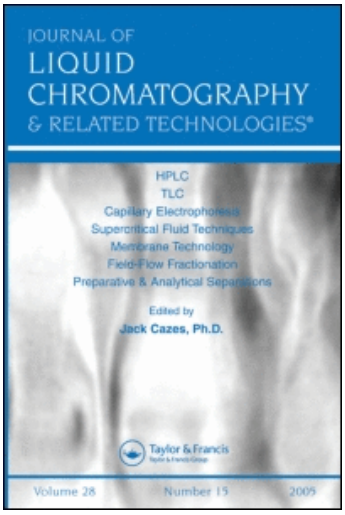

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Separation of Peonidin and Cyanidin, Two Anthocyanidins, in Cranberries by Capillary Electrophoresis

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ABSTRACT

A method by capillary electrophoresis for the separation and quantification of cranberry pigment aglycones was developed. The method employed an acidic buffer consisting of 150 mM phosphoric acid, 3 M urea, and 50 mM β-cyclodextrin (pH 2.11). The acidic pH of the buffer was advantageous in preserving the flavylium cation, maximizing absorbency at 525 nm, and for comparison with high performance liquid chromatography methods. The total analysis time by capillary electrophoresis was under 20 min.

Key Words: Separation; Peonidin; Cyanidin; Anthocyanidin; Cranberries; Capillary electrophoresis; HPLC.

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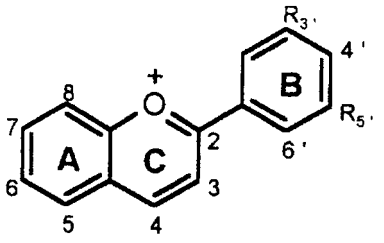
INTRODUCTION

Anthocyanins are natural compounds responsible for the vibrant reds, blues, and violets of flowers, fruits, and vegetables. They are water-soluble glycosides and acylglycosides of anthocyanidins, or aglycones. They are primarily located in the outer layer of plant tissue called the exocarp and are critical for the characterization of specific varieties of cranberries, as well as other plants, since different cultivars may have different anthocyanin profiles. The recent demand for natural food colorants and health supplements by the consumer has stimulated the food industry to use such natural pigments as anthocyanins. Unfortunately, anthocyanins are difficult to isolate and are very unstable in aqueous and slightly acidic or neutral solutions. Recent research has focused attention to address these problems of pigment extraction, isolation, and stabilization.[1–4]

The purpose of this study was to develop a method by capillary electrophoresis (CE) to separate and quantify the pigments in cranberries. The literature on anthcyanin analysis by CE is in its infancy, with only a handful of articles in publication.[5–8] Initial research employed a borate buffer with pH near 8.0, which resulted in the need for very concentrated samples due to decreased peak absorbency and high compound degradation. This study used an acidic buffer to resolve these problems of pigment degradation and high sample concentration.

Because standards were not commercially available for all four of the major anthocyanins in cranberries, it was not possible to identify the peaks from the electropheragram. Therefore, a method was developed to analyze the two anthocyanidins present in cranberries, cyaniding and peonidin (Fig. 1), for which standards were available.



3	5	6	7	3'	4'	5'	Compound
OH	OH	H	OH	OH	OH	H	Cyanidin
OH	OH	H	OH	OCH_3	OH	H	Peonidin

Figure 1. Structures of cranberry anthocyanidins.

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EXPERIMENTAL

Samples

Twelve samples of fresh commercial cranberries from the Maine Department of Agriculture were stored at 38°F for five days, before being extracted in duplicate and analyzed for their anthocyanidin content.

Materials

Fresh cranberries were supplied by the Maine Department of Agriculture. HPLC grade methanol (MeOH), HPLC grade acetonitrile (AN), acetic acid, and hydrochloric acid (HCl) were purchased from EM Science (Gibbstown, NJ). Phosphoric acid, urea, and 2-propanol were obtained from Fisher Scientific Company (Fair Lawn, NJ). Anthocyanidin standards peonidin and cyaniding, 95% purity, were supplied by Indofine Chemical Company (Milford, MA), and distilled water was obtained from our laboratory.

Anthocyanidin Extraction

The procedure applied to the extraction of cranberry pigments was adapted from Sapers et al.,[9] whereas, the acid hydrolysis procedure was adapted from Hong and Wrolstad.[10] Fresh cranberries from the Maine Department of Agriculture were macerated in a Cuisinart (Cuisinart Corp., East Windsor, NJ) and extracted in duplicate. A five-gram sample was weighed into a cylindrical tube and wrapped in aluminum foil to block light. Approximately 20 mL of extraction solvent (95% ethanol: 1.5 M HCl 85 : 15 v/v) was added to the berries and then subjected to a polytron (Kinematica, model CH-6010; Kriens-Luzern, Switzerland) for three minutes at a speed of 45 × 100 rpm. The slurry was then centrifuged (Sorvall RC-5B; Newtown, CT) for 10–12 min at 15,000 × *g*. The supernatant was collected into an amber beaker, and the residue was extracted two more times.

All three supernatants were combined. A 3 mL aliquot of the combined supernatants was combined with 15 mL of 1% HCl in MeOH, in an amber, 50 mL flat-bottomed round flask. The flask was placed in a hot water bath (100°C) for 35–40 min, immediately followed by placing it in a cold water bath until it was sufficiently cooled to be handled (5 min). The liquid was next subjected to a clean up procedure by loading it onto an activated 100 mg C18 Sep-Pak (Waters; Milford, MA) with 5 mL of 2-propanol, 10 mL of distilled water, and the entire sample (ca. 18 mL). Elution of the pigments was done

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with 2 mL of 1% HC1 in MeOH. For CE and HPLC analysis, the pigments were dried under nitrogen and reconstituted in 2.0 mL of 50 : 50 water : MeOH with a pH of 1.0 (HC1). Later, it was determined that the drying step could be eliminated by eluting the pigments from the C18 cartridge with 50 : 50 water: MeOH with a pH of 1.0 ($\pm$ 0.1 pH unit using HC1) solvent. The non drying modification was not used for this study, but has been used in future work. Pigments were not filtered prior to injection due to pigment loss on the filter paper.

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 PhotoDiode Array Detector (DAD) (Hewlett-Packard Company; Waldronn, Germany). Capillary dimensions were 48.5 cm with an effective length of 48.0 cm, an inner diameter of 75.0 mm, a bubble factor of 2.7, and an optical length of 200 mm. The system was conditioned daily with 0.1 M phosphoric acid for 15 min at 40°C, HPLC grade water for 15 min at 40°C, and running buffer for 15 min at 27°C. The concentration of the running buffer was 150 mM phosphoric acid, 3 M urea, and 50 mM β-cyclodrextrin with a pH of 2.11 ($\pm$ 0.1 pH unit) Hydrodynamic injection was employed by applying 50 mBar of pressure for five seconds.

The system was then run at a constant voltage of 20 kV, which resulted in a relatively constant current of 110 A. The temperature was set at 27°C, and the DAD was set to monitor at 525 nm. Impurities were expelled from the capillary during the post-conditioning, which involved flushing the capillary with 0.1 M phosphoric acid for five minutes, HPLC grade water for five minutes, and run buffer for five minutes. All post-conditioning rinses were performed at 27°C. The buffer vials, as well as the post-conditioning vials, were replenished after every fourth injection to maintain a consistent pH. Each sample was injected once, and a standard was injected for every second sample. Standards were made up in 50 : 50 water: MeOH (pH 1.0 with HC1) at a concentration of ca. 0.01 mg/mL.

HPLC Conditions for Anthocyanidin Analysis

Samples were analyzed on an HP 1050 HPLC system equipped with an HP 1050 series isocratic pump and an HP 1040A DAD (Hewlett-Packard Company; Avondale, PA). The column was a Columbus C18; 150 $\times$ 4.6 mm; 5.0 u (Phenomenex; Torrance, CA) with a mobile phase of HPLC grade water,

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acetonitrile, acetic acid, and phosphoric acid in a ratio of 70 : 20 : 7.5 : 1.5. The volume of sample injection was 10.0 μL, the flow rate was 1.0 mL/min, and the wavelength was set at 525 nm. Each sample was injected once, with a standard injected after every third sample. The system was run at ambient temperature. Standards were made up in 1% HC1 in MeOH at a concentration of ca. 0.01 mg/mL. Calculations were determined based on peak areas.

RESULTS

A typical electropheragram and chromatogram of a sample extract of cranberry aglycones are represented in Fig. 2. The order of migration of the two compounds was peonidin, followed by cyanidin. This was different than the order of elution for the same compounds by HPLC, in which cyanidin was eluted first, followed by peonidin. It is evident from the electropheragram that the resolution and peak shape is very good. The two compounds were 100% resolved, which facilitated quantification. The mean migration times and coefficients of variance for 26 consecutive injections of a mixture of peonidin and cyanidin were 14.74 and 18.01 min, respectively, with coefficients of 2.9% and 10.7%. These values support the reproducibility of this method.

Figure 3 shows the results obtained by injecting or introducing the same pigment extracts into both the HPLC and CE systems. The total anthocyanidin content was higher for the HPLC method for every cultivar, but significant differences existed for only two of the twelve cases (17%). Nonetheless, the correlation coefficient was high at 0.967 and suggested that the two methods are comparable for the quantification of cranberry anthocyanidins. Lower results for the CE analysis were most likely due to the decreased sensitivity of the CE on-line detection. The path length of the detection window in CE is much smaller than that found in the HPLC system, which greatly affects sensitivity.

DISCUSSION

The most important parameter regarding CE analysis of anthocyanic compounds was the pH of the run buffer. Initially, a pH of less than 2.0 was tried in order to ensure the compounds were kept in the flavylium cation form, but after 60 min nothing was detected. It was necessary to measure the pH of the run buffer each time it was made, because even slight changes in pH caused shifts in migration time, or affected resolution. The initial pH of the 150 mM phosphoric acid was 1.5. With the addition of 3 M urea, the final pH was around 2.1. It was the addition of the urea that caused the initial separation

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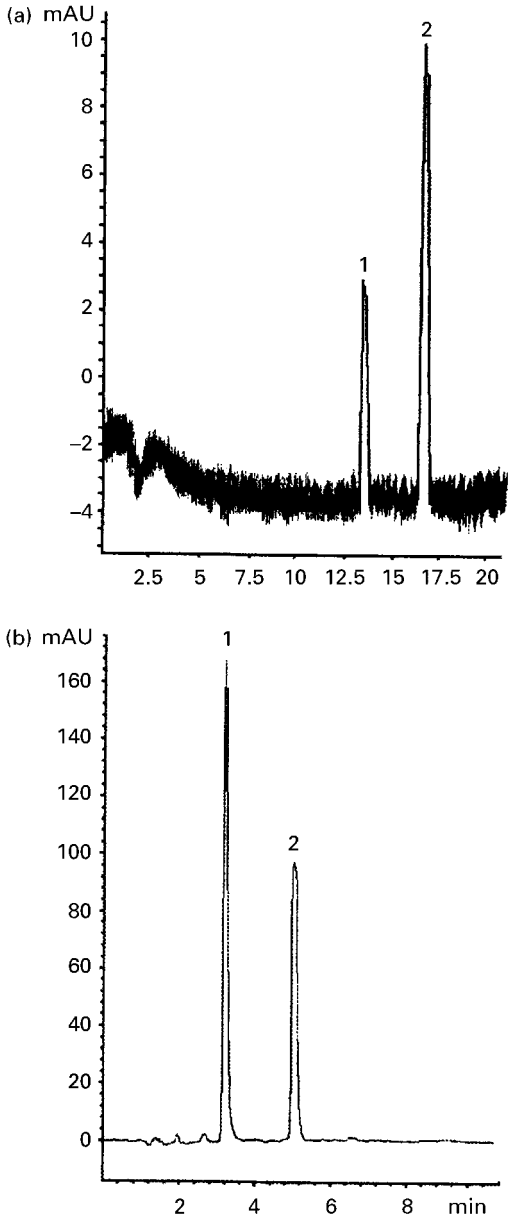

Figure 2. (a) CE electropheragram and (b) HPLC chromatogram of cranberry anthocyanidins.

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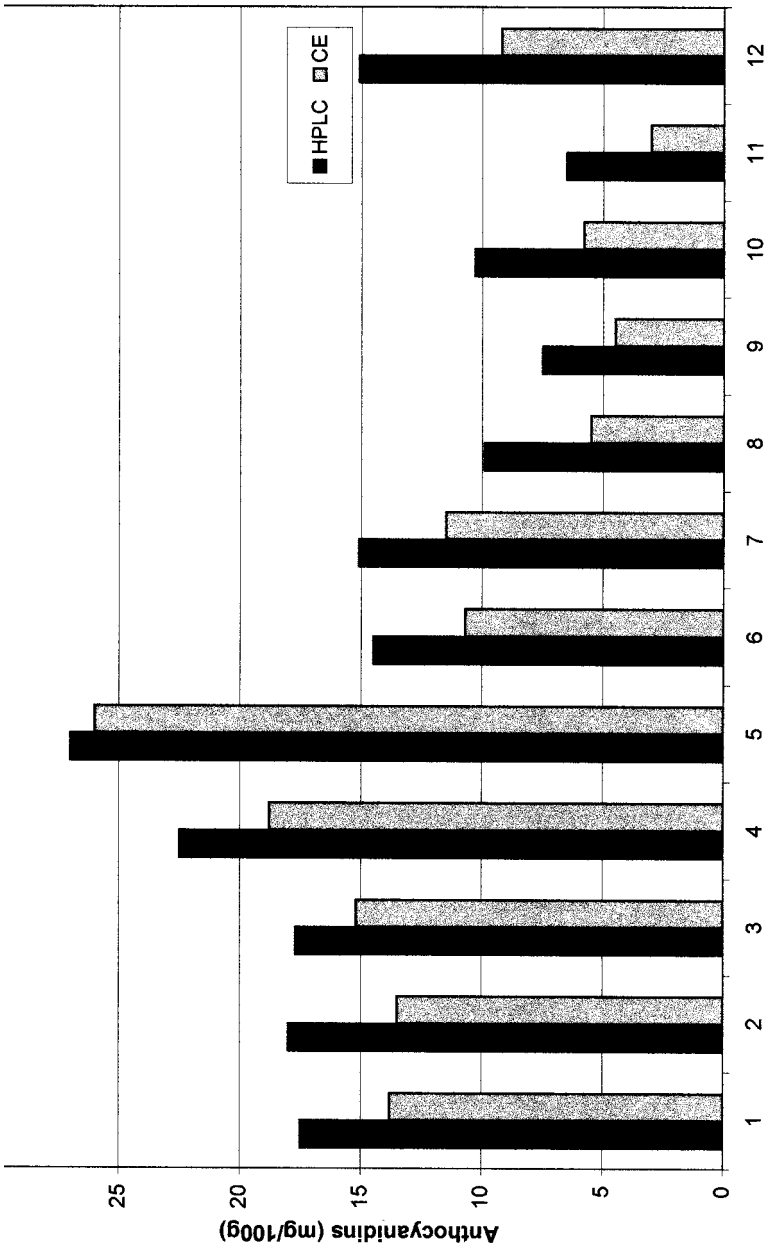

Figure 3. Comparison of the analysis of anthocyanidins by CE vs. HPLC.

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of the two aglycones with a 2 M urea concentration, but levels greater than 3 M urea caused peak broadening, which was most likely due to structural transformations of the pigments at the higher pH values.

The addition of β-cyclodextrin proved beneficial in sharpening the peaks, as well as preserving the compounds. Preservation of anthocyanic compounds from tart cherries by β-cyclodextrin has been investigated by Chandra et al.[11] In this study, several concentrations of β-cyclodextrin were tested to optimize peak shape without compromising migration time. Because β-cyclodextrin added an element of chromatography to CE via the formation of hydrogen bounds between the pigment and the inner hydroxyl groups of the β-cyclodextrin, the more β-cyclodextrin added to the buffer, the longer the run time. There appeared to be no additional increase in peak sharpness with concentrations of β-cyclodextrin greater than 50 mM, but the migration time increased to more than 30 min.

In this application, the main problem with peak shape was that of peak tailing. When looking at the on-line spectra, it was evident that there were different structural forms of the aglycones through the detection window. As can be seen in the electropheragram in Fig. 2, the peaks are 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 electro-osmotic flow), but also increasing the current and sometimes resolution and sensitivity of the analytes.

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