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Separation of blackcurrant anthocyanins by capillary zone electrophoresis

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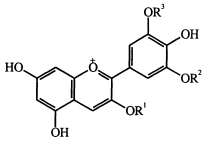
Abstract

The four major anthocyanins present in juice of the blackcurrant (*Ribes nigrum*) may be completely separated by capillary zone electrophoresis under strongly acidic conditions. The separation, resolution and peak shapes of the anthocyanins are critically influenced by the pH of the running buffer and the presence of an organic solvent. Fused-silica and polyacrylamide-coated capillary columns were evaluated for their ability to resolve the closely migrating analytes. Optimum qualitative separation was achieved on a fused-silica capillary with a phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.5. © 1998 Elsevier Science B.V.

Keywords: Blackcurrant; Fruit juices; Food analysis; Anthocyanins; Phenolic compounds

1. Introduction

Anthocyanins are water-soluble pigments that are responsible for the red, purple and blue colors of flowers and fruits of higher plants. Because the anthocyanin profile is distinctive for a given plant, these compounds serve as biochemical markers in plant chemotaxonomy and are used for quality assurance and quality control in the food industry [1]. The fruits of blackcurrant (*Ribes nigrum* spp.) are known to have a high content of anthocyanin pigments. Chandler and Harper [2] were the first to report the isolation and identification of its four major anthocyanins: cyanidin 3-glucoside (CG), cyanidin 3-rutinoside (CR), delphinidin 3-glucoside (DG) and delphinidin 3-rutinoside (DR) (Fig. 1). Anthocyanins are known to occur in different chemical forms that differ in color depending on the pH of the solution (Fig. 2) [3]. At pH 1, the flavylium cation (red colored) is the predominant species (structure I in Fig. 2). Between pH values of 2 and 4, the blue quinoidal species predominate (structures



R^1	R^2	R^3	Common Name
β-D-glucopyranosyl	OH	H	cyanidin 3-glucoside
6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosyl	OH	H	cyanidin 3-rutinoside
β-D-glucopyranosyl	OH	OH	delphinidin 3-glucoside
6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosyl	OH	OH	delphinidin 3-rutinoside

Fig. 1. Structures of the four major anthocyanins found in the blackcurrant juice.

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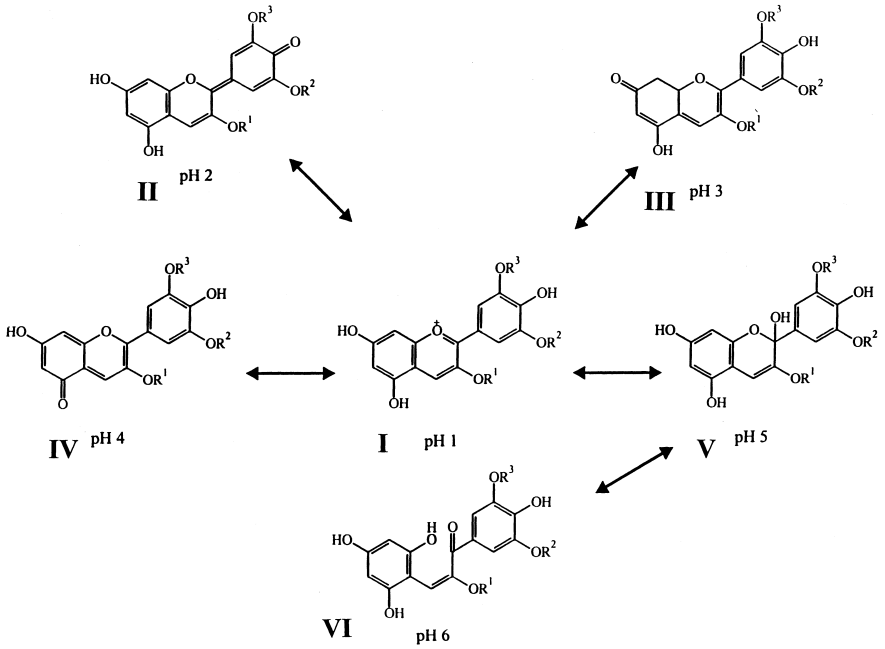

Fig. 2. Equilibrium distribution of a general anthocyanin as a function of the pH of the aqueous solution (modified after Brouillard, 1988 [23]; with permission of the author).

II–IV in Fig. 2). At pH values of 5 and 6, two colorless species form, which have carbinol pseudobase (structure V in Fig. 2) and chalcone structures (structure VI in Fig. 2), respectively. At higher pH values ($\geq$7), the anthocyanin molecules rapidly degrade.

Several reports have been published concerning the separation and identification of blackcurrant anthocyanins using various chromatographic techniques [4–7]. In general, anthocyanins can be separated by high-performance liquid chromatography (HPLC), in their stable flavylium form (red-colored species) using strongly acidic (pH$<$2) mobile phase conditions [8]. Under these conditions, the anthocyanins can be selectively detected by their absorbance at 520 nm.

Recently, Bridle et al. [9,10] used capillary electrophoresis in the free solution mode (capillary zone electrophoresis; CZE) to separate mixtures of anthocyanins qualitatively. In both reports, the separations were performed in uncoated fused-silica capillaries, using borate running buffers at pH 8. The applicability of this method is limited in part by the fact that anthocyanins are pH-sensitive compounds that chemically decompose in basic media. Furthermore, the anthocyanins were detected by absorption at 580 nm, which necessitated the use of very concentrated samples. The lack of a sufficient quantity of absorbing anthocyanin ions at pH 8 hindered the sensitive detection of the analyte ions. A much larger sample concentration (87 times) was required for an equivalent CZE response at pH 8 compared to HPLC at pH 1.8 [10]. One obvious way to prevent the chemical degradation of the anthocyanin compounds during CZE analysis is to separate them using an acidic running buffer. An analysis performed under acidic conditions (pH less than or equal to 2) would also enhance the detection sensitivity by increasing the number of stable flavylium cations in the sample.

In this report, we describe a CZE method for the separation of anthocyanins in a fruit-juice product under strongly acidic conditions. The four major anthocyanins present in blackcurrant juice were

separated on a fused-silica capillary with a sodium phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.5.

2. Experimental[1]

2.1. Instrumentation and equipment

All experiments were carried out on a commercially available capillary electrophoresis system. Detection employed on-column visible absorption at 520 nm. A personal computer provided system control. Data analysis was performed using a chromatography data station. A commercial pH meter was used to check and adjust the pH of the running buffers. Two different capillaries of identical dimensions were tested: an uncoated fused-silica (Polymicro Technologies, Phoenix, AZ, USA) and a linear polyacrylamide (LPA)-coated capillary (Bio-Rad, Hercules, CA, USA). Each capillary was of the following dimensions: total length, L_t, of 75 cm; effective length (length to the detector), L_d, of 70.4 cm and an inner diameter, I.D., of 50 μm. All running buffers were filtered through 0.45 μm disposable syringe-type filter units before use.

2.2. Reagents

All chemicals were of analytical reagent grade, unless otherwise noted. Anhydrous monobasic sodium phosphate and dibasic sodium phosphate were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, chloroform and phosphoric acid were obtained from Mallinckrodt (Paris, KY, USA). Anhydrous diethyl ether was obtained from EM Science (Gibbstown, NJ, USA). Methanol and acetonitrile were of HPLC grade and were purchased from J.T. Baker (Phillipsburg, NJ, USA). Authentic standards of cyanidin 3-glucoside (CG) and cyanidin 3-rutinoside (CR) were purchased from Extrasynthese (Genay, France). A standard of delphinidin 3-glucoside (DG) was obtained from Plantech (Reading, UK). Water from a Millipore Milli-Q system (Bedford, MA, USA) was used for the preparation of samples and buffers.

2.3. Preparation of samples

The blackcurrant extract was a commercial product (Extrenorm) supplied by the Group Fournier (Dijon, France) as a fine powder having a fuchsia color and an odor redolent of blackcurrants. It was originally prepared by crushing the fruits, straining off the clear juice, adding maltodextrin as a stabilizer for the components in the juice, and then spray-drying the treated juice to obtain the powdered extract. The blackcurrant anthocyanin sample was prepared as follows: 1 g of blackcurrant powder was dissolved in 25 ml of water, and this aqueous solution was partitioned against 50 ml quantities of solvents of increasing polarity; chloroform, diethyl ether and ethyl acetate. An equal volume of methanol was added to the remaining aqueous solution. After being kept overnight at 4°C, the mixture was filtered and the white precipitate (maltodextrin) was discarded. The clear filtrate was dried and redissolved in 3 ml of water. A CZE sample was obtained by diluting this stock solution 1:10 (v/v) in water.

2.4. Preparation of standards

A stock solution of each anthocyanin standard was prepared in water at a concentration of 1 mg/ml. Identification of the individual anthocyanins in the blackcurrant samples was accomplished by spiking the samples with the pure anthocyanin standards. CG, DG, and CR were identified based upon spiking experiments, while the slowest migrating anthocyanin, DR, was identified by comparing its UV–visible spectrum (obtained by HPLC analysis with diode array detection) to known spectra. Standards of DR are not commercially available.

2.5. Preparation of running buffers

The aqueous running buffers were prepared by mixing 12.5 ml from two stock solutions of mono-

[1]Disclaimer: Certain commercial equipment, instruments or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified are the best available for the purpose.

basic sodium phosphate (200 mmol/l) and dibasic sodium phosphate (200 mmol/l) and diluting to 100 ml with water. The pH was adjusted with phosphoric acid. For the buffers containing the organic solvent modifier, 10, 20 or 30 ml of either methanol or acetonitrile were added to the buffer before dilution to 100 ml. The apparent pH value was adjusted with phosphoric acid after the addition of the organic solvent.

2.6. Capillary electrophoresis

Separations were performed at 25 kV with the capillary temperature held constant at 20°C. Samples were introduced into the capillary at its anodic end by hydrostatic injection for 4 s at low pressure. The capillaries were rinsed between injections for 60 s at high pressure with each new buffer. Direct visible absorption detection was performed at 520 nm.

3. Results and discussion

Both CZE and micellar electrokinetic capillary chromatography (MECC) have previously been applied to the separation of flavonoid (polyphenolic) compounds [11–16]. The majority of these separations involve the use of running buffers titrated to basic pH values (pH 8.0–10.5). These conditions are suitable for most polyphenols with pK_a values of between eight and ten, but are not suitable for the analysis of pH-sensitive anthocyanins.

3.1. Effect of buffer pH

Initial efforts were focused on resolving the four major anthocyanins (CR, CG, DR and DG) in the blackcurrant juice sample based solely on optimization of the pH of the running buffer. Phosphate-based running buffers titrated to four different pH values (2.8, 2.3, 1.8 and 1.5) were investigated. As the pH value of the running buffer was lowered to strongly acidic values, we observed progressive peak sharpening, a decrease in migration times and improved resolution of the anthocyanin compounds (Fig. 3A–D).

As the buffer pH becomes more acidic, the silanols on the silica capillary wall become progressively more protonated, thereby reducing the charge on the capillary wall and concomitantly decreasing the electroosmotic flow (EOF) [17]. However, in the pH range used in this study (2.8–1.5), the EOF is expected to be low, and the electrophoretic mobility of the analyte becomes a major determinant for the migration behavior of the analytes. The increase in the migration time observed at higher pH values (Fig. 3A,B) was probably due to an increase in the amount of neutral quinoidal bases (structure II–IV in Fig. 2) and, therefore, a decrease in the overall electrophoretic mobility of the components of the sample.

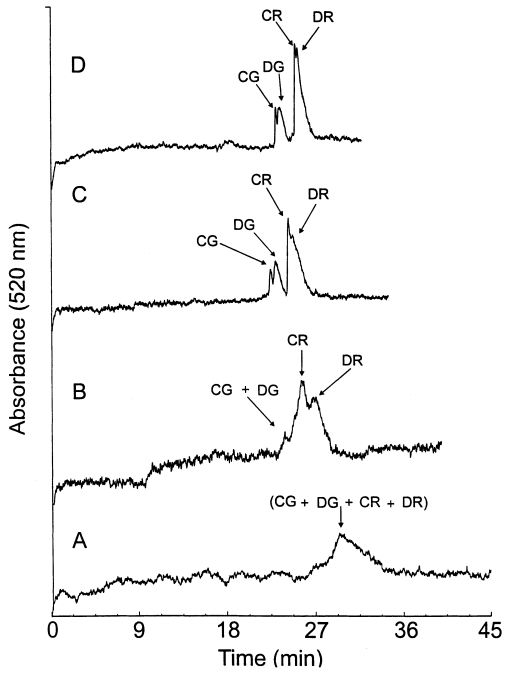

Fig. 3. Influence of running buffer pH on the resolution of the blackcurrant anthocyanins. A=pH 2.8, B=pH 2.3, C=pH 1.8, D=pH 1.5. Separation conditions: running buffer=25 mmol/l NaH_2HPO_4–25 mmol/l Na_2HPO_4, pH variable; capillary=uncoated fused-silica, L_t=75 cm, L_d=70.4 cm, I.D.=50 μm; capillary temperature=20°C; detection=520 nm; injection=pressure (4 s); running voltage=25 kV.

The observed broadening of all of the peaks with increasing pH cannot be attributed to interactions between the wall of the silica capillary and the analytes, because the same peak shape was observed with a polyacrylamide-coated capillary (data not shown). Peak broadening with increasing pH is also

seen in the corresponding HPLC analysis, and has been attributed to slow interconversion between the flavylium cation and the quinoidal forms of the anthocyanin molecules [18]. The phosphate running buffer at an apparent pH of 1.5 provided the best separation of the anthocyanin compounds in the sample, and all further experiments were performed at this pH value. An attempt was made to perform the CZE analysis at a pH value of less than 1.5, but at the voltage used, the current was excessive (over 100 μA).

3.2. Effect of addition of an organic modifier

Addition of organic solvents to the running buffers has been reported to improve the efficiency in some CZE separations [19–21]. To enhance the resolution of the closely migrating anthocyanin compounds, methanol and/or acetonitrile were included in the phosphate running buffer. Running buffers that contained 0, 10, 20 or 30% (v/v) organic solvent at an apparent pH value of 1.5 were used to analyze the blackcurrant sample. In general, as the concentrations of methanol (Fig. 4A–D) and acetonitrile (Fig. 5A–D) in the running buffer increased, there was a corresponding improvement in the anthocyanin peak shape and in peak resolution. The best separation, in terms of peak efficiency and resolution, baseline stability and analyte migration time was obtained with the phosphate running buffer containing 30% (v/v) acetonitrile (Fig. 5D).

Increasing concentrations of organic solvents added to the running buffers have been shown to decrease the EOF, while also shifting the ionization constant of the silanol groups present in the walls of a fused-silica capillary to higher values. This shift was attributed to some extent to the increasing viscosity and/or the decreasing dielectric constant of the running buffer [17]. The migration times for the anthocyanins increased with both organic modifiers, and this effect was more pronounced in the runs with methanol, in agreement with previously reported data [20,21]. The improvement in resolution observed upon addition of organic solvents has been attributed not only to changes in the EOF, but also to changes in the electrophoretic mobility of the analyte. The presence of an organic modifier in the running buffer will have a major impact on the charge density of the

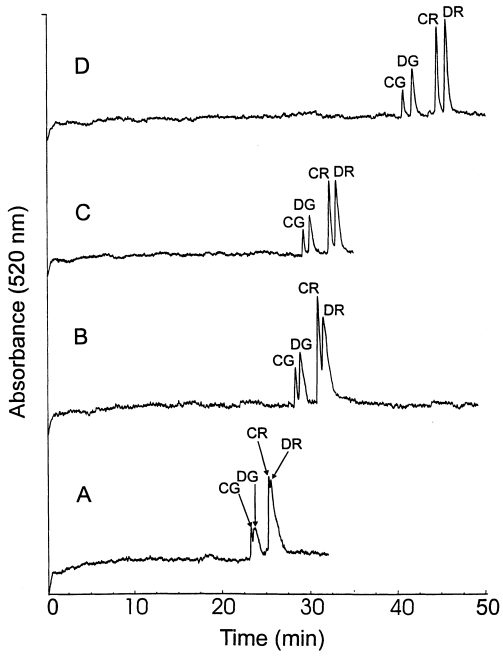

Fig. 4. Effect of methanol buffer modifier on peak efficiency and resolution of the blackcurrant anthocyanins. A=0% (v/v) methanol, B=10% (v/v) methanol, C=20% (v/v) methanol, D=30% (v/v) methanol. Separation conditions: running buffer=25 mmol/l NaH_2HPO_4–25 mmol/l Na_2HPO_4 with a variable methanol content, apparent pH=1.5; capillary=uncoated fused-silica, L_t=75 cm, L_d=70.4 cm, I.D.=50 μm; capillary temperature=20°C; detection=520 nm; injection=pressure (4 s); running voltage=25 kV.

molecule, because it may change the apparent pK_a for ionization of the analyte (the acid–base properties of a buffer change upon introduction of an organic solvent [22]), and generate different solute–analyte interactions [21]. In addition, such modifiers may also affect the electric field gradient by changing the resistivity of the solution, and alter the diffusion coefficient of the solute species by causing changes in the viscosity and hydrophobicity of the solution matrix [21]. Further studies are required to evaluate the contribution of these parameters on the resolution of the anthocyanin molecules.

3.3. Capillary comparison

The best separation of the four anthocyanins was achieved with a fused-silica capillary using a phosphate running buffer with 30% (v/v) acetonitrile at

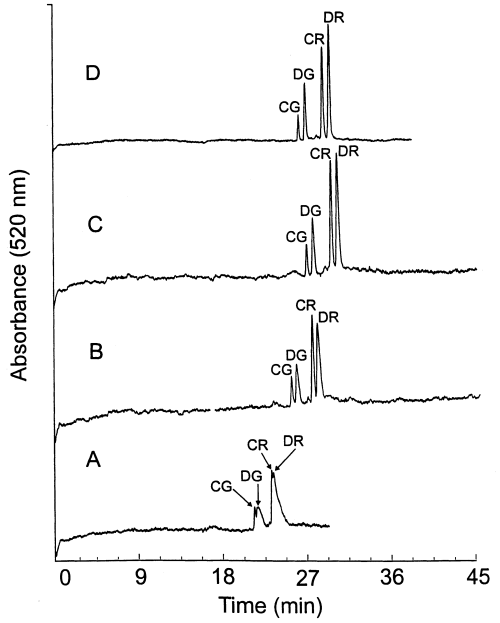

Fig. 5. Effect of acetonitrile buffer modifier on peak efficiency and resolution of the blackcurrant anthocyanins. A=0% (v/v) acetonitrile, B=10% (v/v) acetonitrile, C=20% (v/v) acetonitrile, D=30% (v/v) acetonitrile. Separation conditions: running buffer=25 mmol/l NaH_2HPO_4–25 mmol/l Na_2HPO_4, with a variable acetonitrile content, apparent pH=1.5; capillary=uncoated fused-silica, L_t=75 cm, L_d=70.4 cm, I.D.=50 μm; capillary temperature=20°C; detection=520 nm; injection=pressure (4 s); running voltage=25 kV.

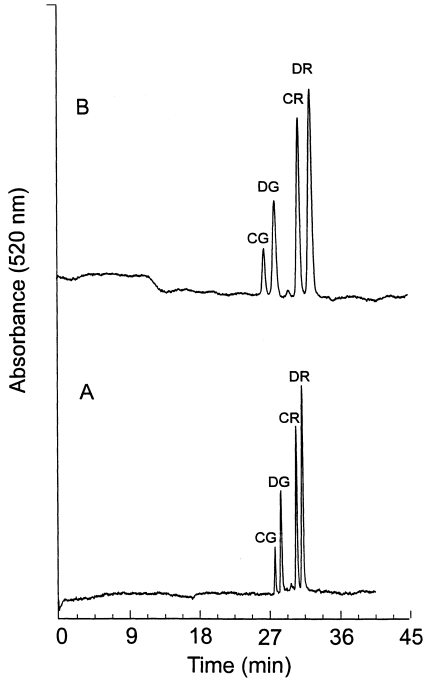

Fig. 6. Comparison of the optimized separation of the blackcurrant anthocyanins on an uncoated fused-silica capillary and on a linear polyacrylamide (LPA)-coated capillary. A=uncoated fused-silica, B=LPA-coated capillary. Separation conditions for A: Running buffer=25 mmol/l NaH_2HPO_4–25 mmol/l Na_2HPO_4–30% (v/v) acetonitrile, apparent pH=1.5; capillary=uncoated fused silica, L_t=75 cm, L_d=70.4 cm, I.D.=50 μm; capillary temperature=20°C; detection=520 nm; injection=pressure (4 s); running voltage=25 kV. Separation conditions for B: Running buffer=25 mmol/l NaH_2HPO_4–25 mmol/l Na_2HPO_4–30% (v/v) acetonitrile, apparent pH=1.8; capillary=LPA-coated, L_t=75 cm, L_d=70.4 cm, I.D.=50 μm; capillary temperature=20°C; detection=520 nm; injection=pressure (4 s); running voltage=25 kV.

an apparent pH of 1.5 (Fig. 6A). To determine if any interaction between the anthocyanin molecules and the fused-silica capillary wall might be occurring, the anthocyanin separation on a LPA-coated capillary was attempted. One of the problems associated with using coated capillaries is their instability, and the LPA coating was not stable under the best conditions (pH 1.5) for the uncoated capillary. Separation of the anthocyanin compounds was not achieved until the apparent pH value of the running buffer was increased to 1.8. At this pH, the coating was stable and gave reproducible separations over a period of several days. The peak shape and resolution on the two columns with the same buffer composition (with and without organic modifiers) at an apparent pH of 1.8 were identical, but the migrations times for the analytes were different on both columns (migration times were 1 to 3 min longer with the LPA column depending on the running buffer). The same peak shape and resolution on both columns is an indication that there is almost no interaction between the silanols on the uncoated capillary and the anthocyanins. The best separation on the LPA capillary was obtained with a phosphate running buffer containing 30% (v/v) acetonitrile at an apparent pH of 1.8 (Fig. 6B). The order of elution for the four anthocyanins was not changed by addition of the organic modifiers or by changing the capillary coating. Additionally, the observed elution order was consistent with the relative charge density of the four compounds.

Using the acidic (pH 1.5) buffer conditions to separate the anthocyanins, we obtained a greater

sensitivity than previously reported using pH 8. We were able to detect our three standards (CG, CR and DG) at concentrations of 25 μg/ml, whereas under basic conditions, 4.0 mg/ml were required for detection of minor peaks [10]. A detailed comparison between the CZE method developed here and a HPLC method for the analysis of anthocyanin pigments in the blackcurrant juice and other plant samples is in preparation.

Acknowledgements

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