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Application of Dispersive Liquid-Liquid Microextraction to Determine Aflatoxin B1 in Tomato Paste Samples

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ABSTRACT

Background: Aflatoxin B1 is a secondary toxic metabolite produced by several aspergillus species. This study was conducted to determine the presence of aflatoxin in tomato paste samples. **Methods:** A total of 30 tomato pastes were analyzed for Aflatoxin B1 contamination via HPLC-FLD. Analyte extraction was done by dispersive liquid-liquid microextraction. **Results:** The limit of detection (LOD) and limit of quantitation (LOQ) were 0.14 and 0.44 µg/kg, respectively. The recovery rates ranged from 91 to 94%. The findings showed all samples were contaminated with Aflatoxin B1 and the average concentration was 1.1 ± 0.02 µg/kg. The amount of aflatoxin B1 in 6 samples was higher than the limit set by the European Union. **Conclusion:** The proposed method was successfully applied to the analysis of tomato paste samples with quantitative results. The main advantages of the developed method include its simplicity in operation, rapid achievement of a very high sample, and low cost.

Keywords: *Mycotoxins; HPLC; Aflatoxin B1; Iran*

Introduction

Mycotoxins are the secondary metabolites produced by different types of fungi (Flores-Flores *et al.*, 2015). Their presence in food depends on a variety of factors, such as temperature, humidity, storage, and Processing (Rawat, 2015). Aflatoxin is one of the most toxic known mycotoxins produced by genus Aspergillus, mostly aspergillus flavus and aspergillus parasiticus species (Yu *et al.*, 2004).

Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are the most important types of aflatoxin. Furthermore, aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) are metabolites of AFB1 and AFB2 in the livestock body (Kumar *et al.*, 2017), which are generally found in a wide range of foods such as grains, pistachio, milk, and nuts (Alshannaq and Yu, 2017). Aflatoxins are highly toxic, mutagenic, teratogenic, and carcinogenic compounds that are known as the causative agents of liver carcinogenesis. The most toxic aflatoxin is AFB1 that was determined by the

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International Agency for Research on Cancer (IARC) as group 1 carcinogen types (Abrar *et al.*, 2013).

The allowed limits of AFB1 and total aflatoxin are 2 and 4 µg/kg in food products in the European Union, respectively (Authority, 2007, Kilicel *et al.*, 2017). Various methods were introduced to detect aflatoxins, such as ELISA and HPLC using mass detection and several other methods. In this regard, HPLC with fluorescence detection (FLD) is one of the most commonly used methods to detect aflatoxin (Espinosa-Calderón *et al.*, 2011).

The dispersive liquid–liquid microextraction (DLLME) technique was introduced in 2006, which is a quick, low-cost, and low-solvent consumption method. This method is used to extract a small amount of the material from aqueous samples. This technique is based on injecting a small amount of a solvent extraction agent (like C2Cl4) and a dispersed solvent (like acetone) into an aqueous solution. The resulting solution is then centrifuged to separate the organic and aqueous layers. This process is useful in extracting organic compounds from aqueous samples (Berijani *et al.*, 2006, Rezaee *et al.*, 2006).

In previous studies over analysis of mycotoxins using the development and rapid sample preparation methods, the suitability of DLLME was investigated for sensitive determination of aflatoxins in samples. HPLC_FLD was used as a determination technique (Campone *et al.*, 2011).

In this study, the aim was to measure the amount of aflatoxin extracted using DLLME method in tomato paste samples collected from the northwest of Iran. Later, the amount of aflatoxin contamination in Iran was compared with the international standards.

Materials and Methods

Chemicals and reagents: Sodium chloride (NaCl), n-hexane, chloroform (CHCL3), and Formic acid (CH2O2) included high purity LC grade provided by Merck (Darmstadt, Germany). The stock solution of AFB1 at a concentration of 1000.0 ng/ml was supplied by Sigma (St.Louis, MO, USA). Methanol and acetonitrile were prepared from Duksan Pure Chemicals (Kyungkido, Korea), deionized extra pure water was also purchased from Shahid Ghazi pharmaceutical company (Tabriz-Iran). To construct a calibration curve, 6 dilutions of AFB1 standards were prepared from 0.6, 1.25, 2.5, 5, 10, and 20 ng/ml by adding appropriate volumes of methanol to obtain these concentrations. The Stock solution of AFB1was kept at 4 ºC in the dark.

Equipment and chromatographic conditions: The HPLC analyses were carried out in a KNAUER HPLC system with a K-1000 pump (Berlin, Germany) coupled with a fluorescence detector. Biotech 2003 degasser (United State) and the analytical column included C18 column 4.6x250 mm (Knauer, Berlin, Germany).

The mobile phase was acetonitrile: methanol: water (24:25:51), de-aerated by an ultrasonic bath for 10 min at a flow rate of 1.5 ml/min in isocratic mode. The fluorescence detector was set at wavelengths of 373 and 450 nm for excitation and emission, respectively.

Method of validation: The developed analytical method was validated based on repeatability (intra-day precision) and reproducibility (inter-day precision), which is also called RSD, linearity, accuracy (as recoveries), selectivity, as well as limits of detection (LODs) and quantification (LOQs). The LOD was calculated as the concentration of analyte, whose peak area was three times the area of the noise of a blank sample ($S/N \geq 3$). LOQ was calculated by taking three replicates of the detection limits when $S/N \geq 10$. The acceptable value for RSD is below 20% and the acceptable range for recovery is 70-120% (Fontana *et al.*, 2016). In measuring linearity, a value of higher than 0.99 is acceptable.

Tomato paste sampling: Tomato paste samples were collected from different stores in 2019 within two months. A total of 30 samples were collected from different brands, including 10 famous Iranian brands, 12 non-famous Iranian brands, 5 non-Iranian brands, and 3 homemade tomato pastes.

Extraction procedure: The extraction procedure of analyte from tomato paste samples was carried out according to a previous study method (Amirkhizi *et al.*, 2015) after applying some modifications. To this end, 5 g well-homogenized tomato paste samples were mixed with 0.1 g NaCl and 20 ml acetonitrile-

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water (80:20, V/V). Later, they were blended by a magnetic stirrer for 10 minutes; 1 g diatomaceous earth was added and mixed for 5 min and the mixture was clarified with Whatman filter paper No.1 (Whatman Inc., Clifton, NJ, USA).

In the next stage, 3 ml of n-Hexane was added to 10 ml of the extracted solution and shortly shaken by Vortex. The n-Hexane was separated by centrifugation for 1 min and cleaned up by DLLME (Amirkhizi *et al*., 2015).

Dispersive liquid–liquid microextraction procedure: A volume of 260 µl chloroform (extracting solvent) was added to 1 ml of the obtained extract (disperser solvent) and the pH was adjusted to about 5.8 by formic acid. The mixture was quickly injected into a 10 ml centrifuge test tube with a conical bottom containing 2.5 ml of distilled water. It was then vortexed for a few seconds to obtain a stable and cloudy solution; so, AFB1 was entrapped into the fine CHCl3 droplets. This ternary component solution was centrifuged at 5000 RPM for 3 minutes after the aqueous upper layer was removed. The precipitated chloroform was transferred to a small tube by a 220 µl sampler and dried at a mild flow of nitrogen gas. The residue was re-dissolved in 20 µl methanol and then injected into the HPLC system (Afzali *et al.*, 2012, Amirkhizi *et al.*, 2015).

Results

Method validation: In order to verify the linearity, six concentrations of the standard B1 0.6, 1.25, 2.5, 5, 10, and 20 ng/ml were injected into the device in three replications and obtained with a calibration curve R2 = 0.9974, which is acceptable. The LOD and LOQ were 0.14 and 0.44 µg/kg, respectively. The recovery and RSD rates were acceptable (**Table 1**).

Testing the method for evaluating the natural occurrence of AFB1 in tomato pastes:

The analytical results of AFB1 in samples are reported in **Table 2**. AFB1 was detected in all samples and quantified in 14 samples. In this study, concentration ranged from 0.47 µg/l to 7.7 µg/l. In this study, 46.6% of the samples were contaminated with AFB1. The average contamination rate was 1.1 ± 0.02 µg/kg. A total of 6 samples contained more than 2 µg/kg AFB1, which is within the limit allowed by the Europe Union. As expected, none of them was famous commercial brands.

Table 1. Recovery rate obtained from three concentrations

Aflatoxin B1 spiked (µg/kg)	Aflatoxin B1 found (µg/kg)	Recovery (%), n=3
0.6	0.55	91.5 ± 0.16[a]
1	0.94	94.0 ± 0.23
4	3.64	91.0 ± 0.42

a: Mean ± SD

Table 2. List of analyzed tomato pastes

Sample no.	Aflatoxin B1 content (µg/kg) (n=3)
1	0.7 ± 0.035[b]
2	< LOQ
3	< LOQ
4	0.47 ± 0.016
5	< LOQ
6	< LOQ
7	< LOQ
8	1.6 ± 0.10
9	< LOQ
10	< LOQ
11	< LOQ
12	1.96 ± 0.017
13[a]	2.33 ± 0.23
14[a]	7.7 ± 0.29
15[a]	4.9 ± 0.40
16[a]	2.9 ± 0.14
17	0.9 ± 0.05
18	< LOQ
19	0.53 ± 0.039
20	< LOQ
21	0.48 ± 0.068
22	< LOQ
23[a]	2.1 ± 0.06
24	< LOQ
25	< LOQ
26	1.9 ± 0.04
27	< LOQ
28[a]	3.7 ± 0.10
29	< LOQ
30	< LOQ

a: Means: above the limit allowed by the Europe Union; *b: Mean ± SD*; *LOQ: limit of quantitation*

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Table 3. Compare this study with other researches

Sample	Determination technique	Pretreatment method	LOD	LOQ	Recovery (%)	country	Ref.
Rice	HPLC-FLD	DLLME	0.009 µg/kg	0.03 µg/kg	85.2-112.0	China	(Lai *et al.*, 2015)
Noodle	HPLC-FLD	QuEChERS	0.51 µg/kg	1.03 µg/kg	85-109	Malaysia	(Moazami and Jinap, 2009)
Cereals	HPLC-FLD	Validated method	0.0125 ng/g	0.05 ng/g	77-104	Brazil	(Rahmani *et al.*, 2010)
Black pepper	HPLC–MS/MS	QuEChERS	Not clearly informed	4 µg/kg	102-104	Belgium	(Yogendrarajah *et al.*, 2013)
Pistachio	HPLC-FLD	(REGULATION, 2006)	0.10 µg/kg	0.11 µg/kg	88.5-89.1	Turkey	(Hepsag *et al.*, 2014)
Tomato paste	HPLC-FLD	DLLME	0.14 µg/kg	0.44 µg/kg	91-94	Iran	This work

Table 4. Comparison of aflatoxin levels with other studies

Samples	No. of samples	% Occurance	Range	Country	Ref.
rice	370	63.5	0.030-20.0 µg/kg	China	(Lai *et al.*, 2015)
Pistachio	3	66.7	81.6 ng/g	Bahrain	(Musaiger *et al.*, 2008)
Sunflower seed Safflower seed	50 123	13 85	< 2-168 ng/g < 5.4 ng/g	Iran	(Beheshti and Asadi, 2013)
Pistachio Sorghum	40 93	52.5 62.0	0.16-122.4 ng/g 0.34–52.9 ng/g	Tunisia	(Ghali *et al.*, 2009)
Chicken liver eggs	50 150	72 58	0.30-16.36 µg/kg 0.30-2.35 µg/kg	Iran	(Amirkhizi *et al.*, 2015)
Tomato paste	30	100 (detected) 46.6 (quantified)	< 0.44-7.7 µg/kg	Iran	This work

Discussion

The advantages of DLLME method include its lower costs due to less solvent consumption, high enrichment factor, and environmental friendliness due to reduced sewage production (Afzali *et al.*, 2012). So far, many methods have been applied for extracting aflatoxin, but the DLLME method is one of the best methods for aflatoxin analysis due to its advantages. **Table 3** shows examples of comparing DLLME with other methods, indicating that DLLME is one of the most appropriate methods for extraction that gives credit to researchers(Chen *et al.*, 2005).

Milk and pistachio are among the most important products which contamination with aflatoxins is frequently reported. Due to the presence of aflatoxin in various foodstuff and toxicity of AFB1, it is essential to study this mycotoxin in one of the high-consumption foodstuffs in Iran. A study in China showed that 235 out of 370 rice samples were contaminated with AFB1. Moreover, 65 rice samples were positive in terms of contamination with AFB2 (Espinosa-Calderón *et al.*, 2011). Contamination of pistachio nuts with aflatoxins is high in some regions such as the Persian Gulf. A study in Bahrain showed that two of three pistachio samples were contaminated with AFB1 and one of these two samples had a very high contamination level (Afzali *et al.*, 2012). In a study from Iran, AFB1 and AFB2 were detected in 13% and 8% of the sunflower seed samples, respectively. Furthermore, aflatoxins B1, B2, G1, and G2 were detected in 85%, 20%, 16%, and 16% of the

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safflower seed samples, respectively. In 10% and 1.6% of the sunflower seeds, the level of AFB1 was above the European Union maximum limit (Campone *et al.*, 2011). In Tunisia, researchers showed that 52.5% of pistachios and 62% of Sorghum included AFB1 positive and the concentrations ranged from < LOQ to 122.4 and 0.34–52.9 ng/g, respectively (Afzali *et al.*, 2012). A study was conducted on 150 eggs and 50 chicken liver samples in Iran. The findings showed that 72% of the chicken liver samples and 87% of the egg samples were contaminated with AFB1 (Kumar *et al.*, 2017). Researchers from the Netherlands and the United Kingdom studied 26 peanuts and found two positive AFB1 samples. **Table 4** contains more information in this regard. These results indicate that the level of aflatoxin in foodstuffs is high throughout the worldwide.

Conclusions

In this study, an appropriate analytical procedure was successfully developed for analyzing AFB1 in tomato paste samples. As far as we know, no study has ever investigated the presence of aflatoxin in Iran's tomato paste samples. In this study, a rapid and simple analytical procedure was successfully developed for the analysis of aflatoxins in tomato paste samples. Based on the sequential application of solid–liquid extraction and DLLME before the instrumental analysis by HPLC-FLD, this novel method provides a sensitive and accurate determination of analytes.

Based on the findings, AFB1 concentration was above the limit allowed by the Europe Union in the six studied brands. Our aim was to develop an appropriate method to determine aflatoxins in foodstuffs for increasing the humans' health.

Authors' contributions

Study concept and design: Nemati M and analysis and interpretation of data: Arabkhani P.; drafting of the manuscript: Safavizadeh V, Mojkar M and Shyrina D.

Conflict of interest

We confirm no known conflicts of interest considering this study.

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