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Development and validation of a capillary zone electrophoresis method for the quantitative determination of anthocyanins in wine

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Abstract

A capillary zone electrophoresis (CZE) method is proposed for the quantitative determination of anthocyanins in wine as an alternative to high-performance liquid chromatography. The CZE separation was carried out using a 46 cm (effective length)×75 μm I.D. fused-silica capillary at 10 °C and a 50 m*M* sodium tetraborate buffer at pH 8.4 with 15% of methanol as modifier. A voltage of 25 kV and a hydrodynamic injection of 300 mbar s were used. The electropherograms were recorded at 599 nm. It was found that SO_2 (antibacterial and antioxidant agent added to wine during its production) increased the absorbance of anthocyanins at 599 nm in a basic medium. Therefore, a concentration of 250 mg/l of SO_2 was added to the samples and the calibration solution before the analysis in order to avoid errors by this matrix effect. The analytical response was linear (R=0.998) between 10 and 700 μg/ml of malvidin-3-*O*-glucoside. The limit of detection and the reproducibility (as a relative standard deviation, n=11) were 1 μg/ml and 1.5%, respectively. Finally, the CZE method was validated by the analysis of synthetic wine samples (errors less than 8%) and by the comparison of the results obtained in the analysis of different monovarietal wines by CZE with those obtained by the standard HPLC method. In this comparison, a good correlation (R=0.998) with a slope of 1.005±0.044 and an intercept of −0.752±6.690 was obtained for malvidin-3-*O*-glucoside.

Keywords: Wine; Food analysis; Anthocyanins; Glucosides

1. Introduction

Anthocyanins are the natural pigments, characterized by the flavilium group, responsible for the red–purple colour in red wine. Wine may contain up to 17 of these pigments depending on the variety. They are the 3-monoglucosides of five anthocyanidins (cyanidin, peonidin, malvidin, petunidin and delphinidin), plus the same compounds acylated with acetic, coumaric or caffeic acid. They have been identified in different chemical forms that differ in terms of colour depending on the pH of the solution [1]. In strongly acidic aqueous media (pH 1) the red-coloured flavilium cation is the predominant species. Moreover, anthocyanins are only stable for pH values where the flavilium cation dominates. Between pH values of 2 and 4, the uncharged blue quinoidal unstable species prevails, and if the pH is increased, the ionization of the hydroxyl groups forms the anionic blue quinoidal unstable species. At pH 5 and 6, two colourless species can also form, which have carbinol pseudobase and chalcone structures, respectively. The chemical stability of anthocyanins depends particularly on the glycosylation of position 3. The anthocyanidins, which have a hydroxyl group in position 3, can bond a water

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molecule and form the pseudobase. At pH 3 the anthocyanidin colour decreases gradually and the coloured form cannot be regenerated by acid addition. On the contrary, the anthocyanin glucosides have a glucoside molecule that bonds the 3-hydroxyl group and the pseudobase is stable and, thus, the red form is completely regenerated by acidification.

The evaluation of the anthocyanin content in grapes and wine is important from a commercial point of view. The colour and the quality of wine depend to a large extent on the anthocyanins present in the grapes, but the winemaking and maturation processes also influence the concentration and the type of anthocyanins in wine.

The standard technique to analyse the anthocyanins is high-performance liquid chromatography (HPLC) [2–8]. Reversed-phase HPLC on a variety of C_{18} columns and mobile phase gradients using methanol or acetonitrile are used. The pH of the mobile phase is normally kept below 2 by the addition of a small amount of formic or acetic acid in order to avoid the peak broadening resulting from the interconversion between the red flavilium cation and the blue quinoidal species. Besides, reversed-phase ion-pair chromatography has been proposed [9] to separate the anthocyanins in red wine. A gradient mode elution with tetrabutylammonium hydrogensulphate as a counter-ion, phosphoric acid and methanol–acetonitrile–water in the mobile phase (pH 2) was used in this method.

Photodiode array detection was used to determine the UV–visible spectral characteristics of the pigments [4,6]. Some differences in the UV–visible spectrum may be used to distinguish the anthocyanins from the derivatives. However, mass spectrometry detection coupled with HPLC allows a rapid and unambiguous identification of the anthocyanins in wine [3,10,11] because HPLC–MS combines the separation of the chromatography with the selectivity and sensitivity of the mass spectrometry detection.

The first analysis of a mixture of standard anthocyanins by capillary zone electrophoresis (CZE) was reported by Bridle et al. [12]. Although the anthocyanins are pH-sensitive compounds that chemically decompose in basic media, an uncoated fused-silica capillary and a borate running buffer at pH 8 were used. Moreover, the low concentration of absorbing anthocyanin ions at pH 8 reduced the sensitivity of the method.

Anthocyanins in strawberry and elderberry extracts have also been separated by CZE using a standard silica capillary and pH 8.0 running buffer [13]. Although there are similar characteristics of the anthocyanins in the elderberry and the strawberry, the separation achieved for elderberry extracts was worse than that obtained for strawberry extracts, possibly due to additional interfering compounds in the extract.

Bicard et al. [14] reported the separation of natural anthocyanins by capillary zone electrophoresis in acidic media in order to prevent their chemical degradation and enhance the detection sensitivity for the first time. They proposed the use of a fused-silica capillary in a system set to run from cathode to anode and a running buffer whose composition was 0.25 m*M* of a cationic surfactant, cetyltrimethylammonium bromide (CTAB), in a 160 m*M* phosphate buffer of pH 2.1. The CTAB, a quaternary ammonium salt with an alkyl long chain, was deposited on the capillary wall in order to avoid the retention of the flavilium ion on the capillary wall and change the direction of the electroosmotic flow. In this paper, four natural anthocyanins (cyanidin-3-monoglucoside, cyanidin-3-monogalactoside, cyanidin-3,5-diglucoside and malvidin-3,5-diglucoside) were prepared in the buffer and their migration times were obtained.

Moreover, the separation of the four major anthocyanins present in blackcurrant juice has been carried out by CZE under strongly acidic conditions [3,15]. Fused-silica and polyacrylamide-coated capillaries were evaluated, and optimum 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. However, under these conditions the migration times were too long (27–32 min).

In this paper, we propose the use of CZE as an alternative to HPLC for the quantitative analysis of wine anthocyanins in order to reduce the analysis time and the solvent consumption. This is the first time that wine anthocyanins have been separated by capillary zone electrophoresis. The CZE conditions were optimised using real wine samples and once a good resolution was obtained, the features of the

method were established. Finally, the method was validated and applied to the analysis of several monovarietal wines.

2. Experimental

2.1. Reagents and samples

Boric acid and disodium tetraborate were supplied by Merck (Darmstadt, Germany), sodium hydroxide by Prolabo (France), phosphoric acid by Carlo Erba (Rodano, Italy) and tartaric acid by Sigma (St. Louis, MO, USA). HPLC-grade methanol and ethanol supplied by Merck and Milli-Q (Millipore, Molsheim, France) ultrapure water were used. All the solutions were filtered through a 0.45 μm filter and sonicated for 15 min before use. The C_{18} SPE cartridges were supplied by Supelco (Bellefonte, PA, USA).

Malvidin-3-*O*-glucoside chloride, cyanidin-3-*O*-glucoside chloride and peonidin-3-*O*-glucoside chloride supplied by Extrasynthese (Genay, France) were used as standards. Other wine anthocyanins were not commercially available.

Stock standard solutions were prepared in ethanol. The use of an organic solvent (methanol or ethanol) is necessary to prepare the stock solutions in order to ensure the solubility of the compounds at high concentrations (1–5 g/l). Degradation of anthocyanins was observed during the CE run when aqueous solutions of the compounds, without the buffer capacity of the wine matrix, were used. Therefore, the calibration solutions were prepared in a synthetic wine matrix by dilution of the ethanolic stock standard solutions in a 6 g/l tartaric acid solution of pH 3.5 and adjusting the ethanol content to 12% (v/v). Besides, each anthocyanin produced several overlapped peaks when the percentage of the organic solvent (methanol) was too high (>50%). These peaks might correspond to the different species resulting from the acid–base equilibriums coexisting at a pH between the wine pH (≈3.5) and the tetraborate buffer pH (8.4).

Potassium metabisulfite (Merck) was added to the calibration solution and the wine samples (250 mg/l of SO_2) in order to avoid the differences caused by SO_2 in the analytical response.

The samples were monovarietal red wines from different subzones of the qualified origin denomination Rioja (D.O.C. Rioja). The red wine varieties included Garnacha, Tempranillo and Graciano.

2.2. Sample preparation

Wine samples were centrifuged at 5000 rev./min for 5 min at room temperature using a 5804 Eppendorff centrifuge (Hamburg, Germany). A part of the supernatant was transferred to a topaz bottle, kept at 4 °C under nitrogen and filtered through a 0.45 mm filter before the HPLC or CZE analysis. The rest of the supernatant was preserved at −40 °C and protected from light.

2.3. CZE separation

CZE was carried out using an Agilent CE instrument (Waldbronn, Germany) equipped with a standard cassette containing an uncoated fused-silica capillary and a diode array detector.

The capillary was conditioned before injection by a first washing with 0.1 *M* sodium hydroxide for 2 min, then with ultrapure water for 2 min, and finally with the running buffer for 5 min. The buffer vials were replenished automatically after each run in order to use fresh buffer solution each time and improve the reproducibility of the migration times.

The running buffer was 50 m*M* sodium tetraborate (pH 8.4) containing 15% of methanol. A capillary with an internal diameter of 75 μm and an effective length of 46 cm, a voltage of 25 kV and an average current of 110 μA were used. The capillary temperature was maintained at 10 °C and the samples were injected by hydrodynamic mode at 50 mbar for 6 s (30 nl sample volume or 6 mm plug length). Electropherograms were recorded at 280 and 599 nm and the spectrum from 200 to 599 nm was also collected for each peak. All the analyses were performed in triplicate and the results were expressed as mean values. Anthocyanins were detected at 599 nm because at pH 8.4 they are presented as the blue quinoidal base.

Anthocyanic compounds at pH 8.4 are expected to be negatively charged and migrate towards the anode. The separation depends on the charge/size ratio and the interaction with the buffer molecules.

2.4. HPLC–UV spectrophotometry

HPLC was performed using a modular Waters liquid chromatograph (Milford, MA, USA) equipped with two 515 HPLC pumps, an on-line degasser, a 717 Plus autosampler and a photodiode array detector, and furnished with a Nucleosil 120 C_{18} column (20 cm×0.46 cm, 5 μm) (Teknokroma, San Cugat del Vallés, Spain).

The mobile phase was a mixture of solvent A (water–formic acid, 95:5, v/v) and solvent B (acetonitrile) according to a step gradient from 10% B to 30% B for 60 min, increasing up to 50% in 1 min and then up to 100% in 1 min. The flow-rate and the injection volume were 1 ml/min and 30 μl, respectively. The chromatograms (Fig. 1) were recorded at 520 nm and the UV spectra were collected from 200 to 650 nm. Chromatographic separation was carried out at room temperature.

The anthocyanins were detected at 520 nm because in an acidic medium they are presented as the red flavilium cation.

2.5. HPLC–mass spectrometry

The system was composed of an Engine 5989-B Hewlett-Packard quadrupole mass spectrometer equipped with a 59987 Hewlett-Packard electrospray ionization source operated in positive ion mode and an 1100 Hewlett-Packard liquid chromatograph (Palo Alto, CA, USA). Chromatographic separation was performed under the same conditions described above but the injection volume was 20 μl. The mobile phase flow was split into a 35:965 ratio between the UV detector and the MS detector in order to introduce the optimal flow-rate (35 μl/min) into the electrospray ionization interface. The eluted compounds were mixed with nitrogen at a 30 l/min flow-rate and at 300 °C in the electrospray ionization interface. The cone voltage was a linear function of the relative molecular mass, starting at 40 V for m/z 100 and ending at 80 V for m/z 600. The compounds were chemically ionised by proton transfer, the positive ions generated were introduced into the mass spectrometer and the abundance of selected m/z corresponding to MH^+ ions of anthocyanin compounds were recorded.

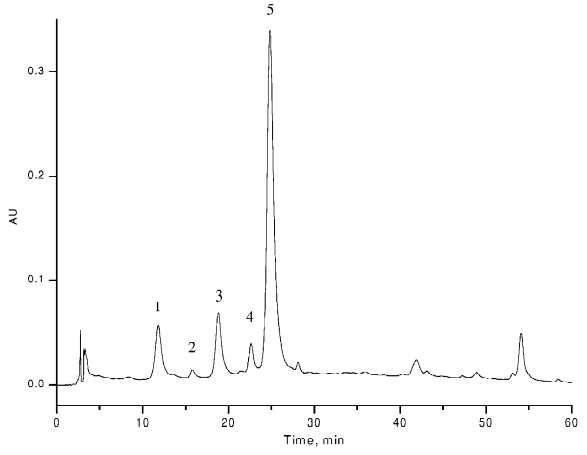

Fig. 1. Chromatogram of a wine sample at 520 nm. Peak identification: 1, delphinidin-3-*O*-glucoside; 2, cyanidin-3-*O*-glucoside; 3, petunidin-3-*O*-glucoside; 4, peonidin-3-*O*-glucoside and 5, malvidin-3-*O*-glucoside. For HPLC conditions see Experimental.

3. Results and discussion

3.1. Optimisation of the CZE separation of wine anthocyanins

A detailed study of the variables affecting the CZE separation was performed using the univariate method. Wine samples were used to study the influence of the electrophoretic variables on the separation.

Two different 20 m*M* buffer solutions were tested: a boric acid buffer and a disodium tetraborate buffer, both at pH 9.2; the latter yielded the best resolution.

The influence of the pH was studied between 8.0 and 10.0. The pH of the disodium tetraborate buffer had a marked influence on the peak resolution and the migration time as the number of peaks and the migration time increased with increased pH. The electropherograms obtained at three pH values are shown in Fig. 2A. As it can be seen, the best separation of the anthocyanins was obtained at pH 8.4.

The CZE separation of wine anthocyanins as cationic species under acidic conditions was also tested using a 72 cm×50 μm fused-silica capillary and a 0.25 m*M* cetyltrimethylammonium bromide in 160 m*M* NaH_2PO_4–H_3PO_4 buffer of pH 2.1 as reported by Bicard et al. [13] for standard solutions

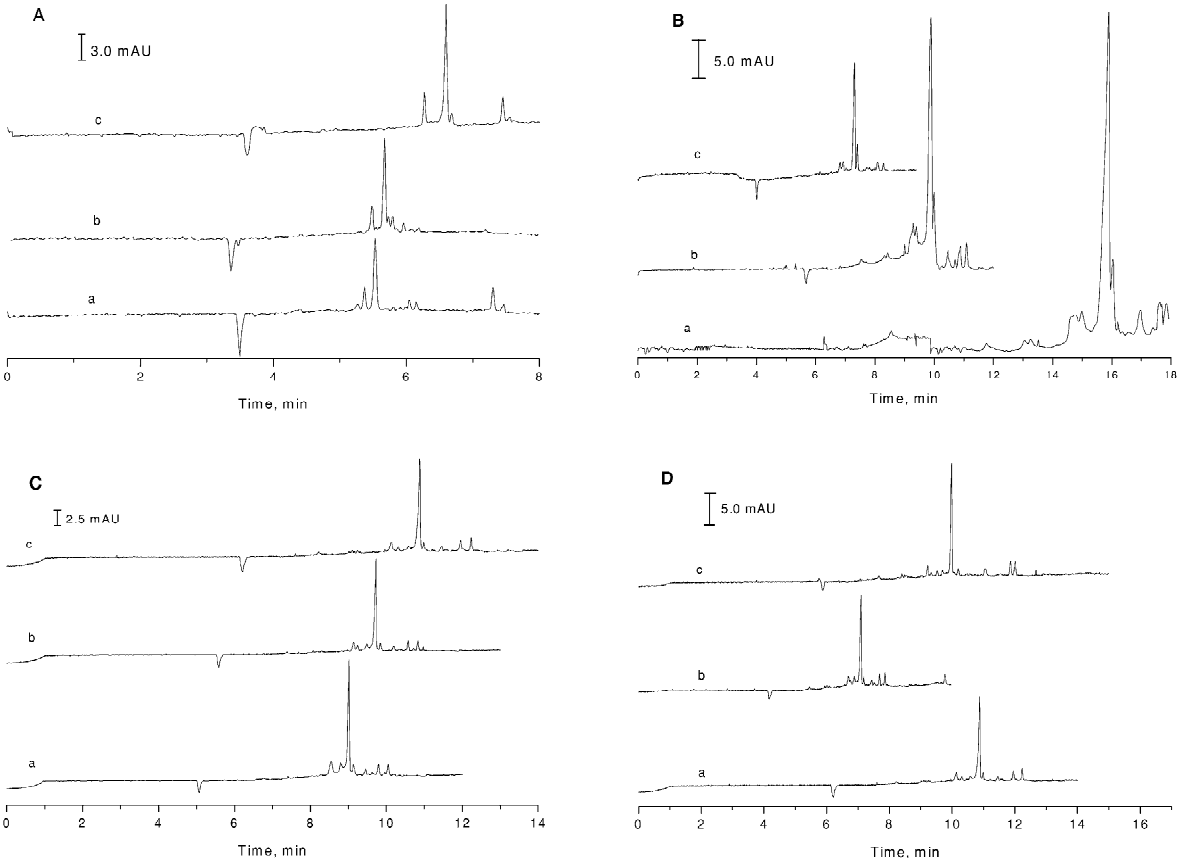

Fig. 2. Optimisation of the CZE variables. (A) Effect of pH on the CZE separation of wine anthocyanins using a sodium tetraborate buffer: (a) pH 8.4, (b) pH 9.3 and (c) pH 10.0. CZE conditions: fused-silica capillary of 56 cm effective length×50 μm I.D. at 25 °C, voltage of 30 kV and hydrodynamic injection of 250 mbar s. (B) Effect of the capillary internal diameter: (a) 100, (b) 75 and (c) 50 μm. CZE conditions: fused-silica capillary of 56 cm effective length at 25 °C and 50 m*M* sodium tetraborate buffer of pH 8.4, voltage of 30 kV and hydrodynamic injection of 100, 150 and 250 mbar s, respectively. (C) Influence of the temperature of the capillary on resolution: (a) 25, (b) 15 and (c) 10 °C. CZE conditions: fused-silica capillary of 56 cm effective length×75 μm I.D., 50 m*M* sodium tetraborate buffer of pH 8.4, voltage of 25 kV and hydrodynamic injection of 200 mbar s. (D) Electropherograms of a wine using (a) a 56 cm capillary, (b) a 46 cm capillary and (c) a 46 cm capillary and 15% (v/v) of methanol as buffer modifier. CZE conditions: 75 μm I.D., 50 m*M* sodium tetraborate buffer of pH 8.4, capillary temperature of 10 °C, voltage of 25 kV and hydrodynamic injection of 200 mbar s.

of other anthocyanins. The electropherogram obtained under these conditions only showed one broad peak. This suggests that, although the acidic conditions could be better than the basic conditions for UV detection, they are not good for the CZE separation of anthocyanins in the wine matrix.

The electroosmotic flow (EOF) can be modified by adjusting the concentration and ionic strength of the buffer solution, and therefore, the effect of the buffer concentration was also studied. The increased buffer concentration decreased the electroosmotic flow, enhancing the mobility differences between the compounds. The buffer concentration was studied within 20–100 m*M*. An increased buffer concentration resulted in an increased resolution but also in increased migration times and thus, a buffer concentration of 50 m*M* was selected for further experiments as a compromise.

In order to increase the signals, the preconcentration of the sample by solid-phase extraction was studied, but the retention of some anthocyanic compounds was not complete. Also, the internal diameter

of the capillary was increased to enhance the sensitivity. Capillaries with internal diameters of 50, 75 and 100 μm were studied. The absorbance signals increase with increasing the capillary diameter because the optical path increases, but the resolution decreases. Nevertheless, increased capillary diameters reduce the adsorption of the compounds on the capillary wall minimizing the tailing peaks. The electropherograms obtained using capillaries with different internal diameters are shown in Fig. 2B. The capillary of 75 μm improved the sensitivity substantially without a significant loss of resolution and thus, this capillary was chosen for further experiments.

The temperature of the capillary had to be controlled because the 75 μm capillary generated a high electric current. The cooling of the capillary has a double positive effect because the viscosity is increased and the diffusion coefficients are reduced with decreased temperatures. Three different temperatures were studied: 25, 15 and 10 °C; the latter provided the best peak resolution as it can be seen in Fig. 2C.

The voltage applied between the anode and the cathode has a direct influence on the electroosmotic speed. In order to evaluate the effect of the voltage on the separation, three different values of this variable were used: 15, 25 and 30 kV. A good separation was obtained at 25 kV. This value was selected as a compromise between the resolution and the migration times.

Since the migration times increased when the capillary temperature was set at 10 °C, the length of the capillary was studied in order to reduce the analysis time. Two capillaries with an effective length of 56 and 46 cm were compared. As it can be seen in Fig. 2D, the migration times were reduced using the 46 cm capillary but with a small loss of resolution.

Buffer modifiers are used to modify the EOF and improve the selectivity. The effect of sodium dodecylsulphate and methanol within a concentration range of 0–20% was studied to use them as buffer modifiers. No improvements on the resolution were observed using sodium dodecylsulphate. On the contrary, 15% (v/v) of methanol in the sodium tetraborate buffer improved the resolution but with some increase in the migration times, as it can be seen in Fig. 2D.

The sample volume injected by hydrodynamic injection mode is smaller when the temperature of the capillary is reduced, and therefore the injection time was also studied. This variable was studied within a range of 200–400 mbar s. The optimum value found was 300 mbar s because it gave rise to a high signal–noise ratio preserving a good resolution. A lack of resolution was observed for the last peaks when larger injection volumes were used.

3.2. Identification of anthocyanic compounds in the electropherograms

Before the CZE analysis, 16 wine anthocyanins were identified by high-performance liquid chromatograpy–mass spectrometry. The identification of the peaks in the chromatogram was based on the MH^+ ion signal, the UV spectrum obtained at the retention time, and the elution order reported in Refs. [4,5,10].

On one hand, fractions of the HPLC effluent were collected at the retention time for malvidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside. The HPLC fractions were passed through a C_{18} cartridge, washed with 9 ml of water, eluted with 4 ml of methanol, and the extracts were concentrated to a volume of about 500 μl under nitrogen. Finally, the concentrated fractions were analysed by CZE in order to identify the compounds in the electropherogram. On the other hand, the identification of the peaks for peonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and malvidin-3-*O*-glucoside was carried out by injecting the standard solutions of these anthocyanins. In this way, the five wine monoglucoside anthocyanins were identified in the electropherogram. The migration times found for malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside were 10.20, 10.60, 12.00, 12.25 and 12.40 min, respectively. An electropherogram is shown in Fig. 3.

Anthocyanins are negatively charged at pH 8.4, so the migration order can be explained by the charge/size ratio. The anthocyanins with a higher relative molecular mass have a smaller charge/size ratio and therefore their migration towards the anode is slower (opposed to the EOF, the main driving force under our conditions, and the detector) and show shorter migration times. Considering only the relative molecular mass, the migration order should be:

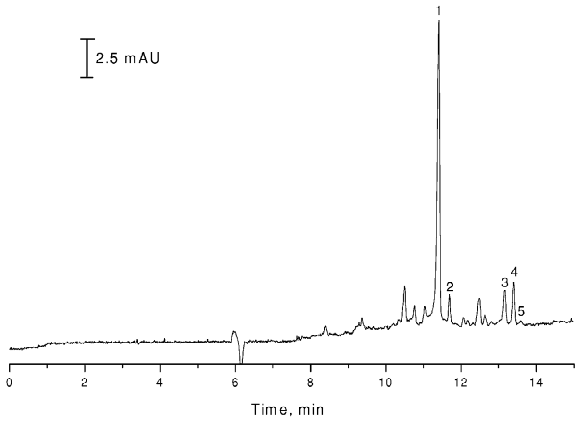

Fig. 3. Electropherogram of a wine sample at 599 nm. Peak identification: 1, malvidin-3-*O*-glucoside; 2, peonidin-3-*O*-glucoside; 3, petunidin-3-*O*-glucoside; 4, delphinidin-3-*O*-glucoside and 5, cyanidin-3-*O*-glucoside. For CZE conditions see Experimental.

malvidin-3-*O*-glucoside ($M_r = 493$), petunidin-3-*O*-glucoside ($M_r = 479$), delphinidin-3-*O*-glucoside ($M_r = 465$), peonidin-3-*O*-glucoside ($M_r = 463$) and cyanidin-3-*O*-glucoside ($M_r = 449$).

There is a contradiction between the migration order found experimentally and that based on the M_r. An explanation of the migration order found could be the differences in the negative charge density and the potential complex formation between tetraborate and anthocyanins with orthodihydroxylation. Malvidin-3-*O*-glucoside and peonidin-3-*O*-glucoside cannot form complexes with tetraborate molecules because they do not have two hydroxyl groups in the *ortho* position, whereas petunidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside do have them and can bond tetraborate molecules. The formation of a complex with tetraborate seems to increase the charge/size ratio. Although the size increases, the negative charge also increases, and therefore, the complex migrates towards the cathode with longer migration times compared to the free anthocyanin. This could explain the delay of petunidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside against peonidin-3-*O*-glucoside.

3.3. Features of the CZE method without SO_2 addition

In order to study the linearity of the CZE response, stock solutions of 5 g/l of malvidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside and 1 g/l of peonidin-3-*O*-glucoside in ethanol were prepared. Dilutions of 10–800, 5–50, and 5–300 mg/l in a synthetic wine matrix were made for malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, respectively, and were analysed in triplicate using the previously described CZE method.

Linear graphs were obtained for cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, but in the case of malvidin-3-*O*-glucoside, a linear behaviour was not observed in the whole range. The malvidin-3-*O*-glucoside signal was adjusted to a polynomial of second degree with a correlation coefficient of 0.997, $A = 0.00055C^2 + 0.168C - 2.439$, where A and C are the peak area and the concentration in mg/l, respectively. Besides, the malvidin-3-*O*-glucoside concentration range studied was divided into two linear ranges as shown in Table 1. The linear range, the equation, the slope and intercept standard deviations, the correlation coefficient and the limits of

Table 1
Features of the CZE methods

Compound	Linear range, mg/l	Equation[a]	s_{slope}	$s_{intercept}$	r	LOD (mg/l)	LOQ (mg/l)
Without SO_2 addition							
Malvidin-3-*O*-glucoside	10–100	$A = 0.189C - 0.81$	0.003	0.19	0.997	6.3	7.2
Malvidin-3-*O*-glucoside	100–800	$A = 0.638C - 78$	0.041	19	0.98	–	–
Cyanidin-3-*O*-glucoside	5–50	$A = 0.201C - 0.08$	0.003	0.08	0.9992	2.0	2.8
Peonidin-3-*O*-glucoside	5–300	$A = 0.276C - 0.63$	0.002	0.23	0.9996	3.7	4.4
With SO_2 addition							
Malvidin-3-*O*-glucoside	10–700	$A = 1.544C + 13.0$	0.015	4.5	0.998	1.2	1.4
Cyanidin-3-*O*-glucoside	5–50	$A = 0.859C + 0.14$	0.016	0.44	0.9993	0.07	0.25
Peonidin-3-*O*-glucoside	5–300	$A = 1.771C + 0.18$	0.020	2.5	0.9996	0.26	0.37

[a] A and C denote the peak area and concentration in mg/l, respectively.

detection (LODs) and quantification (LOQs) for the three wine anthocyanins can be seen in Table 1.

Besides, the reproducibility of the CZE method was evaluated by the analysis of 11 solutions containing 100 mg/l of malvidin-3-*O*-glucoside and 50 mg/l of cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside in the synthetic wine matrix. The peak area reproducibility expressed as a relative standard deviation was 2.0, 7.4 and 8.5% for malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, respectively. The relative standard deviation of the migration time was 0.2, 1.3 and 1.6% for malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, respectively.

Synthetic wine samples containing malvidin-3-*O*-glucoside were prepared and analysed in order to assess the accuracy of the method. The results are shown in Table 2. The relative errors were less than 8% using the relevant linear models in Table 1. However, the relative errors ranged from −43 to 26% when the quadratic equation was used for malvidin-3-*O*-glucoside.

In addition, a recovery study of malvidin-3-*O*-glucoside was carried out. Six wine samples of three types of variety containing malvidin-3-*O*-glucoside concentration ranging from 25 to 300 mg/l were spiked with two different malvidin-3-*O*-glucoside levels (9.3 and 18.7 mg/l). The results obtained are shown in Table 3. It can be seen in the table that the concentrations found were generally in good agreement with the initial concentration plus the added concentration, with recoveries between 91 and 112%.

Table 2
Determination of malvidin-3-*O*-glucoside in synthetic wine samples without and with SO_2 addition

Sample	Conc. (mg/l)	Conc. found[a] ± SD ($n=3$) (mg/l)	Relative error (%)
Without SO_2 addition			
1	32.6	33.4±0.9	2.5
2	60.6	64.3±0.5	6.0
3	93.3	96.0±3.1	2.9
4	139.9	150.8±0.9	7.8
5	233.2	215.5±1.1	−7.6
6	326.4	323.8±1.2	−0.8
With SO_2 addition			
1	23.3	23.9±0.7	2.4
2	51.3	47.4±1.8	−7.5
3	102.6	97.8±0.8	−4.6
4	172.5	170.3±0.7	−1.3
5	233.2	232.8±5.0	−0.1
6	326.4	320.4±6.3	−1.8

[a] Concentration calculated using the corresponding linear equation from Table 1.

A comparison between the concentration of malvidin-3-*O*-glucoside found in real wine samples by the CZE method developed and those obtained by the traditional HPLC method was carried out. A set of 11 monovarietal wines was analysed using both methods. The malvidin-3-*O*-glucoside concentrations ranged from 25 to 300 mg/l. A correlation coefficient of 0.95 was found between both methods, but no agreement was found between the results proving that the CZE results were affected by an error by excess. The regression line indicates a slope of 1.343 with a confidence limit of ±0.339 and an intercept of 109.8 with a confidence limit of ±51.5 ($t_{(n-2)} = 2.262$, $P = 0.05$).

Since no significant errors were found in the analysis of the synthetic samples or in the recovery study, and the results of the comparison with HPLC showed a systematic error, it was concluded that there would be a component in the real matrix (not in the synthetic matrix of the calibration solutions) capable of forming complexes with anthocyanins and modifying the pH-depending equilibriums in a basic medium. SO_2 was identified as the potential interference by an anomalous result obtained for an ecological wine (with a minimal concentration of SO_2 added during its production) and by the fact that SO_2 bonds anthocyanins. Therefore, a further study of the influence of SO_2 on the CZE signal for malvidin-3-*O*-glucoside was done.

3.4. Influence of SO_2

The formation of complexes between SO_2 and anthocyanins has been proposed [16] and thus, SO_2 is expected to play an important role in the displacement of the equilibriums among the different anthocyanin forms (anionic and neutral blue quinoidal forms, flavilium cation and colourless pseudobase and chalcone forms).

The influence of SO_2 on the CZE signal of malvidin-3-*O*-glucoside was studied. Standard solutions containing 300 mg/l of malvidin-3-*O*-glucoside and different concentrations of SO_2, ranging from 0

Table 3
Recovery study of malvidin-3-*O*-glucoside spiked to wine samples without and with SO_2 addition

Wine	Conc.±SD ($n=3$) (mg/l)	Conc. added (mg/l)	Conc. found±SD ($n=3$) (mg/l)	Recovery (%)
Without SO_2 addition				
Gn1	25.2±1.9	9.3	35.7±0.7	112.0
		18.7	45.7±1.1	109.9
Gn2	184.6±0.8	9.3	194.3±0.2	103.9
		18.7	202.8±0.7	97.5
T1	200.6±1.1	9.3	209.2±5.7	91.4
		18.7	219.1±5.2	98.9
T2	216.2±1.4	9.3	225.3±1.7	98.1
		18.7	236.4±0.6	108.5
Gr1	268.3±1.4	9.3	277.0±1.6	93.6
		18.7	286.5±3.3	97.6
T3	282.8±9.0	9.3	292.0±11.4	98.8
		18.7	302.6±6.0	106.4
With SO_2 addition				
Gn3	130.5±2.1	18.7	148.1±1.0	94.4
		28.0	157.3±1.7	95.9
T4	144.3±5.4	18.7	161.5±0.9	92.4
		28.0	170.4±1.2	93.3
T5	144.4±3.0	18.7	164.4±0.9	107.4
		28.0	172.0±1.9	98.5
T6	161.5±1.4	18.7	179.5±1.6	96.6
		28.0	191.4±2.1	106.7
T7	195.9±1.2	9.3	205.3±1.1	100.9
		18.7	214.9±2.3	102.0
Gr2	235.9±0.9	9.3	245.2±1.5	99.4
		18.7	255.3±2.8	103.8

T, Gn and Gr denote the grape varieties: Tempranillo, Garnacha and Graciano.

to 320 mg/l, were prepared in the synthetic wine matrix and analysed by CZE. The peak area increased by increasing the SO_2 concentration until it achieved a plateau at 200 mg/l SO_2, as it can be seen in Fig. 4. Therefore, the SO_2 concentration should be above 200 mg/l in order to avoid differences between the calibration solution and the samples.

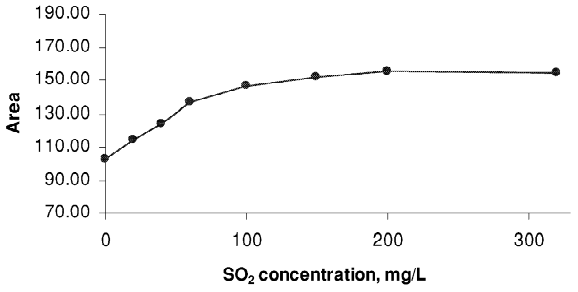

Fig. 4. Influence of the SO_2 concentration on the CZE response of malvidin-3-*O*-glucoside. For CZE conditions, see Experimental.

There were no differences in the migration times for the monoglucoside anthocyanins. A slight peak broadening was observed for increased SO_2 concentrations. However, it did not affect the anthocyanin separation at the working SO_2 concentration level (300–350 mg/l). Only when the SO_2 concentration was around or higher than 800 mg/l, the CZE resolution was affected.

3.5. Features of the CZE method with SO_2 addition

Two hundred and fifty mg/l of SO_2 were spiked to the samples and the calibration solutions just before the CZE analysis. Remarkable improvements in the linearity and the sensitivity were achieved with the addition of SO_2, as it can be seen in Table 1.

A linear graph was obtained for the whole range of malvidin-3-*O*-glucoside concentration studied (10–700 mg/l). The limits of detection and quantification calculated as the noise plus three and 10 times the relative standard deviation of the noise for a blank were 1.2 and 1.4 mg/l for malvidin-3-*O*-glucoside, respectively; but a concentration of 1 mg/l for malvidin-3-*O*-glucoside could be detected since its peak area was more than three times the blank signal. The LOD and LOQ of peonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside were more than 10-fold lower than those obtained without SO_2 addition.

The repeatability study showed a relative standard deviation of 1.5% ($n=11$) for the migration time and the peak area for 100 mg/l of malvidin-3-*O*-glucoside. The relative standard deviations ($n=11$) of the migration time and the peak area for 90 mg/l of peonidin-3-*O*-glucoside were 3.4 and 3.9%; and for 20 mg/l of cyanidin-3-*O*-glucoside 3.3 and 5.6%, respectively.

In addition, the accuracy of the method was checked by analysing six synthetic wine samples with levels of malvidin-3-*O*-glucoside, ranging from 23 to 326 mg/l (the usual levels in wine). The relative errors were less than 8%, as it can be seen in Table 2. Besides, the recoveries found for malvidin-3-*O*-glucoside spiked to real wine samples were between 92 and 108%, as it can be seen in Table 3. The results obtained in the determination of peonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside in synthetic wine samples are shown in Table 4. The relative errors were also less than 8%.

3.6. Validation of the method

The applicability of the method was checked by analysing real wine samples. The results are shown in Table 5.

The HPLC method was used for the statistical comparison of the results obtained by the CZE method. The results are also shown in Table 5. The *t*-paired test applied to both sets of results for each compound showed that the calculated *t*-values were lower than the critical *t*-values ($P=0.05$) as can be seen in Table 6. Thus, it can be assumed that the method provides comparable results. In addition, the results obtained with the CZE method proposed are in good agreement with those found by the HPLC method. The regression equation between the CZE and HPLC results showed coefficients of correlation higher than 0.998 for the three compounds; and the confidence intervals of their slope and intercept includes the ideal unity and zero values, respectively, as listed in Table 6. These results prove the good agreement between both methods.

Table 4
Determination of peonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside in synthetic wine samples with SO_2 addition

Sample	Conc. (mg/l)	Conc. found[a] ± SD ($n=3$) (mg/l)	Relative error (%)
Peonidin-3-*O*-glucoside			
1	5.1	5.0±0.1	−2.0
2	13.9	15.0±0.4	7.9
3	27.9	27.9±0.5	0.0
4	32.6	31.8±0.7	−2.5
5	46.4	49.6±0.7	6.9
6	185.7	195.8±4.6	5.4
Cyanidin-3-*O*-glucoside			
1	4.6	4.5±0.1	−2.2
2	18.5	18.1±0.5	−2.2
3	23.2	24.9±1.1	7.3
4	32.4	35.0±1.1	8.0
5	37.1	38.2±2.1	3.0
6	46.3	48.2±2.0	4.1

[a] Concentration calculated using the corresponding linear equation from Table 1.

Table 5
Comparison of the anthocyanin-3-*O*-glucoside concentrations[a] (mg/l) found in wine samples by CZE and HPLC methods

Sample	Malvidin-3-*O*-glucoside		Peonidin-3-*O*-glucoside		Cyanidin-3-*O*-glucoside	
	HPLC	CE	HPLC	CE	HPLC	CE
1	60.3±3.0	61.9±0.4	4.94±0.03	4.7±0.5	–	–
2	171.5±2.8	173.0±2.1	12.4±0.2	12.6±0.3	2.93±0.07	3.0±0.2
3	202.3±1.5	196.8±2.1	8.3±0.7	8.2±0.4	0.9±0.1	0.9±0.1
4	153.4±2.6	153.1±8.8	8.1±0.3	8.2±0.4	1.36±0.04	1.3±0.1
5	155.2±0.9	157.7±6.3	9.11±0.02	8.9±0.5	1.16±0.02	1.20±0.03
6	95.2±7.3	94.3±3.3	5.9±1.1	5.5±1.2	–	–
7	52.2±1.0	51.0±0.4	5.08±0.06	5.4±0.4	0.74±0.04	0.7±0.1
8	128.8±2.2	130.8±2.2	10.4±0.1	10.2±0.5	1.27±0.03	1.2±0.1
9	88.8±7.7	87.8±0.3	9.2±0.5	9.1±0.1	–	–
10	248.7±4.7	255.0±0.9	17.7±0.3	17.8±0.7	1.48±0.06	1.9±0.1
11	184.5±0.8	178.0±11	5.6±0.1	5.1±0.5	–	–

[a] Mean±standard deviation ($n=3$).

Table 6
Results of the *t*-paired test and regression study applied to the CZE and HPLC values

	t-Paired test		Regression	
	t-Value	Critical *t*-value	Slope	Intercept
Malvidin-3-*O*-glucoside	0.110	2.228	1.005±0.044	−0.752±6.690
Peonidin-3-*O*-glucoside	1.312	2.228	1.028±0.044	−0.342±0.423
Cyanidin-3-*O*-glucoside	0.933	2.447	1.046±0.109	−0.006±0.135

4. Conclusions

A CZE method for the quantitative determination of anthocyanins in wine samples has been proposed for the first time, proving the analytical possibilities of CZE in a basic medium for anthocyanic compounds.

The basic medium allowed a faster separation than the acidic medium. It has been proved that the anionic blue anthocyanin forms are stable enough for quantitative analysis during the separation process if the calibration solution and the sample pH are around 3.5 before the analysis. Moreover, the sensitivity increased with the addition of SO_2.

The CZE method presented a good sensitivity, reproducibility and accuracy. It provided results comparable to the HPLC method, but besides, had a minimal set-up time, reduced the costs and the consumption of reagent and gave better separation efficiencies in a shorter analysis time. Therefore, it can be concluded that CZE is an advantageous alternative to HPLC for the quantitative analysis of wine anthocyanins.

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