Simultaneous determination of *cis*- and *trans*-resveratrol in wines by capillary zone electrophoresis

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A capillary zone electrophoresis method for determining *cis*- and *trans*-resveratrol isomers is proposed. Optimal conditions for the quantitative separation were investigated. A background electrolyte solution consisting of 40 mM borate buffer adjusted to pH 9.5, hydrodynamic injection and 5 kV of separation voltage were used. Good linearity and precision were obtained for the two isomers. Detection limits of 0.06 mg L^{-1} for *trans*-resveratrol and 0.08 mg L^{-1} for *cis*-reveratrol were obtained. The developed method is rapid and sensitive and it has been applied to determine *cis*- and *trans*-resveratrol in several red wines. The samples were purified and enriched by passing them through a preconditioned C_{18} cartridge and eluting the isomers with acetonitrile–water (3 + 7).

Introduction

The phytoalexin *trans*-3,5,4′-trihydroxystilbene (resveratrol) was first reported in the skins of grapes[1] and later in wines.[2–10] Research on the resveratrol content in wines has been due to the interest in the prevention of cancer and heart disease by ingestion of chemical agents that reduce the risk of carcinogenesis.[11–13] Resveratrol inhibits cellular events associated with tumour initiation, promotion and progression. The compound also functions as an antimutagen and has anti-fungal properties.

A number of investigations on the resveratrol concentrations of commercial wines have been conducted. HPLC techniques are the most commonly used procedures[3,4,7,9,14–17] but gas chromatographic,[2,18,19] GC-MS[6,20,21] and capillary zone electrophoresis (CZE)[22] techniques have also been proposed.

Resveratrol exists in two isomeric forms (Fig. 1) that can be present in variable amounts in commercial wines. The *trans* isomer is transformed to the *cis* form under UV light.[3] The physiological activity of the *cis* form has not been studied previously, so it is important to distinguish it from the *trans* isomer and to quantify each separately.

Methods to assay *cis*-resveratrol are less numerous, though more recently various HPLC techniques also became available.[19,23–25] The problem with this isomer is the lack of a suitable standard. The only published procedure to synthesize resveratrol yielded the stable *trans* isomer as the main product[6,26,27] and only this isomer is commercially available at the present time. We have applied similar procedures to those reported previously[24,25] to overcome this limitation and to propose the first CZE method to assay both isomers simultaneously.

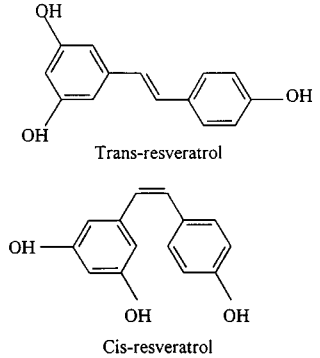

Fig. 1 Structures of *cis*- and *trans*-resveratrol.

The determination of *cis*- and *trans*-resveratrol in wine generally requires the use of extraction and preconcentration techniques prior to CZE, in order to simplify the electropherograms. This is because those compounds are present in wine at very low concentrations and the matrix of the wines is highly complex. Firstly, liquid–liquid extraction procedures have been proposed for sample preparation.[28,29] More recently, solid phase extraction has been applied to separate *trans*-resveratrol and other phenolic compounds[13,22,24,25] using mainly solvents such as methanol and ethyl acetate to elute these analytes from C_{18} columns.

In this paper, we propose a rapid method for CZE to determine *cis*- and *trans*-resveratrol. We used, prior to the electrophoretic separation, a C_{18} cartridge for the extraction and preconcentration of these components in wine. The proposed method is simple and faster than by the described HPLC methods previously used for determining *cis*- and *trans*-resveratrol.

Experimental

Reagents

The organic solvents were HPLC grade (methanol and acetonitrile, Sharlau, Barcelona, Spain). Milli-Q water (Millipore, Watford, Herts., UK) was used throughout the study.

Trans-resveratrol was purchased from Sigma (St. Louis, MO, USA). A stock standard solution of 250 mg L^{-1} was prepared in acetonitrile and stored in the dark. *Cis*-resveratrol was prepared from the *trans* isomer by UV-irradiation.[24] Working standard solutions were prepared by diluting the stock standard solution with purified water or wine.

A 100 mM sodium tetraborate stock buffer solution was used as background electrolyte. A solution of 0.1 M sodium hydroxide was used for conditioning the capillary. The extraction of *trans*- and *cis*-resveratrol was performed in a reverse phase cartridge C_{18} (Waters Sep-pak, Milford, MA, USA). The cartridge was conditioned before use by means of 5 ml of methanol followed by 5 ml of buffer solution, pH = 7.00 (potasium phosphate monobasic–sodium phosphate dibasic).

Apparatus

A Beckman (Fullerton, CA, USA) P/ACE System 5510 capillary electrophoresis equipped with a diode-array UV/VIS detector and controlled by a Dell DIMENSION P133V with P/ACE station software was used. The separation was made using a fused silica capillary (25 cm × 75 μm id) maintained in a cartridge with a detection window of 100 × 800 μm.

Operating conditions

The capillary was conditioned prior to its first use by flushing with 0.1 M NaOH for 10 min then with water for 5 min and finally with the electrolyte solution for 5 min.

The running buffer was 40 mM sodium tetraborate (pH = 9.5) with a voltage of 5 kV, average current of 40 μA and temperature of 25 °C. Samples were injected by hydrodynamic injection for 5 s. Electropherograms were recorded at 320 nm.

The capillary was flushed between two separations with 0.1 M NaOH (1 min), ultrapure water (1 min) and fresh buffer (2 min).

Duplicate injections of the solution were performed and average peak areas corrected (area/migration time) were used for the quantitation.

Sample preparation

Wine (25 ml) was poured into a beaker and neutralized to pH 7.00 by means of NaOH. The cartridge was then slowly loaded with the neutralized wine. After, the cartridge was washed with 2 ml of water, 2 ml of buffer solution, pH 7.00, and 4 ml acetonitrile–water (1 + 9). *Cis*- and *trans*-resveratrol were then eluted with 4 ml acetonitrile–water (3 + 7). The cartridge was then washed with 5 ml of methanol and 5 ml of buffer solution.

Results and discussion

Optimization of the electrophoretic procedure

Effect of electrolyte pH. The pH of the running electrolyte had a significant impact on the ionization of the acidic silanols of the capillary wall and on the electrophoretic mobilities of the isomers studied. A pH in the range 7.0–10.7 was chosen for method development to separate *cis*- and *trans*-resveratrol by CZE. In order to determine the optimum pH value, a set of electrolytes at several pH values were tested. The influence of the electrolyte pH on the migration times of the studied isomers is depicted in Fig. 2.

A pH of 9.5 was selected as optimum in order to minimize analysis times with a good resolution between peaks.

Effect of ionic strength of electrolyte. The optimum ionic strength of the electrolyte must be a balance between an acceptably low current to minimize the noise and a good peak efficiency. The effect of the concentration of buffer solution from 10 to 50 mM on the migration time of the isomers is shown in Fig. 3. As can be seen, when the concentration of buffer increases the migration times of *cis*- and *trans*-resveratrol also increase. A concentration of 40 mM of buffer was selected to maintain good peak shape and low current in order to minimize the noise and baseline aberrations.

Effect of voltage applied. The electroosmotic flow-rate (EOF) and the velocity of migrating isomers are proportional to the applied voltage used for separation. Application of a high voltage reduces analysis times, but may lead to significant losses of resolution and peak efficiencies, because excessive heating occurs within the capillary. The choice of operating voltage should be optimized in conjunction with the choice of electrolyte concentration, capillary dimensions and temperature to produce an acceptable current level.

The influence of the applied voltage upon the migration times is shown in Fig. 4. As expected when the voltage increases from 3 to 10 kV, the migration times of both isomers decrease as well

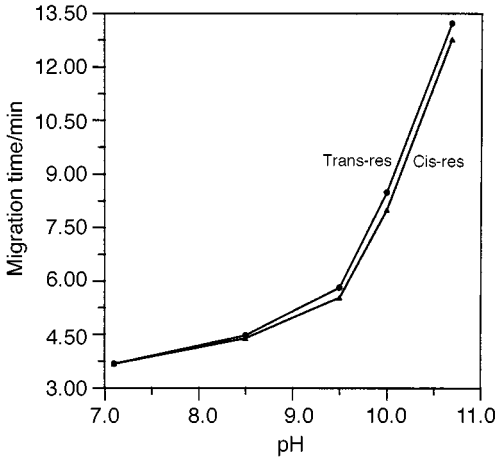

Fig. 2 Influence of electrolyte pH. Operating conditions: variable pH buffers (40 mM), 5 kV and 25 °C.

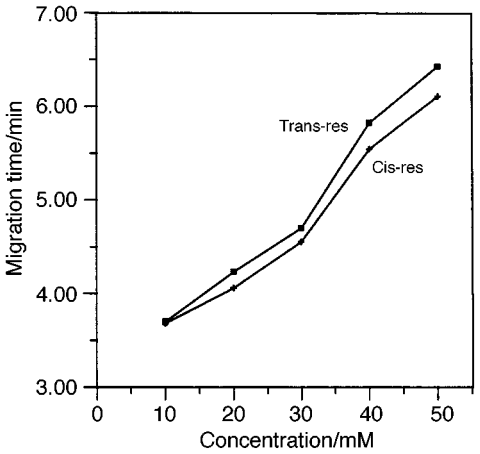

Fig. 3 Influence of buffer molarity. Operating conditions: variable buffer molarity (pH = 9.5), 5 kV and 25 °C.

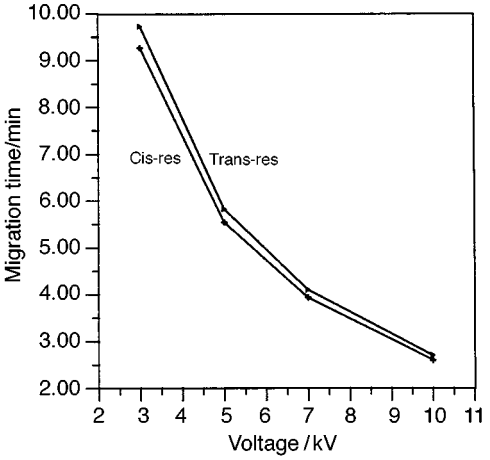

Fig. 4 Influence of voltage. Operating conditions: 40 mM borate buffer, pH = 9.5, variable voltage and 25 °C.

as the resolution between peaks. Therefore, the earlier value of 5 kV was employed in all the studies because good resolution between peaks was obtained and with higher values high currents were observed.

Optimization of the washing step. It is important to maintain a consistent EOF from run to run since any variation results in poor migration time precision. Sample components can become adsorbed onto the capillary surface and change the effective charge on the wall, resulting in a change in EOF. To prevent difficulties owing to adsorption and to ensure a consistent EOF, the capillary is flushed between injections with a dilute NaOH solution that strips the top surface of the capillary wall. A 0.1 M NaOH solution was used to rinse the capillary beween injections.

Samples were analysed following varying rinsing times from 1 to 5 min. Good precision in the migration times were observed in this interval of time. A time of 1 min was selected as suitable to obtain repeatable migration times (ten sequential injections).

After the rinsing with 0.1 M NaOH, a 2 min rinsing with the electrolyte was chosen for the preparation of the capillary before performing the sample injection.

From these studies, the following electrophoretic conditions were selected: electrolyte, 40 mM $Na_2B_4O_7$, pH 9.5; voltage, 5 kV; capillary, fused-silica (25 cm × 75 μm id); injection, hydrodynamic, 5 s; temperature, 25 °C; detection signal, 320 nm.

Fig. 5 shows the electropherogram corresponding to the standards of *cis*- and *trans*-resveratrol. From this electropherogram, it can be ascertained that the selected electrophoretic procedure is excellent for the separation of both isomers. As it can be seen in this figure, the determination of *cis*- and *trans*-resveratrol in wines is faster by the proposed method in this work (CE) than LC (typical analysis times of 6.5 min in CE and at least 10 min in LC[13,23–25]).

Solid-phase extraction in a reversed phase cartridge

First, the method was applied to the analysis of wines which had not been subjected to any special treatment, but due to the presence of a large quantity of various interferent compounds and the low concentration of resveratrol, it was necessary to extract the compounds of interest to obtain a cleaner electropherogram. C-18 cartridges were used to extract *cis*- and *trans*-resveratrol. Variables such as organic solvent, proportion and volume of organic solvent : water ratio in order to elute the analytes free from interferences were studied.

A cleaner electropherogram was obtained when acetonitrile–water (3 + 7) was utilized to desorb the analytes; previously, the cartridge charged with the wine sample was washed with 2 mL of pH 7.0 buffer solution and 4 mL of acetonitrile–water (1 + 9) in order to minimize the interferences. Finally *cis*- and *trans*-resveratrol were eluted with 4 mL acetonitrile–water (3 + 7). This volume of eluent was found to be enough to elute quantitatively the analytes at the concentration levels present in the wine samples.

Fig. 6 shows a representative electropherogram resulting from the analysis of a red wine. As can be seen, good resolution between the interferent compounds of wine and the compounds analyzed was obtained.

Perfomance of the method

Stability of the solutions. Although this test is often considered as part of the ruggedness of the procedure, it should be carried out at the beginning of the procedure validation because it determines the validity of the data of the other tests.

At the beginning, a stock standard solution of *trans*-resveratrol was obtained by dissolving appropriate amounts of this compound in 96% ethanol (v/v). This solution was protected from light and stored at 4 °C. The stability was established by preparing fresh solutions daily. The stock solution was diluted with water by a factor of 20 in a calibrated flask and carrying out a CZE separation step at 25 °C using a potential of 5 kV, 40 mM borate, pH 9.5 and 5 s of injection time. Analyses were repeated every day for 3 d and then at 7 and 14 d.

It could be seen that, for 3 d, the *trans*-resveratrol stock solution showed unchanging electropherograms but, for a week, retention time of resveratrol increased considerably. In order to confirm this fact, the UV absorption spectra were obtained at the apex peaks. The overlay spectra are presented in Fig. 7. Different spectra were obtained as might have been expected.

A similar study was carried out for resveratrol in acetonitrile. In this solvent, the response factors of standard solutions were found to be unchanged for at least up to 10 d. Less than a 0.2% concentration difference was found between the solutions freshly prepared and those aged for 10 d.

Linearity. The calibration graphs for *trans*-resveratrol were produced from results obtained by injecting standard solutions in the range 0.5–20 mg L^{-1}.

For *cis*-resveratrol calibration, aliquots of the *trans* isomer stock solution were diluted in water to cover the range 2–30 mg L^{-1}. A portion of each standard was irradiated for 30 min at

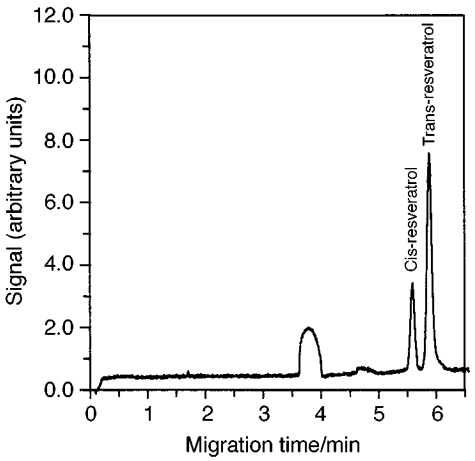

Fig. 5 Electropherogram of a standard mixture of *cis*- and *trans*-resveratrol. Operating conditions: 40 mM borate buffer, pH = 9.5, 5 kV and 25 °C.

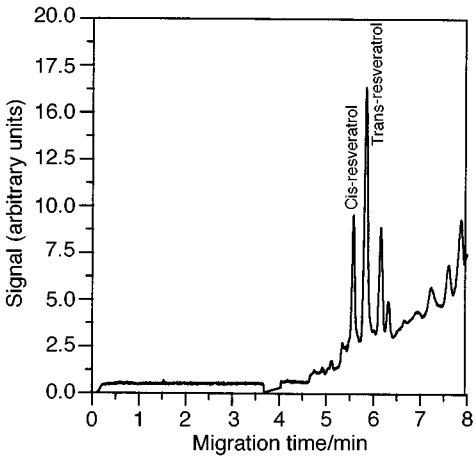

Fig. 6 Electropherogram of a sample of red wine. Operating conditions: 40 mM borate buffer, pH = 9.5, 5 kV and 25 °C.

254 nm. Whereas the non-irradiated standard only contained the single peak of *trans*-resveratrol, this peak was diminished upon irradiation and was preceded by an earlier peak which was shown to be *cis*-resveratrol in an amount identical to the decrease in the *trans*-isomer (Fig. 8). Values for the *cis*-resveratrol standards were therefore assigned on the basis of the decrease in *trans*-resveratrol following irradiation.

Each point of the calibration graph corresponded to the mean value obtained from three independent area measurements. The corresponding regression equation and other characteristic parameters for the determination of both isomers are show in Table 1. The regression line passed through the origin with $t_{calc} = 0.35$ for *cis*-resveratrol and 1.93 for *trans*-resveratrol ($P > 0.05$).

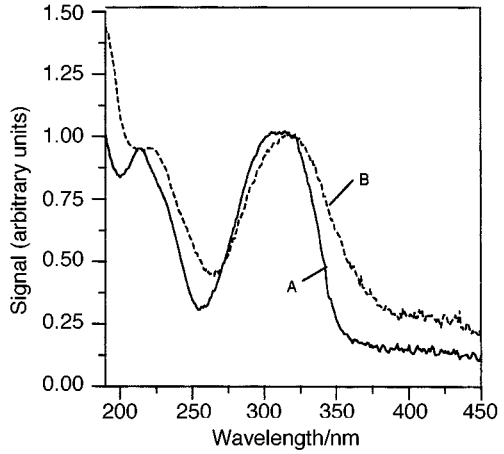

Fig. 7 Absorption spectra of *trans*-resveratrol (8 mg L^{-1}) (A) freshly prepared in ethanol and (B) after seven days prepared in ethanol.

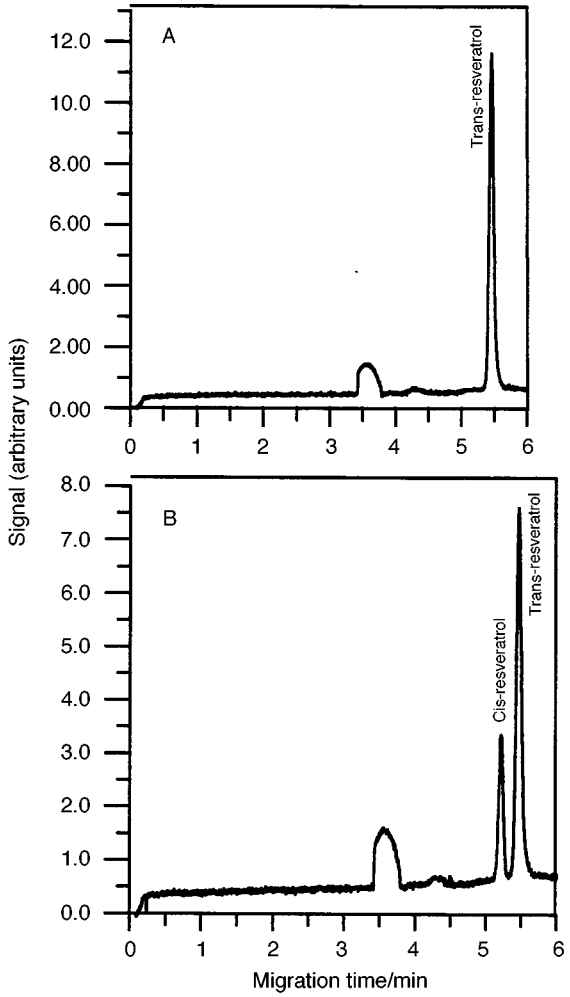

Fig. 8 (A) Electropherogram of a standard of *trans*-resveratrol; (B) electropherogram of a standard of *trans*-resveratrol irradiated for 30 min.

Precision. The precision of the proposed method for determining *cis*- and *trans*-resveratrol is expressed in terms of relative standard deviation (RSD).

In order to test the precision of the electrophoretic procedure, eight injections of a standard of 4 and 5 mg L^{-1} of *cis*- and *trans*-resveratrol, respectively, were carried out sequentially. This operation was repeated over 3 d. The precision of the migration time and peak area corrected were excellent with RSD (%) values ($n = 24$) of 0.25 and 0.8 for migration time and 1.3 and 1.8 for peak area corrected for *trans*- and *cis*-resveratrol respectively.

To evaluate the extraction method, a wine sample spiked with 2 mg L^{-1} of *trans*- and *cis*-reveratrol were analysed independently six times. This analysis was repeated over two days. The average of the recoveries from the spiked wine was 104.17 and 108.60 with RSD (%) of 1.95 and 6.50 for *trans*- and *cis*-resveratrol respectively. Furthermore, six replicate analyses were performed on a wine with *cis*- and *trans*-resveratrol, the procedure was repeated over two days. The average content of *trans*- and *cis*-resveratrol was 1.60 ± 0.06 mg L^{-1} and 1.07 ± 0.06 mg L^{-1}, respectively, with the confidence intervals evaluated at $P = 0.05$. These values are in agreement with the extrapolated values found using the method of the standard additions.

Recovery. In order to test the accuracy of the proposed method, several aliquots of irradiated *trans*-resveratrol standards were added into a wine that had none present. These samples were analysed using the extraction and electrophoretic procedure described in this work. Good results were obtained, as can be seen in Table 2. In all the cases triplicate analyses were made.

Limits of detection (LD) and quantitation (LQ). The LD and LQ were calculated by measuring six blanks, using the maximun sensitivity allowed by the system and calculating the standard deviation of this response. LD was estimated by multiplying the standard deviation by a factor of three. The LQ was defined as ten times the standard deviation.

The LD and LQ obtained considering a concentration factor of 6.25 for each isomer, are summarized in Table 1. The LQ was subsequently validated separately by the analysis of six standards prepared at 0.25 mg L^{-1} for *trans*-resveratrol and at 0.30 mg L^{-1} for the *cis*-isomer.

Analysis of wine samples

To demonstrate the usefulness of the extraction and CZE methods developed, several wines produced in Castilla-La Mancha (Spain) were analyzed.

Table 1 Statistical parameters

	cis-Resveratrol	*trans*-Resveratrol
Equation	$Y = (-80.84 \pm 230) + (1296.91 \pm 53)X$	$Y = (-847 \pm 440) + (1750.79 \pm 36)X$
r	0.9967	0.9991
LD/mg L^{-1}	0.080	0.064
LQ/mg L^{-1}	0.30	0.25

Table 2 Recovery[a]

	trans-Resveratrol			*cis*-Resveratrol		
	Added/mg L^{-1}	Found/mg L^{-1}	Recovery (%)	Added/mg L^{-1}	Found/mg L^{-1}	Recovery (%)
Sample 1	0.9	0.95 ± 0.07	106.1 ± 9.5	0.2	0.23 ± 0.01	115.0 ± 4
Sample 2	1.5	1.63 ± 0.16	108.0 ± 12.9	0.4	0.34 ± 0.05	89.5 ± 4.8
Sample 3	2.0	1.95 ± 0.22	97.5 ± 10.9	0.7	0.8 ± 0.02	111.1 ± 2.2
Sample 4	2.5	2.66 ± 0.10	106.6 ± 4.2	0.96	1.06 ± 0.02	110.3 ± 2.1

[a] Results are mean ± s (n = 3).

Table 3 Analysis of wines

	Standard addition/mg L^{-1}		Direct measurement/mg L^{-1}	
Wine	*trans*-Resveratrol	*cis*-Resveratrol	*trans*-Resveratrol	*cis*-Resveratrol
Rias bajas	0.40	0.29	0.43 ± 0.07	0.32 ± 0.04
Señorio Llanos	1.46	0.98	1.60 ± 0.06	1.07 ± 0.06
Estola	2.18	—[b]	2.05 ± 0.07	—
Viña Albali	0.6	0.53	0.65 ± 0.07	0.49 ± 0.02
D. Eugenio	—	—	—	—
Viña Cuerva	—	—	—	—
Yuntero	—	—	—	—
Tomillar	0.73	0.25	0.74 ± 0.07	0.3 ± 0.02
Cason Histórico	—	—	—	—
Campo Bello	—	—	—	—
Pata Negra	0.88	0.11	0.91 ± 0.02	0.12 ± 0.02
Caserio Vigón	—	—	—	—

[a] Mean value ± s (n = 3). [b] Not detected.

The use of a photodiode detector allowed us to confirm the identity of the peak not only by its migration times, but also by the overlay of the UV-VIS spectra with a standard.

The techniques studied for validating the peak purity corresponding to *cis*- and *trans*-resveratrol in the analysed wines were: normalising and comparing spectra from several peak sections; and absorbance at two wavelengths. Both techniques demonstrate the purity of the obtained peak in all the cases.

The results obtained from wine samples are given in Table 3. All determinations were carried out in triplicate. In order to evaluate the possible matrix effect, the method of standard addition was used for the determinations of these isomers in wines. In both cases the application of the *t*-test for the slopes of the calibration graphs showed no significant statistical differences. Consequently there is no evidence of systematic error affecting the determination of *cis*- and *trans*-resveratrol in wine by the proposed method. The concentrations found by using this method are shown in Table 3 and, as can be seen, they coincide with those obtained without standard addition by the proposed method.

Conclusion

In this work, a method is described for the extraction and determination of *trans*- and *cis*-resveratrol in wine by CZE. Although *trans*-resveratrol has been previously determined in wine by CZE, this is the first report on the determination of *cis*-resveratrol concentrations in wine by CZE. It could be concluded that CZE can be an alternative to traditional existing methods for the determination of *trans*- and *cis*-resveratrol in wine. The proposed method is faster than those previously proposed for the determination of both isomers. The results obtained concerning linearity, recovery, precision and sensitivity were highly satisfactory and comparable to those obtained by the proposed methods in the literature. The developed method allows the determination of *cis*- and *trans*-resveratrol at low levels with detection limit of 0.08 mg L^{-1} for the *cis*-isomer and 0.06 mg L^{-1} for *trans*-resveratrol.

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