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Determination of Aflatoxin B_1 Contamination in Wheat and Rice Flour Collected from Iranian Market Using Simple and Reliable HPLC Method

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Abstract Aflatoxin B_1 (AFB_1), as the most toxic mycotoxin, has a large variety of toxic effects to human and animals. To evaluate incidence of AFB_1 in wheat flour and rice flour sold in Iranian markets, 40 samples including 16 wheat flour samples and 24 rice flour samples were collected from commercial brands commonly marketed in Iran and analyzed for their AFB_1 content using post-column photochemical derivatization and HPLC. The mobile phase consisting of water, methanol and acetonitrile mixture (53:28:19, v/v/v). The sample preparation was done with simple extraction with acetonitrile and water (80/20, v/v) accompany with immunoaffinity column cleanup. Our results indicated a good recovery (minimal 90% in wheat flour and 97 % in rice flour) in spike range 0.2-8 $\mu g.kg^{-1}$ with RSDs lower than 5%. The results showed both high incidence and high levels of AFB_1 in rice flour samples compared to wheat flour. AFB_1 was detected in 18.5% with maximum level of 0.26 $\mu g.kg^{-1}$. AFB_1 was found in 100% of rice samples with a mean and maximum level of 4.09±2.565 $\mu g.kg^{-1}$ and 10.16 $\mu g.kg^{-1}$, respectively. The results indicate that although the level of AFB_1 in wheat flour is not comparatively critical point regarding quality of wheat flour, high incidence and high contamination level of AFB_1 in rice flour samples could make serious health problems for Iranian consumers.

Keywords: *Aflatoxin B_1 (AFB_1), HPLC, Wheat and rice flour, photochemical derivatization*

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1. Introduction

Aflatoxins (AFs), as secondary metabolites are produced by *Aspergillus* genus with high potential risk for contamination of a wide variety of agricultural commodities and foodstuffs, especially in cereals [1,2]. Among of cereals, wheat and rice are extensively contaminated with Aflatoxin B_1 (AFB_1) both in the tropics and semi-tropics area during cultivation, storage and transfer of crops [3,4]. AFB_1 is a potent carcinogen, teratogen and mutagen which is listed on group I carcinogen by the International Agency for Research on Cancer (IARC) and proven as the important cause of hepatocarcinoma [5]. Previous studies suggested the contamination of wheat and rice, flour and products derived from them such as biscuit containing wheat flour, breakfast cereals and baby foods cause health problems in consumers, particularly in children [2,6,7].

AFs are low molecular mass polar compounds, which considerable UV absorption and fluorescence properties. Therefore, liquid separation techniques are predominated in their analysis [3]. With the development of high performance liquid chromatography (HPLC) methods, fluorescence detection using post-column is still used as golden choice in official analyses in foodstuffs [1,8,9]. Recently, application of automated photochemical online post-column methods is recommended as the best methods in derivatization of AFB_1 and AFG_1 due to simplicity, sensitivity and reproducibility methods compared to other chemical derivatization techniques. This is achieved by passing the HPLC column elution through a reaction coil wound around a UV light at ambient temperature, which causes hydration of AFB_1 and AFG_1 to their respective hemiacetals [3].

Wheat and rice are the most popular and consuming foodstuffs by Iranian population which used up to 2.5 and 1.7 folds more than developing countries (60 kg) and advanced countries (96.5 kg) every year, respectively [10,11].

Human exposure to aflatoxins is mainly due to food intake [12,13,14]. It seems that higher consumption level of Iranian people is related to numerous varieties of

wheat-based products such as bread, confectioneries, pasta and etc. Further, rice consumption is 7 folds more than European Union countries with 36.6 kg per capita consumption every year in contrast to 5.3 kg for EU members. The world's average rice consumption is 57.2 kg per capita; with 68.1 kg in developing and 12.4 kg in advanced countries. The rice is used in varieties of food forms and flour. Rice flour can utilize for making rice Soup, rice noodles some pancakes and some dessert. It can be used to thicken soups and stews, as well as providing an alternative to wheat flour in cakes and biscuits. As it is gluten-free, so it can't be used to make yeasted loaves of bread. Although rice flour usually prepared at home, nowadays, rice flour is increasing using the ready to use. Natural amount of aflatoxins in wheat flour and rice has been published by other investigators [4,15,16,17,18,19].

Since, wheat flour and rice are the most widely consumed in Iranian population, contamination with AFB_1, even as low level, in long term can cause serious health problems for consumers. For this reason, we determined AFB_1 in wheat flour and rice flour samples consumed in Iran using photochemical online post-column derivatization technique and HPLC method.

2. Material and Methods

2.1. Sampling

A total of 40 samples were randomly collected from marketed in Iran including 16 wheat flour samples from 8 different brands and 24 rice flour samples from 12different brands. All samples were preserved in their original packaging at -20°C and analyzed before the expiration date.

2.2. Materials

The AFB_1 standard was obtained by Sigma-Aldrich (Munich, Germany) after dissolving in methanol as a stock solution (1000 ppb) and preparation of working standard from the intermediate stock (20 ppb). Methanol and acetonitrile (both HPLC grade) were purchased from Merck (Darmstardt, Germany). Sodium chloride, potassium chloride, potassium dihydrogen phosphate and anhydrous disodium hydrogen phosphate were purchased from Merck in analytical grade (Darmstardt, Germany) for preparation phosphate buffer (PBS) according to Brera et al. , 2003 [15]. Immunoaffinity columns (IAC) Afla Test WB columns were purchased from Vicam (Watertown, MA, USA).

2.3. Apparatus

Detection and quantification were performed using an HPLC system (KNAUER, Berlin, Germany) equipped with fluorescence detector (KNAUER, Berlin, Germany), solvent degasser (KNAUER**,** Berlin, Germany), Smartline Pump 1000 (KNAUER, Berlin, Germany**)** and On-line photochemical derivatization (UVETM LCTech GmbH, Dorfen, Germany) with a 254 nm low-pressure mercury lamp and a 1 ml knitted reaction coil, fitted around the UV lamp. The wavelengths setting used were at 365 and 435 nm as excitation and emission wavelength, respectively, in AFB_1 determination.

2.4. Extraction Methods and Preparation

AFB_1 was quantified according to the method of AOAC Official Method (NO.999.07) with minor modification. Briefly, 5g of sample was mixed with 0.5 g of sodium chloride and 25 ml solvent which composed of acetonitrile/water (80:20, v/v). The shaking of solution (5 min) was done by high-speed blender (Waring blender, USA) and 15 ml of clear solution was filtrated by Whatman No. 4 filter paper and then diluted with water to 150 ml. Then, 50 ml of diluted solution was passed through the IAC column that was conditioned with 10 ml PBS (Flow rate=1-2 drops/second). The column was washed with 10 ml ultrapure water. Aflatoxin was eluted slowly with 2 ml methanol and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with a 1 cc mobile phase and injected into the HPLC system (Figure 1).

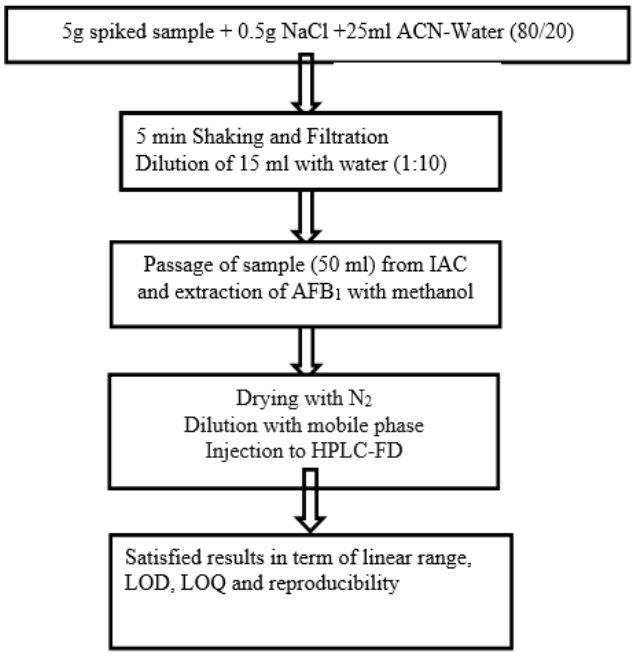

Figure 1. The schematic procedure of AFB_1 extraction from rice and wheat flour samples

2.5. HPLC Analyses and Chromatographic Conditions

The separation was performed on a C18 RP-HPLC column (250 x 4.6 mm i.d, particle size 5µm; KNAUER, Germany) using an HPLC system equipped with fluorescence detector and a post-column photochemical derivatization reactor. The column temperature was set at 25°C. The mobile phase was composed of water, methanol and acetonitrile mixture (53:28:19, v/v/v) that was filtered through a 0.45 mm membrane and degassed by sonication process before use with flow rate 1 ml/min. The fluorescence detector was operated at excitation wavelength of 365 nm and emission wavelength of 435 nm and maxim emission wavelength of the photochemical derivatization reactor was 254 nm. The injection volume

for both and standards and samples were 100 µl. The run time and the retention times were 10 min and 6.9±0.2 min, respectively.

3. Results and Discussion

3.1. Performance Characteristics of the Proposed Method

Based on literature review, HPLC method with IAC is the best method for determination of AFB_1 in wheat and rice flour samples [16]. On-line photochemical derivatization technique caused to obtained new derivative structures B_2a and G_2a with stable and higher fluorescence signal of AFB_1 and AFG_1 aflatoxin, because of their reaction with hydroxyl radical by ultraviolet radiation [8]. Recent studies suggested that this equipment exhibits simplicity, linearity of response, reproducibility, no need of chemical reagents, and additional pumps or electrochemical cells [8,20]. Therefore, we investigated the effect of on/off–line photochemical derivatization in height peak and severe response of detector. The HPLC chromatograms of AFB_1 in spiked samples (2µg.kg^{-1}) in two different form of photochemical derivatization (on-line and off-line) have been shown in Figure 2.

For achieving of optimized performance characteristics of chromatographic conditions the precision, accuracy, calibration data and linearity of calibration curve, LOD, LOQ, selectivity and real sample analysis were evaluated in present investigation. Linear calibration curve was constructed using six AFB_1 standards at the range of 0.05-8 µg.kg^{-1} with a correlation coefficient (R^2) greater than 0.9998.

The LODs and LOQ based on the signal-to-noise ratio of 3 and 10 were 0.015 and 0.05 µg.kg^{-1}, respectively, for both wheat flour and rice samples. The recovery experiments were performed by spiking the wheat and rice blank sample at four levels (0.2, 2, 4 and 8 µg.kg^{-1}) with three replicates for each level.

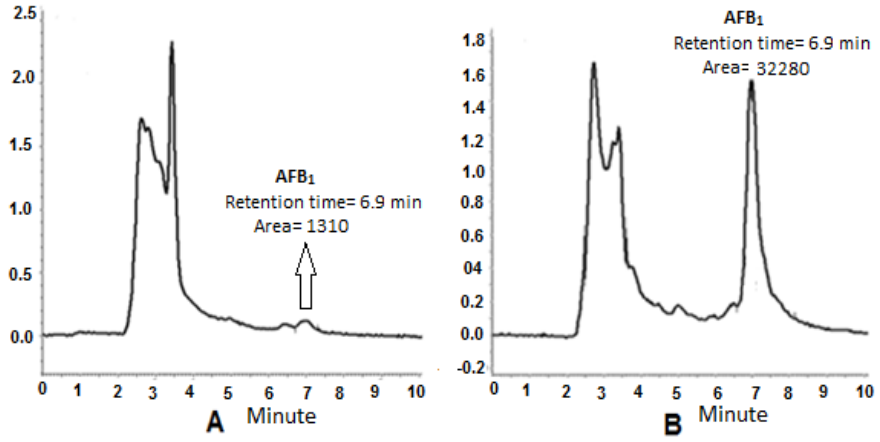

Figure 2. HPLC chromatograms of AFB_1 in spiked samples (1µg/kg): A: off-line photochemical derivatization; B: on-line photochemical derivatization.

The results of recovery experiments were between 92-106 % for wheat spiked samples and 97-109% for rice spiked samples. The RSD of the recoveries value ranged less than 5% and less than 3% for wheat flour spiked samples and rice flour spiked samples, respectively. The HPLC chromatograms of AFB_1 spiked samples (2 µg.kg^{-1}) for our suggested method have been shown in Figure 3. The results of validation methods for AFB_1 contamination in wheat and rice flour are shown in Table 1.

Table 1. Precision and accuracy for determination of aflatoxin B_1 in wheat and rice flour (n=3)

Sample	Spiked level (µg.kg^{-1})	Intra- day Recovery (%)		Inter- day Recovery (%)	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Wheat flour	0.2	91.96±3.70	4.02	90.83±3.06	3.37
	2	97.49 ±2.04	2.09	97.49±2.11	2.16
	4	102.08±3.63	3.56	99.75±3.35	3.36
	8	105.15±1.72	1.64	105.91±2.56	2.41
Rice flour	0.2	97.50±1.50	1.54	97.83±1.30	1.33
	2	99.82±1.15	1.15	104.58±3.11	2.97
	4	103.75±12.3	1.19	108.75±1.28	1.17
	8	108.15±0.79	0.73	109.49±1.94	1.77

3.2. Application of Method to the Real Samples

Mycotoxins especially Aflatoxins attract worldwide attention due to their impact on human health, animal productivity and trade. These secondary metabolites caused contamination of agricultural crops in worldwide in the field prior to harvest or during inappropriate storage conditions, under moisture and insect infestation [21]. It has been detected in various commodities especially cereals such as wheat and rice and products derived from them [19,22,23].

The results of the AFB_1 analysis for wheat flour and rice flour samples are shown in Table 2. According to the results, AFB_1 was detected in 3 out of 16 wheat flour samples with maximum level of 0.26 µg.kg^{-1} which is lower than the maximum allowed according to EC (2 µg.kg^{-1}) and Institude of Standards and Industrial Research of Iran (ISIRI) (5 µg.kg^{-1}). Our results were in agreement with the following studies, Yazdanpanah et al. (2013) reported that none of the 18 wheat flour samples contained detectable amounts of AFB_1 [16]. The results of Ghasemi-Kebria et al., 2013 in wheat flour of Golestan province in Iran showed the mean AFB_1 contamination in 100 samples was 0.53 µg.kg^{-1} [24]. The obtained results

are in contrast with Buyukunal et al (2007) and Abdullah *et al*. (1998). Buyukunal et al (2007) analyzed 100 wheat flour samples in Turkey and found 57% of the samples were contaminated to AFB_1 and in 5% of the samples level AFB_1 was exceeding the maximum limits defined by the Turkish [25]. Abdullah *et al*. (1998) reported that 1.2% of the 83 analyzed wheat flour samples in Malaysia were positive to AFB_1 at a concentration of 25.6 $\mu g.kg^{-1}$ [26]. The results of studies in Iran revealed that the rate of AFB_1 in wheat flour is not comparatively critical point, regarding to the quality of wheat flour.

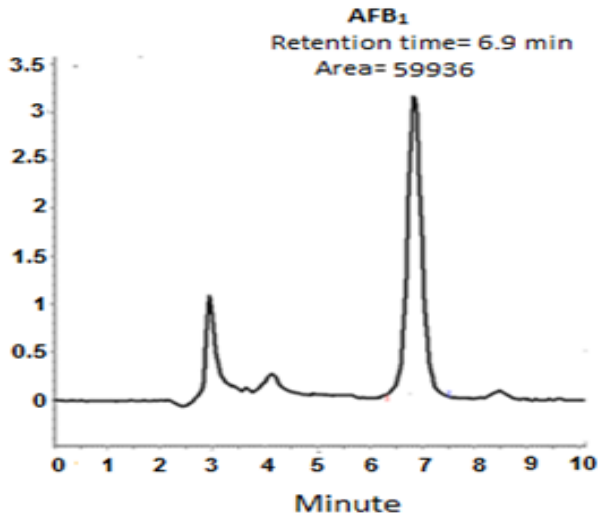

Figure 3. HPLC chromatograms of AFB_1 spiked samples (2 $\mu g.kg^{-1}$)

Regarding the rice flour samples, our results showed that all of the samples were contaminated to AFB_1 at the range of 0.46-10.16 $\mu g.kg^{-1}$. The level of AFB_1 in 33.3% of samples was higher than the maximum allowable level according to EC and ISIRI (5 $\mu g.kg^{-1}$) [27]. To the best of our knowledge, there is no study on AFs contamination in ready to use rice flour, so we compared our results with the studies which evaluated AFB_1 in rice samples. High frequency of AFB_1 was reported by some investigators in Iran [16,28,29].

The frequency of AFs in rice has been previously reported in Iran. According to Mohammadi et al. (2012), 75% of 152 imported rice samples were contaminated with AFB_1 with the mean of 0.46 $\mu g.kg^{-1}$ [30]. In another study, Yazdanpanah et al. (2013) reported that AFB_1 was detected in 50% of 18 rice samples with the mean of 4.2 $\mu g.kg^{-1}$ for positive samples. The contamination level of one sample was found higher than 5 $\mu g.kg^{-1}$ [16]. According to Nazari et al 2014, 21.5% of rice samples were positive for AFB_1 with a mean level of and 3.9 $\mu g.kg^{-1}$. Three rice samples exceeded the ($\mu g.kg^{-1}$) in the range of 5.8–30.8 $\mu g.kg^{-1}$ [31]. Feizy et al.2010 reported that 69% of the samples contained detectable amounts of AFB_1 is higher than 0.2 $\mu g.kg^{-1}$ [32]. The data of Joshaghani et al., 2013 research showed AFB_1 level in all of samples (35 samples) were lower than Iranian standard committee [33]. These studies were in agreement with our result confirming that the occurrence of AFB_1in most of the samples is high. The differences between reports might be resulting of various conditions during harvesting, transport, granulation and storage [34,35].

Table 2. Aflatoxin B_1 occurrence in rice and wheat flour samples collected in Iran

Flour type	No. of samples	Positive (%)	Mean± SD ($\mu g.kg^{-1}$)	Range($\mu g.\ kg^{-1}$)	No. of samples > 2 $\mu g.kg^{-1}$ [a]	No. of samples > 5 $\mu g.kg^{-1}$ [b]
Rice	24	24(100)	4.09±2.56	0.46-10.16	18	8
Wheat	16	3(18.75)	0.19±0.08	0.1-0.26	0	0
Total	40	27(67.5)	4.02±2.71	0.1-10.16	18	8

a: 2µg/kg: maximum established level of EU regulations for cereals
b: 5 µg/kg: maximum established level of Iranian standard organization regulations for cereals flour.

4. Conclusion

Wheat and rice flour can be used as a base compound in foodstuff. On the other hand, there is no secondary process which decreases of AFB_1 level in these products. Therefore, determination of AFB_1 level in wheat and rice flour is an important concern for the public health. In this study, an accurate, simplicity, sensitive, reproducibility and modified analytical method are reported for the determination of AFB_1 in wheat and rice flour. This study involved optimization of HPLC-FD and on-line photochemical derivatization with satisfied results in term of linear range, LOD, LOQ and reproducibility using the spiked samples. The priority of our suggested method is related to lower solvent and sample usage in extraction stage, analysis time, better recovery, suitable accuracy and precision which caused to have a reliable method in determination of AFB_1 in wheat flour and rice flour. Despite applying the proposed method, low values of AFB_1 was found in wheat flour samples and seems this value of AFB_1 in wheat flour is not comparatively critical point regarding quality. On the other hand, the results showed that the high occurrence and high contamination level of AFB_1 in most of the rice flour samples.

It should be regarded that contaminations in rice flour, which is one of the most popular and consuming foodstuffs in the daily diet of Iranian people, has a potential hazard for the community health. It revealed that Implementation of food security and quality as well as standard agricultural and hygiene applications in harvest, storage and transport can provide reduction or abolishment of the community health hazards.

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