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Natural Occurrence of Ochratoxin 'A' in Raisins in Pakistan

Zuzzer A. Shamsuddin*, Mobeen A. Khan and Aftab Ahmed
Food and Feed Safety Laboratory, PCSIR Laboratories Complex, Karachi-75280, Pakistan

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Abstract. Grape raisins have become an important commodity in Pakistan. Ochratoxin 'A' and aflatoxin B_1 are important mycotoxins from the human health point of view, which were determined in uncleaned grape raisins collected from local vendors during 1999-2002. A total of 160 samples of these raisins were analyzed for the quantitative detection of ochratoxin 'A' and aflatoxins. No aflatoxins were detected in any of the raisin samples above the detection limit of 1µg/Kg. Ochratoxin 'A' was determined using two different methods. Method–I involved methanol-aqueous extraction of the raw unclean raisins, followed by the adjustment of the extract to pH 2 with 2 N hydrochloric acid, and partitioned into chloroform. Method-II involved methanol-equeous extraction, followed by cleanup with the clarifying agent, ammonium sulfate, and partitioned into chloroform. The minimum amount of ochratoxin 'A' detected in the samples, with method-I, was 3.2 ± 0.9 µg/Kg, while with method-II the minimum value noted was 9.7 ± 0.8 µg/Kg. The maximum amount determined in the samples analysed using method-I was 16.8± 2.7 µg/Kg, while with method-II the maximum value was 29.2 ± 2.9µg/Kg.

Keywords: raisins, ochratoxin 'A', aflatoxin B_1, mycotoxins, afflatoxins, carcinogenic metabolites

Introduction

Ochratoxin 'A' was first discovered during a pure culture study of *Aspergillus alutaceus* (*A. ochraceus),* which occurs naturally in contaminated grains and oilseeds (Hohler, 2000; Shotwell *et al.,* 1969; Ward and Diener, 1961). Although all *Aspergillus* species produce ochratoxin 'A', the highest quantity of this toxin is produced by *A. carbonarius* (Pitt *et al.,* 2002; Pitt, 2000). *A. carbonarius* grows at high temperatures and is associated with maturing fruits, especially grapes. The fungus is highly resistant to sunlight, survives sundrying, and is a source of ochratoxin 'A' in fresh grapes, dried wine fruits, and wine (Bakker and Pieters, 2002). Ochratoxin 'A' is known to possess, nephrotoxic, hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive properties (Marquardt *et al.,* 1990; Roschenthaler *et al*.,1984; Steyn, 1984). Ochratoxin 'A' has been shown to cause Balkan endemic nephropathy, a chronic renal disease found in closed areas of Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, and the former Yugoslav Republic of Macedonia (Abouzied *et al.,* 2002).

The presence of ochratoxin 'A' in the red grape juice and wine in Europe has sparked the discussion of health risks involved. Ochratoxin 'A' has been detected prior to the preparation of juice and the wine fermentation process, the presence of which has been traced to surface of the berries and not from the inside of the fruits (Bau *et al.,* 2004). Occurrence and risk assessment of ochratoxin 'A' in table wine and grape juices have been evaluated (Zimmerli and Dick, 1996). It has been reported that the level of ochratoxin 'A' in bottled red wines ranges between 0.08 and 4.83 µg/l in 17 out of 18 samples (Tateo and Bononi, 2001). Ochratoxin 'A' has also been reported in Italian wine (Pietri *et al.,* 2001). In another survey, the levels of ochratoxin 'A' have been established in 122 representative varieties of wine cultivars from various wine producing regions of South Africa. (Stander and Steyn, 2002).

Natural cooccurrence of ochratoxin 'A' with aflatoxins was first reported in corn samples collected from northern Iran (Yazdanpanah *et al.,* 2001). A study conducted in the UK indicated that no aflatoxin was found in any sample of retail dried vine fruits analysed (MacDonald *et al.,* 1999), whereas ochratoxin 'A' was found in excess of 0.2 µg/Kg in 19 out of 20 currants, 17 out of 20 sultana and 17 out of 20 raisin samples examined, giving an overall incidence of 88% with the maximum level of ochratoxin 'A' found to be 53.6 µg/Kg. Ochratoxin 'A' has been encountered as a contaminant in various foodstuffs such as composite samples of cereals, pastry, coffee and raisins (Malir *et al.,* 2001). It has been detected as a contaminant in cereals, assorted beans and maize (Steyn, 1984), in poultry-feed (Thirumala-Devi *et al.,* 2002), Polish cereal grains (Czerwiecki *et al.,* 2002), and animal feed mostly in the countries with a temperate climate (Dalcero *et al.,* 2002).

The multi-faceted and potent toxicity of ochratoxin 'A' to experimental animals, its accepted role as a disease determinant in porcine nephropathy, its putative role in the idiopathic human disease, the Balkan endemic nephropathy and the asso-

*Author for correspondence

ciated urinary tract tumors, and its widespread occurrence usually in trace amounts in foods, has focused the attention on minimizing human intake of ochratoxin 'A' (Mantle, 2002). Many countries have set limits for certain mycotoxins in products for human and animal consumption. Several countries have also established concentration limits for ochratoxin 'A' in different food products, such as for EU countries, non-processed cereal food products (5 µg/Kg), cereals and processed food products (3 µg/Kg), and dried wine fruits OTA (10 µg/Kg) (FAO, 2003).

Importance of mycotoxins in food and feed has attained much priority in Pakistan. Many quests for better ways to control the contamination of aflatoxins, in particular, and other mycotoxins, in general, during the last ten years, have given a boost to the food and feed sectors in Pakistan. Raisins are used locally in various delicious food recipes and dassert dishes. In view of the economical and health consequences and the nature of ochratoxin 'A', a study was undertaken to assess its levels in the locally vended raisins.

Materials and Methods

Aflatoxins and ochratoxin 'A' standards were from Sigma Chemical Company, MO, USA. Precoated thin layer chromatography (TLC) plates of silica gel 60 (layer thickness 0.25 mm, 20x20 cm) on glass or aluminum, without fluorescent indicator were from E. Merck, Darmstadt, Germany. Analytical grade chemicals and solvents were used for all analyses.

Samples of raisins were collected from local vendors and were divided into representative samples by following the statistical procedure of 'coning and quartering method'. Raisin samples (2 Kg) were mixed, reduced and tipped into the centre. It was scooped to turn the heap of raisins from the edge towards the centre. The heap was broken occasionally and reconstructed several times to ensure thorough mixing. After mixing the raisins, the top of the cone was flattened by using a board so that the height was less then that of the width. The heap of raisins was divided into two approximately equal halves, which were then reduced in the same fashion. Two diagonally opposite quarters of the raisins were eliminated and the remaining raisins were mixed thoroughly as described above. Dust or small particles associated with each quarter were carefully collected together with the raisins accordingly. The procedure was repeated many times in order to reduce the sample to 50 g size.

Aflatoxin B_1 and ochratoxin 'A' were determined quantitatively in 160 samples of the raisins. Aflatoxins were analysed in accordance with method 975.36 (AOAC, 2000). The presence of aflatoxins was detected under longwave UV light, in an enclosed viewing cabinet, and quantified by visual comparison with standards on TLC plates (method 968.22 F(d) AOAC, 2000). The presence of aflatoxin B_1 was confirmed by spraying the TLC plates with 50% sulphuric acid (method 980.20 F; AOAC, 2000) and/or making the derivative with trifluoroacetic acid (method 978.15 F; AOAC, 2000).

The following procedures were used for the determination of ochratoxin 'A'.

Method-I. The method-I was adapted from method 973.37 (AOAC, 2000). Ochratoxin 'A' was extracted from raisin samples with methanol-aqueous extraction followed by pH adjustment (pH = 2) of the extract with 2 N hydrochloric acid and partitioning into chloroform. The ochratoxin 'A' was entrapped in a column containing diatomaceous earth impregnated with a basic aqueous solution. Interfering compounds were removed with hexane and chloroform washes, and the ochratoxin 'A' was eluted with a benzene-acetic acid solution. The column eluate was evaporated to near dryness in rotary evaporator. Extracts were dissolved in 100 µl chloroform and were mixed on vortex mixer. Different quantities of extracts were spotted on silica gel TLC plates. Plates were developed first in toluene : ethyl acetate : acetone (3:2:1, v/v), followed by plate-development in the same direction with the developing solvent system of toluene : ethyl acetate : acetone (3:2:1, v/v) with 1% formic acid. The plates were observed under longwave UV light. Positive spots for ochratoxin 'A' were confirmed by exposing the TLC plates to ammonia vapour and noting the colour change of the ochratoxin 'A' spot methods (49.6.02, 975.38G; AOAC, 2000; 1990).

The identification procedure was accasionaly confirmed by forming methyl or ethyl ester derivatives of the ochratoxin 'A' and reanalysing by TLC.

Method-II. Representative samples of 50 g raisins were blended with 270 ml of methanol and 30 ml aqueous 4% potassium chloride solution for 3 min. It was filtered through fluted qualitative filter paper. The clean up of samples was done by the addition of 150 ml of 20% ammonium sulphate solution. It was filtered and 150 ml filtrate was mixed with equal quantity of water and extracted with two portions of 10 ml chloroform. The chloroform was reduced to near dryness under gentle stream of nitrogen, redissolved in chloroform and analysed by thin-layer chromatography, using longwave UV light for visualization of the fluorescent ochratoxin 'A' spots and confirmation as described for method-I.

Results and Discussion

No aflatoxin B_1 was detected ($\leq$ 1µg/Kg) in any sample of the raisins. Similar results were reported for aflatoxins in samples

of dried vine fruits such as currants, raisins and sultanas, during studies in UK (MacDonald *et al.,* 1999). However, in one report (Abdel-Sater and Saber, 1999), aflatoxin B_1 was detected in one out of 20 samples of dried raisins.

Both the methods were evaluated by using spiked samples. The naturally contaminated samples were spiked to levels of 20 µg/Kg ochratoxin 'A'. Only for method-I the filtrate was acidified to pH 2.0. The spiked sample was transferred to separatory funnel and ochratoxin 'A' was determined as described, repeated thrice. Recoveries are shown in Table 1 (a & b). For method-I, the recoveries ranged from 85.8% ± 3.4% to 92.2% ± 3.2% and the coefficient of variation ranged from 2.52% to 6.01%. For method-II, the recoveries ranged from 88.8% ± 2.7% to 109.8% ± 5.5% and the coefficient of variation ranged from 1.32% to 5.8%. These values were achieved for both methods at 20 µg/Kg. The limits of detection for both the methods is approximately 3 µg/kg for raisins. In the present study, 80 samples were analysed for ochratoxin 'A' by method- I, while other 80 samples were analysed by method-II. The amount of ochratoxin 'A' detected by the two methods are given in Table 2 (a & b), which show the percentage of contamination of raisin samples, along with the minimum and maximum amount of ochratoxin 'A' detected during different years. The minimum and maximum levels of ochratoxin 'A' detected during the period 1999-2002 with method-I were 3.2 ± 0.9 and 16.8 ± 2.7 µg/Kg, respectively. While using method-II, the minimum and maximum amount of ochratoxin 'A' detected for the same years were 9.7 ± 0.8 to 29.2 ± 2.9 µg/Kg, respectively.

Several analytical methods are available for the determination of ochratoxin 'A' in food, feed, assorted grains, cereals, dry fruits, and from other biological samples. Generally, the major steps in all the methods are the same which include sampling, sample preparation, extraction, clean-up, determination, and confirmation. The sampling and sample preparation steps for the two methods used in the present study were the same. In method-II, instead of acidifying the sample extract, the clean-up of the sample was done by addition of ammonium sulphate solution as the clarifying agent. The use of ammonium sulfate resulted in the absence of additional spots and the elimination of streaks, which are the two characteristics considered desirable for TLC. This was achieved successfully with the use of ammonium sulphate in the clean-up step for the detection of ochratoxin 'A' in raisins, which was also used for the purpose by Soares and Rodriguez-Amaya (1985) for samples of corn, peanut, beans, rice and cassava.

It was observed during the present study that the fluorescence detection sensitivity of ochratoxin 'A' on the developed TLC plates could be greatly improved by exposure to ammonia vapours. This converts the ochratoxin 'A' to its ammonium salt and as a result of that it shifts the absorption maxima from 330 to 370 nm and enhances the fluorescence emission at 460 nm. In contrast to the findings of Soares and Rodriguez- Amaya (1985), it was observed in the present study that ammonia vapours significantly enhanced the fluorescence of ochratoxin 'A' spots on the TLC plate, while according to these authors exposure to ammonium vapours actually decreased the fluorescence.

Table 1(a). Percentage recoveries of ochratoxin 'A' by method-I[*,**]

Year	Recovery (%)
1999	87.2 ± 2.2
1999	86.8 ± 3.5
1999	86.0 ± 4.3
2000	92.2 ± 3.2
2000	89.6 ± 5.3
2000	89.8 ± 5.4
2001	92.0 ± 3.0
2001	88.0 ± 4.2
2001	90.0 ± 4.5
2002	86.6 ± 5.2
2002	85.8 ± 3.4
2002	86.4 ± 2.2

*all the values are the average of five replicates; **spiked standard: ochratoxin 'A' added (20µg/Kg); ±: standard deviation

Table 1(b). Percentage recoveries of ochratoxin'A' by method-II[*,**]

Year	Recovery (%)
1999	93.8 ± 2.2
1999	89.6 ± 1.3
1999	109.8 ± 5.5
2000	91.4 ± 5.3
2000	98.8 ± 1.3
2000	104 ± 2.9
2001	89.8 ± 2.3
2001	94.8 ± 1.5
2001	101.6 ± 1.7
2002	88.8 ± 2.7
2002	95.8 ± 3.2
2002	102.2 ± 3.4

*all the values are the average of five replicates; **spiked standard: ochratoxin 'A' added (20µg/Kg); ±: standard deviation

Table 2(a). The amount of ochratoxin 'A' detected by method-I

Year	Samples found to be contaminated* (%)	Minimum amount detected (µg/Kg)	Maximum amount detected (µg/Kg)
1999	85	3.2 ± 0.9	14.4 ± 1.8
2000	90	6.3 ± 1.4	16.8 ± 2.7
2001	20	12.2 ± 1.4	15.0 ± 1.6
2002	55	4.4 ± 2.1	16.6 ± 2.4

*average of 20 samples; ±: standard deviation

Table 2(b). The amount of ochratoxin 'A' detected by method-II

year	Samples found to be contaminated* (%)	Minimum amount detected (µg/Kg)	Maximum amount detected (µg/Kg)
1999	85	9.7 ± 0.8	23.7 ± 2.5
2000	90	11.8 ± 0.9	29.2 ± 2.9
2001	20	13.6 ± 0.6	21.8 ± 2.4
2002	55	9.7 ± 0.9	18.5 ± 1.1

*average of 20 samples; ±: standard deviation

In several investigations, ochratoxin 'A' has been reported to be determined in dried fruits like currants, raisins, sultanas, by using acidic methanolic extraction, immunoaffinity, chromatography clean-up and HPLC determination (Pascale *et al.,* 2000; Chung *et al.,* 1999, Degelmann *et al.,* 1999; IARC, 1982). In the early 1990s an AOAC method received the widest acceptance (AOAC, 1990). Several multimycotoxin methods have been developed (Shotwell, 1976; Eppley, 1968) in which chloroform extract was applied directly on to silica gel column, and in the first instance aflatoxin and zearalenone were eluted followed by the elution of ochratoxin 'A' with acetic acid and benzene. Ochratoxin 'A' was then determined as a free acid by TLC and estimated by visual comparison, or by TLC densitometry. In the present study, we also used acidic methanolic extraction, but instead of immunoaffinity chromatography clean-up and HPLC determination, which are quite expensive, we performed the liquid/liquid partition followed by thin layer chromatography. The time involved for the whole procedure in both the methods was more or less the same. In acidified commodities, ochratoxin 'A' is readily soluble in many organic solvents, so that this characteristic has been used as the principle of extraction in several methods. In the present study, method-I was based on the same principle.

Bearing in mind the fundamental role of the clean-up step in identification, sensitivity and suitable quantification by TLC, the initial efforts were aimed to describe the cleaning step. Clarifying agent ammonium sulfate was selected as to its relative low cost and for being more rapid as compared with nearly all other systems, except for the expensive pre-packed and clean-up columns or cartridges. The test with ammonium sulfate was noted to be adequate. Its use yielded better recoveries with an average of 96.7%. Copper sulphate was also used for the extraction of ochratoxin 'A' in raisins, however, the recovery was less than 60% (unpublished data).

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