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Research Note

Survey for Co-occurrence of Ochratoxin A and Aflatoxin B_1 in Dried Figs in Turkey by Using a Single Laboratory-Validated Alkaline Extraction Method for Ochratoxin A

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ABSTRACT

A survey was carried out to determine the co-occurrence of ochratoxin A and aflatoxin B_1 in dried figs from Turkey. Samples from two seasons of crops (2003 and 2004) intended for export to the European Union and the 2004 crop obtained from the domestic Turkish market were analyzed. Affinity column cleanup methods were employed for determining separately ochratoxin A and aflatoxin B_1, but for ochratoxin A an alkaline extraction procedure was employed (in contrast to the conventionally employed acidic extraction), which gave consistently higher toxin recovery. In-house validation of the ochratoxin A method gave a limit of detection of 0.15 ng/g and a limit of quantification of 0.5 ng/g with a repeatability of 5.8% in the range 5 to 10 ng/g (with a mean recovery of 94% for spiked samples). Positive results for ochratoxin A were confirmed by liquid chromatography–mass spectrometry. For the 2003 export figs (58 samples), 7 samples contained only aflatoxin B_1, 2 samples contained only ochratoxin A, and 2 samples contained both toxins (with maximum concentrations of 35.1 ng/g for aflatoxin B_1 and 13.0 ng/g for ochratoxin A). Similarly for the 2004 export figs (41 samples), 16 samples contained only aflatoxin B_1, 4 samples contained only ochratoxin A, and 2 samples contained both toxins (with maximum concentrations of 20.6 ng/g for aflatoxin B_1 and 26.3 ng/g for ochratoxin A). Of 20 retail samples of dried figs from Turkey, only one sample contained ochratoxin A (2.0 ng/g) and none were contaminated with aflatoxin B_1. This survey revealed a 14 to 15% incidence of occurrence of ochratoxin A for 2 years, which is higher than previously reported.

Dried figs are economically important in Turkey; production of 46,500 tonnes in 2003 represented about 60% of the world dried fig production *(12).* Dried figs are predominantly exported to the European Union (EU), although domestic consumption also is important. For export to the EU, dried figs must comply with a regulatory limit of 2 ng/g for aflatoxin B_1 (4 ng/g total aflatoxins). To achieve this demanding standard, figs are individually screened under UV light for blue, green, and yellow (BGY) fluorescence *(14),* and those figs showing any signs of contamination are rejected. The screened figs are then tested for aflatoxins following the sampling plan set out in EU regulations *(3),* which state that for lot weights ≥15 tonnes, 30-kg samples (with 100 subsamples) should be blended to a homogeneous paste. Only those batches that pass this testing regimen are cleared for export, and those with higher aflatoxin concentrations are returned to be screened again until the concentrations are below the required limit of 2 ng/g aflatoxin B_1. EU regulations also set limits for ochratoxin A in dried vine fruit; presence of this toxin is a recognized problem because of toxin formation during sun drying of these commodities *(8, 13).* EU limits for ochratoxin A in dried vine fruits are currently set at 10 ng/g *(5),* but these limits are under review and a reduction to lower levels may be implemented in 2005. Dried figs are not at present included in the list of commodities for which EU controls are applied for ochratoxin A, which is limited to currants, raisins, and sultanas.

Although ochratoxin A in dried figs has not been considered a significant problem, the available survey data are limited and methods previously employed have lacked adequate sensitivity. Ozay and Alperden *(10)* analyzed 103 fig samples from the 1988 crop in Turkey and reported only a 3% incidence of occurrence of ochratoxin A, but the limit of detection of the thin-layer chromatography procedure was not given. In subsequent work *(11),* no ochratoxin A was detected in any of 32 samples of dried figs, but again the limit of detection of the method was not given and the aim of the study was to identify the optimum procedures for handling and drying rather than to determine the presence of ochratoxin A. Bayman et al. *(1)* analyzed 50,000 individual samples of figs grown in California, and those found to contain toxigenic fungi were analyzed for ochratoxin A. Although with this approach the incidence of contamination was only 0.1 to 0.6% over three seasons (1997 through 1999), the limit of detection of 10 ng/g for och-

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ratoxin was relatively high. Where contamination was found, ochratoxin concentrations as high as 1,850 ng/g were present in individual figs *(1).* These findings are consistent with those in other reports *(2),* where contamination of individual figs with ochratoxin A at concentrations as high as 9,600 ng/g were found, although it is not clear whether these decayed figs would have normally been included in products intended for human consumption. The most extensive compilation of survey data on ochratoxin A is contained in the European Commission's 2002 Scientific Co-operation Project report *(4).* Despite analysis of large numbers of samples of a variety of foods, the only data on dried figs were for 20 samples imported into The Netherlands; only 2 of these 20 samples contained ochratoxin A at a maximum concentration of 0.8 ng/g, and the remainder contained <0.1 ng/g.

Few surveillance data on ochratoxin A in dried figs are available, and the high reporting limits used in earlier studies may have distorted the picture in terms of the true incidence of occurrence. Many laboratories have used acid extraction methods for dried fruit, which results in toxin recoveries averaging only 70% *(7).* In contrast, when alkaline extraction is used, recoveries exceeding 90% can be achieved *(9),* which can provide better insight into the true incidence of occurrence of ochratoxin A in dried figs.

The aim of the work reported here was to validate a method for detection of ochratoxin A in dried figs using alkaline extraction conditions and to apply this method for a small survey of ochratoxin A in dried figs in Turkey. We also tried to establish when ochratoxin A is found in dried figs. Additionally it was thought to be of interest to establish when ochratoxin A is found in dried figs and the extent to which there is co-occurrence with aflatoxins, and thus aflatoxin analysis was also carried out on the same samples.

MATERIALS AND METHODS

Samples. Samples of dried figs (1 kg) were obtained from 2003 and 2004 production of various Turkish fig exporters. The samples were obtained after the figs had been individually screened under UV light at the stage of testing for compliance with EU limits. All samples were taken following EU regulations *(3).* Three 10-kg fig samples per container were collected as 100 incremental subsamples of 300 g each. Each 10-kg sample of figs was homogenized individually in a Robot Coupe R-23 high-speed blender (Robot Coupe, Jackson, Miss.) without addition of any solvent or water. Following homogenization, each paste sample was divided into three equal subsamples, one of which was taken for analysis. Samples of dried figs (approximately 1 kg) were also obtained in 2004 from local markets by random sampling of retail lots. All samples for ochratoxin A analysis were prepared as a slurry by initially mincing before being blended as one part dried fruit with one part water (wt/wt). All samples for aflatoxin analysis were homogenized in a Robot Coupe R-23 high-speed blender without solvent.

Ochratoxin A analysis: conditioning of immunoaffinity columns. Immunoaffinity columns (R-Biopharm Rhône, Glasgow, UK) were at room temperature prior to conditioning. For conditioning, 10 ml of phosphate-buffered saline (PBS) was passed through the column by gravity. A small portion (0.5 ml) of the PBS was retained on the column until the sample solution was applied.

TABLE 1. *In-house validation data for ochratoxin A in dried figs*

		Within batches		Between batches	
Ochratoxin A (ng/g)	Recovery (%)	RSD (%)[a]	No. of replicates	RSD (%)	No. of batches
5	98.1	4.2–5.1	8	4.9	3
10	90.4	5.1–9.0	8	6.8	3

[a] RSD, relative standard deviation.

Ochratoxin A analysis: extraction. The sample (40 g) was weighed into a blender jar, 100 ml of methanol was added, the sample was blended at high speed for 1 min, 80 ml of 2% sodium bicarbonate was added, and the sample was blended again for 2 min at high speed. The sample was then filtered through Whatman no. 4 filter paper (Whatman, Clifton, N.J.), the filtrate was collected in a 100-ml conical flask, 10 ml of the clear filtrate (equivalent to 1 g of sample) was transferred into a beaker, and 20 ml of PBS was added.

Ochratoxin A analysis: immunoaffinity column cleanup. The diluted filtrate (30 ml) was passed through the column by gravity. The column was then washed with 20% methanol (20 ml) applied in 10-ml portions at a maximum flow rate of 5 ml/min and dried by passing air through the column with a syringe for 10 s.

Ochratoxin A was eluted using a two-step procedure. Methanol plus acetonitrile (98:2, vol/vol) (1.5 ml) was applied to the column and passed through by gravity. Distilled water (1.5 ml) was then passed through the column and collected in a 3-ml calibrated volumetric flask. Clear solutions were used directly for high-pressure liquid chromatography (HPLC) analysis. Cloudy solutions were passed through a disposal filter unit (0.45 μm) prior to injection into the HPLC apparatus.

Ochratoxin A analysis: HPLC. A validated ACE 5 (4.6 mm by 25 cm by 5 μm) C-18 column was used with fluorescence detection at excitation of 333 nm and emission of 443 nm for ochratoxin A. The mobile phase was acetonitrile:water:acetic acid (47:51:2), flow rate was 1 ml/min, and column temperature was 40°C. Under these conditions, the retention time of ochratoxin A is approximately 13.2 min.

Ochratoxin A analysis: in-house validation of method. Three batches of dried figs each comprising eight spiked samples, two blanks, and a quality control sample were analyzed at spiking levels of 5 and 10 ng/g (total of 66 determinations). Samples (40 g) in a blender were spiked with either 0.1 or 0.2 ml of a solution of ochratoxin A in methanol (1,000 ng/ml) and allowed to equilibrate for 1 h in the dark. The three batches for each of the two spiking concentrations were analyzed using the method described above over a period of 3 days. Table 1 contains the within-batch and between-batch recovery and precision values. The detection and quantification limits were determined by spiking blank samples for the concentration of 0.6 ng/ml ochratoxin A standard and were calculated by using S/N equal to 3 and 9, respectively.

Ochratoxin A analysis: calibration. Fresh standard solutions were prepared daily from stock solutions (ready to use: 1,000 ng/ml), and 7-point calibration graphs were prepared for each batch by including a set of standards at both the beginning and the end of each run. Calibration curves were constructed using the area response for each standard concentration employed, and correlations were always higher than 0.995.

TABLE 2. *Ochratoxin A in dried fig samples*

Sample type	No. of samples at concentration range (ng/g) of:					
	<0.1	<0.1–1.0	1.0–5.0	5.0–10.0	10.0–15.0	>15.0
2003 export	51	2	1	1	3	0
2004 export	35	2	1	0	0	3
2004 retail	19	0	1	0	0	0

TABLE 3. *Aflatoxin B_1 in dried fig samples*

Sample type	No. of samples at concentration range (ng/g) of:					
	<0.1	<0.1–1.0	1.0–5.0	5.0–10.0	10.0–15.0	>15.0
2003 export	49	2	4	1	1	1
2004 export	24	8	7	1	0	1
2004 retail	20	0	0	0	0	0

Ochratoxin A analysis: confirmation. Mass spectra were obtained on an Agilent 1100 benchtop mass spectrometer (Agilent, Palo Alto, Calif.). The optimum conditions were obtained by using flow injection analysis. The instrument was operated in positive electrospray mode with fragmentor voltage of 35 eV, source temperature of 350°C, capillary voltage of 3,500 V, nebulizer gas (N_2) flow of 10 liters/h, and nebulizer pressure of 50 psig. The protonated molecule ion [M+H] at *m*/*z* 404 and the chlorine isotopic ion at *m*/*z* 406 (which was not used in calibration graphs) were monitored. HPLC was performed throughout using a Zorbax Eclipse XDB C18 column (4.6 mm by 50 mm by 3.5 μm; Agilent) connected to an Agilent 1100 binary pump, autosampler, and thermostatted column compartment. The mobile phase A was 0.04 M ammonium acetate adjusted to pH 4.5 with glacial acetic acid, and the mobile phase B was acetonitrile (45:55). Under these conditions, the retention time of ochratoxin A is approximately 3.7 min. Calibration graphs of ochratoxin A peak areas (*m*/*z* 404) versus concentration (1 to 20 ng/ml) were plotted. The identity of ochratoxin A was confirmed in all samples containing >4.0 ng/g.

Aflatoxin analysis. Aflatoxin analysis was based on a published method (Aflaprep IFU P07.v14, 16 April 2003; R-Biopharm Rhône Ltd.) using immunoaffinity column cleanup with HPLC/fluorescence detection and postcolumn bromination (Kobra Cell, Rhône Diagnostics, Glasgow, UK). The laboratory was ISO17025 accredited for the analysis of aflatoxin B_1 in dried figs using this method.

Analytical quality assurance. Analytical quality assurance measures for ochratoxin A involved inclusion in each batch of 10 samples duplicate samples spiked at 5 ng/g, two blank fig samples, and a quality control sample (Food Analysis Performance Assessment Scheme [FAPAS] test material). Analytical quality assurance measures for aflatoxins involved inclusion in each batch of 20 samples one sample spiked at 10 ng/g total aflatoxin, one blank fig sample, and a quality control sample (FAPAS test material). Batches of samples were deemed acceptable when blanks were <0.1 ng/g and spiked samples indicated more than 85% recovery.

RESULTS AND DISCUSSION

Performance of method for ochratoxin A analysis. The results of the in-house validation of the alkaline extraction method for ochratoxin A are shown in Table 1. Average recoveries of 90 and 98% at 5 and 10 ng/g were equivalent to recoveries of 85 to 115% using a similar alkaline extraction procedure for dried vine fruits *(9)*. However, these recoveries were better than those reported for acid extraction in a survey of dried vine fruits (63 to 71%) *(8)* and in a recent collaborative study of dried figs (73%) *(7)*. The limit of detection of the method reported here was 0.15 ng/g, and the limit of quantification of 0.5 ng/g is comparable to the limit of quantification of 0.1 ng/g for dried fruit *(9)*.

Survey of ochratoxin A in dried figs. The results of the surveys of ochratoxin A in 2003 and 2004 figs are shown in Table 2. In the survey of 2003 dried figs for export (58 samples), 2 samples contained only ochratoxin A and 2 samples contained both aflatoxin B_1 and ochratoxin A (with maximum concentrations of 13.0 ng/g for ochratoxin A and 35.1 ng/g for aflatoxin B_1). Similarly, for the 2004 crop of dried figs for export (41 samples), 4 samples contained only ochratoxin A and 2 samples contained both toxins (with maximum concentrations of 26.3 ng/g for ochratoxin A and 20.6 ng/g for aflatoxin B_1). Of 20 retail samples of dried figs from Turkish markets, only 1 contained ochratoxin A (2.0 ng/g). The incidences of contamination with ochratoxin A of 14 and 17% in 2003 and 2004 export samples is higher than has been previously reported *(10, 11)*, which is a reflection of the lower limits of quantification used in this survey.

Survey of aflatoxins in dried figs. The results of the surveys of ochratoxin A in 2003 and 2004 are given in Table 3. In the survey of 2003 dried figs for export (58 samples), 7 samples contained only aflatoxins and 2 samples contained both toxins (with maximum concentrations of 35.1 ng/g for aflatoxin B_1 and 13.0 ng/g for ochratoxin A). Similarly, for the 2004 dried figs for export (41 samples), 16 samples contained only aflatoxin $B_1$and 2 samples contained both toxins (with maximum concentrations of 20.6 ng/g for aflatoxin B_1 and 26.3 ng/g for ochratoxin A). Of 20 retail samples of dried figs from Turkey, none were contaminated with aflatoxins. The samples analyzed for aflatoxin B_1 were intended for export and had been screened for BGY fluorescence under UV light to remove any contaminated figs. Seven samples from 2003 and nine samples from 2004 did not comply with EU limits for aflatoxin and would have been withdrawn from export for further screening. This testing assumes that the samples analyzed are representative of the batch (20 tonnes), which despite rigorous sampling nevertheless involves a 20% producer risk that samples will be rejected even if they are below the EU limit of 2 ng/g for aflatoxin B_1.

Implications for ochratoxin A in dried figs. This is the first extensive survey for ochratoxin A in dried figs using sensitive methodology, and the results indicate a more widespread occurrence than previously supposed and cooccurrence with aflatoxin B_1 in some instances. Because extremely high concentrations of ochratoxin A have been found in individual fruits *(1, 2)* and because BGY fluorescence screening to eliminate aflatoxin-contaminated figs cannot identify ochratoxin A hot spots, further work is needed that will focus on ochratoxin A concentrations in

individual fruits. Although in this study ochratoxin A and aflatoxin B_1 were determined in separate laboratories, the analysis of aflatoxins and ochratoxin A simultaneously should be considered. In a recent study *(6)*, ochratoxin A, aflatoxins, and zearalenone were isolated using a multianalyte affinity column. Provided extraction and HPLC conditions for the two different toxins can be optimized without compromising either, simultaneous determination would be a cost-effective approach to monitoring.

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