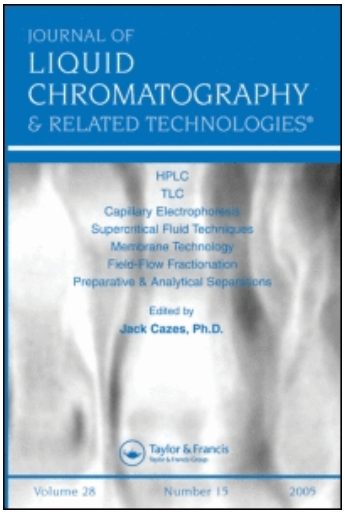


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Anthocyanins by Capillary Zone Electrophoresis

P. Bridle[a]; C. García-Viguera[a]; F. A. Tomás-Barberán[a]
[a] Lab Fitoquímica Dept. Ciencia y Tecnología de Alimentos, Murcia, Spain

To cite this Article Bridle, P. , García-Viguera, C. and Tomás-Barberán, F. A.(1996) 'Analysis of Anthocyanins by Capillary Zone Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 19: 4, 537 — 545
To link to this Article: DOI: 10.1080/10826079608005518
URL: http://dx.doi.org/10.1080/10826079608005518

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 537–545 (1996)

ANALYSIS OF ANTHOCYANINS BY CAPILLARY ZONE ELECTROPHORESIS

P. Bridle,[+] C. García-Viguera,* F.A. Tomás-Barberán

Lab. Fitoquímica
Dept. Ciencia y Tecnología de Alimentos
CEBAS-CSIC Apdo.Correos 4195
30080 Murcia, Spain.

ABSTRACT

A simple mixture of anthocyanins was analysed by CZE, using a standard silica capillary with a pH 8 borate running buffer. Generally, the anthocyanins separated according to the nature of the aglycone and degree of glycosylation. A concentrated injection solution was required for detection at 580 nm, due to the small proportion of coloured quinonoidal base in the anthocyanin equilibrium mixture at this pH, the small injection volume and the short detector path-length used in this technique.

It was observed that the ability to maintain a satisfactory current during analysis was critically dependent on the pH of the injection solution.

[+] Permanent address: Institute of Food Research, Reading Laboratory, Whiteknights Road, Reading RG6 6BZ, U.K.

INTRODUCTION

The CZE analysis of various groups of flavonoid compounds has been reported by several authors. SEITZ et al.[1] used this technique to separate pharmaceutical flavonols, McGHIE[2] analyzed sugarcane flavones and GIL et al[3] studied phenolic compounds in Spanish red wines. The effect of molecular structure on the electrophoretic mobility of flavonols was reported by McGHIE and MARKHAM.[4] The effect of different aglycone substituents and glycosylating groups on the CZE separation of flavonol-7-glycosides was described by MORIN et al.[5-6] A comparison of HPLC and CZE analysis of non-colored phenolics in red wines has recently been completed.[7] Other authors[8-12] have studied flavonoids, using micellar electrokinetic capillary chromatography (MECC) and isotacophoresis.[13] As far as we are aware, this is the first report describing the analysis of anthocyanins by CZE

Anthocyanin analysis using traditional chromatographic techniques is well established[14-15] and high performance liquid chromatography (HPLC) is now a standard technique for analyzing anthocyanins and other natural food colorants,[15-16] offering good separation and excellent quantitation. However, with the advent of capillary electrophoresis, a completely new technique for the separation of a wide variety of natural products has emerged. This preliminary study aimed to discover whether it was possible to analyze anthocyanins by CZE, as a prologue for a future comparison with other techniques.

MATERIALS AND METHODS

Capillary electrophoresis was performed using a Beckman P/ACE System 5510, with diode-array detection at 580 nm (detector path-length 75μm). A fused-silica capillary, 50 cm (to the detector) x 75 μm I.D., was used at 25°C, with a running buffer of 150 mM sodium borate (pH 8). Anthocyanins were introduced by hydrodynamic injection for 2 s (9nL injected) and run at a voltage of 25 kV, producing a current of approximately 45μA. The system was configured to run from anode to cathode. Electropherograms were processed using Beckman System Gold , PC-based chromatography data system software. All anthocyanin standard compounds used were purified from plant sources[17] in our laboratory at Reading, except malvidin 3,5-diglucoside (malvin) (Aldrich Chem. Co., Wisconsin, USA). Anthocyanins were dissolved in a mixture of 25 mM phosphate buffer (pH 2.5) and methanol (3:1). A suitable solute concentration for detection using our conditions was 1.2 mg/mL of each compound (2.5 mM expressed as malvidin 3-glucoside). The capillary was conditioned by washing with methanol for 5 min followed by freshly prepared 1

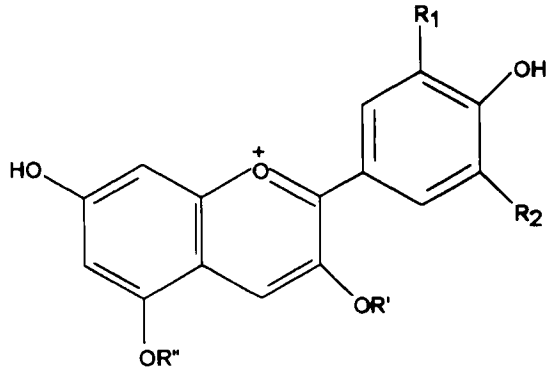
M sodium hydroxide (5 min), 0.1 M sodium hydroxide (5 min), distilled water (3 min) and fresh electrophoretic buffer (3 min). To optimize migration time and peak shape reproducibility, the capillary was flushed between analyses with 0.1 M sodium hydroxide (3 min) and distilled water (2 min).

RESULTS AND DISCUSSION

Anthocyanin standards were selected for analysis by CZE on the basis of their differing structural features (e.g. degree of hydroxylation or methoxylation and class of glycosidic substituent) so that any relationship between structure and electrophoretic behavior might be readily established. (Figure 1)

Analyses were performed at pH 8. Anthocyanins can exist in several equilibrium forms in aqueous solution, depending on the pH; the nature of these structures has been thoroughly discussed.[18-20] The red-colored flavylium cation is the familiar anthocyanin form which predominates at low pH values. Hence all chromatographic methods (paper (PPC), thin layer (TLC), column (CC) and HPLC) for anthocyanin analysis are based on acidified solvent systems, where a low pH is maintained. This colored anthocyanin species is particularly easy to detect selectively from other (colorless) compounds, either visually (PPC, TLC, CC) or at a visible wavelength setting on a HPLC detector. At higher pH values, the colorless carbinol base dominates in an equilibrium with the chalcone; near neutral pH and above, the blue-colored quinonoidal form also makes a small contribution to the total anthocyanin concentration. Thus, freshly made neutral or alkaline solutions are violet or blue, but their colors fade slowly. Furthermore, at pH values above neutrality, ionization of hydroxyl groups on the flavylium molecule may occur; depending on the position of hydroxyl substitution on the molecule, pKa values have reported to lie between 8.2 and 12.5 for analogous flavonols.[4] However, it should be noted that it is impractical to obtain accurate values for anthocyanins bearing multiple ionisable hydroxyl groups, since observation of deprotonation in alkaline solution is obscured by the instability of the pigment, which more or less rapidly disappears to a colorless equilibrium form.[21]

Various CZE parameters can be selected for analyzing anthocyanins, but in this first approach, we used analytical conditions similar to those used successfully for the analysis of non-colored phenolics in red wines.[3] Other variants in methodology are currently being explored, and we will report on these in due course. The separation of anthocyanins under these conditions is affected by three factors: charge-mass ratio, the influence of the electro-osmotic flow (EOF) and the possibility of borate (buffer) complexation, since borate is



R_1	R_2	R'	R"	Compound
H	H	Glucose	H	Pelargonidin 3-glucoside
OCH_3	OCH_3	Glucose	H	Malvidin 3-glucoside
OCH_3	OCH_3	Glucose	Glucose	Malvidin 3,5-diglucoside
OH	H	Glucose	H	Cyanidin 3-glucoside
OH	H	Rhamnose-glucose	H	Cyanidin 3-rutinoside
OH	OH	Glucose	H	Delphinidin 3-glucoside

Figure 1. Structures of anthocyanins.

known to complex readily with ortho-dihydroxyl groups.[22] It is evident from pH equilibria theory, that the anthocyanins should be present as neutral or negatively charged (ionization of hydroxyl groups) species, running from anode to cathode in the CZE capillary at varying rates according to charge density and influence of EOF. Under our conditions (CZE with borate buffer), anthocyanins with the highest negative charge density (charge/mass ratio) will have the greatest electrophoretic mobility (migration towards the anode), and therefore will be more strongly retained in the capillary. The main driving force under these conditions is the EOF, which causes all the anthocyanins in

the sample to elute at the cathode (detector end of the column). Anthocyanins with the least negative charge density elute first; conversely, anthocyanins having the greatest number of ionized phenolic hydroxyls at the buffer pH, and those which are able to complex with borate, will have a greater negative charge and be more strongly retained on the capillary, consequently eluting with longer migration times.

The effect of increasing glycosylation in anthocyanins with the same aglycone, is to shorten the migration time (MT), since the charge/mass ratio is lowest in the most highly glycosylated forms (Figure 2). This is illustrated by comparing malvidin 3,5-diglucoside (MT=5.07) with malvidin 3-glucoside (MT=6.34) or cyanidin 3-rutinoside (MT=6.79) with cyanidin 3-glucoside (MT=7.26); the anthocyanins with the greater mass (but the same charge), elute with shorter migration times in both examples. These results accord well with earlier studies of CZE analysis of flavonol O-glycosides.[23-24]

When examining the migration order of the four anthocyanin 3-monoglucosides, the behaviour of malvidin 3-glucoside (MT= 6.34) compared to pelargonidin 3-glucoside (MT= 6.30), is not explained by the charge/mass ratio theory. In this case, both anthocyanins have the same number of ionizable phenolic hydroxyls and the same possibilities for borate-complex formation; however, malvidin 3-glucoside has the lower charge/mass ratio due to the higher molecular weight of malvidin compared with pelargonidin. Thus, theoretically, malvidin 3-glucoside should elute with a shorter MT than pelargonidin 3-glucoside. However, the reverse situation occurred (Figure 2). Thus, another explanation is required for this anomalous behavior; for instance, it is possible to speculate that the acidity of the 4'-hydroxyl of both anthocyanins may differ due to the presence of adjacent methyl ethers in malvidin 3-glucoside. This could affect the ionization of this hydroxyl group and hence the overall electrophoretic mobility.

The migration order of the remaining 3-monoglucosides accords with theory; consequently the increase in migration time of cyanidin 3-glucoside (MT=7.26) and delphinidin 3-glucoside (MT=7.41), when compared with pelargonidin 3-glucoside (MT= 6.30), is due to the additional free phenolic hydroxyls on the B-ring, capable of ionization at pH 8. Additionally, the possibilities of borate-complex formation are increased, thus delphinidin 3-glucoside elutes later than cyanidin 3-glucoside, which in turn elutes later than pelargonidin 3-glucoside.

The pH of the solvent used for sample preparation is of vital importance. To dissolve the anthocyanins for analysis, it was necessary to use a low pH

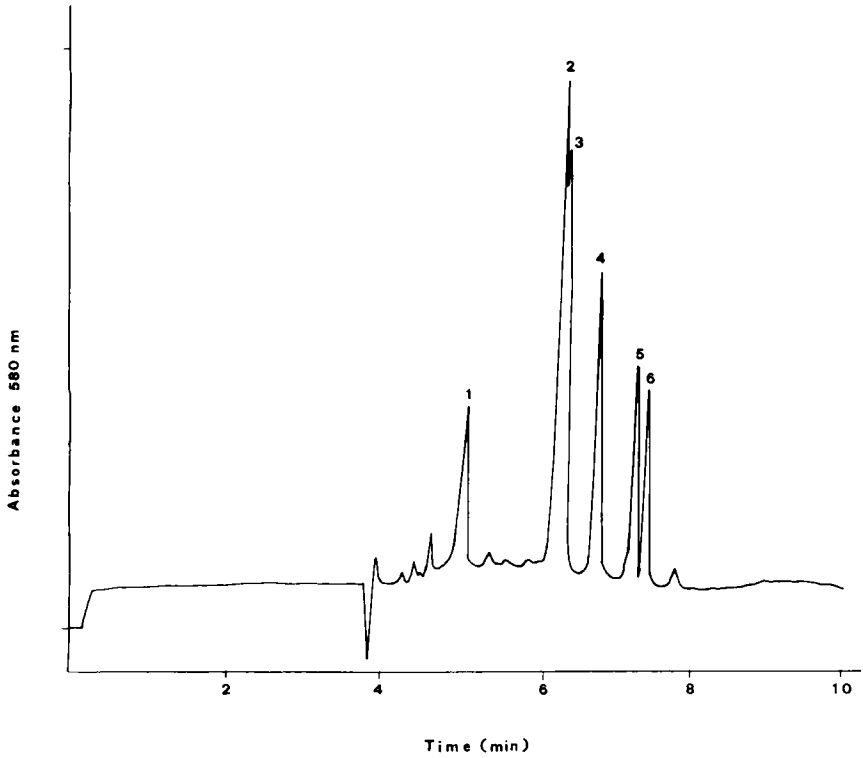


Figure 2. CZE electropherogram of anthocyanin standards. 1) Malvidin 3,5-diglucoside, 2) Pelargonidin 3-glucoside, 3) Malvidin 3-glucoside, 4) Cyanidin 3-rutinoside, 5) Cyanidin 3-glucoside, 6) Delphinidin 3-glucoside.

buffer (pH 2.5), with or without an addition (<25%) of methanol. Our general observation was that 3-glycosides dissolved more readily than 3,5-diglucosides. These conditions provided a good compromise between dissolving the anthocyanin at a pH so high that the sample became unstable and degraded on standing, and a pH so low that there was insufficient running current after injection. Even traces of (formic) acid in the injection solvent had a deleterious effect on the current.

Peaks were adequately monitored at 580nm, a suitable wavelength for detecting the quinonoidal anthocyanin form. The exact pH of the running sample is not certain, but we believe it was very close to pH 8, since the CZE spectrum of cyanidin 3-glucoside matched closely that of the anthocyanin

dissolved in pH 8 buffer and measured on a spectrophotometer. Similar CZE spectra were recorded for the other anthocyanins analyzed. It is difficult at this stage, to estimate how useful spectra taken at pH 8 could be for characterizing different anthocyanins, compared to the more familiar and distinctive spectra typically obtained at acidic pH values. However, in this first approach, the main objective was to obtain a separation of the different anthocyanins, and this means of detection gave adequate information to achieve this end.

In conclusion, we were able to obtain satisfactory separation of a simple anthocyanin mixture at pH 8 using CZE with spectrophotometric detection. The optimization of the analytical parameters for the separation of this interesting group of compounds in an acidic buffer is currently being investigated.

ACKNOWLEDGEMENTS

The authors wish to thank the Spanish Ministerio de Educación y Ciencia and the British Council for financial support (Acciones Integradas 1994/6: 155 B-220B & 561-1338), also J. Aliphon (UK), A. Díez-Cascón and E. Torija (Spain), Beckman Instruments, for technical advice and discussion.

REFERENCES

1. U. Seitz, P.J. Oefner, S. Nathakarnkitkool, M. Popp, G.K. Bonn, Electrophoresis, **13**, 35-38 (1992).

2. T.K. McGhie, J. Chromatogr., **634**, 701-772 (1993).

3. M.I. Gil, C. García-Viguera, P. Bridle, F.A. Tomás-Barberán, Z. Lebensm.Unters Forsch., **200**, 278-281 (1995).

4. T.K. McGhie, K.R. Markham, Phytochem. Anal., **5**, 121-126 (1994).

5. Ph. Morin, F. Villard, M. Dreux, J. Chomatogr., **628**, 153-160 (1993).

6. Ph. Morin, F. Villard, M. Dreux, P. Andre, J. Chromatogr., **628**, 161-169 (1993).

7. C. García-Viguera, P. Bridle, Food Chem., **54** (in press) (1995).

8. P.G. Pietta, P.L. Mauri, A. Rava, G. Sabbatini, J. Chromatogr., **549**, 367-373 (1991).

9. C. Bjergegaard, S. Michaelsen, H. Sorensen, J. Chromatogr., **608,** 403-411 (1992).

10. C. Bjergegaard, S. Michaelsen, K. Mortensen, H. Sorensen, J. Chromatogr., **652**, 477-485 (1993).

11. C.L. Ng, C.P. Ong, H.K. Lee, S.F.Y. Li, Chromatographia, **34**, 166-172 (1992).

12. F. Ferreres, M.A. Blázquez, M.I. Gil, F.A. Tomás-Barberán, J. Chromatogr., **669**, 268-274 (1994).

13. U. Seitz, G. Bonn, P. Oefner, M. Popp, J. Chromatogr., **559**, 499-504 (1991).

14. G. Hrazdina, "Anthocyanins", in **The Flavonoids: Advances in Research**, Eds. J.B. Harborne, T.J. Mabry, Chapman and Hall, London and New York, 1982, pp.135-188.

15. D. Strack, V. Wray, "Anthocyanins", in **Methods in Plant Biochemistry, Vol. 1, Plant Phenolics**, Ed. J.B. Harborne, Academic Press, London, 1989, pp. 325-356.

16. A.G.H. Lea, "HPLC of Natural Pigments in Foodstuffs", in **HPLC in Foodstuff Analysis**, Ed. R. Macrae, Academic Press, London, 1988, pp. 277-337.

17. J.B. Harborne, "The Anthocyanin Pigments", in **Comparative Biochemistry of the Flavonoids**, Academic Press, London, 1967, pp 1-36.

18. R. Brouillard, J.E. Dubois, J. Am. Chem. Soc., **99**, 1359-1364 (1977).

19. R. Brouillard, B. Delaporte, J. Am. Chem. Soc., **99**, 8461-8468 (1977).

20. R. Brouillard, B. Delaporte, **Protons and Ions Involved in Fast Dynamic Phenomena**, Ed. P. Laszlo, Elsevier, Amsterdam, 1978, pp. 403-412.

21. R. Brouillard, " Chemical Structure of Anthocyanins" in **Anthocyanins as Food Colours**, Ed. P. Markakis, Academic Press, London, 1982, pp. 1-40.

22. K.R. Markham, "Ultraviolet-visible Absorption Spectroscopy" in **Techniques of Flavonoid Identification**, Academic Press, London, 1982, pp. 36-49.

23. Ph. Morin, M. Dreux, J. Liquid Chromatogr., **16,** 3735-3755 (1993).

24. F.A. Tomás-Barberán, Phytochem. Anal., **6**, 177-192 (1995).

Received April 27, 1995
Accepted September 15, 1995
Manuscript 3863