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Incidence of Ochratoxin A in Raw and Salted Dried Fruits Using High Performance Liquid Chromatography

[1]Farnaz Nayebpoor, [2]Manouchehr Momeni and [1]Farhad Safarpoor Dehkordi

[1]Young Researchers Club, Shahrekord Branch, Islamic Azad Universirty, Shahrekord, Iran
[2]Biotechnology Research Center, Shahrekord Branch, Islamic Azad Universirty, Shahrekord, Iran

Abstract: Study the presence of Ochratoxin A (OTA) in foods shows the quality of food products and manufacturer's commitment to ethical issues. This present study was carried out in order to investigate the incidence of OTA in raw and salted dried fruits using High Performance Liquid Chromatography (HPLC). Totally, 134 raw and salted dried fruits were collected from the supermarkets in Isfahan province, Iran. Foodstuffs were: raisins, salted and raw pistachio, hazelnut, almonds, dried fig and walnut. All samples were immediately transferred to the laboratory in dried and sterile condition. Samples were analyzed for contamination with OTA using the HPLC method. The limit of quantification (LOQ) (S/N, 10:1) of OTA was 0.02 ng/g in pistachio and hazelnut and 0.03 ng/g in walnut, dried raisins, almonds and dried figs. The incidence of occurrence of OTA in raisins and dried fig was 10.41% and 14.73%, respectively. Analytical results showed that pistachio, salted and raw pistachio, hazelnut, almonds and walnut samples contained no detectable OTA, but concentrations ranged from 2.3±14.2 to 7.9±4.3 ng/g in raisins and from 7±3.8 to 2.9±18.2 in dried figs. Results showed also that 16.41% of total number of dried fruits samples was positive OTA. This is the first report on the occurrence of OTA in dried fruits in Iran.

Key words: Incidence Study · Ocratoxin A · Dried Fruits · High Performance Liquid Chromatography · Iran

INTRODUCTION

Mycotoxins are secondary metabolites principally produced by molds of genera *Penicillium*, *Aspergillus* and *Fusarium.* Nowadays, more than 300 mycotoxins are known. The most known and important mycotoxins are ochratoxin A (OTA), aflatoxins and *Fusarium* toxins [1]. The OTA, chemically known as N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl}-3-phenyl-L-alanine, is a ubiquitous secondary fungal metabolite primarily produced by the genera of *Aspergillus* and *Penicillium*. Several *Aspergillus* species (*Aspergillus* spp.) have been described as producers of OTA, including strains of *A. ochraceus*, *A. ostianus*, *A. alliaceus*, *A. sulphureus*, *A. sclerotiorum*, *A. foetidus, A. melleus*, *A. albertensis*, *A. niger, A. glaucus*, *A. awamori*, *A. petrakii*, *A. carbonarius*, *A. wentii* and *A. auricomus* [2]. In colder climates, *Penicillium* spp. such as *P. verrucosum*, *P. nordicum*, *P. aurantiogriseum* have been described as producers of OTA.

The OTA is one of the most studied mycotoxins because of the wide range of foodstuffs it contaminates and also because its occurrence has been reported in foodstuffs all around the world [3, 4]. The OTA has been widely detected in pulses [5], cereals [6], foodstuffs of animal origin [5], cacao [5], grape juice [5, 7], green coffee beans [8], wheat grain [9], barley [9], corn [9], bread [10], rice [11], dried fruits [11], wine [12], beer [12] and fruit juice [12]. Since, contamination and infection of foods and human being have been reported from Spain [13], Netherlands [14], France [15], Tunisia [3], the Czech Republic [16], Egypt [17], Argentina [18], Morocco [19] and Australia [20].

The OTA has teratogenic (reproductive), immunosuppressant and carcinogenic effects and a clear connection has been shown between nephropathy (kidney disease) and exposure to OTA in humans and animals. In addition the genotoxicity of OTA has been described previously [21]. Consumption of food contaminated with OTA during pregnancy and/or

Corresponding Author: Manouchehr Momeni, Biotechnology Research Center,
Islamic Azad University, Shahrekord Branch, Shahrekord, Iran, P.O. Box: 166.
Tel: +989132805063, Fax: +983813381892.

childhood is suspected to induce lesions in testicular DNA that puberty could promote testicular cancer [22]. Several studies have been focused on detection of OTA in dried fruits in Iran [23, 24] but there is no extensively report on incidence of OTA in dried fruits in Iran. Therefore, the present study was carried out in order to study the incidence of OTA in raisins, salted and raw pistachio, hazelnut, almonds, dried fig and walnut using High Performance Liquid Chromatography (HPLC) in Iran.

MATERIALS AND METHODS

Chemical and Reagents: Crystalline OTA was purchased from Sigma (St. Louis, MO, USA). Stock standard solution with concentration of 500 µg/mL was prepared in methanol kept at –20°C and in amber glass (GFF VidraFOC, Barcelona, Spain). Solid phase C8 (50 µm) and nylon acrodisk (0.45 µm) were from Análisis Vínicos (Tomelloso, Spain). Silanized glass wool was supplied by Panreac (Barcelona, Spain). Standard working solutions were prepared by appropriate diluting in the same solvent and stored in glass-stoppered tubes at -20°C. Methanol for liquid chromatography, hydrochloric acid (35%) and acetic acid (glacial) for analysis, were supplied by Merck (Darmstadt, Germany). Formic acid (98-100%), reagent grade ACS, was supplied by Scharlau (Barcelona, Spain). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

Samples Collection: One hundred and thirty four samples of dried fruits including raisins (n=48), salted and raw pistachio (n=12), hazelnut (n=12), almonds (n=12), dried fig (n=38) and walnut (n=12) were randomly collected from supermarkets of Isfahan province, Iran from January to October 2012. All samples were milled and then divided with a subsample divider. A 200 g subsample was collected in a plastic bag and kept at –20°C until analysis.

Extraction Procedure: The procedure used for OTA analysis in dried fruits was the method of Blesa *et al.* [25]. Briefly, samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2.5 g) of the sample was placed into a mortar (50 mL capacity) and gently blended with 1.5 g of the solid phase (C8) for 5 min using a pestle, to obtain a homogeneous mixture.

This mixture was placed into a 100 mm×9 mm i.d. glass chromatographic column with a coarse frit (No. 2) and covered with a plug of silanized glass wool at the top of the column. OTA was eluted with 20 mL methanol-formic acid (99:1, v/v) with a vacuum manifold. The elute was evaporated to 3 mL with a gentle stream of N_2 at 45°C and then, it was filtered through a nylon acrodisk (0.45 µm) and centrifuged at 5000 rpm for 10 min with centrifuge 5810-R Eppendorf (Madrid, Spain). The extract was filtered again and evaporated to 0.5 mL with N_2 at 45°C.

Liquid Chromatography-Fluorescence Detection: A Shimadzu (Kyoto, Japan) SCL-6A system LC equipped with two LC-6A pumps, a Rheodyne Model 7125 injector (20 µL loop) and a Shimadzu RF-10A XL fluorescence detector was used. An LC column Phenomenex (5 µm) (150 mm×4.6 mm i.d.) was used with a mobile phase of methanol–formic acid 0.1 M (70:30, v/v) at a flow rate of 0.4 mL/min. Detection of OTA was carried out using 333 and 464 nm as wavelengths for excitation and emission, respectively.

To estimate the limit of detection (LOD) and the limit of quantification (LOQ), dried fruit samples spiked at 8.0, 4.0, 1.0 and 0.5 ng OTA/g were extracted and analyzed. The LOD and the LOQ values were calculated according to s/n=3 and s/n=10, respectively.

Method Performance: Mean OTA recovery from dried fruits (n=6) spiked with 3.0 ng OTA /g was 94% (RSD=6%). These values are in agreement with the EU Commission Directive 2002/26/EC for methods of analysis of OTA in foodstuffs [26]. Intra-day (n=5) and interday (5 different days) variation values at a fortification level of 3.0 ng/g were 6.2 and 7.1%, respectively. These values are below 15% which is the maximum variation for certification exercises for several mycotoxins. The LOD and the LOQ were 0.017 ng/g and 0.051 ng/g, respectively. All positive samples were confirmed by OTA methyl ester formation. The resulting OTA methyl ester (OTA-Me) has a quite different retention time than OTA, which allows the confirmation of OTA identity by an almost complete disappearance of the first OTA peak and the presence of a new one (Fig. 1).

Occurrence of OTA in Dried Fruits: Results of occurrence of OTA in analyzed dried fruit samples (Table 1) showed contamination of 48 out of 100 total analyzed samples with OTA greater than the LOQ. Fig. 2a shows a chromatogram of a naturally contaminated sample of dried fruit.

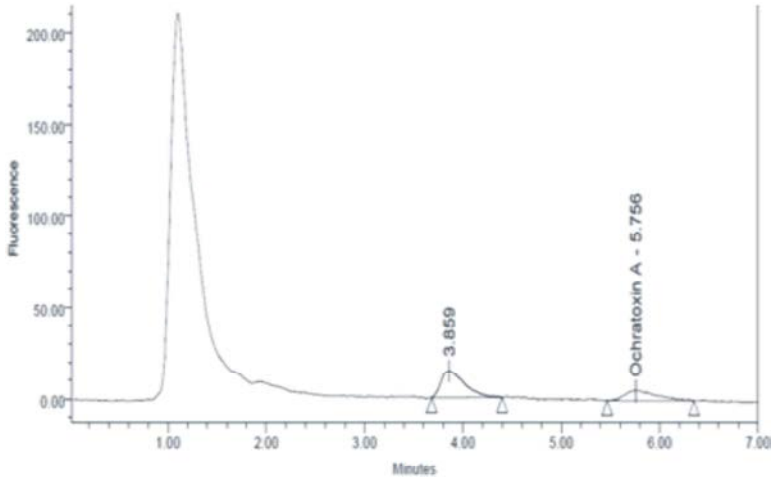

Fig. 1: OTA standard chromatogram in various samples

Table 1: Recoveries and limit of quantification (LOQ) for OTA in different analyzed matrixes

Samples	Recovery (%)	LOQ (ng/g)
Raisins	8.64	10.4
Salted pistachio	4.94	0
Raw pistachio	4.94	0
Hazelnut	4.94	0
Almonds	4.94	0
Dried fig	8.64	44.7
Walnut	4.94	0

Table 2: Incidence of OTA in various samples using HPLC

Samples	No. of samples	Positive results for presence of OTA (%)
Raisins	48	5 (10.41)
Salted pistachio	6	0
Raw pistachio	6	0
Hazelnut	12	0
Almonds	12	0
Dried fig	38	17 (44.73)
Walnut	12	0
Total	134	22 (16.41)

Levels of OTA in positive samples ranged between 0.14 and 149 ng/g, where the average level of OTA in positive samples was 13±1.5 ng/g. The incidences of OTA in dried fruit from Rabat, Témara, Meknès, Salé and Casablanca were 53.1, 54.5, 33.3, 37.5 and 61.5%, respectively. The averages for OTA in positive samples of dried fruit were 17.00±2.35, 7.10±0.40, 5.40±1.54, 10.00±1.68 and 25.50±2.14 ng/g, respectively. The highest frequency of positive samples (61.5%) and the most contaminated dried fruit sample (149 ng/g) were found in the Casablanca area.

RESULTS AND DISCUSSION

Recoveries for OTA on samples spiked at a level of 10 ng/g for raisins, salted and raw pistachio, hazelnut, almonds, dried fig and walnut at was summarized in Table 1. The OTA standard chromatograph was shown in Figure 1. The limit of quantification (LOQ) (S/N, 10:1) of OTA was 10.4% ng/g in raisins and 44.7% ng/g in dried fig. As shown, the limit of quantification (LOQ) allows OTA determination at the maximum levels indicated in the European legislation. Results of the study reflected the analysis of OTA in dried fruits (Figure 2).

The incidence of OTA in various dried fruits has been shown in Table 2. This present study showed that the OTA has the high ability to dried fruit's contamination. This is an important public health problem. Besides, this toxin reduces the nutritional values of dried fruits. Similar studies have been performed on Morocco and Brazil [10, 27]. The limit of quantification (LOQ) (S/N = 10:1) of OTA was 0.02 ng/g in rice, 0.03 ng/g in pistachio, peanut and walnut and 0.03 ng/g in dried raisins and dried figs in Brazil which was in harmony with our study. One hundred and seventeen dried fruit samples including black sultanas, white sultanas, dates, dried plums, dried figs and apricots were studied for presence of fungi and OTA in Morocco and it was found that *Aspergillus niger* was predominant, with 406 isolates, of which 15% were OTA producers. They were followed by *A. ochraceus*, with 15 isolates and 87% ochratoxigenics and *A. carbonarius*, with only five isolates of which 60% were OTA producers [10]. The incidences of occurrence of OTA in dried raisins, walnuts, peanuts, dried figs and rice were 30%, 35%, 25%, 65% and 90%, respectively, in Brazil [27] which was higher than our results. Thirty percent and 3.33% of examined apricot samples and

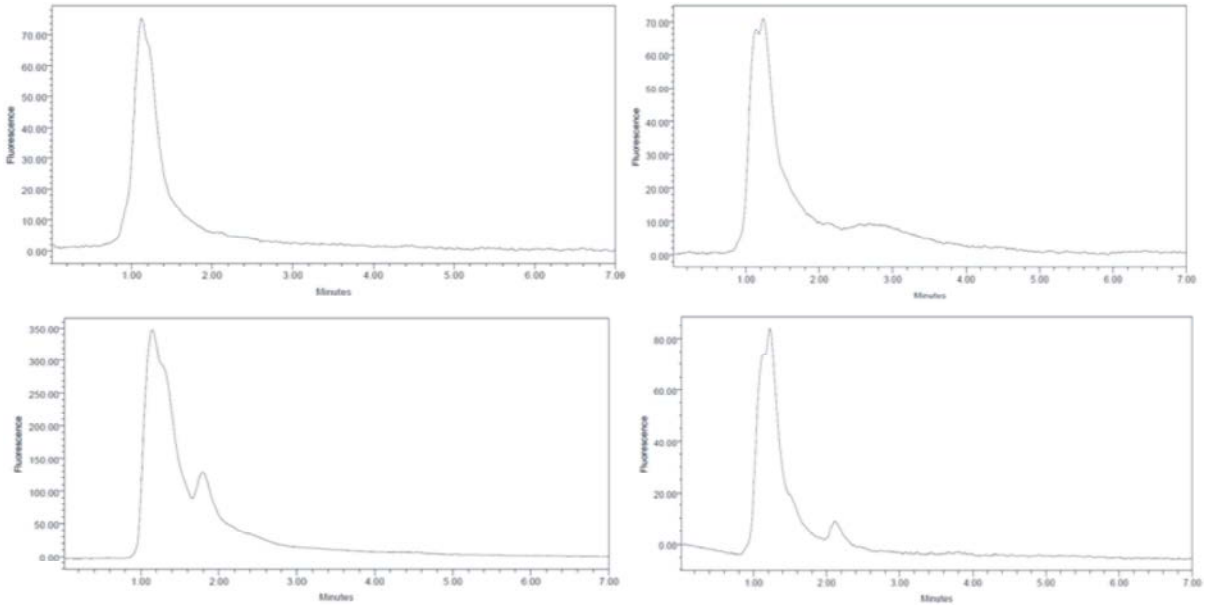

Fig. 2: Detection of OTA in dried fruits using HPLC method

13.33% and 20% of examined prunes samples contained aflatoxin B(1) and OTA more than 0.2Â ngÂ $g^{(-1)}$ in previous report from Iran [23].

Ninety eight dried figs, 53 sultanas and 20 dried apricots destined were examined for presences of OTA and aflatoxins and it was recognized that only 2 (4%) of the sultanas exceeded the 10 ng $g^{(-1)}$ maximum limit set, 28 (53%) of 53 sultana samples contained detectable levels of OTA, in the range of 0.51-58.04 ng $g^{(-1)}$, Eighteen of 98 (18%) dried figs contained detectable levels of OTA, in the range of 0.87-24.37 ng $g^{(-1)}$ and finally one of the 20 dried apricots was contaminated, with 0.97 ng $g^{(-1)}$ OTA [28]. The higher average recoveries of toxins in dried fruits have been reported from Iran (91.1% and 98.5% for aflatoxin B and OTA, respectively, while the detection limit was 0.2Â ngÂ/g for both mycotoxins) [23].

Our results showed that 10.41% of raisins were contaminated with OTA while previous UK surveys reported an incidence of OTA in raisins of 85% [29]. A German survey [30] reported a 95% overall incidence of OTA in raisins and currants. Similar incidences have been reported from Finland (71%) [31], France (46%) [31], Canada (79%) [32] and Netherlands (10%) [31].

The OTA which was detected in dried fruits of this present study can causes several poisoning in humans. Studies showed that the OTA is the most abundant and hence the most commonly detected toxin [33, 34]. A study in Tunisia showed that the OTA has been detected in food and blood samples of patient with the clinical signs of poisoning [4]. Similar recovery rates for OTA have been reported previously: 82.4% at 10 ng $g^{-}1$ [11], 80%–84% at 2, 5 and 10 ng g^{-1} [35] and from 80% to 85% at 1, 7.6 and 29.6 ng g^{-1} [27] in dried fruits.

We suggested processing of dried fruits with sanitary methods and using modern techniques for inspection the toxic quality of dried fruits. Keeping dried fruits in cool and dry places and away from sunlight are help to prevent fungal growth and OTA production.

As far as we know, this is the first report that has shown the contamination of raisins, salted and raw pistachio, hazelnut, almonds, dried fig and walnut available in Iran by OTA. The acceptable level of OTA was exceeded in 16% of analyzed samples of dried fruits in Iran. It can be concluded that the occurrence of OTA in some dried fruits samples is due to the fact that may be some food safety and quality standards (good agricultural practices (GAPs), good manufacturing practices (GMPs) and the hazard analysis and critical control point (HACCP) system need to be applied and performed in most of Iranian food units to control growth of moulds and mycotoxin production during harvesting, distribution and storage periods. The moisture content of dried fruits should be monitored and timetable schedules for sun-drying periods, storage and transport should be modified according to what has been observed in the models. Therefore, many studies should have been performed on different Iranian foods for study the presence of OTA.

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