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Comparison of ELISA and capillary electrophoresis with laser-induced fluorescence detection in the analysis of Ochratoxin A in low volumes of human blood serum

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Abstract

In this paper the determination of Ochratoxin A (OTA) in low volumes of human blood serum by enzyme-linked immunosorbent assay (ELISA) is compared with an appropriate capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) method. In order to use ELISA for high-throughput analysis in epidemiological studies no sample cleanup was performed. Both methods showed a limit of detection (LOD) of 0.5 ng/mL. Comparing the precisions of both methods, the data show that the quantified concentrations in ELISA are higher than the corresponding concentrations in the CE-LIF method. Using a matrix calibration curve instead of a standard calibration curve the reproducibilities of both methods are comparable. No additional matrix effect could be observed by adding phenylalanine as probable matrix compound to the serum.

Keywords: Capillary electrophoresis; CE-LIF human blood serum; Low sample volume; Ochratoxin A; ELISA

1. Introduction

Ochratoxin A (OTA) is a naturally occurring mycotoxin which is produced by several species of the genera *Aspergillus* (e.g. *Aspergillus ochraceus*) and *Penicillium* (e.g. *Penicillium verrucosum*). OTA is found mainly in food derived from plants, such as cereals [1], coffee [2,3], nuts [4], dried fruits, spices [5], and wine and grapes [6,7]. The intake of OTA through contaminated feed may lead us to its occurrence in the blood, kidneys and liver of pigs and poultry [8]. The consumption of several foods, including cereal products, wine, beer, as well as meat and sausage of pork, can also cause high plasma levels of OTA in human blood [9]. OTA has been reported to have nephrotoxic [10], carcinogenic [11], genotoxic [12] and immunotoxic effects [13,14]. Moreover, OTA is suspected to be the causing agent behind the Balkan Endemic Nephropathy, a kidney disease in south-eastern Europe [15].

Several methods have been developed to determine OTA in various matrices, like milk [16], certain tissues [17], animal feeds [18], urine [19] and human blood serum [20]. Because of the low concentrations of OTA usually found in human blood serum, its analytical determination calls for sensitive techniques.

To analyze OTA in human blood serum with RP-HPLC, various sample cleanup procedures have been developed [9,16]. In most studies, OTA was extracted from blood serum or plasma by chloroform after acidification to a pH below 2.5 [16]. Recently published methods using immunoaffinity or RP-SPE columns have been developed for the determination of OTA in blood and tissues [21,22]. Depending on sample cleanup and instrumentation, limit of detection (LOD) between 5 pg/mL and 50 pg/mL has been achieved.

In addition to these methods, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been employed. Corneli and Maragos developed a method for the determination of OTA in coffee, corn and sorghum using CE-LIF [23]. A LOD of 0.2 ng/g has been achieved with this method [23,24]. However, capillary electrophoresis has the disadvantage that the injection volumes are very small. To overcome this, several injection techniques have been developed [25–28].

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One technique to enhance the sensitivity of detection is on-line sample concentration by sample stacking with reversed polarity [29–31]. On the basis of these techniques recently a method for the determination of OTA in human blood serum was established [32]. This method was developed to analyze human blood serum starting with a sample volume of 50 μL. In planned investigations concerning the internal exposure of children to OTA only such small volumes of serum were available because of the broad spectrum of parameters to be analyzed and the limited availability of sample material because of ethical questions. This biomonitoring is absolutely essential in order to assess the existing risk in indoor environments containing mould.

Another technique often used for the analysis of OTA is enzyme-linked immunosorbent assay (ELISA) [22,33] The ELISA technique is useful for high-throughput analysis. Additionally, only a small volume of sample is necessary for the analysis. Therefore, this technique should be suitable for the biomonitoring of children, where only small sample volumes are available. Unfortunately commercially available ELISA test kits for OTA are validated only for corn and not for human blood serum.

The aim of this study was to compare the recently published CE method [32] with an ELISA method. The test kit is a direct competitive ELISA in a microwell format for detection and quantification of OTA concentrations in parts per billion (ppb). Due to the low sample volume, sample cleanup ought to be avoided. In the published method [33], the extraction of 2 mL serum followed by a solid phase extraction protocol is performed. However, avoiding cleanup step is desirable to maintain the high-throughput feature of ELISA.

2. Experimental

2.1. Material and reagents

Ochratoxin A (from *A. ochraceus* [303-47-9]), dansylphenylalanine (DNS-Phe) and coumarin-3-carboxylic acid (C3A) were purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (Suprasolv grade), dichloromethane (Lichrosolv grade), sodium chloride and phosphoric acid (80%) were obtained from Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate-7-hydrate was purchased from Riedel-de-Haën via Sigma. Veratox® ELISA-test-kit for Ochratoxin A was purchased from Neogen.

2.2. CE-LIF

For the analysis of OTA in low volumes of human blood serum, the recently published method of Koeller et al. [32] was used. A HP3D CE system (Agilent, Waldbronn Germany) equipped with a laser-induced fluorescence detection system (15 mW helium-cadmium ion laser with an excitation wavelength of 325 nm from Picometrics, Toulouse, France) was used. Separation was performed on a fused silica capillary (60 cm × 50 μm; 45 cm to detector). The capillary was thermostated at 20 °C by air. Samples kept in the autosampler were also thermostated at 20 °C.

Prior to first use, the capillaries were conditioned by rinsing with methanol for 15 min, with water for 5 min, with 1N NaOH for 20 min, with 0.1N NaOH for 10 min and finally with separation buffer for 20 min. The capillaries were prepared for daily use by rinsing with water for 10 min, with 1N NaOH for 10 min, with 0.1N NaOH for 10 min and with separation buffer for 15 min. Before each analysis the capillary was flushed with 0.1N NaOH for 1 min and with separation buffer for 2 min.

A 30 mM phosphate buffer (pH 7.0) was used as separation buffer. Injection was performed hydrodynamically at a pressure of 50 mbar for 60 s. Subsequently a voltage of −20 kV was applied to the column. After 30 s the voltage was switched to +20 kV. The run time of each analysis was 12 min.

2.3. Sample cleanup for CE-LIF

Twenty microliters of 0.2 M sodium hydrogenhydrocarbonate solution was added to 50 μL serum. In order to remove neutral and basic matrix components the sample was shaken twice with 100 μL dichloromethane. The organic layer was discharged. Subsequently, the water phase was acidified with 100 μL 10% phosporic acid and extracted with 100 μL dichloromethane by agitation in a vortex mixer for 5 min at room temperature. The mixture was centrifuged at 14,000 × *g* for 2.5 min. Three phases were obtained: aqueous (upper), white ring (middle) and dichloromethane (lower). The lower phase was transferred into a vial fitted with conical inset. The aqueous layer and the white precipitate were extracted with 100 μL dichloromethane again. The combined dichloromethane fractions were evaporated to dryness and reconstituted in 20 μL of the injection solution.

2.4. ELISA

Veratox® for ochratoxin (Neogen) is a direct competitive ELISA in a microwell format for detection and quantification of OTA concentrations in parts per billion. This ELISA was used and strictly performed according to the manufacturer's instructions. Briefly, ochratoxin is extracted from the sample with a 50% (v/v) methanol–water solution. Following this, free ochratoxin in the samples and controls is allowed to compete with enzyme-labeled ochratoxin (an OTA-horse radish peroxidase conjugate) for the antibody binding sites. After a wash step, substrate is added which when catalyzed by the antibody-bound peroxidase reacts to produce a blue color. Following exactly 10 min of incubation, stop solution is added to terminate the enzymatic reaction. Immediately thereafter the test is read in a spectrophotometer (SpectraImage) to yield optical densities at a wavelength of 650 nm, as stated in the manufacture's instructions. The optical densities of the controls form the standard curve, and the samples optical densities are plotted against the curve to calculate the concentration of OTA. The data were quantitatively analyzed using a 4-parameter logistic curve-fit for the standard curve (easyWIN kinetics V6.0a, TECAN, Crailsheim, Germany).

2.5. Experimental design

For analyses, we used a pooled sample of human sera which were specified as OTA free. This sample was used for the preparation of all dilutions of OTA.

Each series is composed of six calibration points and three quality control samples. The concentration levels of the calibration points ranged from 0 ng/mL up to 12.5 ng/mL (0 ng/mL; 0.02 ng/mL; 0.1 ng/mL; 0.5 ng/mL; 2.5 ng/mL; 12.5 ng/mL). It was expected that ELISA has a lower LOD than the CE method. Therefore, two calibration levels (0.02 ng/mL and 0.1 ng/mL) were defined.

The concentrations for the quality control samples were 1 ng/mL, 5 ng/mL, and 10 ng/mL.

Due to the phenylalanine moiety of OTA it was suspected that phenylalanine might act as a competitor for the OTA antibody in the ELISA immunoassay. In order to estimate these cross reactions between OTA and phenylalanine, in series 3 and 4 a sample containing 5 ng/mL OTA and 5 ng/mL phenylalanine were added.

The above-specified samples were aliquoted into sterile 1.5 Eppendorf tubes and stored until analyses at −20 °C to simulate the circumstances under which samples may be obtained before the analyses can be carried out. The sample volume of all aliquots was 50 μL. Randomly selected representatives of the different aliquots were taken for analyses in both methods.

3. Results and discussion

3.1. Calibration

According to the validation of the used CE-LIF method [32], concentrations lower than 0.5 ng/mL were not detectable. Therefore, the calibration curve consisted of three points only in all four series. In order to check the interday performance each series was analyzed on another day. The calibration curves on all four days are summarized in Table 1. The last row of Table 1 the mean calibration curve, constructed from the mean value of the datapoints in each calibration level, is shown. The variance of each calibration level ranged between 5.1% and 17.6%. It can be seen from Table 1 that the slopes of the calibration curves are reproducible from day to day.

Generally in ELISA the calibration is performed by a 4-parametric logistic curve which is typically for ELISA. To do so, six standard control samples, where OTA is dissolved in methanol, were plotted against their optical densities at 650 nm. As stated in the manual instructions all samples of the series were quantified with this curve. In Fig. 1 the calibration curves of the standard control samples and the serum calibration samples are shown. It can be seen that differences occurred which can be addressed to matrix effects. The calibration curve with serum calibration samples shows a plateau in the low concentration range. Therefore quantification below 0.5 ng/mL was not possible. Under these circumstances the LOD was set to 0.5 ng/mL.

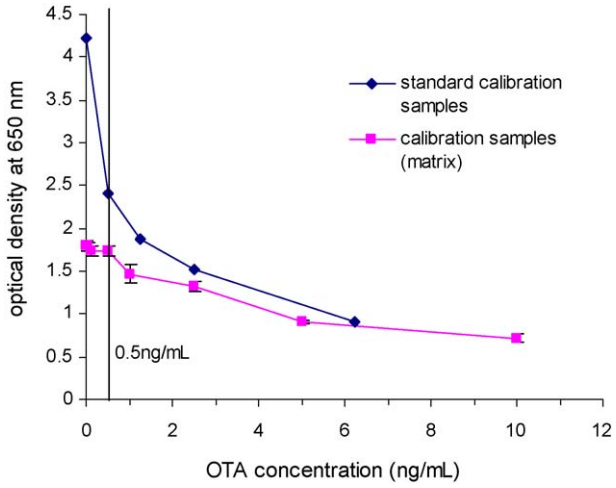

Fig. 1. ELISA calibration curves without matrix (diamonds) and with human blood serum as matrix (rectangles).

This result can be explained by contamination of the original serum with OTA or matrix effects. The contamination with OTA can be excluded because the CE analysis did not find OTA in the blank sample and in the samples spiked with 0.02 ng/mL, and 0.1 ng/mL.

3.2. Reproducibility and precision

The results of the quantification of the quality control samples are summarized in Table 2. Analyzing the QC-samples with CE the measured concentrations of OTA agree well with the expected QC-sample concentration. The reproducibility of all QC-samples was below 20%, as a rule lower than approximately 15%.

According to instruction manual, using the standard sample calibration curve the reproducibility of ELISA method is very poor. It ranges between 11% and 32% (Table 3). To improve the reproducibility the matrix calibration curves were used for quantification. Every QC-sample is quantified with the matrix calibration curve of its own series. The reproducibility with this calibration is below 20%. This shows that matrix components in

Table 1
Calibration curves on 4 days obtained by CE analysis

	Calibration curves	r^2
Series 1	$y = 0.0525x + 0.0753$	0.9999
Series 2	$y = 0.048x + 0.1259$	0.9995
Series 3	$y = 0.0548x + 0.1410$	0.9971
Series 4	$y = 0.0552x + 0.883$	0.9976
Mean ($n = 4$)	$y_{mean} = 0.0524x + 0.1076$	0.9972

Table 2
Quantification of samples analyzed by CE

OTA samples	CE	
	Mean concentration ($n = 4$) (ng/mL)	Variation (%)
1 ng/mL (LQC)	1.06	15.9
5 ng/mL (MQC)	5.01	6.8
10 ng/mL (HQC)	9.80	9.9

Table 3
Quantification of samples analyzed by ELISA

OTA samples	ELISA with standard sample calibration		ELISA with matrix sample calibration	
	Mean concentration ($n=4$) (ng/mL)	Variation (%)	Mean concentration ($n=4$) (ng/mL)	Variation (%)
1 ng/mL	1.73	31.9	1.73	19.6
5 ng/mL	7.21	11.1	7.21	9.8
10 ng/mL	12.80	17.4	12.06	10.0

Data were quantified with the standard calibration curve (no matrix) and with matrix calibration curve.

ELISA can become significant in spite of washing. The found concentrations for the quality control samples are higher than expected in ELISA. The phenomenon that ELISA overestimates the OTA concentrations was also observed by other groups [34]. In Fig. 2 the found concentrations of the control samples of ELISA method are plotted against those found with CE method. The slope of the linear curve is bigger than 1. This leads to a concentration-dependent overestimation of the OTA concentration by the ELISA. Additionally, the axis intercept is 0.7 ng/mL. This concentration-independent factor amplifies the overestimation. One reason for that might be the nonlinear calibration. Additionally, in competitive ELISA small changes in the lower concentration range cause high changes of the optical density. On the other hand, small changes in optical density show high changes in the OTA concentration.

3.3. Crossreaction with phenylalanine

In order to study the matrix effects phenylalanine was chosen. Blood serum from healthy humans contains 2–10 ng/mL phenylalanine. Due to the structural similarity of phenylalanine and OTA (Fig. 3) it was suspected that phenylalanine can mimic the OTA binding site of the antibody. Therefore, in each of the series 3 and 4 a sample containing 5 ng/mL OTA as well as phenylalanine (also in a concentration of 5 ng/mL) was measured. In samples containing phenylalanine analyzed by CE OTA were found at concentrations of 4.95 ng/mL and 5.17 ng/mL, respectively. Concentrations obtained from ELISA were 7.53 ng/mL and 6.57 ng/mL. The values obtained by ELISA are higher than those expected. Nevertheless, this value agrees very well with the OTA concentrations of those samples not spiked with phenylalanine. The quantification revealed that neither significant additive nor subtractive effects of phenylalanine interfere with the analysis of OTA. This means that the observed matrix effects cannot be explained by the presence of phenylalanine in serum.

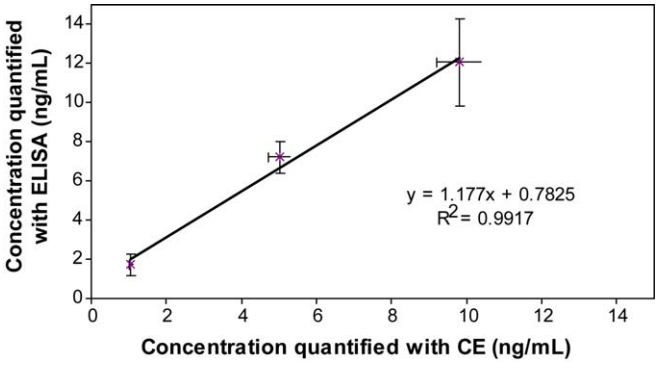

Fig. 2. Plot of quantified samples analyzed by CE vs. ELISA.

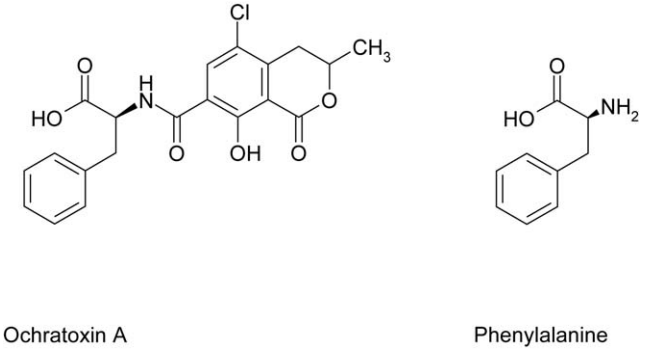

Fig. 3. Ochratoxin A (OTA) and phenylalanine.

4. Summary

Based on the experimental results it can be concluded, that generally CE and ELISA can be used to analyze OTA in serum. The analysis of 50 μL serum was possible in the calibrated concentration range from 0.5 ng/mL to 12.5 ng/mL. The LOD of 0.5 ng/mL agreed well with the earlier published method validation [32]. The working range of the ELISA method was estimated from 0.5 ng/mL up to 12.5 ng/mL. Using the standard calibration curve leads to low reproducibility. To increase the reproducibility a calibration curve constructed with matrix containing samples has to be used. However, the precision of ELISA is worse than those of the CE method. In order to mimic the matrix effects measurements with phenylalanine were done. The results showed that the presence of phenylalanine in equimolar concentrations has no effect on the ELISA process. Obviously other matrix compounds can interact with the antibody or interfere with the staining reaction. Doubtless ELISA is a good method to analyze low volume samples. In epidemiological studies with high sample throughput ELISA can be used as a fast semi quantitative screening method, in order to preselect the samples. On the other hand, actually there are no ELISA kits for the analysis of OTA in human blood serum commercially available. Using ELISA kits validated on other matrices (e.g. cereals) matrix effects and poorer LOD might be expected. Therefore, for the exact determination of the OTA concentration a chromatographically method (HPLC or CE) and a sample cleanup is required.

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