APB
Advanced Pharmaceutical Bulletin, **2012**, 2(1), 123-126
doi: 10.5681/apb.2012.018
http://apb.tbzmed.ac.ir/
TUOMS PG

Determination of aflatoxins in nuts of Tabriz confectionaries by ELISA and HPLC methods

Siahi Shadbad Mohammad Reza[1], Ansarin Masoud[2], Tahavori Ali[3,4], Ghaderi Faranak[1,5], Nemati Mahboob[1,2*]

[1] *Department of Pharmaceutical and Food Control, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

[2] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*

[3] *Students' Research Committee, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

[4] *Research Center for Pharmaceutical nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran.*

[5] *Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 10 April 2012
Accepted: 29 April 2012
ePublished: 10 May 2012

Keywords:
Aflatoxins
Nuts
Confectionery
ELISA
HPLC

ABSTRACT

***Purpose*:** Aflatoxins (AFs) are a group of mycotoxins and secondary metabolites of various species of *Aspergillus*. There are various forms of aflatoxins including B1, B2, G1, G2, M1 and M2 types. Aflatoxins cause important health problems and have high potential effect on liver cancer. Therefore, numerous investigations have been conducted during last three decades. The aim of this work is to determine the contamination levels of nuts used by the confectionaries in Tabriz. ***Methods*:** A total of 142 samples including 35 almond , 26 walnut, 4 seeds of apricot, 6 sunflower seeds kernel, 6 sesame seed, 6 peanuts , 32 pistachio,13 hazelnuts and 14 cashews samples were collected from Tabriz confectionaries. The ELISA method was employed for the screening of total aflatoxins. ***Results*:** In 13 cases (28.1% of pistachios, 5.1% of walnuts and 7.1% of cashews) contamination rate of higher than 15 ppb were observed. The HPLC method was applied for the confirmation of ELISA results. Aflatoxin B1 was the highest detected AFs. ***Conclusion*:** The overall results of the tested samples indicated that the rate of contamination of pistachios is higher than the other tested samples.

Introduction

Aflatoxins (AFs) are a group of mycotoxins and secondary metabolites of strains of the fungi *Aspergillus spp.*[1] There are various forms of aflatoxins including B1, B2, G1, G2, M1 and M2 types.[2] AFs have commonly been found to contaminate a wide variety of food stuffs such as nuts, rice, wheat, barley, cereal and some other crops.

AFB_1 known as a powerful hepatocarcinogenic and genotoxigenic substance and has been classified as group 1 carcinogen.[3] The 8, 9- epoxide aflatoxin B_1 is the active ingredient to the carcinogenesis effect of AFB_1.[4]Generally tropical condition such as high temperature and moisture lead to fungal proliferation and production of AFs .[5] Poor storage condition can also contribute to fungal growth and increase the risk of aflatoxin contamination .[5] Aflatoxin effects are very strong in liver carcinogenesis .[6] Therefore AFs have an important role in public health. Nuts and their products are consumed as well as part of the ingredient of our daily diet. So it is important to control their occurrence and level in food.

AFs are regulated in more than 76 countries. Currently the worldwide MTL for AFB_1 and total AFs are 1- 20 ng/g and 0-35 ng/g respectively .[7] European Commission regulating set limits for AFB_1 and total AFs 2 and 4 ng/g respectively in nuts, groundnuts, dried fruit and cereals since 1998 .[8,9] Research on Food products has demonstrated that AFs are still being found frequently in food product at level that are of significant concern for consumers .[10]

Due to the significant health risks associated with the presence of aflatoxins in foods, it is important to establish a data collection on the occurrence of these toxins in nuts as valuable foods. The aim of this study was to screen the content of AFs in nut used in the confectionary products in Tabriz market as a leader for these products in Iran and also a first report in this regard.

Materials and Methods

Chemicals

AFB_1, B_2, G_1 and C_2 standard solution was prepared from Supellco (USA). aflatoxins concentrations

***Corresponding author:** Mahboob Nemati (PhD), Department of Pharmaceutical and Food Control, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz 51664, Iran. Tel: +98 (411) 3341315, Fax: +98 (411) 3344798, E-mail: nematim@tbzmed.ac.ir

Copyright © 2012 by Tabriz University of Medical Sciences

wereAFB1 1.00µg/mL, AFB2 0.200µg/mL, AFG1 1.00µg/mL, AFG2 0.200µg/mL. The standard solutions were in 100% methanol. Solvents and chemicals used were of highest purity and analytical grade. For clean-up step immunoaffinity column (IAC) AflaCLEAN^{TM} (LC Tech, Germany) were used.

Sampling

A total of 142 different types of nuts included 26 samples of walnut, 35 samples of almond, 32 samples of pistachio, 13 samples of hazelnut, 4 samples of seeds of apricot, 14 samples of cashew, 6 samples of sunflower seeds kernel, 6 samples of sesame seeds and 6 samples of peanut were purchased from Tabriz market and confectionaries and stored frozen at – 20° c until started analysis. Samples transferred to room temperature and then approximate value homogenized completely.

Enzyme – linked immunosorbent assay (ELISA) analysis.

For analysis of samples by ELISA, Euroclon kit was used with detection limit of 4 ppb. Analysis was performed according to the protocol of the manufacture. ELISA microplate reader (State Fax® 2100, Awareness, USA) was used to read the absorbance of samples and standard solutions at 450 nm. The extraction of aflatoxins from nuts was carried out using a 33% methanol solution. After filtering with filter paper (whatman No. 1, USA) 500 μl of the filtrate and 500 μlit of 33% methanol were transferred to microtubes and stored at – 20 ^{oC} until started analysis. 50 μl distilled water was added in to the mixing wells. Then 50 μl of standards (0.1, 0.2, 0.5, 1, 4 μg/kg) were added to standard wells and 50 μl of samples were added to other wells. 50 μl enzyme conjugate placed in each of wells and 100μl antibody (anti –aflatoxin) was added. After mixing slowly, wells were incubated at room temperature (20 – 25 ^{oC}) for 20 min. The content of the antibody – coated well was emptied out and washed five times with washing buffer. Then, 200 µl of chromogen solution was added to each wells and incubated at room temperature for 20 min. Finally 50 μl of stop solution was added to each well and mixed prior to absorbance reading at 450 nm.

Apparatus

ELISA microplate reader (State Fax® 2100, Awareness, USA) was used to read the absorbance of samples and standard solutions at 450 nm. The LC system consisted of a KNAUER equipped with model K-1000 pump, auto sampler spark triathlon and fluorescence detector RF-550 (Shimadzo, Japan). The guard column, Hichrom ODS, 5 mm (Hichrom Ltd., Reading, Berkshire, UK) was placed between the auto sampler and the column, Hichrom ODS (250 ×4.6mm I.D., 5 mm, Hichrom Ltd., Reading, Berkshire, UK) column. Post column derivatization of Aflatoxin was carried out by a photochemical reactor, UVE (LC Tech, Germany).

Extraction procedure

Aflatoxins were analyzed in nuts according to the method reported by Stroka *et al* .[10] Briefly, in 10 g of fatty samples (pistachio, peanut, and walnut)), 1 g of NaCl added and blended with 40 ml of methanol: water (80:20) and 20 ml of n-hexane for 3 min. After separation of the two phases, n-hexane was eliminated. Nonfat samples were extracted only with 40 ml of methanol: water (80:20). The extract was filtered with filter paper (whatman No. 1 USA) and 10 ml of filtrate was passed by glass microfiber filter.

Immunoaffinity column clean-up

An aliquot of 7 ml was diluted with 43 ml of PBS buffer (pH 7.4). An immune affinity column AflaCLEAN^{TM} (LC Tech, Germany) was conditioned with 10 ml of PBS buffer by gentle syringe pressure at a flow rate of 1 ml/min. Then, the mixture of the filtrate diluted extract (50 ml) was applied to the IAC column (1- 2 drops per second), followed by a washing with 20 ml of bidistilled water and then dried with air. Aflatoxins were then slowly eluted from the IAC with 2 ml methanol into a glass vial.

HPLC determination of aflatoxins

After the ELISA screening, HPLC confirmation proceeded with contaminated samples (≥ 15µg/kg total AFs). In brief 5 g of homogenized samples was extracted with 0.5 g NaCl and 30 ml methanol: water, (2: 8) by high – speed blender. (In fatty samples n – Hexan was added in order to remove fat). The mobile phase consisted of acetonitrile: methanol: water (17:29:54, v/v/v). The mobile phase was degassed by sonication. The Hichrom ODS (250× 4.6 mm I.D., 5 mm, Hichrom Ltd., Reading, Berkshire, UK) column was connected as LC column. The column was eluted with a flow rate of 1 ml/min. The aflatoxins were detected at the excitation and emission wavelengths of 365 and 435 nm, respectively. The injection volume was 20 ml. For the quantization of aflatoxins in samples, a separate calibration curve was established for each aflatoxin. Triplicate samples were used for setting calibration curve, determining LODs and extraction recovery.

The limit of detection (LOD) was defined as the analyte concentration that gives a signal equal to: y_b + 3.3sb

where y_b is the signal of the blank and sb is its standard deviation. Similarly, the limit of quantitation (LOQ) was defined as: y_b + 10sb

In the unweighted least-squares method, it is quite suitable in practice to use sxy instead of sb, and the value of the calculated intercept instead of y_b. Thus: LOD = 3.3 s_{xy}/b and LOQ = 10 s_{xy}/b where b is the slope of the regression line (19, 20). Based on the above equations, the LOD and LOQ values for aflatoxins are shown in table 1.

Copyright © 2012 by Tabriz University of Medical Sciences

Table 1. Validation of aflatoxins determination by HPLC analysis.

Aflatoxin	LOD (µg/kg)[a]	LOQ (µg/kg)[b]	Calibration curve[c]	R^2
AFB1	0.38	1.26	Y=30729x+7714	0.999
AFB2	0.08	0.28	Y=64499x+12356	0.999
AFG1	0.42	1.4	Y=11004x+8230	0.996
AFG2	0.05	0.19	Y=27323x+7461	0.997

[a] Limit of detection (LOD).
[b] Limit of quantification (LOQ).
[c] x = concentration of aflatoxins (µg/kg) and y = intensity.

The efficacy of method was examined by the determination of the recoveries of the AFs. The recoveries were ascertained by spiking 10, 20, 30 µg/ml of AFB_1 and AFG_1 and 0.2, 0.4, 0.6 µg/ml of AFB_2 and AFG_2 and the extraction result were expressed as percentage that were ranging from 85.6 to 112.3%.(table2.)

Table 2. Recoveries of aflatoxins in edible nuts.

Aflatoxins	spiked aflatoxin µg/ml	Recovery (%)	RSD
AFB1	10	95	2.1
	20	104.7	2.1
	30	90.1	4.2
AFB2	0.2	90.3	3.5
	0.4	97	2.9
	0.6	112.3	3.5
AFG1	10	85.6	2.5
	20	107.2	1.9
	30	100.2	3.1
AFG2	0.2	90.1	3.1
	0.4	97.4	3.1
	0.6	94.2	3.8

Precautions in handling and decontamination of aflatoxins

The handling of preparation of standards, working solutions and samples must be done in fume hood with suitable protective clothing (laboratory cover, mask and gloves). All glass wares of laboratory accessories should be immersed in a 10% sodium hypochlorite before cleaning or discarding. Accendital spill and laboratory surface should be cleaned with 1% sodium hypochlorite .[14]

Results and Discussion

The contamination of food with AFs is a very important case of focus of health scientists. Nuts are food samples which are susceptible for this contamination because of their composition and storage conditions. The incidence of AFs in different types of nuts and nutty products has been studied by several authors from different countries.

Chun et al (2007) found that nut samples in South Korea were contaminated with AFs (10.6% of incidence) in the range of 0.20 – 28.2 µg/kg [11] In China peanut was reported contaminated with AFs being than average level of 80.3µg/kg and the highest level being 437µg/kg .[4] In Turkey peanut butter was contaminated with AFs in the range of 8.16 – 75.7 ng/g which AFB1 was in the range of 2.06 – 63.7 µg/kg [12] Cheraghali and et al (2007) reported that 11.8% and 7.5% of 10068 Iran pistachio nut samples were above the MTL of AFB1 and AFs respectively .[13] Yin – Hui and et al (2008) reported that nut and nutty products were contaminated with AFs ranging in level from 16.6 µg/kg up to 711 µg/kg in Malaysia .[14]

In this study, At first ELISA method was used for screening contaminated samples. ELISA analysis is suitable for determination of contaminant in a large number of samples in a shorter time. According to the results, between 142 samples, 13 samples were contaminated above the maximum tolerated level (15 µg/kg). The most contaminated samples were walnuts (76.9%) and pistachio (53.12%). the least amount of contamination found in samples of almonds, hazelnuts and apricots.

Table 3. The results for confirmation of Presence of aflatoxins in different samples by HPLC analysis.

Sample category	Incidence			Aflatoxins [a] (µg/kg)			
	No [b]	%	Total [c]	AFB1	AFB2	AFG1	AFG2
Almond	5/35	14	<5	ND [d]	ND	ND	ND
Walnut	20/26	76	1-54	15.4-35.1	4-8.1	1.4-8.2	ND-5.1
Apricot	1/4	25	<5	ND	ND	ND	ND
Sesame	0/6	0	ND	ND	ND	ND	ND
Sunflower	0/6	0	ND	ND	ND	ND	ND
peanut	2/6	33	<5	ND	ND	ND	ND
Hazelnut	1/13	7	1-13	ND	ND	ND	ND
pistachio	17/32	53	1-54	9.5-43.8	0.9-9.4	ND-19.7	ND- 7.1
Cashew	2/14	14	11-20	18.3	2.7	ND	ND

[a] AF concentration was not corrected for the recovery result.
[b] Incidence no was represented by positive/total sample of the particular category.
[c] Total was represented by the summation of aflatoxin B1, B2, G1 and G2 levels.
[d] Not detected. (The samples with total Aflatoxin lower than 15 µg/kg were not tested by HPLC)

Copyright © 2012 by Tabriz University of Medical Sciences

The HPLC used for confirmation and quantification of AFs levels in contaminated samples. From 13 samples with contamination upper than 15 µg/kg, 9 samples were pistachio, 3 walnut and 1 cashew samples. The HPLC method used for determination of AFs in the above mentioned samples successfully confirmed the results of ELISA method. Also the results of HPLC showed that contamination with AFB_1 were higher than other AFs, which is a potent carcinogen (table 3).
In the present study some samples were collected as Powder form .the contamination in these kinds of samples were higher compared to intact ones. Probably that is due to their higher surface area and also storage conditions in confectionaries which make samples more susceptible for the fungal growth and contamination.
The higher level of AFs in some nuts, especially about AFB1, suggests that a good supervision is necessary about the production and storage of nuts which are used in confectionaries.

Conclusions

This study presents useful information about the risk of AFs hazard in different types of nuts and their products. The high occurrence of AFs emphasizes the need for regular monitoring and a more stringent food safety system in order to control the AFs at the lowest possible level.

Acknowledgement

This article was written based on a dataset of PharmD. Thesis (No. 3552), registered in Tabriz University of Medical Sciences.

Conflict of interest

The authors report no conflicts of interest.

References

1. Hajdu S, Obradovic, A Presterl, E,Vecsei V. Invasive mycoses following trauma. *Injury* 2009; 40(5):548-554.
2. Cole R J, Cox R H. Handbook of toxic fungal metabolites. New York, Academic Press, 1981.
3. International Agency for Research on Cancer. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC monographs on the evaluation of carcinogenic risk to humans,* Lyon, IARC, 1993, 56: 489–521.
4. Wang J, Liu XM.Surveillance on contamination of total aflatoxins in corn, peanut, rice, walnut and pine nut in several areas in China. *Chinese journal of preventive medicine* 2006;40(1):33-7
5. Bhat R V, Vasanthi S. Mycotoxin food safety risk in developing countries In: Food safety in food security and food trade. International Food Policy Research Institute. 2003; Focus 10, vision 2020, Brief 3.
6. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American journal of clinical nutrition* 2004;80(5):1106-22
7. Food and Agriculture Organization of the United Nations. Worldwide regulations for mycotoxins in food and feed in 2003. FAO food and nutrition paper 2004; 81. FAO, Rome.
8. Commission Directive 98/53/EC of 16 July 1998 laying down the methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs. *Official Journal of the European Communities* 1998, L201:93-101.
9. Commission Regulation (EC) No. 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communities* 2001; L77: 1–13.
10. Stroka J, Anklam E. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *Trends in Analytical Chemistry* 2002, 21(2): 90–95.
11. Chun HS, Kim HJ, Ok HE, Hwang JB, Chung DH. Determination of aflatoxin levels in nuts and their products consumed in South Korea. *Food Chemistry* 2007;102(1): 385–391.
12. Yentür G, Er B, Ozkan MG, Oktem AB. Determination of aflatoxins in peanut butter and sesame samples using high-performance liquid chromatography method. *European Food Research Technology* 2006; 224(20): 167–170.
13. Cheraghali AM, Yazdanpanah H, Doraki N, Abouhossain G, Hassibi M, Aliabadi S, et al. Incidence of aflatoxins in Iran pistachio nuts. *Food and Chemical Toxicology* 2007; 45(5): 812–816.
14. Yin-Hui L, Noryati I, Aishah AL, Rosma A. Aflatoxin occurrence in nuts and commercial nutty products in Malaysia . *Food Control* 2010; 21(3): 334–338.

Copyright © 2012 by Tabriz University of Medical Sciences