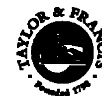
Food Additives and Contaminants, 2001, Vol. 18, No. 9, 830–835



Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India

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(Received 17 July 2000; revised 14 January 2001; accepted 18 January 2001)

Ochratoxin A (OA) contamination of black pepper, coriander seeds, powdered ginger and turmeric powder was estimated using indirect competitive ELISA. Samples (1 g) were extracted with 0.5% potassium chloride (KCl) in 70% methanol (5 ml) and diluted subsequently to give two-fold to ten-fold step-wise dilutions in phosphate-buffered saline containing 0.05% Tween 20 and 0.2% bovine serum albumin (PBS-T BSA). For extracts from the spices analysed, ELISA estimates of OA concentrations were compared with those made by HPLC. All estimates were within 1–2 standard deviation of the ELISA values. More than 90% of OA added to spice samples was recovered from samples containing between 5 and 100 μg/kg OA. Extracts of OA-free spice samples contained substances that interfered with ELISA, presumably because of non-specific reactions. This effect was avoided by preparing all the test solutions in extracts of OA-free spice samples. In 126 samples obtained from retail shops, OA was found to exceed 10 μg/kg in 14 (in the range of 15–69 μg/kg) of 26 black pepper samples, 20 (in the range of 10–51 μg/kg) of 50 coriander samples, two (23 μg/kg and 80 μg/kg) of 25 ginger samples and nine (in the range of 11–102 μg/kg) of 25 turmeric samples. This is the first record in India of the occurrence of OA in what are some of the most widely used spices in Indian cooking.

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Keywords: ochratoxin A, indirect competitive ELISA, HPLC, black pepper, coriander, ginger, turmeric

Introduction

Ochratoxins are a group of toxic secondary metabolites produced by certain fungi in the genera *Aspergillus* and *Penicillium* (Steyn 1971, Applegate and Chipley 1973, Chu 1974, Harwig 1974). Among them, ochratoxin A is the most toxic. It has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic, mutagenic and to be an immunosuppressive agent (Kuiper-Goodman and Scott 1989). Of greatest concern for human health is its implication in an irreversible and fatal kidney disease referred to as 'Balkan Endemic Nephropathy' (Krogh 1974). OA has been found to occur in foods of plant origin, in edible animal tissues, and in human milk, blood sera and tissues (Creppy *et al.* 1995).

OA has been detected in diverse food and feed commodities (Veldman *et al.* 1992, Oyelami *et al.* 1996, Zimmerli and Dick 1996), including spices. Spices found by HPLC to contain relatively high (>5 μg/kg) levels of OA were Chinese red pepper (Akiyama *et al.* 1998) and paprika and nutmeg (Vrabcheva *et al.* 1998). The moulds isolated from spices are predominantly *Aspergillus* and *Penicillium* species (Flannigan and Hui 1976) and these are implicated in the production of several mycotoxins.

Spices and condiments are extensively used in Oriental and Indian cooking. The only record of ochratoxin A contamination in spices from India is its detection by ELISA in chillies (Thirumala-Devi *et al.* 2000). In this paper, we report the occurrence of OA in many samples of four of the most commonly used spices in India, namely black pepper, coriander, ginger and turmeric by both ELISA and HPLC methods.

Food Additives and Contaminants ISSN 0265–203X print/ISSN 1464–5122 online © 2001 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/0265203011004492 1

Materials and methods

Materials

Ochratoxin A, ochratoxin A-BSA conjugate, goat anti-rabbit IgG-ALP conjugate, *p*-nitrophenyl phosphate, and bovine serum albumin (BSA), were all purchased from Sigma Chemical Co., St Louis, USA. Microtitre plates (Maxi-sorp F96) were obtained from Nunc (Nalge Nunc International, Denmark) and immunoaffinity columns from Vicam, Watertown, MA, USA. Chloroform, acid acetic and orthophosphoric acid were analytical grade; acetonitrile, methanol and toluene were HPLC grade. All other chemicals were reagent grade or chemically pure.

Collection of samples and preparation for ELISA

Black pepper (26 samples), coriander (50 samples), ginger (25 samples), and turmeric (25 samples) were purchased from retail shops in 200 g quantities. Black pepper and coriander were purchased as seeds and turmeric and ginger were bought as powders. Black pepper and coriander were ground to a fine powder in a Waring blender and ginger and turmeric powders were used without any further grinding. Samples of 200 g were thoroughly mixed and three 15 g sub-samples were taken. Each sub-sample was extracted with 75 ml of a mixture of 0.5% KCl in 70% methanol by blending in a Waring blender. Extraction was followed by shaking for 30 min and filtration through Whatman No. 41 filter paper. The filtrate was diluted two- to ten-fold in phosphate-buffered saline containing 0.05% Tween 20 and 0.2% bovine serum albumin (PBS-T BSA) prior to ELISA.

Antibodies

OA polyclonal antibodies were those described by Thirumala-Devi *et al.* (2000). Antibodies were highly specific for OA and did not cross react with ochratoxin B, coumarin, 4-hydroxy coumarin or L-phenylalanine.

Indirect competitive ELISA procedure for processing the various spice samples

An indirect ELISA procedure similar to that reported for aflatoxins (Devi *et al.* 1999) and ochratoxin A (Thirumala-Devi *et al.* 2000) was used. Microtitre plate wells were coated with 1 μg/ml of OA–BSA in 0.2 M sodium carbonate buffer, pH 9.6 (150 μl/well) overnight at 4°C. Subsequent steps were at 37°C for 1 h. Antiserum was diluted in PBS-T BSA, held for 45 min at 37°C and added in 50 μl volumes to 100 μl of OA standards ranging from 100 ng/ml to 100 pg/ml, prepared in a diluted extract from spices that did not contain OA (verified by HPLC). Spice samples were extracted with aqueous methanol–KCl as described above, filtered and used at a 1:10 dilution in PBS-T BSA, except for ginger which was diluted to 1:8. One hundred μl of each sample were added to wells containing 50 μl of antiserum diluted to 1:100 000. Goat antirabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect rabbit antibodies attached to OA-BSA. *p*-nitrophenyl phosphate was used as a substrate at 1 mg/ml. Absorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titertek Multiskan, Lab Systems, Finland) after incubation at room temperature for 1 h.

Standard curves were obtained by plotting $\log_{10}$ values of OA concentration against optical density at A_{405}. The concentrations of OA in samples were determined from the standard curves and expressed in μg/kg using the formula: OA concentration (ng/ml) in sample extract × dilution with buffer × extraction solvent volume used (ml) /sample weight (g). In order to test the recovery of OA, 15 g spices were mixed with pure OA to give concentrations ranging from 1 to 100 μg/kg. Spiked samples were extracted and assayed as for unknown samples.

Determination of OA by HPLC

Ochratoxin A was extracted by using an adaptation of the extraction procedure of the Technical Committee of European Committee for Standardization (CEN/TC 275 1998). Spice powder samples (50 g) were transferred into a 500 ml PTFE (polytetrafluoro ethylene) container mixed with 200 ml chloroform and 20 ml 0.1 M orthophosphoric acid. This mixture was triturated for 3 min with the

Table 1. Recovery of OA from artificially contaminated spice samples as determined by HPLC.

Concentration of OA used for spiking (μg/kg)	Concentration of OA estimated (μg/kg)[a]	Percent recoveries of OA from spiked samples[b]
0.4	0.36 ± 0.01	90.1 ± 0.03
1.0	0.89 ± 0.01	88.9 ± 0.01
2.4	2.08 ± 0.03	86.5 ± 0.01
6.0	5.28 ± 0.07	87.9 ± 0.01
10.0	9.05 ± 0.28	90.5 ± 0.03

[a] Samples were spiked with a known concentration of OA and assayed. Data represent mean of three replications ± SD.
[b] Determined by the formula, detected OA (μg/kg) divided by the concentration of OA used for spiking and multiplied by 100. Values are means ± SD.

Ultra-Turrax CAT × 620 (Staufen, Germany) fitted with a T17-V shaft at 13 500 rpm to produce a slurry. After centrifugation for 10 min under 820 *g* at 5–10°C, the chloroform phase was transferred to a 500 ml beaker. The remaining part was extracted again with 200 ml chloroform and 20 ml of 0.1 M orthophosphoric acid. The combined chloroform phases (*ca* 350 ml) were evaporated to dryness by rotary evaporation at 30–40°C. The residue was dissolved in 100 ml of 0.5 M $NaHCO_3$ and transferred to a 120 ml PTFE container. After 10 min centrifugation at 820 *g* at 5–10°C, 20 ml of sample was passed through an Ochratest™ immunoaffinity column (Vicam, Watertown, MA, USA) at about 1–2 ml/min. Before loading the extract, the Ochratest™ column was conditioned with PBS, pH 7.4 (20 ml). Twenty ml of de-ionized water was used to wash the loaded immunoaffinity column and OA was then eluted with 2 ml methanol and 2 ml de-ionized water. Atmospheric air (*ca* 20 ml) was passed through the column to collect all the eluate. A 1 ml sample of the eluate was then filtered through a 0.45 μm microfilter (Millex®-HV) for HPLC analysis. For HPLC, 50 μl samples were injected by full loop injection. The chromatographic system consisted of a Perkin-Elmer LC049 isocratic pump (Norwalk, CT, USA) equipped with a Rheodyne model 7125 NS injection valve (50 μl) (Rheodyne, Cotati, CA, USA), an RF-551 fluorescence spectrophotometer detector (Shimadzu, Kyoto, Japan) equipped with a 150 W xenon lamp ($\lambda_{excitation} = 332$ nm and ($\lambda_{emission} =$ 462 nm) and a Spectra-Physics SP4290 chromato-integrator (San José, CA, USA). The analytical column was an Hypersil™ BDS reversed-phase C_{18} (150 × 4.0 mm i.d., 3 μm particle size) (Tracer Analytical, Barcelona, Spain). The column was left at ambient temperature. The mobile phase was acetonitrile–water–acetic acid (45:54:1 v/v/v) eluted at a flow rate of 1.0 ml/min. OA was assayed by measuring peak height at the ochratoxin A retention time and comparing it with the relevant calibration curve (five points, in the range 1–5 ng of ochratoxin A/ml, $r^2 = 0.9992$). Standard solutions for the calibration curve were prepared in the mobile phase from a stock solution containing 20 μg/ml of OA in toluene–acetic acid (99:1).

Within the tested range of spiking samples (0.4–10 μg OA/kg), the procedure showed good precision (RSD = 3.1%) and accuracy with an overall recovery of 89 ± 1.6% (table 1). The detection limit of ochratoxin A was 10 ppt (based on the ratio signal/noise = 3/1) and the quantitation limit was 35 ppt (based on the ratio signal/noise = 10/1).

Results and discussion

Comparison of estimates of OA concentration by ELISA and HPLC

The OA contents of samples spiked with pure OA were estimated by ELISA and HPLC. The results show good correspondence (table 2). HPLC estimates were within 2 SD of ELISA estimates, although always less.

Effect of extracts from various spices in ELISA

The ELISA procedure reported for OA estimation in chillies (Thirumala-Devi *et al.* 2000) gave a non-

Table 2. Determination of OA in selected spice samples by ELISA and HPLC.

	OA contamination	
Sample type	ELISA[a]	HPLC[a]
Black pepper	4.2 ± 1.2	ND
Black pepper	103.2 ± 5.1	98.3
Coriander	8.3 ± 0.9	7.8
Coriander	59.0 ± 2.4	53.0
Ginger	25.3 ± 0.8	23.3
Ginger	77.8 ± 4.3	71.3
Turmeric	32.4 ± 1.9	29.1
Turmeric	54.0 ± 3.6	47.4

[a]ELISA values are means of three estimates ± SD; HPLC values are for single estimates.

specific reaction, presumably due to interference by substances present in the spice extracts. To confirm that interference in ELISA was due to substances in spice samples, we compared OA standards prepared in PBS-T BSA with those prepared in spice extract shown by ELISA to contain the equivalent of less than 10 µg/kg OA. Curves were found to be influenced by substances present in different spice extracts (figure 1). As a result, it was essential to prepare the standard solutions of OA in extracts of spices. Chillies have been shown to contain substances that bind to aflatoxins (Shantha 1999) and also that interfere in the estimation by indirect ELISA of OA (Thirumala-Devi *et al*. 2000). Thus, toxin standards were prepared in spice extracts shown to be substantially free of OA.

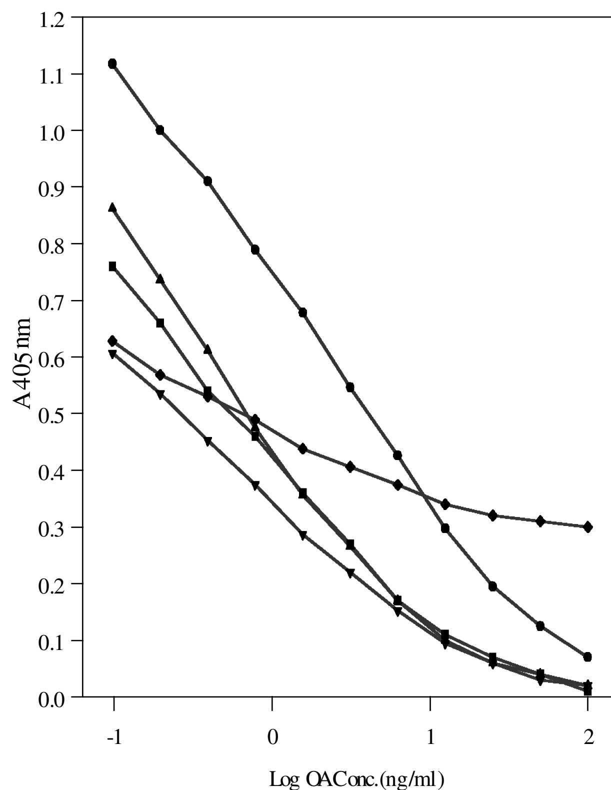

Figure 1. Effect of extracts of spices on the standard curve of OA by indirect competitive ELISA. OA standard curves prepared in 7% methanol (●), black pepper (◆), coriander (▲), ginger (■), and turmeric (▼) extracts.

Recovery of OA from spiked spice samples

To confirm that the extraction procedures were effective, pure OA was added to powdered spice samples and extracted in 70% methanol–KCl. Recoveries from samples of black pepper, coriander and turmeric samples estimated by ELISA were greater than 90% (table 3). However, recovery from ginger samples was >90% only when extracts were diluted eight-fold.

Analysis of spice samples collected from retail shops

The results of OA analysis of spices collected from retail shops are shown in table 4. Three replicates of 126 samples of black pepper, coriander, ginger and turmeric were measured. The results showed that 45 samples contained more than 10 µg/kg OA in amounts that ranged up to 110 µg/kg (table 3).

OA contamination occurred at higher levels in black pepper, turmeric and coriander than in ginger. High levels of OA (110 µg/kg) were detected in turmeric, which is one of the most widely used spices in Indian cooking.

The production of OA and the growth of the fungi responsible are dependent upon factors such as temperature, humidity, handling during harvesting and conditions during storage. The majority of spices are produced in countries with tropical climates, which favour growth of the fungi. Unfortunately, methods that would be suitable for particular spices have not been developed. We also presume that lack of sufficient surveillance data on the occurrence of OA in spices in India can be attributed to the

Table 3. Recovery of OA from artificially contaminated spice samples as determined by ELISA.

No.	Concentration of OA used for spiking (μg/kg)	Concentration of OA estimated (μg/kg)[a]	Percent recoveries of OA in spiked samples[b]
Black pepper			
1	5	4.80 ± 0.39	96.0 ± 8.1
2	10	9.60 ± 0.82	96.0 ± 8.6
3	50	49.2 ± 0.88	98.4 ± 1.8
4	100	104 ± 4.08	103 ± 4.0
Coriander			
1	5	5.90 ± 0.57	118.0 ± 9.7
2	10	10.5 ± 0.82	105.3 ± 7.8
3	50	52.5 ± 2.1	105 ± 4.0
4	100	108 ± 7.9	108 ± 7.4
Ginger			
1	5	4.73 ± 0.29	94.7 ± 6.2
2	10	9.30 ± 0.73	93.0 ± 7.8
3	50	49.5 ± 0.72	99.1 ± 1.5
4	100	104 ± 5.5	104 ± 5.3
Turmeric			
1	5	4.97 ± 0.05	99.3 ± 1.0
2	10	10.0 ± 0.08	100 ± 0.82
3	50	52.8 ± 3.1	106 ± 5.9
4	100	98.7 ± 1.0	98.7 ± 1.0

[a] Each sample was spiked with a known concentration of OA, extracted in 70% methanol and assayed. Data represent mean of three replications ± SD.
[b] Determined by the formula, detected OA (μg/kg) divided by the concentration of OA used for spiking and multiplied by 100. Values are Means ± SD.

Table 4. Incidence and range of OA in selected spice samples as determined by indirect competitive ELISA.

	OA contamination		No. of samples with OA (μg/kg) contents in the ranges of			
Sample type[a]	Incidence	% contamination	10–29	30–49	50–100	110
Black pepper	14/26	54	8	5	1	0
Coriander	20/50	40	16	3	1	0
Ginger	2/25	8	1	0	1	0
Turmeric	9/25	36	3	2	3	1

[a] See text for details.

non-availability of inexpensive diagnostic tools. The present study clearly showed that four important spices can harbour OA at levels beyond those permitted by public health authorities. Our results emphasize the need for surveillance of OA contamination in spices and suggest that strategies should be developed that can lead to contamination being minimized.

Acknowledgements

This document is an output from a project (R7083) funded by the UK Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of DFID. KT-D and DVRR are supported by

ICRISAT. MAM acknowledges financial support from the Scottish Executive Rural Affairs Department.

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