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## **ANALYSIS OF NATURAL ANTHOCYANINS BY CAPILLARY ZONE ELECTROPHORESIS IN ACIDIC MEDIA**

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### **ABSTRACT**

Four natural glycosylated anthocyanins were detected at 520 nm, in their colored flavylium cation form using the capillary zone electrophoresis mode in acidic media. For analyses, 0.25 mM cetyltrimethylammoniumbromide in 160 mM  $\text{NaH}_2\text{PO}_4/\text{H}_3\text{PO}_4$  as running buffer was used in a 72 cm  $\times$  50  $\mu\text{m}$  internal diameter fused silica capillary column. The pH of the electrophoretic media was of the order of 2.1. It is demonstrated that hydrophobic interactions between the chromophore and the alkyl chain of the surfactant on one hand and influence of the positive charge of the surfactant on the capillary wall on the other hand, are responsible for the detection of the colored flavylium cation form of anthocyanins in acidic media.

## INTRODUCTION

Anthocyanins belong to the flavonoid-type of phenolic molecules and there are about 300 naturally occurring known structures. They are the most important group of water soluble plant pigments visible to the human eye and they are largely responsible for the colors of flower petals and fruits.<sup>1</sup> However, anthocyanins also appear in roots, stems, leaves, and bracts, usually accumulating in the vacuoles of epidermal or sub-epidermal cells.

Chromatographic techniques such as high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) have been widely used for qualitative and quantitative flavonoids analysis.<sup>2</sup>

Capillary electrophoresis (CE), however, offers now new advantages over HPLC for its faster separation times, smaller samples, and reagents requirements, for instance.

This is why CE, either in the capillary zone electrophoresis (CZE) mode or in the micellar electrokinetic capillary (MEKC) mode, has attracted much attention in the case of polyphenolic compounds analysis.<sup>3-7</sup> The ionization of the hydroxyl groups of the flavonoid aglycone provides a possible separation of such molecules down to pH 7, since they have thus their own electrophoretic mobility.

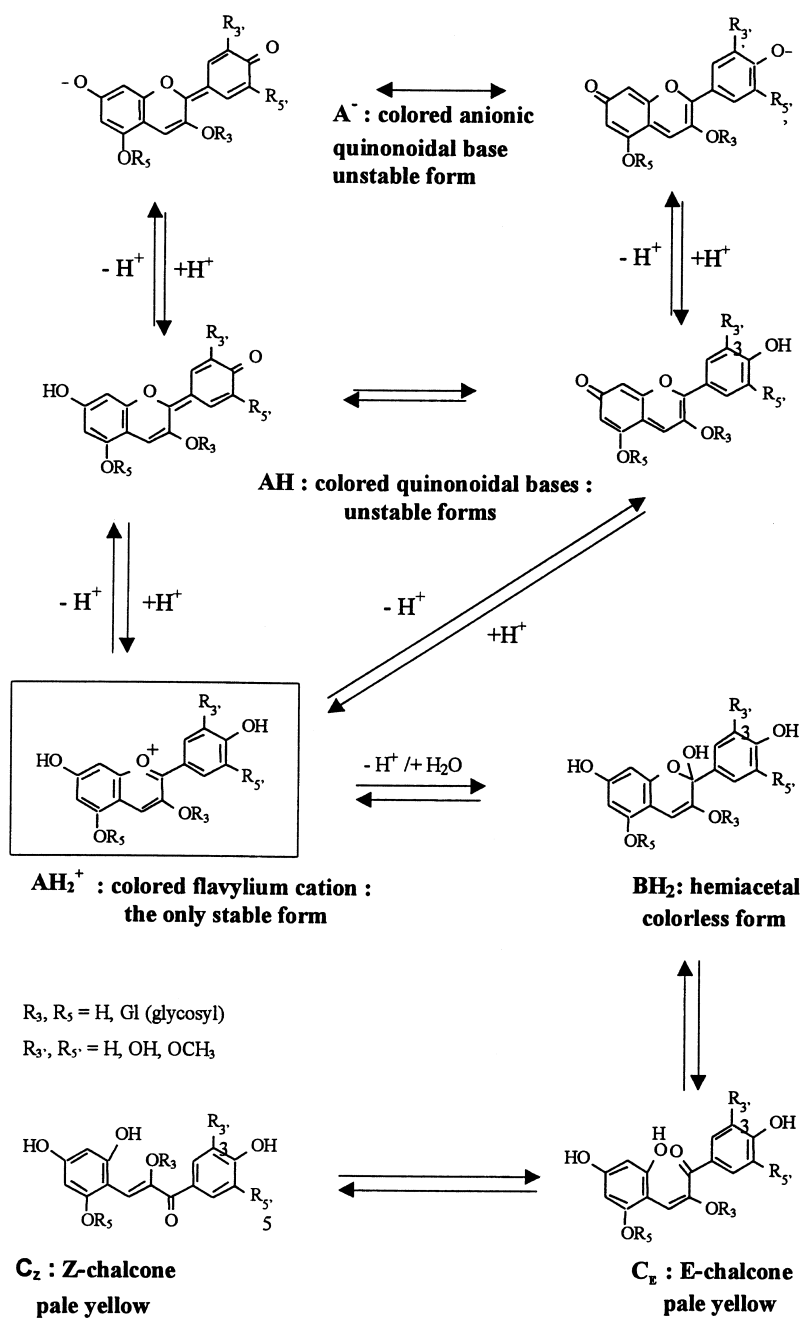
Also, Morin et al.<sup>5,6</sup> separated flavonoid O-glycosides by adding to the electrophoretic buffer a complexing agent (borate) giving negatively charged derivatives.

Only very recently, Bridle et al.<sup>8</sup> described the analysis of anthocyanins by CZE in basic medium (sodium borate, pH 8), where the anionic quinonoidal base could be monitored at 580 nm in small quantities (anthocyanin structural transformations in aqueous solution are presented in Figure 1).

In order to reach the anthocyanin dominating colored structure (flavylium cation), which only occurs in acidic to fairly acidic media, we propose here, a way of analyzing anthocyanins precisely in the acidic range.

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**Figure 1 (right).** Anthocyanin structural transformations in aqueous solution.



## EXPERIMENTAL

We used a Hewlett Packard 3D CE apparatus with diode-array detection. Electropherograms were run at 520 nm using a 72 cm (to the detector)  $\times$  50  $\mu$ m internal diameter fused silica capillary with a 3X bubble cell.

The system was configured to run from cathode to anode. Separations were performed at 25°C with a running buffer whose composition was 0.25 mM of a cationic surfactant, the cetyltrimethylammonium bromide (CTAB) in 160 mM phosphate buffer ( $\text{NaH}_2\text{PO}_4$ ,  $2\text{H}_2\text{O}$  /  $\text{H}_3\text{PO}_4$ ) (pH 2.1) at a voltage of  $-25$  kV. The current generated was about  $-70$   $\mu$ A.

Anthocyanins were injected in a hydrodynamic way for 8 s at 50 mbar pressure.

Four natural anthocyanins were investigated: cyanidin 3-monoglucoside (purified from the black glutinous rice *Oriza sativa* L.), cyanidin 3-monogalactoside, cyanidin 3,5-diglucoside, and malvidin 3,5-diglucoside, all three commercially available from Roth, Karlsruhe (Figure 2).

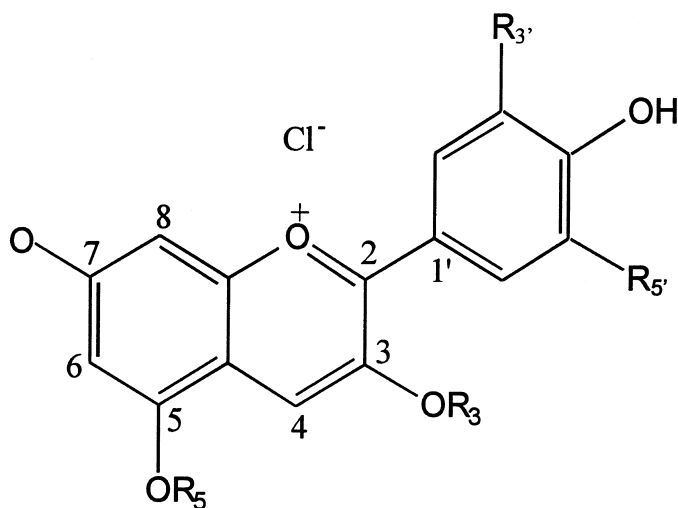
We also injected a crude extract of black glutinous rice. Anthocyanin solutions were prepared using citric acid (100 mM) with disodiumhydrogenophosphate (200 mM) so that the pH value was 2.1. Finally, 30 % (v/v) of methanol were added to the sample.

Anthocyanin concentrations were 0.5 mg / mL for malvidin 3,5-diglucoside, 0.3 mg / mL for cyanidin 3,5-diglucoside, 0.1 mg / mL for cyanidin 3-monoglucoside, 0.05 mg / mL for cyanidin 3-monogalactoside, and in the order of 1 mg / mL for the crude extract.

Running buffers and sample buffers were both filtered prior to injection through a polyvinylidene fluoride filter (0.2  $\mu$ m pore size and 13 mm diameter) from Acrodisc.

Before the first injection, the capillary was washed at 40°C with freshly prepared 1 M sodium hydroxide (10 min), then with 0.1 M sodium hydroxide (10 min), followed by ultrapure Millipore water (10 min) and finally by the running buffer itself, this time, at 25°C (15 min).

Between two sample injections, the capillary was flushed at 25°C with 0.1 M sodium hydroxide (3 min), then with ultrapure Millipore water (3 min) and finally with the running buffer (5 min).



Anthocyanin	R <sub>3'</sub>	R <sub>5'</sub>	R <sub>3</sub>	R <sub>5</sub>
Cyanidin 3-monogalactoside	OH	H	galactosyl	H
Cyanidin 3-monoglucoside	OH	H	glucosyl	H
Cyanidin 3,5-diglucoside	OH	H	glucosyl	glucosyl
Malvidin 3,5-diglucoside	OCH <sub>3</sub>	OCH <sub>3</sub>	glucosyl	glucosyl

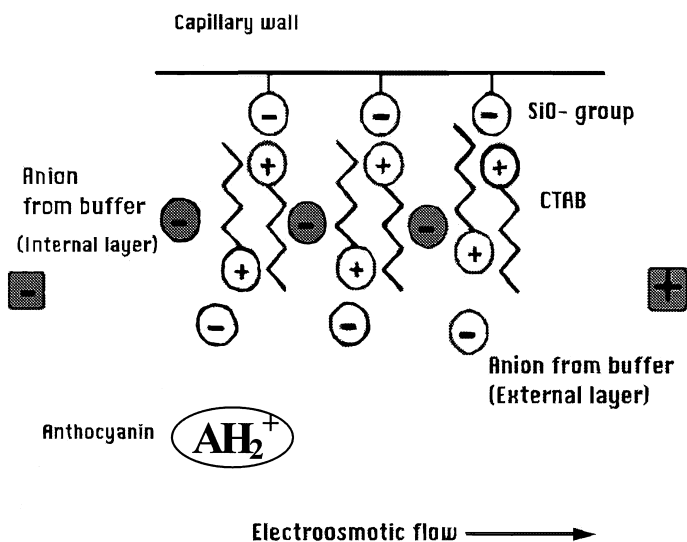
**Figure 2.** Structures of the natural anthocyanins investigated in this work.

## RESULTS AND DISCUSSION

The color of any natural anthocyanin solution is very variable, in tint and intensity, according to the free acidity or pH of the solution.

In strongly acidic aqueous media, anthocyanins are in the orange to red flavylium cation form. Moreover, they are only stable for pH values where the flavylium form dominates.

When an aqueous solution of anthocyanin is brought from pH 2 to neutrality, the uncharged violet quinonoidal bases are formed in small quantities, fading rapidly to give essentially the uncharged colorless hemiacetal (Figure 1).



**Figure 3.** Representation of the inner capillary wall interacting with the cationic surfactant CTAB.

At high pH values, only a little amount of the colored quinonoidal base remains at equilibrium. The whole thermodynamic and kinetic parameters of the equilibria shown on Figure 1 have been established by Brouillard and his collaborators.<sup>9-11</sup>

Until now, analysis of anthocyanins by CE have been made in basic medium at pH 8 with a borate running buffer.<sup>8</sup> At this pH, the quinonoidal bases are the only detectable species in the visible range. Thus, it would be better to detect and identify anthocyanins in their flavylium form, not only because of the good visible light absorption properties of that structure but also because of the easiness of working quantitatively in acidic medium.

We first used slightly acidic solutions (pH 3 to 4), cyanidin 3,5-diglucoside being the anthocyanin. The electropherograms were run in the normal CZE mode (detection at the cathode). Unfortunately, the flavylium cation could not be detected at any wavelength.

We thought that the cation could have been retained on the capillary wall, due to possible ionic interactions between the negatively charged silanoate group SiO<sup>-</sup> covering the inner capillary and the positively charged

flavylium ion. In order to avoid this retention, we deposited on the capillary wall a quaternary ammonium salt with an alkyl long chain, the cetyltrimethylammoniumbromide (CTAB), a cationic surfactant.

Since this is a flow modifier, the direction of the electroosmotic flow was changed; anions from the buffer are now attracted by the quaternary ammonium, and their motion pulls the buffer toward the anode, see Figure 3. The concentration used was only a quarter of the CTAB critical micellar concentration ( $\text{cmc} \cong 1 \text{ mM}$ ), in order to avoid micelles initially.

The sample was prepared in a buffer at the same pH as the running buffer, to keep the anthocyanin in the cationic flavylium form during the whole injection. Under such experimental conditions, all flavylium structures were easily detected at 520 nm (Figure 4).

In order to determine the contributions of both the hydrophobic part and the positively charged polar head of the cationic surfactant in the successful detection of flavylium ions, we attempted two additional experiments. In the 160 mM phosphate running buffer (pH 2.1), we first replaced the CTAB with an alkyl short length quaternary ammonium salt, in this case, the tetramethylammonium chloride. Several buffers were prepared using different concentrations of it going from 5 to 200 mM. After two hours, no peak could be detected whatsoever the wavelength.

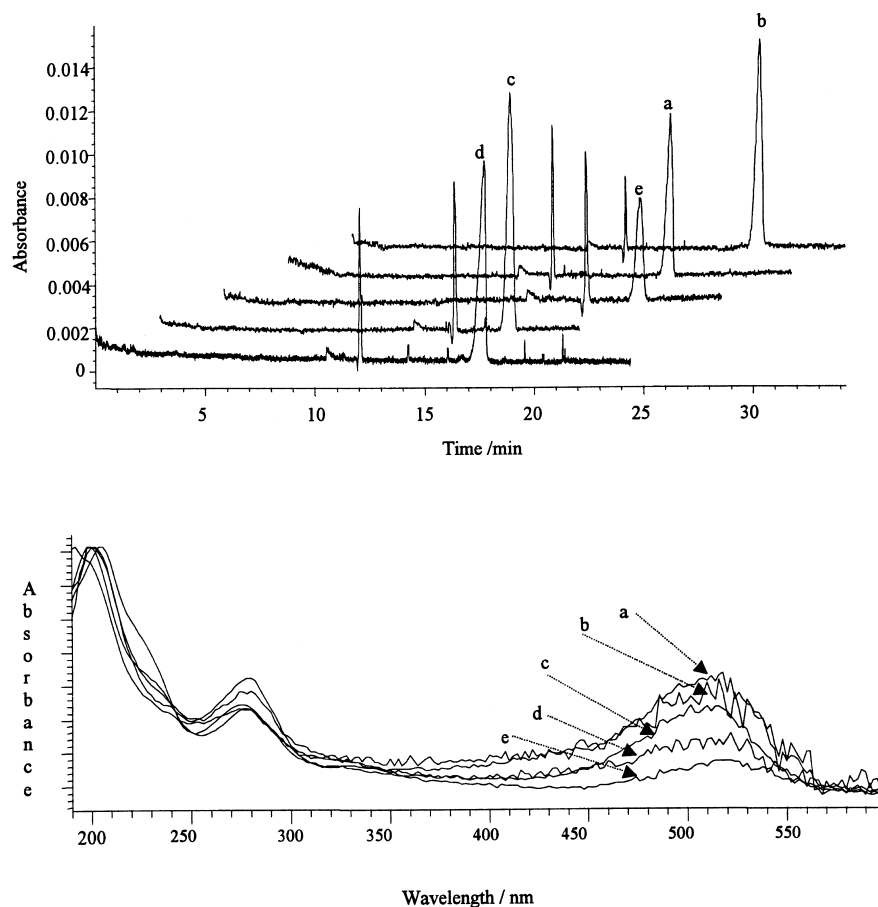
In a second time, instead of using a cationic surfactant in the electrophoretic medium, we used sodium dodecyl sulfate (SDS) in the 160 mM phosphate running buffer. Like for CTAB, we worked at a concentration smaller than the SDS cmc (4 mM,  $\text{cmc} \cong 8 \text{ mM}$ ).

Again, no peak could be detected and we came to the conclusion that flavylium cations are only detected in the presence of an alkyl long-chain quaternary ammonium salt such as CTAB.

Neither the presence of alkyl short length quaternary ammonium salt, nor the presence of alkyl long chain sulfate in the running buffer, produce conclusive effects.

Consequently, the influence of the positive charge of the surfactant on the whole capillary wall on one hand, and the influence of the alkyl chain of the cationic surfactant causing hydrophobic interactions between the chromophore and CTAB on the other hand, are responsible for the detection of flavylium structures in acidic media.





**Figure 4.** Electropherograms at 520 nm (upper part) and related UV-visible absorption spectra of cyanidin 3-monoglucoside (a), cyanidin 3-monogalactoside (b), cyanidin 3,5-diglucoside (c), crude extract of black glutinous rice (d) and malvidin 3,5-diglucoside (e) (lower part).

Flavonoids analysis by CE is generally being achieved with basic running buffers because flavonoids are weak acids whose  $pK_a$  values are in the order of 9-10. With anthocyanins, analysis have to be fulfilled in more acidic media since their ionization is observed at pH values in the range 3 to 5.<sup>12</sup> We will now apply the CE detection method to beverages made out of fruits which all represent acidic media. In particular, attention will be given to the observation of anthocyanins occurring in wines.

**ACKNOWLEDGMENT**

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