailing peaks. The chromatographic data in this study was very good for quantitative evaluation because of the sharp peaks and minimal tailing.

**FIGURE 20.**

**TOTAL & INDIVIDUAL ANTHOCYANIDIN CONTENTS IN FROZEN WILD MAINE CRANBERRIES***




* 1998 Harvest.

The main goal of this research project was to develop a method to separate and quantify ACY by capillary electrophoresis (CE). Because standards were not available, it was not possible to identify the ACY peaks from the electropherogram since the order of migration in CE may not be the same as the order of elution in HPLC. Therefore, a method was developed to analyze the two aglycones present in cranberries. The extraction procedure and CE conditions were outlined in the 'Methods & Materials' section, but are presented here in Table 13.

TABLE 13.

CAPILLARY ELECTROPHORESIS
CONDITIONS FOR CRANBERRY ANTHOCYANIDIN ANALYSIS

| CAPILLARY | BUFFER | INJECTION | ELECTRIC FIELD |
|---|---|---|---|
| L=48.5 cm<br>l=40.0 cm<br>id=75 mm | 150 mM $H_3PO_4$,<br>3 M Urea<br>50 mM β-cyclodextrin<br>pH 2.11 | Hydrodynamic<br>5 seconds<br>50 mbar | 25 kV<br>~80 μA |
| **TEMPERATURE**<br>25° C<br>**WAVELENGTH**<br>525 nm | **CONDITIONING**<br>0.1 M HCl, 15min<br>0.1 M $H_3PO_4$,15 min<br>Buffer, 15 min | **BTWN RUNS**<br>0.1 M $H_3PO_4$, 3 min<br>Buffer, 5 min | **SAMPLE SOLVENT**<br>50% MeOH<br>50% HPLC $H_2O$<br>pH 1.0 w/HCl |

Two of the methods previously described in the literature review under 'Anthocyanin Content: Capillary Electrophoresis' by da Costa et al. (1998) and Bicarrd et al. (1999) were applied to the analysis of anthocyanidins for cranberries in this study, but the compounds were not separated in some instances or detected in others. The method outlined in Talbe 13 has not been applied to the analysis of anthocyanic compounds or any other compounds to my knowledge. It was the product of several different methods applied to an array of compounds: β-cyclodextrin (Chandra et al., 1993; Luong & Nguyen, 1997; Esaka & Kano, 1997); and urea (Terabe, 1991). Organic solvents such as methanol and acetonitrile added to the run buffer did not prove helpful in the resolution of the aglycones.

The most important parameter regarding CE analysis of anthocyanins was the pH of the run buffer. Initially a pH of less than 2.0 was strived for to ensure that the compounds were kept in the flavylium cation form, but after 60 minutes no peaks were detected. Once the present method was developed, it was necessary to measure the pH of the run buffer each time it was made. Even slight changes in pH caused shifts in migration time or affected resolution of the aglycones. Therefore, it was necessary to also accurately weigh the amount of urea that was added to the buffer solution since it caused the increase in pH. The initial pH of the 150 mM phosphoric acid was ~1.5, but with the addition of 3 M urea, the final pH was ~ 2.1. It was the addition of the urea that caused the initial separation of the two aglycones once a level of 2 M was reached. When more than 3 M of urea was added to the solution peak broadening occurred, which was most likely due to structural transformations of the pigments at the higher pH values.

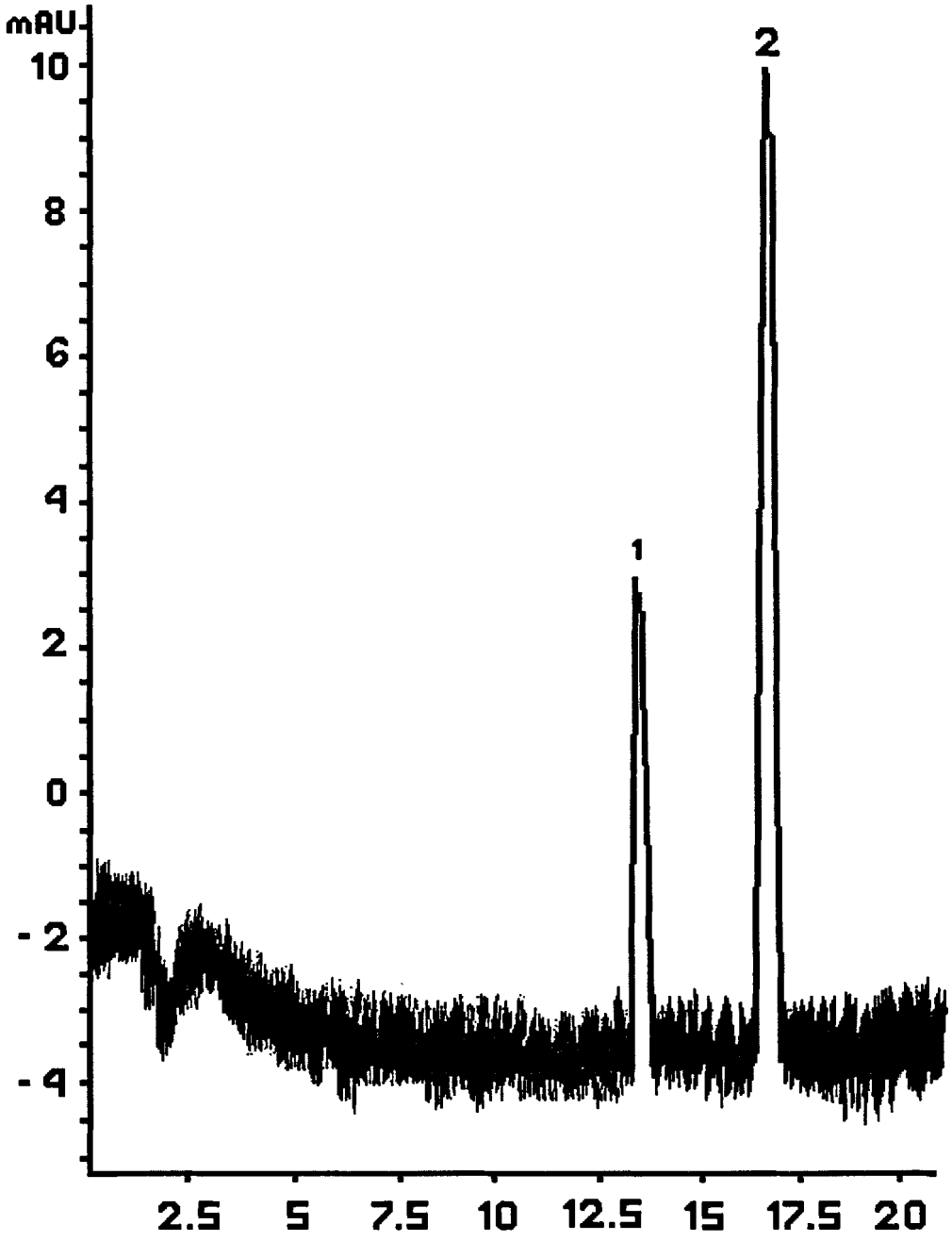
The β-cyclodextrin proved beneficial in sharpening the peaks as well as preserving the compounds. Preservation of anthocyanic compounds by β-cyclodextrin was investigated by Chandra et al. (1993). In this study, several concentrations of β-cyclodextrin were experimented with to obtain the best peak shape without compromising migration time. Because β-cyclodextrin added an element of chromatography to CE in the form of size exclusion and hydrophobic interaction, the more β-cyclodextrin that was added to the buffer the longer the run time became. There appeared to be no additional increase in peak sharpness with concentrations of β-cyclodextrin higher than 50 mM, but the migration time increased to ~30 minutes.

In this application, the main problem with peak shape was that of peak tailing. When looking at the on-line spectra, it was evident from continued detection at 275 nm

that there were several structural forms of the aglycones passing through the detection window. This wavelength is the maxima of the degraded products. Therefore, higher concentrations of β-cyclodextrin did not resolve the problem of peak tailing. It is evident from the electropherogram represented in Figure 21, that the peaks were sharp and rather symmetrical. This was achieved by increasing the voltage of the system. Increasing the voltage has been known to not only decrease migration time (by increasing the EOF), but also increasing the current and sometimes resolution and sensitivity of the analytes (Baker, 1995). It was beneficial to get the migration time as short as possible without compromising resolution because anthocyanic compounds degrade quickly. Table 14 lists the average migration times for each compound, along with their respective %CV's. Based on one day's values of successive runs, the small %CV values showed promise for this CE method. Because the cyanidin aglycone appeared to experience more fluctuation in migration time, and it was significantly larger in concentration than peonidin, it would be of interest of investigate the effect of sample concentration on different CE parameters. It has been reported that the sample plug length does affect resolution of analytes and possibly other parameters of the analysis (Baker, 1995). This may be due to the "stress" placed on the system when the voltage is applied.

FIGURE 21.

ELECTROPHEROGRAM OF CRANBERRY ANTHOCYANIDINS*




* Analysis performed on commercial cranberries.
Peak assignment: 1 – Peonidin, 2 – Cyanidin.

**TABLE 14.**

**AVERAGE MIGRATION TIMES (IN MINUTES) FOR CRANBERRY ANTHOCYANIDINS BY CE**

| Date | Peonidin | Cyanidin | Stats |
|---|---|---|---|
| 11/19/99 | 26 | 26 | # of cases |
| | 14.736 | 18.014 | Mean |
| | 2.9 | 10.7 | % CV |

There was a large amount of background noise, as evident from the noisy baseline in the electropherogram. This decreased the sensitivity of the method and hindered the detection of peonidin, which is sometimes present in cranberries in smaller concentrations than cyanidin. Peonidin also appeared to be more labile than cyanidin to processing conditions, which increased the need for speedy extraction and analysis times. This was seen in the comparison of CE to HPLC. Two of the 23 samples showed no peonidin detection by CE, but were detected by the HPLC method. Consequently, the HPLC method had minimal baseline noise. The noisy baseline of the CE method may also have resulted from a dirty capillary. The capillary used for this application had been previously used for other applications for which alkaline run buffers were employed. Normally, one capillary is used for one application, and run buffers of acidic nature are kept separate from those of neutral or basic run buffers. Switching from low pH to high pH with the same capillary is very stressful on the capillary and requires long equilibration time as well as thorough clean up. Unfortunately, capillaries are costly, and it was not feasible to get another capillary.

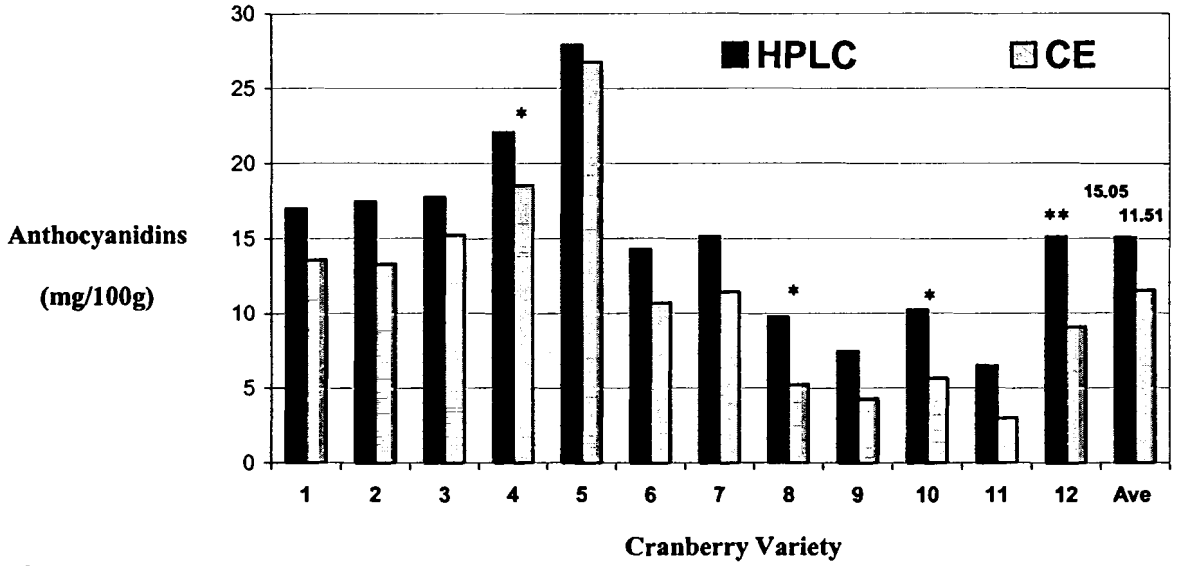
Another point to mention involves the extraction procedure. The final step of the acid hydrolysis of the ACY was to clean up the pigments on an activated C18 Sep-Pak® and to elute them with 1% HCl in MeOH. In this solvent, the pigments were injected onto the HPLC system, but with the CE system, they were first dried down and reconstituted in 50:50 water:MeOH (pH 1.0 with HCl). While being dried down and stored in the freezer overnight, the pigments experienced some breakdown, peonidin more so than cyanidin. Therefore, for future analysis it may be necessary to elute the pigments off of the C18 cartridge with the CE solvent to eliminate the drying step. This would be advantageous for a couple of reasons: it would cut down on extraction time and decrease the risk of pigment breakdown.

It is also evident form Figure 21 that the order of detection was reversed in CE compared to that of HPLC. Because peonidin is more hydrophobic due to the methoxy group at position 3', it eluted second from the HPLC reversed-phase column. In CE where compounds traveled under the influence of charge and electro-osmotic flow and since capillary wall-analyte interactions were almost eliminated, peonidin was the first to migrate through the capillary, followed by cyanidin.

Figure 22 shows the results obtained by injecting or introducing the same pigment extracts into both the HPLC and CE systems. (The pigments originally injected into the HPLC were in the 1% HCl in MeOH solvent, but because of the degradation of the pigments upon drying for the CE system, they were reinjected in the HPLC system in the 50:50 water:MeOH (pH 1.0 with HCl) solvent; the same pigment extract that was analyzed by CE.) Therefore, the two methods of anthocyanidin analysis were able to be compared quantitatively.

## FIGURE 22.

## COMPARISON OF ANTHOCYANIDIN CONTENTS BY HPLC & CE[a]




[a] 1999 Fresh commercial berries.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test.

For every cultivar, the total anthocyanidin content was higher for the HPLC method than for the CE method, but significant differences existed for only three of the twelve cases (25%), which are represented by asterisks (*) in Figure 22. Nonetheless, the correlation coefficient was high at 0.967 and suggested that the two methods were indeed comparable for the quantitation of cranberry anthocyanidins. Further studies should include analyses on a variety of fruits that have more complex anthocyanidin profiles than the two aglycones presented here to determine the true applications of this method. Will other aglycones be able to be resolved by this method? I would have liked to conduct these trials if time had allowed.

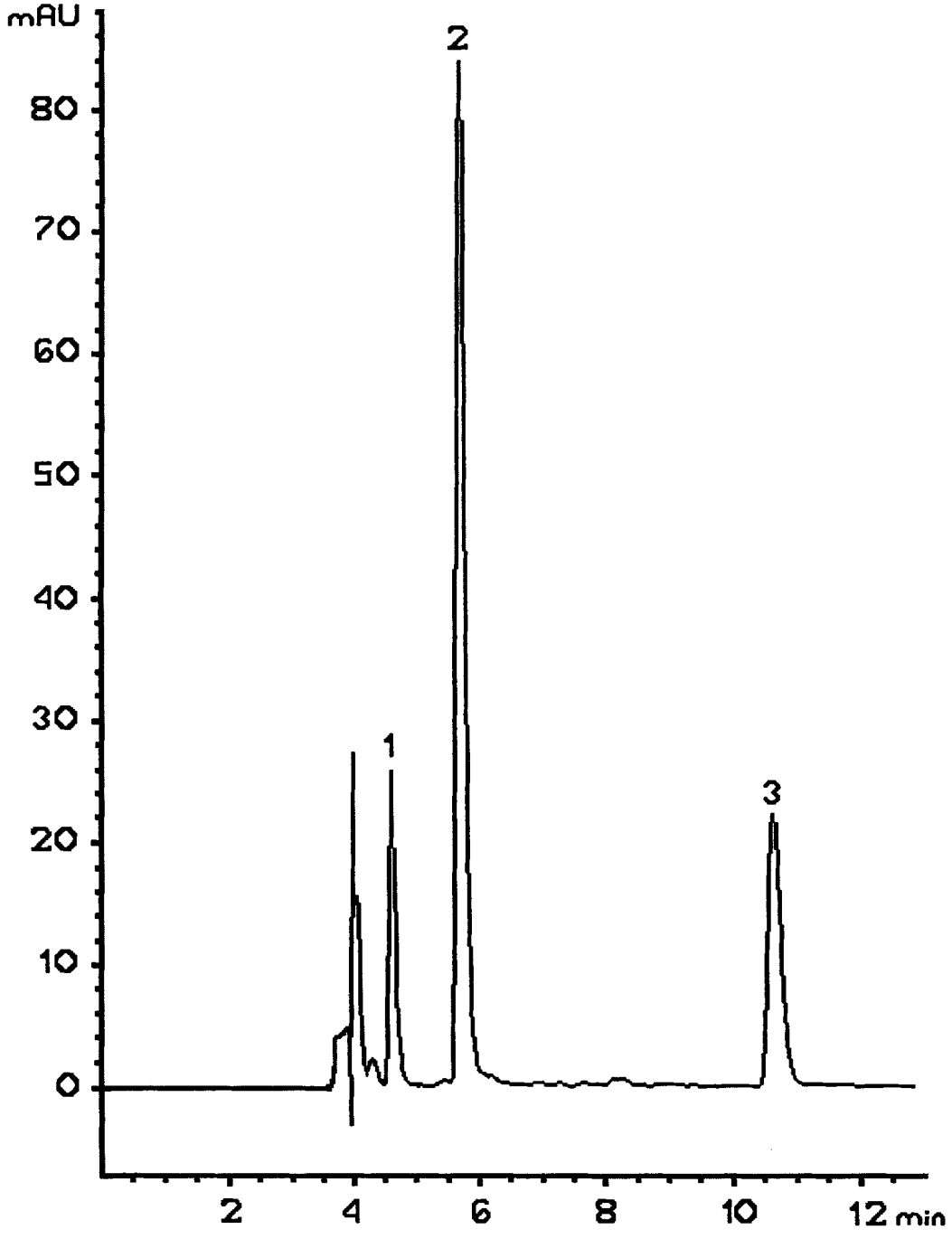
# ORGANIC ACID CONTENT

Three of the five major organic acids in cranberries (L-ascorbic acid is discussed in the next section) (16 fresh; 13 frozen) were analyzed by HPLC. Benzoic acid was not detected by this method and was believed to have adsorbed on to the column wall. A typical chromatogram is illustrated in Figure 23. The extraction procedure followed that outlined by Riaz and Bushway (1994); whereas, the HPLC conditions originated from studies conducted by Keystone Scientific, Inc. on their BetaBasic® 18 column (Keystone Scientific, Inc., 1998).

The mean analysis time differed between the fresh and frozen runs by nearly five minutes. From Table 15, it can be seen that average retention times for quinic and malic acids differed within one minute, but citric acid differed within five minutes. Possible reasons might be slight differences in mobile phase composition from batch to batch, changes in temperature of the room and thus the HPLC instrument, which would affect analyte-column interactions, or changes in column wall integrity. It was interesting to note, though, that the %CV values for the individual retention times for both fresh and frozen trials were very small. This suggested that the method was reproducible and that each compound eluted off of the column at nearly the same time for each run.

FIGURE 23.

HPLC CHROMATOGRAM OF THE
MAJOR ORGANIC ACIDS IN CRANBERRIES*




* Chromatogram of fresh cranberries from 1998 harvest.
Peak assignment: 1 – Quinic acid, 2 – Malic acid, 3 – Citric acid.

TABLE 15.

AVERAGE RETENTION TIMES
FOR CRANBERRY ORGANIC ACIDS BY HPLC

| ORGANIC ACID | RETENTION TIME (min) | % CV |
|---|---|---|
| Quinic Acid | 4.65[a], 5.16[b] | 4.5[a], 3.1[b] |
| Malic Acid | 5.80, 6.72 | 4.9, 2.8 |
| Citric Acid | 10.80, 14.62 | 6.9, 2.1 |

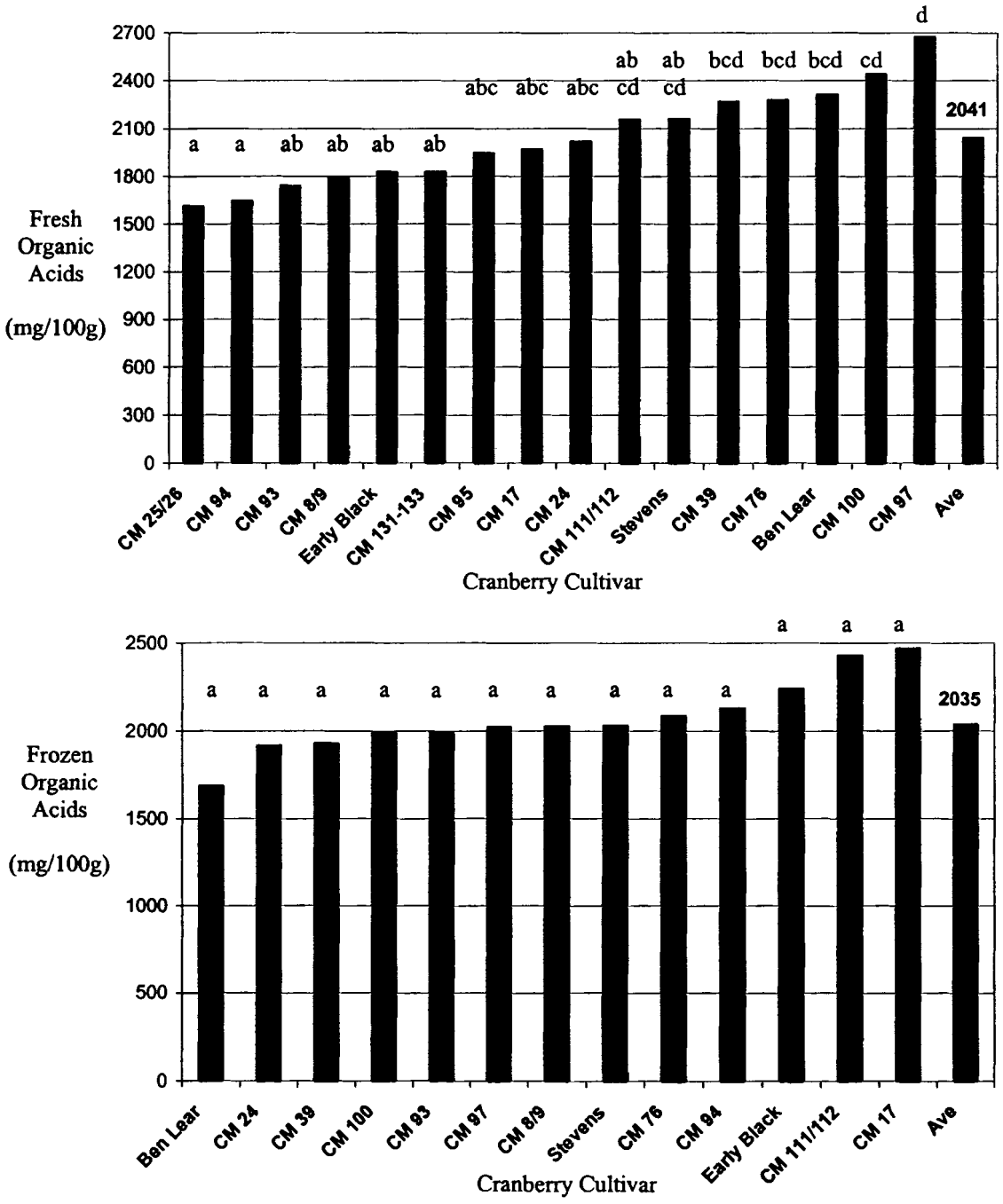
[a] Average retention time and % CV of fresh cranberries.
[b] Average retention time and % CV of frozen cranberries.

The chromatographic results in this study were similar to those by Coppola et al. (1978) on cranberry juice, with acids eluting from the column in the same order and at approximately the same retention times. Research by Hong and Wrolstad (1986) also analyzed cranberry acids and detected four compounds: quinic, malic, citric, and shikimic. Shikimic acid is a compound metabolically related to quinic acid. Shikimic acid was not detected in this study.

The content of organic acids ranged from 1467-3040 mg/100g and 1605-2469 mg/100g for fresh and frozen samples, respectively. The mean acid content was 2041 mg/100g for fresh samples and 2035 mg/100g for frozen samples. Significant differences ($p<0.05$) existed among cultivars and between fresh and frozen samples for their acid content (Figure 24 & 25). Cultivar 'CM 97' expressed the highest acid content in fresh berries; whereas, 'CM 17' was highest in frozen samples. 'CM 25/26' and 'Ben Lear' were lowest in acid content for fresh and frozen samples, respectively. Analysis by ANOVA showed no relationship existed between the acid content and pigment concentration (data not shown).

## FIGURE 24.

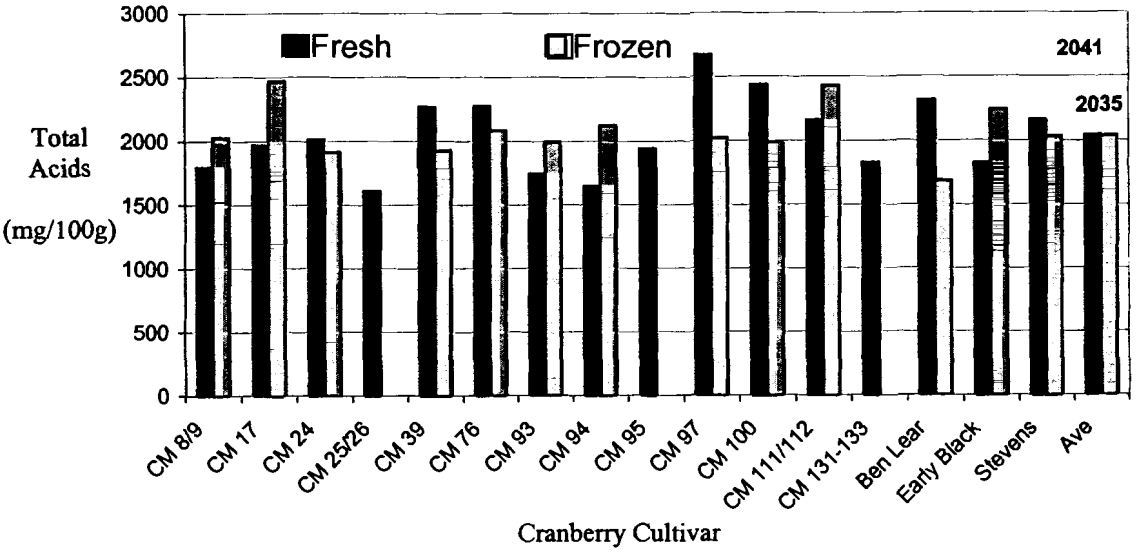
## SIGNIFICANT DIFFERENCES IN TOTAL ORGANIC ACIDS IN FRESH & FROZEN WILD MAINE CRANBERRIES*




* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

## FIGURE 25.

## TOTAL CONTENT OF ORGANIC ACIDS IN FRESH & FROZEN WILD MAINE CRANBERRIES[a]



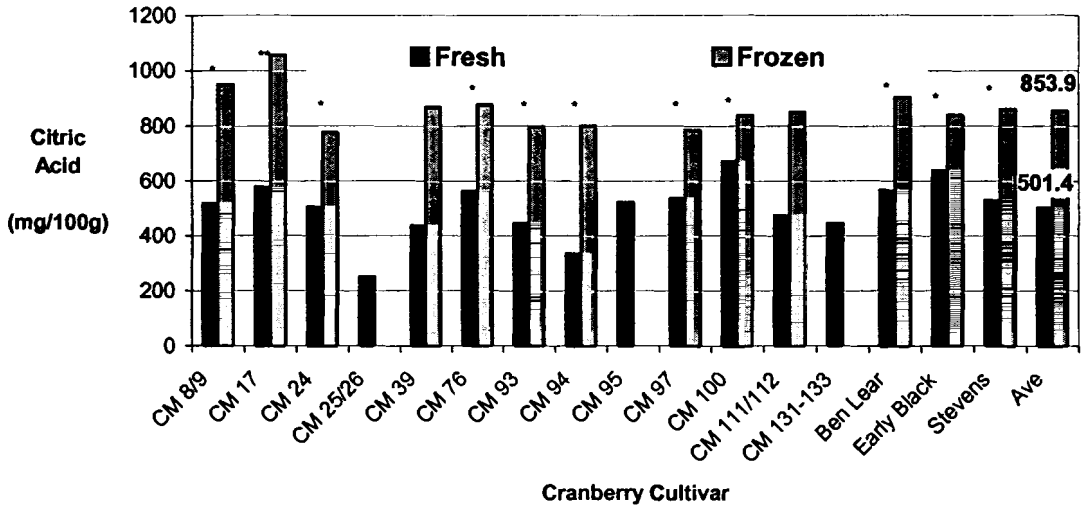

[a] 1998 Harvest.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test for CM17, CM25/26, CM39, CM111/112, CM131-133.

Unlike the ACY in which it appeared that no significant metabolic processes occurred between the fresh and frozen berries, significant changes occurred with the organic acids. Despite the small differences in total acids from fresh to frozen samples, large differences existed among individual acids. Individual acid contents were compared for fresh and frozen samples by the two-tailed t-test ($p<0.05$) when sample size allowed for more than one trial in a set (Figure 26). Out of ten comparisons, only two pairs of fresh and frozen berries were significantly different for total acid content; whereas, eight pairs were significantly different for citric acid, seven pairs for malic acid, and two pairs for quinic acid. Significant differences ($p<0.05$) for individual acids in the fresh state among the 16 different cultivars can be seen in Figure 27. There was little

variation between different cultivars for the frozen samples – citric acid showed no significant differences; malic acid was significantly lower in the "Ben Lear" cultivar; and few differences existed for quinic acid. This may have been due to the small number of sample replicates for each cultivar as sample size diminished with each analysis performed. Fresh samples, on the other hand, showed a greater number of significant differences. "CM 100" was much higher in citric acid content than the other cultivars; whereas "CM 25/26" and "CM 94" were significantly lower in citric acid content. Malic acid appeared to vary less in the fresh berries, but was split into two groups of high and low content. For malic acid, "CM 97" was higher in content than 11 of the other fresh samples. Quinic acid showed only four significant differences among all comparisons of means in fresh berries.

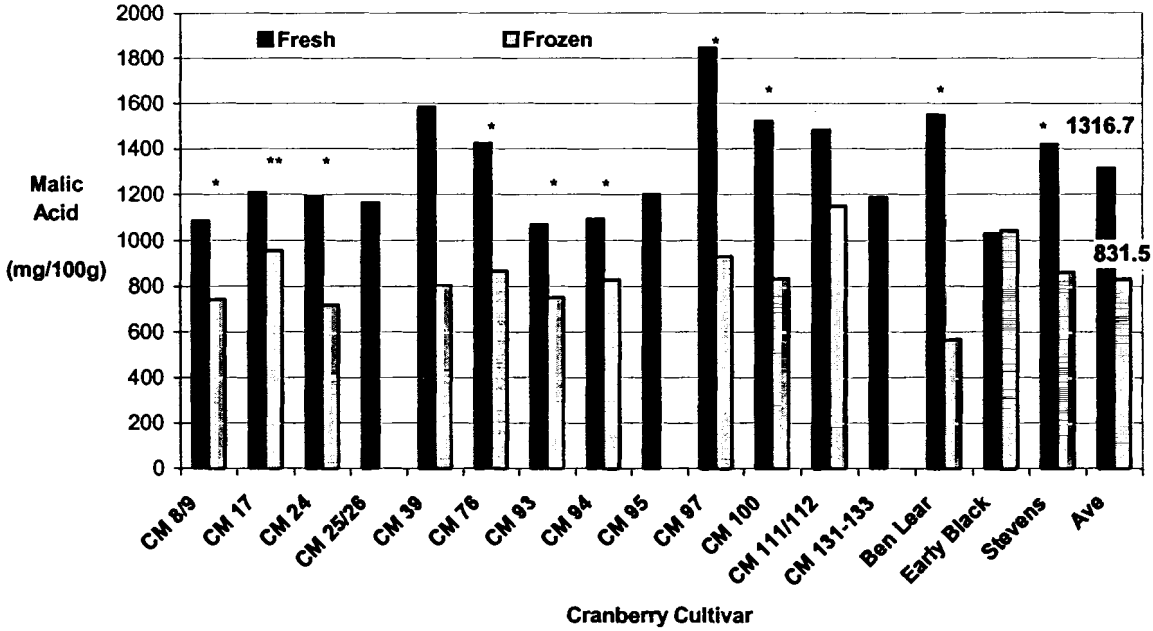
**FIGURE 26.**

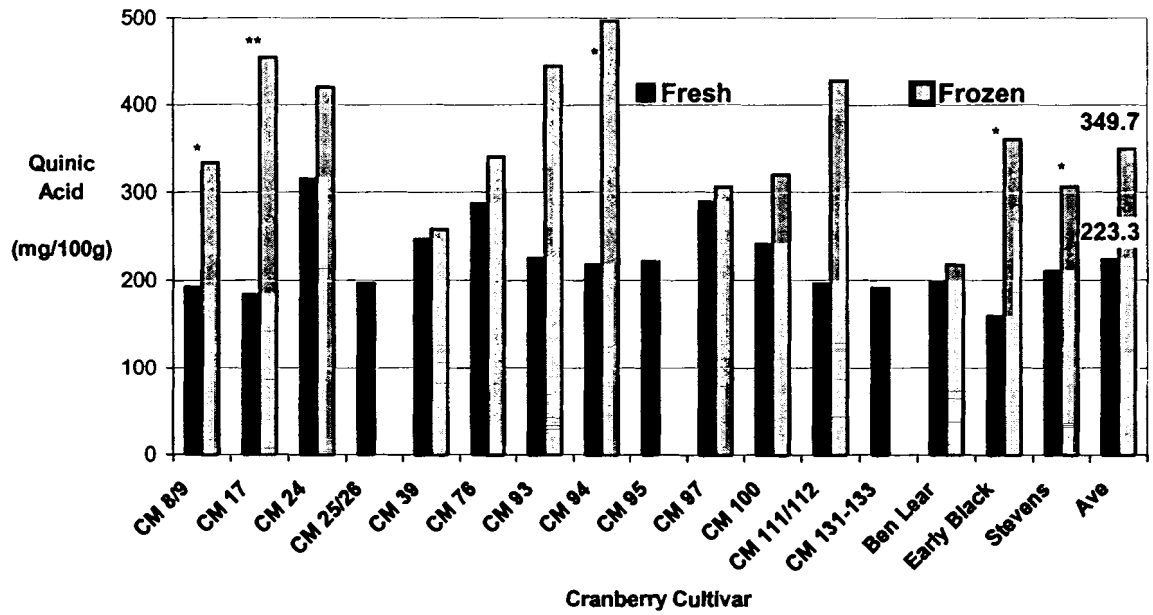
**COMPARISON OF INDIVIDUAL ORGANIC ACIDS IN FRESH & FROZEN WILD MAINE CRANBERRIES[a]**




[a] 1998 Harvest.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test for CM17, CM25/26, CM39, CM95, CM111/112, CM131-133.

## FIGURE 26. CONTINUED

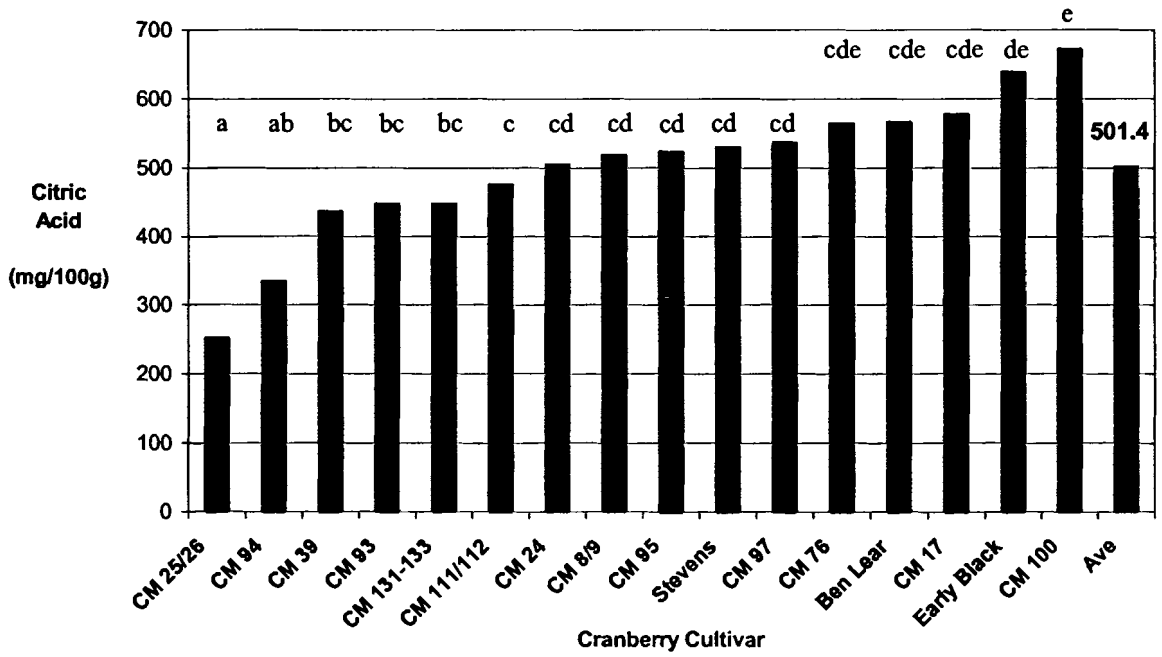


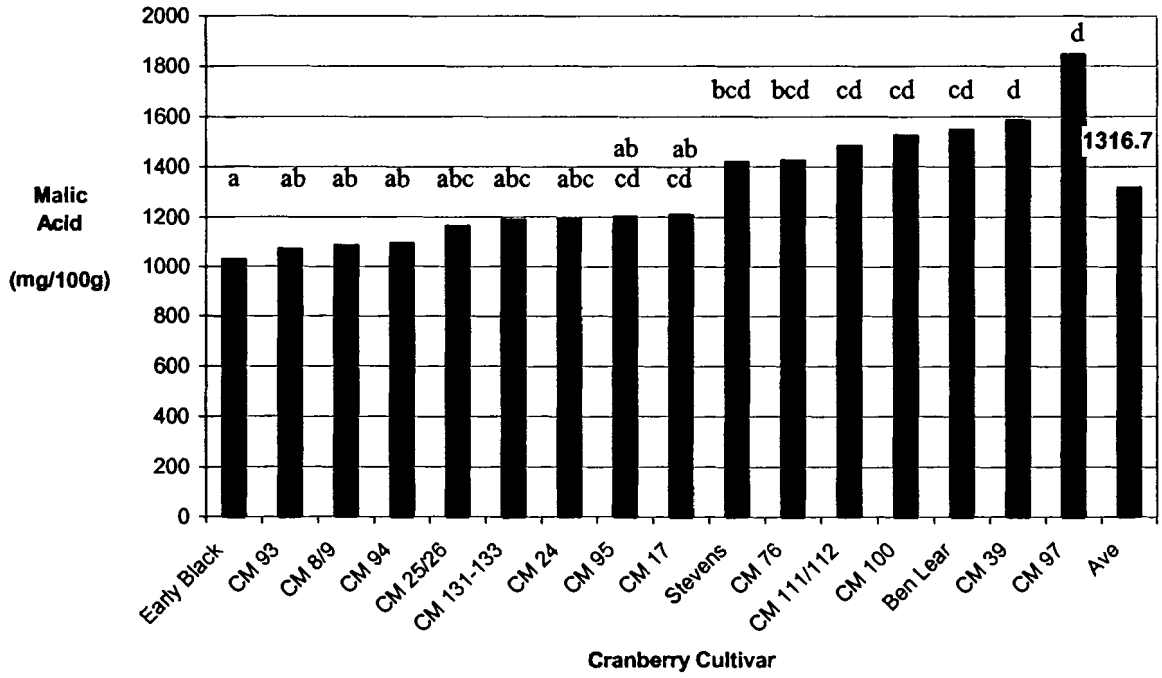




[a] 1998 Harvest.
* Significantly different, $p<0.05$.
** Not enough data to conduct t-test for CM17, CM25/26, CM39, CM111/112, CM131-133.

## FIGURE 27.

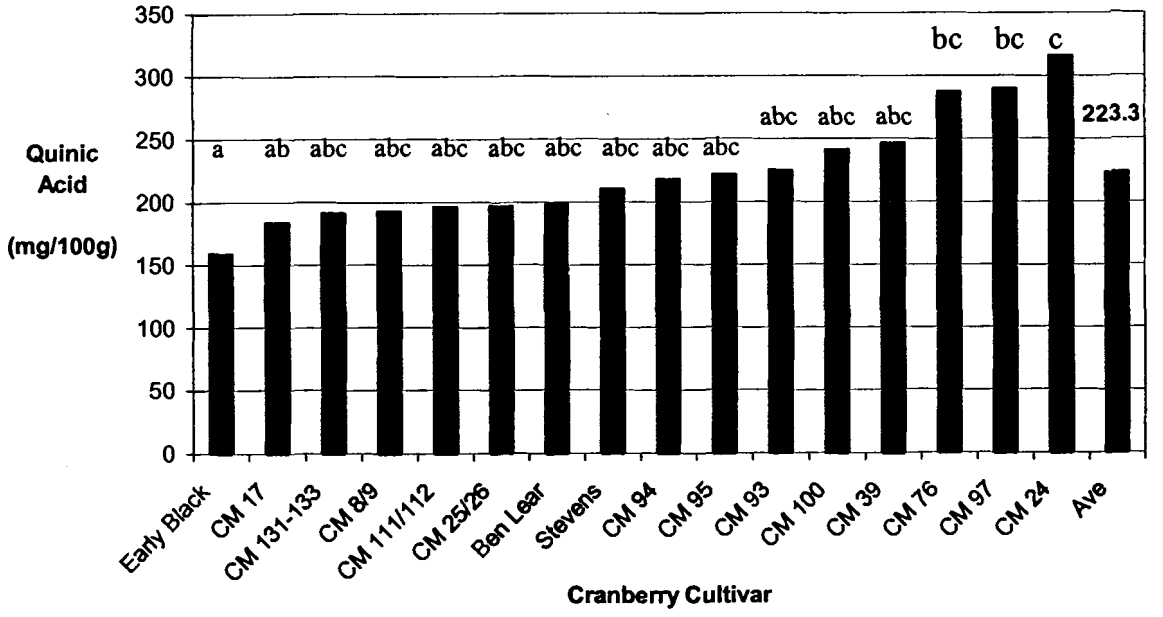
## SIGNIFICANT DIFFERENCES IN INDIVIDUAL ORGANIC ACIDS IN FRESH WILD MAINE CRANBERRIES*




* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

**FIGURE 27. CONINTUED**

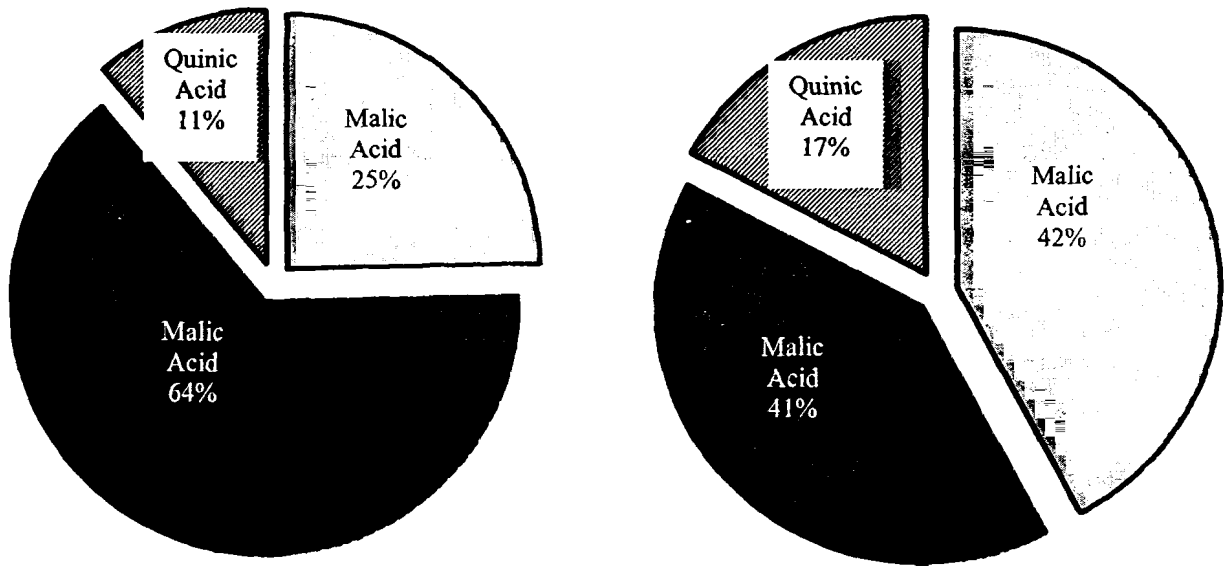



* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

In the fresh cranberry, malic was the dominant acid comprising 64% (1317 mg/100g) of the total, followed by citric at 25% (501 mg/100g) and quinic at 11% (223 mg/100g) (Figure 28). In the frozen cranberry, the proportion of acids changed to reflect a decrease in malic acid, which was offset by increases in citric and quinic acids. The composition of the frozen berry was 42% (854 mg/100g) citric, 41% (832 mg/100g) malic, and 17% (350 mg/100g) quinic. On the large scale, the mean total acid content of the fresh and frozen berries varied slightly; but on the small scale, individual acid composition underwent significant changes. Reasons for these changes between fresh and frozen berries were most likely metabolic in nature. I did not find documentation or further explanation of this observation in cranberries or other fruits. It may be that earlier studies conducted analysis on frozen whole cranberries rather than macerated berries.

FIGURE 28.

AVERAGE % OF TOTAL ORGANIC ACIDS
IN FRESH & FROZEN WILD MAINE CRANBERRIES*




Fresh Frozen

* 1998 Harvest.

The results obtained in this study differed from those of Hong and Wrolstad (1986) on single strength cranberry juice. They determined the proportion of total acids to be 32.3% (860 mg/100g) citric, 27.2% (720 mg/100g) malic, and 39.2% (1050 mg/100g) quinic. Table 16 compares results from this study and that of Hong and Wrolstad (1986). Quinic acid was the dominant acid in their study and more than doubled the mean percentage of total acids reported in this study. It is important to take into account the methods of analysis followed by each researcher in order to help explain any differences in results that may arise.

**TABLE 16.**

**COMPARISON OF CRANBERRY ORGANIC ACIDS FROM TWO STUDIES**

| STUDY | ORGANIC ACID | | |
|---|---|---|---|
| | Citric | Malic | Quinic |
| Watson, 1999 | | | |
| Fresh | 501.4[a] (25%[b]) | 1316.7 (64%) | 223.3 (11%) |
| Frozen | 853.9 (42%) | 831.5 (41%) | 349.7 (17%) |
| Hong & Wrolstad 1986 | 860.0 (32%) | 720.0 (27%) | 1050.0 (39%) |

[a] mg/100g.
[b] % of total acids.

Even though differences in results existed between the two studies, earlier work by Morse (1930) concluded that total acid content varied little among regions and cultivars of cranberries. To explain these contradictions in results, differences in extraction procedures between the studies should be looked at, as well as the type of cranberry. Cranberries used in this study were young and wild; whereas, the cranberries used by Hong and Wrolstad (1986) were frozen commercial berries from Ocean Spray Cranberries, Inc.

# L-ASCORBIC ACID CONTENT

L-ascorbic acid (L-AA), better known as vitamin C, is abundantly found in fruits. Table 1 (pp. 4) listed the typical vitamin C content of cranberries to be in the range of 7.5-10.5 mg/100g. But just as environmental factors and growing conditions affect the

quality and composition of plant foods, such as pigments and organic acids, so too do they affect vitamin C content.

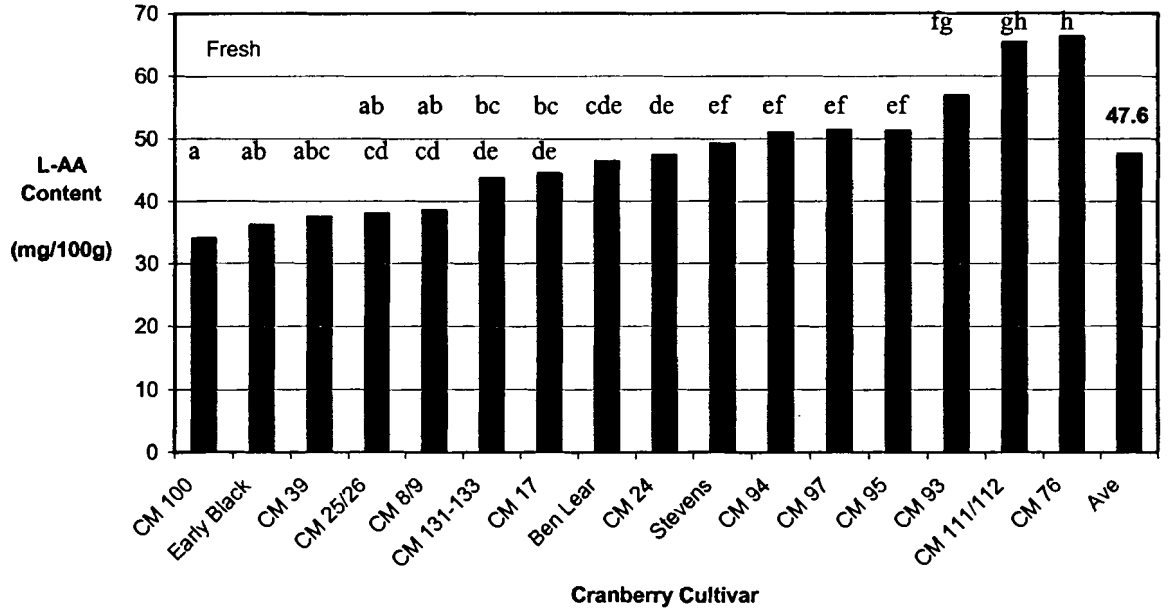
The results obtained in this study by titration gave a range of 32.9 to 72.3 mg/100g for fresh berries and 6.8 to 22.2 mg/100g for frozen berries. The mean L-AA content was 47.6 and 13.2 mg/100g for fresh and frozen berries, respectively. The huge difference in L-AA content from fresh to frozen samples was expected since vitamin C is labile to light, extremes in temperature, oxygen, other food constituents, changes in pH, and metals. The large value for the fresh berries was uncharacteristic, but not unheard of since there are many factors affecting the metabolism of plants, especially the unique growing conditions of cranberries, which has previously been described in 'Cranberry Growth and Harvesting.' There may also have been other substances in the L-AA solution that reacted with the 2,6-dichlorophenolindophenol titrant, which would result in larger than actual values for vitamin C content.

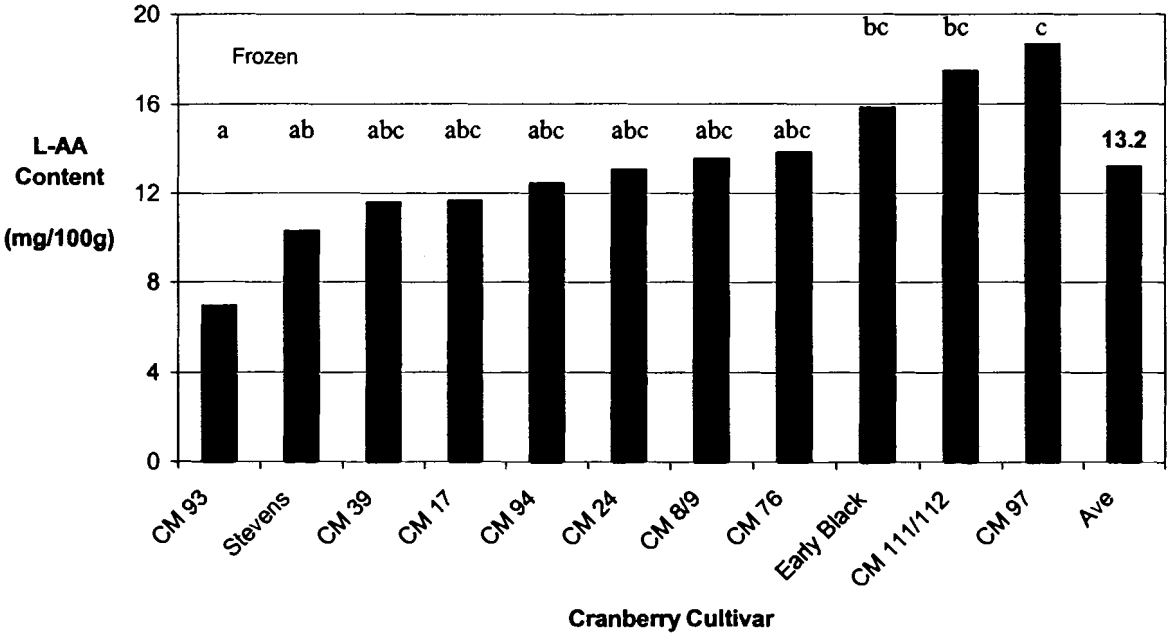
Figure 29 shows the sorted order of L-AA content in fresh and frozen samples. Bars with different letters were significantly different for L-AA content, $p<0.05$. Despite the lack of data to perform the two-tailed t-test for all pairs of fresh and frozen berries, it was assumed that all pairs were significantly different (Figure 30). Data that was available for seven of the cultivars showed uncontested significant differences ($p<0.05$) between fresh and frozen samples. It was interesting to note that the average loss of L-AA in frozen samples was 72.2%.

Except for "CM 111/112," "CM 76" was significantly higher in L-AA content for fresh berries. At the other end of the scale, "CM 100" was significantly lower than 73% of the other cultivars. In the frozen berries, L-AA content varied little.

## FIGURE 29.

## SIGNIFICANT DIFFERENCES IN L-ASCORBIC ACID CONTENT IN FRESH & FROZEN WILD MAINE CRANBERRIES*

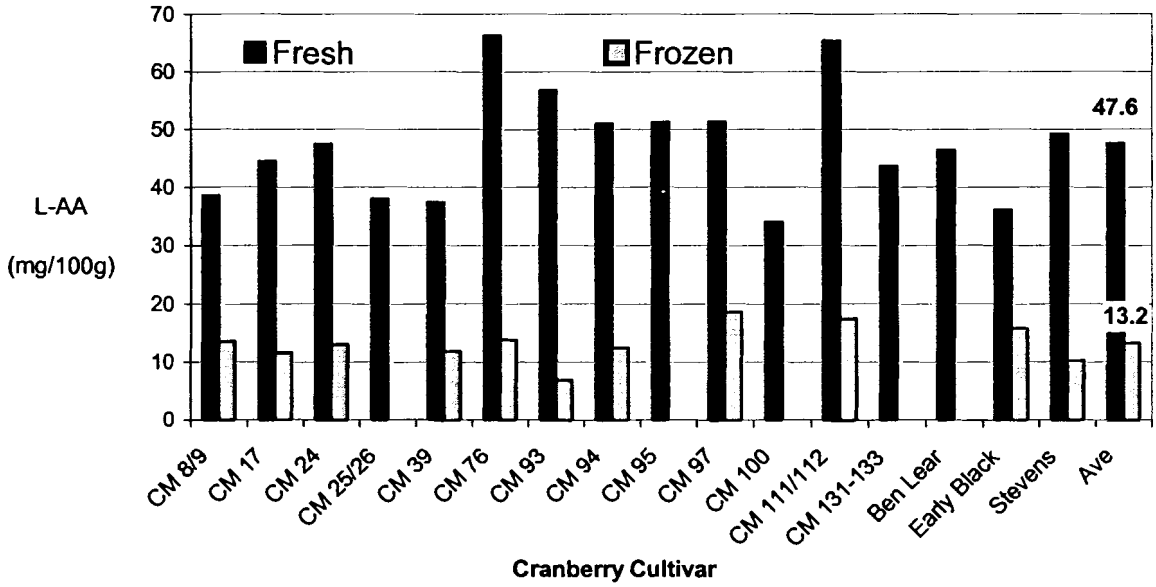



* 1998 Harvest.
Bars with different letters are significantly different, $p<0.05$.

## FIGURE 30.

## L-ASCORBIC ACID CONTENT IN FRESH & FROZEN WILD MAINE CRANBERRIES*




• 1998 Harvest.

# MOISTURE CONTENT

Determination of moisture content in cranberries was a very simple procedure, yet one of importance. It allowed for accurate and consistent calculations of other components, such as the ones researched for this study. Moisture content may vary from one cultivar to another, as well as one berry to another; therefore, it was more reliable to report values on a dry weight basis. The moisture content for the berries in this study averaged 87% for both fresh and frozen states. Significant differences existed for fresh berries, but the overall variation was less than 1% (0.7% CV). The frozen berries showed no significant differences even though the %CV was larger, 2.2%.

# CONCLUSIONS

It has been shown through the research conducted and reported in this paper that wild Maine cranberries are comparable to other cranberries reported in the literature in their anthocyanin, organic acid, and moisture contents. Despite the immaturity of these cranberries (<5 years), they showed great potential for breeding with established cultivated berries. The fresh berries expressed a high average anthocyanin content of 46.26 and 72.65 mg/100g in the harvests from 1998 and 1999, respectively. The average anthocyanin content for the 1999 harvest season was already above the level Mazza and Miniati (1993) described as optimal for cranberry quality (>67 mg/100g).

A method for separating and quantitating cranberry pigments (aglycones) by capillary electrophoresis was developed. The method employed an acidic run buffer, which was advantageous in the analysis of anthocyanic compounds because it exploited the flavylium cation. The method proved to be comparable to its HPLC counterpart with a 0.967 correlation coefficient. The method would be more suitable and efficient in quantitating the anthocyanidins if a reduction in baseline noise could be achieved.

The major organic acids in cranberries, citric, malic, and quinic, were analyzed by HPLC. The average total content for fresh and frozen berries from the 1998 harvest were 2041 and 2035 mg/100g, respectively. The average total organic acid content did not change from fresh to frozen samples, but the individual acid content did change. Citric acid contributed 25% of the total in the fresh berries and 42% in the frozen berries. Malic acid made up 64% and 41%; and quinic acid made up 11% and 17% in the fresh and frozen berries, respectively. No explanation was found in the literature for this change in

individual acid composition between the fresh and frozen samples. It was most likely due to metabolic processes since the berries were macerated before frozen storage. Further research should be conducted.

Because the presence and abundance of organic acids are responsible for cranberries characteristically low pH, which aid in the stabilization and protection of anthocyanins, a relationship between the two groups was looked into. A very low correlation coefficient showed there was little or no effect of the presence of one group on the other.

Vitamin C, or L-ascorbic acid, content of cranberries was also analyzed. The method used was a titration method. Results were very high for the 1998 harvested berries with the fresh berry samples averaging 47.6 mg/100g. There was a significant loss in vitamin C content in the frozen berries, which averaged 13.2 mg/100g. This loss was expected since vitamin C is a very labile compound.

The research conducted and reported in this paper offers information about the hardiness and quality of cranberries grown in the wild in Maine. Continuing research should be conducted on the maturing crops to determine if the berries express certain traits that may enhance the quality of commercial cranberries.

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# BIOGRAPHY OF THE AUTHOR

Dolly J. Watson was born in Presque Isle, Maine on July 26, 1975. There she attended and graduated from Presque Isle High School. She continued her education at the University of Maine where, in May of 1997, she received a Bachelor of Science degree in Human Nutrition. In the fall of 1997, she was accepted into the masters program in Food Science and Human Nutrition at The University of Maine.

During her education at the University of Maine, Dolly was an active member of Kappa Omicron Nu and Phi Kappa Phi honor societies. She was also involved in the Food Science Club and attended professional meetings: Institute of Food Technologists annual meeting in July, 1999 and CESONE in Fall, 1998. She is expected to publish two separate papers on the work she completed while she was a research assistant with the Food Science Department. Dolly is a candidate for the Master of Science degree in Food Science and Human Nutrition from The University of Maine in August, 2001.