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Comparison between capillary electrophoresis and HPLC-FL for ochratoxin A quantification in wine

E. González-Peñas [a,*], C. Leache [a], A. López de Cerain [b], E. Lizarraga [a]

[a] Organic and Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Navarra, C/Irunlarrea s/n, 31008 Pamplona, Spain
[b] Food Science and Toxicology Department, Faculty of Pharmacy, University of Navarra, C/Irunlarrea s/n 31008 Pamplona, Spain

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Abstract

A capillary electrophoresis method with DAD detection has been developed and validated for ochratoxin A (OTA) determination in wine and compared with a previously validated HPLC-FL method. Although the use of UV detection has originated a high value for the quantification limit and, therefore, this method can not be used yet in the determination of OTA in wine in real samples, the analytical performance of the CE-DAD method is fully satisfactory. The more important advantage of CE is the use of the economical and ecological aqueous borate buffer in the separation process. A good correlation of the results has been obtained when 27 fortified wine samples were analyzed by CE-DAD and HPLC-FL. This work demonstrates that the CE technique is a valuable alternative to HPLC-FL analysis of OTA in wine if fluorescence detection is available.

Keywords: Mycotoxins; Ochratoxin A; Capillary electrophoresis; HPLC; Wine

1. Introduction

Ochratoxin A (OTA) (Fig. 1) is a mycotoxin produced by several fungal species from *Aspergillus* and *Penicillium* genera that grow spontaneously in a wide range of commodities, most commonly in cereals, but also in beans, coffee, dried fruits, wine, etc. OTA is known to have nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (International Agency for Research on Cancer (IARC), 1993). The European Commission has fixed maximum limits for OTA in cereals, dried wine fruits, cereal-derived products, infant and young children's foods, coffee and wine as well as their derivatives (Regulation (CE) No. 123/2005). The maximum permitted level for wine (white wine, rosé wine and red wine) is 2.0 ng/ml.

For more than 30 years, considerable research has been devoted to developing methods for the detection and determination of mycotoxins in foods, feed, and biological fluids (Scott & Trucksess, 1997), due to the fact that they are needed for compliance with guidelines, for monitoring and surveying work, and for research. In the case of OTA, the technique used in most studies is HPLC with fluorescence detection (HPLC-FL) because OTA has natural fluorescence (Valenta, 1998). During the past few years, a tendency to research the use of more economical and noncontaminating techniques has been observed; efforts have been made to attempt to reduce the use of organic solvents as they result in higher economical and ecological costs (Corneli & Maragos, 1998).

Capillary electrophoresis (CE) has been recognized as a suitable separation technique that offers the advantages of faster method development, higher efficiency and lower consumption of solvents and reagents. Some methods using CE have been developed for mycotoxin

* Corresponding author. Tel.: 34 948 425653; fax: 34 948 425652.
E-mail address: mgpenas@unav.es (E. González-Peñas).

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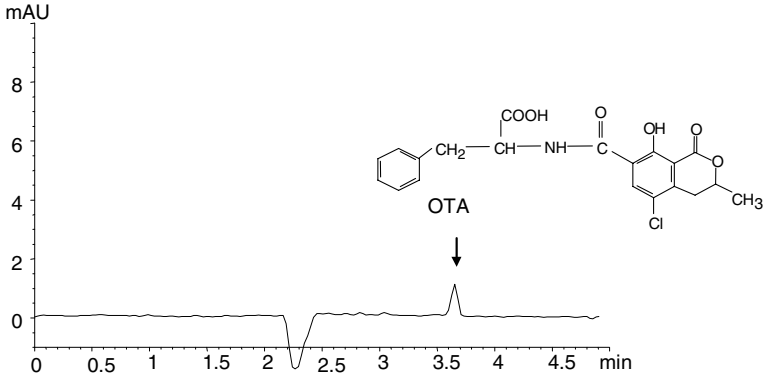

Fig. 1. Chemical structure of OTA and electrophoregram obtained from a sample of wine fortified at 60 ng OTA/ml level.

analysis: fumonisin B1 in corn (Hines, Brueggemann, Holcomb, & Holder, 1995; Maragos, 1995); aflatoxins in corn (Maragos & Greer, 1997) and ochratoxin A in coffee, corn and sorghum (Corneli & Maragos, 1998), but up to now said methods are few in number and no method using CE has been reported for the determination of OTA in wine.

In this work, an analytical method using CE with Diode Array Detection (DAD) for OTA determination in wine has been developed and validated. It was compared with a previously validated HPLC-FL method with respect to the difficulty in sample preparation, development and validation of the method, as well as with regard to the cost of analysis and the results obtained when the samples were analyzed by the two different techniques.

2. Experimental

2.1. Chemicals, reagents and wine samples

Ochratoxin A was purchased from Sigma (St. Louis, MD, USA). All of reagents were pro-analysis grade. Monobasic potassium phosphate, di-basic sodium phosphate, sodium hydroxide, sodium tetraborate, sodium acetate, orto-phosphoric acid 85% and hydrochloric acid 35% were purchased from Panreac (Barcelona, Spain). Chloroform was obtained from Scharlau (Barcelona, Spain), while acetonitrile and methanol HPLC grade were obtained from Riedel de Häen (Seelze, Germany). Ochratest Immunoaffinity Columns (IAC) were purchased from Vicam Inc. (Watertown, MA, USA). Millipore type I water was used to prepare all of the aqueous solutions. Wine was purchased at the local market.

2.2. Preparation of standards

A stock solution of 100 μg/ml of OTA in methanol was prepared and the concentration was verified spectrophotometrically (M_w: 403.8, $\varepsilon_{333\ nm}$ 5500 $M^{-1}cm^{-1}$) (Bacha et al., 1988). Sample spiking solutions and standard curve solutions were prepared from appropriate dilutions of the stock solution with methanol. All of the solutions were stored at −20 °C.

2.3. OTA analysis by HPLC-FL

The chromatographic analysis of OTA in wine was as described in Lopez de Cerain, González-Peñas, Jiménez, and Bello (2002). Briefly, HPLC-FL analysis was performed in an Agilent technologies 1100 high-performance liquid chromatograph coupled to a fluorescence detector (model G1321A) and controlled by Chemstation 3D software. The chromatographic conditions were: column: Tracer Extrasil ODS-2, 5 μm, 25 × 0.4 cm, Teknokroma (Barcelona, Spain) at 40 °C; mobile phase: 29:29:42 (v/v) methanol–acetonitrile–sodium acetate 5 mM (pH 2.2 with phosphoric acid) at 1.5 ml/min. Volume of injection: 100 μl. Fluorescence detection: λ_{ex} = 225 nm; λ_{em} = 461 nm and t_R = 4.9 min.

Using 7 ml of chloroform, OTA was extracted from 7 ml of the wine sample, acidified to pH 2.5 with the addition of 10 drops of HCl 1 N. After being stirred manually and centrifuged at 3000 rpm for 10 min at 25 °C, 5 ml of the organic phase were evaporated to dryness under a stream of nitrogen at 40 °C. The residue was resuspended in 0.2 ml of methanol, diluted with 5 ml of PBS (phosphate buffer saline) and applied directly onto an immunoaffinity column that had been pre-conditioned with 10 ml of PBS. Next, with the use of a syringe, the IAC was washed with 10 ml of water and 10 ml of PBS and then dried by passing air for 10 s. OTA was eluted with four 1 ml portions of methanol at a flow rate of 20–30 drops/min. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C, and the residue was redissolved in 300 μl of methanol. This method presents a recovery of 101% (assayed at three concentration levels in repeatability and reproducibility conditions) and the limit of quantification was 0.07 ng OTA /ml.

2.4. Capillary electrophoresis conditions for OTA analysis

CE-DAD analysis was performed in an Agilent technologies capillary electrophoresis system coupled to a DAD detector (model G1602A) and controlled by Chemstation 3D software.

2.4.1. Method development

(a) *Electrophoretic buffer*. Different buffers have been assayed. Phosphate buffer (20 mM, pH 6.5, 7 and 7.5) with different percentages of methanol (15%, 20% and 30%) produced bad reproducibility in migration times and OTA peak areas. Carbonate/bicarbonate buffers (20, 30, 40 and 50 mM) with and without acetonitrile (10%) originated changing current values, probably due to the presence of microbubbles. Borate Buffer (10, 20 and 30 mM), at different pH values (8.6 and 9.3) and mixed with acetonitrile (30%) were also assayed, obtaining the best results when using the 10 mM borate buffer at pH 9.3.

(b) *Solvent for injection*. Samples were reconstituted and injected using the following solvents: mixtures of phosphate buffer and methanol (50, 70, 30 and 20%), acetonitrile, acetonitrile–water (40% and 60%), methanol, methanol–water (60% and 40%), bicarbonate buffer (1% and 3%) and borate buffer. Only the use of methanol as the sample solvent has prevented the OTA peak deformation.

(c) *Capillary temperature*. Samples were injected into a thermostatized capillary at 6.5 10, 15, 20, 25, 27.5, 30, 35, 37, 40, 45 and 50 °C and it was observed that the shape of the OTA peak improved as capillary temperature decreased.

(d) *Capillary*. The capillary chosen for separation had a bubble-cell in order to increase sensitivity. The use of a capillary with a total length of 48.5 cm (effective length of 40 cm) and internal diameter of 50 μm (Agilent Technologies) has reached the best values of sensitivity and migration time.

(e) *Injection*. Hydrodynamic injection was used at 50 mbar. Different injection times were assayed (6, 8, 10, 20 and 30 s). The best results (greater peaks and no resolution problems) were achieved with 20 s.

(f) *Detection*. 380 nm was fixed as detection wavelength instead of 210 nm in order to increase the selectivity of the analysis.

2.4.2. Final method conditions

The electrophoretic analysis conditions were as follows: a fused silica capillary with a "bubble cell" of 48.5 cm total length (40 cm effective length) × 50 μm ID (Agilent technologies) maintained at 10 °C; the electrophoretic buffer was sodium tetraborate (10 mM). Hydrodynamic injection was performed at 50 mbar for 20 s. The voltage applied for separation was 20 kV. UV detector was fixed at 380 nm. Before injection of each sample, the capillary was rinsed for 0.5 min with sodium hydroxide 0.1 N at 5 bar followed by 0.5 min with water at 5 bar and, finally, 1.5 min with electrophoretic buffer at 7 bar. Extraction of OTA from wine samples was as described for the chromatographic determination of OTA.

3. Results and discussion

3.1. Validation of the CE-DAD method

The use of immunoaffinity purification techniques gives selectivity to the method. Moreover, selectivity was studied by analyzing six wine samples of different origins without OTA and, at the OTA migration time (Fig. 2), no peaks that could interfere in their quantification had appeared. Linearity was assessed in the range of 60–210 ng/ml. All of the criteria used to verify linearity have been matched in the concentration range studied: Curve equation: $y = 2.44x - 0.10$, r: 0.997, RSD

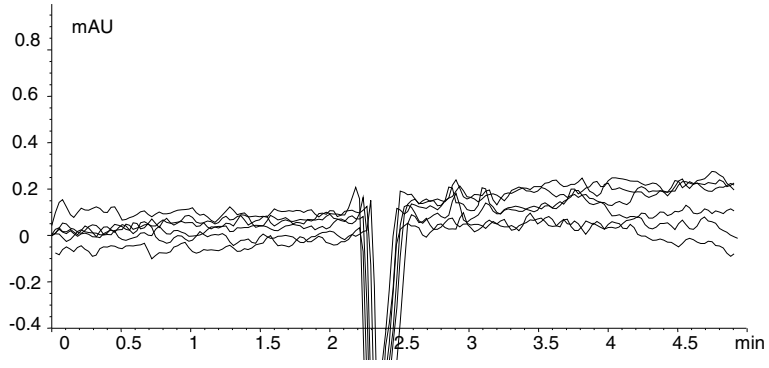

Fig. 2. Superposed electrophoregrams obtained from six samples of wine.

among response factors 3.6 (<5%), slope interval (p = 95%) not including zero (2.22–2.65) and intercept interval (p = 95%) including zero (−0.61 to 0.41). Within-day and between-day precision and recovery of the method have been studied at three OTA levels of the range (60, 120 and 150 ng/ml) in spiked wine; for each concentration level, 3 replicates have been analyzed in one day and on three different days. The recovery has been determined by comparing the peak area of OTA obtained from the wine spiked samples with the OTA peak area obtained from the calibration standards. An ANOVA test ($p > 0.814$) determined that the recovery values obtained were homogeneous throughout the entire range of the method (among the OTA concentrations assayed, RSD of recovery values was 0.9%) (Table 1). The mean value of OTA recovery from wine was 95.7%. The quantification limit (LOQ) was calculated as the lowest concentration for which acceptable recovery (>90%) and precision data was obtained (<10%). The limit was 60 ng OTA/ml wine. This level of sensitivity is much lower than that reported in the reference literature (about 100 μg/ml) when using CE-DAD in OTA analysis (Bohs, Seidel, & Lindner, 1995; Holland & Sepaniak, 1993), but it is still not adequate enough for measuring the low levels of OTA in naturally contaminated wine. Only one report (Corneli & Maragos, 1998) reached a low detection limit (0.2 ng/g) that was more comparable to those of HPLC-FL when analyzing OTA by CE, due to the use of LIF detection.

The CE-DAD method has also been validated taking into account aspects such as electrophoretic buffer stability, stability of the samples in the injector module of the CE system, and robustness. Ten samples of 10 μg/ml of OTA in methanol have been analyzed using the background electrolyte after being prepared and maintained in room or refrigerated conditions for 4, 23 and 30 days. Peak areas have been recorded and their RSD has been calculated. Ten analyses were carried out from one 10 μg/ml of OTA in methanol sample before changing the electrophoretic buffer in the vials at each end of the capillary. Results are shown in Table 2 and they demonstrate (RSD < 5%) that the buffer solution has remained stable for one month in both storing conditions and also that the buffer solution has remained stable during the analysis. This last experience, which lasted 5 h, demonstrates that the samples in the injector were stable for at least this time.

Robustness of the method with respect to changes in the capillary batch or in the concentration of the electrolyte buffer has been studied. Three different batches of capillary have been assayed by the analysis of four samples of OTA in methanol in the range 0.5–2 μg/ml. A RSD of no more than 5% between peak areas has been obtained for each OTA level when the samples are analyzed in different capillary batches. In order to study the influence of the buffer concentration on the quantification of OTA, four samples of 10 μg/ml of OTA in methanol were analyzed using a sodium tetraborate buffer 8, 10 and 12 mM. The OTA peak areas obtained in each case showed a significant difference (RSD > 5%, ANOVA test sig. 0.000). Therefore, the concentration of the electrophoretic buffer is a critical point in the method.

Table 2
Electrophoretic buffer stability

	0 days	4 days	23 days	30 days	
Maintained in laboratory conditions					
Mean OTA peak areas	112.8	116.8	119.2	117.7	RSD (%) 2.3
RSD (%)	0.8	0.9	0.5	2.3	
Maintained in refrigeration					
Mean OTA peak areas	114.2	118.6	117.9	118.4	RSD (%) 1.8
RSD (%)	1.2	0.9	2.8	1.9	
After several injections					

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Table 1
Precision and recovery of the CE-DAD method

ng/ml	Sample	Recovery (%)				
		1st day	2nd day	3rd day		
60	1	93.3	88.5	97.9	Mean (%) 96.5	
	2	97.8	93.5	100.9	RSD (%) 4.4	
	3	97.6	102.5	96.2		
120	1	103.7	90.3	99.1	Mean (%) 94.8	Mean (%) 95.7
	2	97.3	88.6	98.7	RSD (%) 8.6	RSD (%) 0.9
	3	95.9	77.4	101.8		
50	1	101.9	92.4	95.5	Mean (%) 95.9	
	2	93.0	90.1	102.1	RSD (%) 4.2	
	3	95.8	97.0	95.1		

RSD, relative standard deviation.

Table 3
Comparison between CE-DAD and HPLC-FL

Sample (ng/ml)	n	1st day					2nd day					3rd day				
		CE-DAD		HPLC-FL		E[b] (%)	CE-DAD		HPLC-FL		E (%)	CE-DAD		HPLC-FL		E (%)
		Mean	SD[a]	Mean	SD		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
60	3	59	1	54	1	8	60	2	57	4	5	59	4	53	6	10
120	3	127	5	126	5	1	128	2	122	6	5	110	9	110	18	0
150	3	149	7	132	13	13	151	5	153	4	1	143	5	134	3	6

Levels measured (ng/ml) by both techniques in the fortified samples.
[a] SD, standard deviation.
[b] Error as $\frac{\text{CE value}-\text{HPLC value}}{\text{CE value}} \times 100$.

3.2. Sample analysis

Nine fortified wine samples (three samples of each: 60, 120 and 150 ng/ml), were assayed and quantified by HPLC-FL and CE-DAD procedures, and this process was repeated over three days. Due to the high concentration level of OTA in the samples, prior to their analysis by HPLC-FL, a dilution 1/500, with mobile phase, was carried out from the 300 μl of the final extract of OTA in methanol after being injected into the CE system. The results obtained for both processes are similar (Table 3). They have been compared by a regression procedure because it can detect both proportional and constant systematic errors (Nguyen Minh Nguyet et al., 2004). A linear relationship is obtained when representing the results obtained from the HPLC-FL method vs. those obtained from the CE-DAD method; this is evidenced by the good correlation coefficient obtained ($r = 0.98$), a slope close to 1 (0.98) (confidence interval 95%: 0.91–1.06) and an intercept value near 0 (6.58 ng/ml) (confidence interval 95%: −1.80 to 14.97 ng/ml). This is a good data correlation, especially when considering the extradilution process for HPLC-FL samples.

3.3. Methods comparison

The cost and the time employed in setting up the method in CE-DAD have been similar to when using the HPLC-FL method. In the HPLC-FL method described, OTA has a retention time (t_R) of 5 min and the analysis time required is approximately 8 min. In the case of the CE-DAD method, OTA has a migration time (t_M) of 3 min, but 7.5 min are needed for each analysis due to the capillary rinse processes. Therefore, CE-DAD and HPLC-FL methods present similar, low analysis times. The more important difference of the two methods has been the lower cost of solvents and the less hazardous waste generated in the CE-DAD method. In the HPLC-FL method, a flow of the mobile phase (58% organic solvent) of 1.5 ml/min has been used, whereas in the CE-DAD method, the electrophoretic buffer is 100% aqueous solution and no more than 3–5 ml is used per day.

4. Conclusions

Wine contaminated with OTA continues to be a problem, and the EU legislation has established a maximum level of tolerance in wine (2 ng/ml). Therefore, validated methods for determining OTA in wine are needed for both control and research. In addition, there is special interest in that these methods be based on inexpensive and nonpolluting technologies. The CE-DAD method described meets all of the preestablished validation parameters. The cost of the analysis is much lower than that of an HPLC-FL method, taking into account not only economical, but also environmental criteria. The results obtained in analyzing samples by both methods indicate that the CE-DAD method compares well with the HPLC-FL method and offers the advantage of using a smaller quantity of solvent. The quantification limit is lower in the HPLC-FL method due to the use of CE with DAD detection. Therefore, in the determination of OTA in wine in real samples, this method can not be used yet. However, if fluorescence detection is available, the CE technique is a valuable alternative to HPLC-FL analysis of OTA in wine.

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