Note

Solid phase extraction on sax columns as an alternative for ochratoxin A analysis in maize

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Summary

A sensitive and reliable method is described for the determination of ochratoxin A (OTA) in maize. An extraction and clean-up procedure was used, with chloroform-phosphoric acid as the extractant, and liquid-liquid partition and anion-exchange chromatography (SAX columns) for the clean-up. Quantification of toxin is achieved by high performance liquid chromatography (HPLC). Recoveries were between 81-94 % at 3-90 ng/g levels. The detection limit was 0.02 ng.

Key words Analysis, Anion-exchange, SAX, SPE, HPLC, Maize, Ochratoxin A

Empleo de una columna de intercambio iónico con relleno SAX como método alternativo en el análisis de ocratoxina A en maíz

Resumen

Se describe la determinación de ocratoxina A en maíz mediante un método sencillo y con buena sensibilidad. Dicho método consiste en una extracción con una mezcla de cloroformo y acido fosfórico, seguida de una purificación mediante una extracción líquido-líquido y una limpieza final utilizando una columna de intercambio iónico (columna con relleno SAX). La muestra así obtenida es analizada por cromatografia líquida de alta resolución (HPLC). Las recuperaciones obtenidas oscilan entre 81 y 94 % en los niveles estudiados (3-90 ng/g). Se determina un limite de detección de 0,02 ng.

Palabras clave Análisis, Intercambio aniónico, SAX, SPE, HPLC, Maíz, Ocratoxina A

Ochratoxin A, a naturally occurring mycotoxin, is produced by certain species of *Aspergillus* and *Penicillium* fungi. The natural occurrence of this toxin in grains and other plant products has been widely reported [1-3]. Feeding experimental diets containing ochratoxin A have a deleterious effect on a number of animal species. Biological and biochemical effects associated with ochratoxin A include: nephropathy, teratogenicity, enhancement of lipid peroxidation, partial inhibition of ATP-dependent calcium uptake, carcinogenicity and inhibition of cell-mediated immune response [2,3].

Human exposure to ochratoxin A can occur directly by consumption of contaminated plant derived food or indirectly by consumption of animal tissues from livestock exposed to contaminated materials [4]. It has also been associated with nephropathy in Bulgaria and former Yugoslavia [5].

Many analytical methods for the determination of ochratoxin A have been developed, mainly involving the use of thin-layer chromatography or high-performance liquid chromatography [6]. Recently two collaborative studies to check methods for determining ochratoxin A in different cereal matrices have been carried out [7,8]. Although published methods employ a range of extraction and purification steps, only one method presented recently in the IX International IUPAC Symposium on Mycotoxins and Phycotoxins [9] has studied the possibility of using SAX cartridges during the clean up of the sample.

The aim of this study was to develop an HPLC method for determining ochratoxin A in corn using a SAX minicolumn in the clean-up step.

MATERIALS AND METHODS

Reagents. Ochratoxin A was obtained from Sigma Chemical Co. (USA). A stock solution of 200 µg/ml in methanol was diluted with methanol to a concentration of 10 µg/ml and assayed spectrophotometrically (e333 nm= 6640) [10]. Aliquots of this working standard solution were then diluted in methanol to appropriate concentration for spiking. The same volume (90 µl) of spiking solution was used for each test portion. Different concentrations of standard solutions were also prepared using acetonitrile:water:acetic acid (275+250+5 v/v/v) and used to prepare a standard curve at start of analysis.

Preparation of Test Portions. We obtained corn from a local feed store. It was ochratoxin-free as determined by repeated analyses using the method described below. Corn was ground in a grain mill. Test portions (20 g) of grain were weighed into a 250 ml erlenmeyer

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flask and spiked with the corresponding spiking solution. Then samples were shaken for 10 min and then sealed.

Ochratoxin A analysis. Two hundred milliliters of chloroform and 25 ml of phosphoric acid 0.1 M were added to the erlenmeyer flask containing the 20 g corn sample. The mixture was magnetically stirred for 30 min and then transferred into a 500 ml separatory funnel. Fifty milliliters of the lower layer were transferred to a round bottomed flask and evaporated to dryness on a rotary evaporator. The residue was dissolved in 5 ml of hexane and 5 ml of methanol:water (1+1 v/v). The solution was transferred to a 50 ml separatory funnel and shaken gently. The lower phase was collected in a 25 ml erlenmeyer flask and the upper phase extracted twice more with 5 ml of methanol:water (1+1 v/v) which had been used to rinse the round bottomed flask containing the residue.

All the lower phases were combined and added at a flow rate of 1 ml/min to a SAX column (Analytichem Bond-Elut, Varian, USA) previously conditioned with 5 ml of methanol and 5 ml of methanol:water (1+1 v/v). The column was then washed with 8 ml of methanol:water (3+1 v/v) and 3 ml of methanol at 2 ml/min flow rate. These washes were discarded and ochratoxin A was eluted with 2x5 ml of methanol:formic acid (98+2 v/v) at a flow rate of 2 ml/min. Both solutions were combined and evaporated to dryness under nitrogen with minimal heating and the residue dissolved in 500 µl of acetonitrile:water:acetic acid (275+250+5 v/v/v) by mixing on a vortex mixer and filtering through a 0.2 mm microfilter (PTFE, Lida Manufacturing Corp, USA).

Quantification of ochratoxin A was achieved by reversed phase high performance liquid chromatography (HPLC) using an Applied Biosystem Series 400 pumping system (ABI, Analytical Kratos Division, USA).and a SPHERISORB ODS-2 10 mm column, 3.9x25 cm (Tracer Analitica, Spain) with acetonitrile:water:acetic acid (275+250+5 v/v/v) and a flow rate of 1.5 ml/min. A model 980 fluorescence detector (ABI, Analytical Kratos Division,USA), with an excitation wavelength of 330 nm and an emission cut-off of 418 nm, was used. Toxin concentrations were quantified by comparing with external standard solutions.

Statistical Analysis. The data were processed using the Statgraphics 2.0 program (Statistical Graphics Corp., USA).

RESULTS AND DISCUSSION

The method described uses chloroform-phosphoric acid as an extraction system and two purification steps. First of all a liquid-liquid extraction permits the elimination of the lipidic part, then the aqueous solution is cleaned up by using a SAX cartridge rendering the sample ready to analyze by HPLC.

As table 1 shows, recoveries at the three levels studied, 3, 10 and 90 ng/g, ranged from 80.7 to 93.7 %. These results are in the same order or slightly higher than most of the methods described until now for this kind of analysis, only Biacardi and Riberzani [9] claim in their poster higher recoveries. Thus, in the collaborative study carried out by Nesheim *et al.* [7] OTA recoveries from corn artificially contaminated at 10, 20 and 50 ng/g ranged from 53 to 97% with a within-laboratory relative standard deviation of 20.1% whereas the between-laboratories relative standard deviation were 20.7-331.7%. In this method the use of sodium bicarbonate extraction implies a centrifugation step. C18 columns were used by

Table 1. Results of ochratoxin A recoveries in maize artificially contaminated.

Ochratoxin A levels (ng/g)	Number of samples	Ochratoxin A recoveries Mean % (CV)
3	3	81.5 (9.54)
10	3	93.7 (6.44)
90	3	80.7 (2.58)

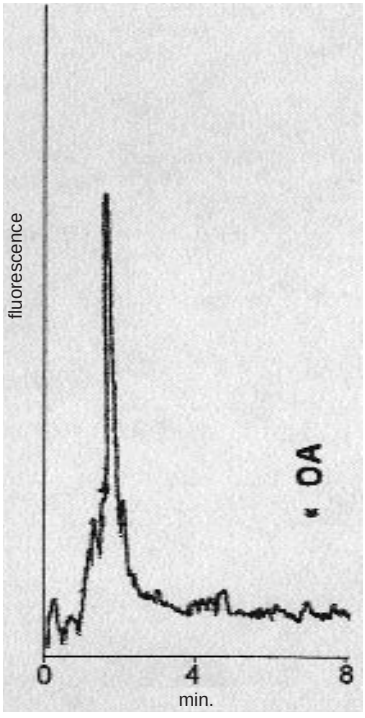

Figure 1. Chromatogram of maize sample (blank sample). OA indicates the retention time for ochratoxin A.

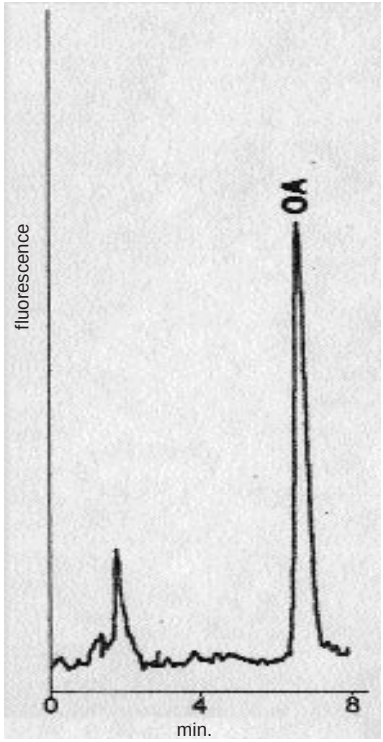

Figure 2. Chromatogram of maize sample spiked to contain 10 ng/g of ochratoxin A. OA indicates the retention time for ochratoxin A.

all the participants in the purification step.

In the collaborative study carried out in the EU using wheat as a substrate [8] different extraction and purification systems were examined. The authors concluded that the clean-up step had a stronger influence on the variation of results than the extraction solvent. At 13 ng/g level recoveries were found to range from 25 to 100% and in the clean up step the laboratories using immunoaffinity columns gave low recoveries.

Seidel *et al.* [11] carried out a study on OTA recoveries from contaminated maize at 1-50 ng/g. When silica SPE columns were used recoveries were about 75%, but dropped to lower figures when C18 columns, fluorisil columns or liquid-liquid partioning techniques were used.

The clean-up method described by Dunne *et al.* [12] using a gel permeation clean-up step gave a 77.6% recovery at 16 ng/g contamination level.

It is worthwhile noting that the recoveries shown in table 1 are only obtained if the process until HPLC analysis is carried out on the same day. We have observed that when ochratoxin A is kept in the methanol/formic acid solution for one day recoveries drop to 60 % or less. Also, maintaining the flow rate indicated during the SAX clean up step seems to be important to obtain good recoveries.

Figures 1 and 2 also show that the described method gives a very clean sample from which we were able to reach a detection limit of 0.02 ng absolute amount (signal:noise ratio= 3:1), resulting in limits of determination of 0.1 ng/g. These values are comparable to the ones described by Hald *et al.* [8], Seidel *et al.* [11] and Biacardi and Riberzani [9], and higher than the ones found by Dunne *et al.* [12].

CONCLUSION

The proposed method permits the quantitation of ocratoxin A in maize samples between the ranges studied. Recoveries are in accordance with many published methods. Also, our clean-up procedure allows the possibility of using the whole solution obtained after the liquid-liquid partition resulting in a very clean sample for the HPLC analysis.

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