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Research Article

Analysis of Aflatoxin Types in Red Pepper Flakes Samples by HPLC

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Abstract

Aflatoxins produced by Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius and Penicillium and Rhizopus molds are a mycotoxin derivative and are harmful to human health. In this study, the aflatoxin B1, B2, G1 and G2 types sold in Kayseri street market were determined in red flakes pepper samples by high performance liquid chromatography (HPLC). Firstly, the aflatoxin types were extracted by shaking technique. A mixture of methanol and water (80:20 v/v), at the 30 ºC temperature. The chromatographic separation was conducted on ODS-2 with Fluorescence Detector, ex: 360 nm, em: 440 nm at 30 ºC for 30 min. For the determination of aflatoxin B1, B2, G1 and G2, standard solutions of analytes at known concentration were prepared according to the official method AOAC 999.07. The calibration line was prepared according to the standards and the results were given according to the calibration line. The detection limit was determined as 0.216 µg L^{-1} for B1 and G1 and 0.0648 µg L^{-1} for B2 and G2. While the highest concentration was analyzed for AFB1 as 7.32, for AFB2 as 2.39 and for AFG1 as 1.23 µg L^{-1}, it was observed that the concentration for AFG2 remained below the quantification limit in samples. The quantification limits were 0.720 µg L^{-1} for aflatoxins B1 and G1, and 0.216 µg L^{-1} for B2 and G2. Recovery studies are in the range of 90-104%. The %RSD was calculated as ≤ 5% (n=11).

Keywords: Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Red Pepper Flakes, HPLC

Kırmızı Pul Biber Örneklerinde HPLC İle Aflatoksin Türlerinin Analizi

Öz

Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius ve Penicillium ve Rhizopus küflerinin ürettiği aflatoksinler bir mikotoksin türevi olup insan sağlığına zararlıdır. Bu çalışmada, Kayseri semt pazarında satılan kırmızı pul biber örneklerinde aflatoksin B1, B2, G1 ve G2 türleri yüksek performanslı sıvı kromatografisi (HPLC) ile belirlenmiştir. Öncelikle aflatoksin türleri 30 ºC sıcaklıkta metanol ve su karışımında (80:20 v/v) çalkalama tekniği ile ekstrakte edilmiştir. Kromatografik ayırma, Floresan Dedektörü ile ODS-2 kolonu üzerinde, ex: 360 nm, em: 440 nm, 30 ºC'de 30 dakika boyunca gerçekleştirildi. Aflatoksin B1, B2, G1 ve G2'nin tayini için, bilinen konsantrasyondaki analitlerin standart çözeltileri, resmi yöntem AOAC 999.07'ye göre hazırlandı. Kalibrasyon doğrusu standartlara göre hazırlanmış ve çalışma aralığına göre sonuçlar verilmiştir. Gözlenebilme sınırı B1 ve G1 için 0,216 µg L^{-1} ve B2 ve G2 için 0,0648 µg L^{-1} olarak belirlendi. En yüksek konsantrasyon AFB1 için 7,32, AFB2 için 2,39 ve AFG1 için 1,23 µg L^{-1} olarak analiz edilirken, örneklerde AFG2 konsantrasyonunun gözlenebilme sınırının altında kaldığı belirlendi. Tayin sınırları, aflatoksin B1 ve G1 için 0.720 µg L^{-1} ve B2 ve G2 için 0.216 µg L^{-1} idi. Geri kazanım çalışmaları %90-104 aralığındadır. Çalışmanın bağıl standart sapması %RSD ≤ %5 (n=11) olarak hesaplandı.

Anahtar Kelimeler: Aflatoksin B1 (AFB1), Aflatoksin B2 (AFB2), Aflatoksin G1 (AFG1), Aflatoksin G2 (AFG2), Kırmızı Pul Biber, HPLC

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1. Introduction

It is possible for the food to be contaminated in the stages starting from the harvest period until it reaches our table. This risk is less in fruits and vegetables consumed fresh. Accumulation of aflatoxins in foods can be caused by environmental factors such as humidity, temperature and insects, on the other hand, aflatoxin accumulation may occur during plant growing, harvesting, drying, processing and storage processes. Aflatoxins produced by Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius and some Penicillium and Rhizopus species are a mycotoxin derivative. They are metabolites with toxic properties in humans and animals, can have acute hepatoxin effective, immunosuppressive and carcinogenic effects. In addition, aflatoxins can cause developmental disorders and hereditary disorders (Moss et al., 1992).

Aflatoxins are mostly found in spices, legumes, shelled and unshelled nuts, oil seeds, dried vegetables and fruits. In addition to these products, aflatoxins can be found in meat, meat products, milk and dairy products (Goldblatt, 1969; Heperkan, 2014). In order to preserve the nutritional quality of foods for a long time, it is necessary to limit enzymatic and microbiological changes. This long time that lasts in a short time in determining the period; in the cold such as preservation, freezing, drying, preservative addition various maintenance trainings and cause deterioration Maintaining quality by minimizing the speed of reactions (Topdaş et al., 2011). Drying technique is one of the methods used to prevent enzymatic and microbiological spoilage of foods. Drying technique is important in the formation of aflatoxin. Some producers carry out drying in the field without harvesting the crop. This situation (Roberts & Patterson 1976; Romer 1973) may cause soil-borne fungal contamination and aflatoxin formation. In red pepper production, dried unopened fungal colonization of peppers a suitable environment for aflatoxin formation (Demircioğlu & Filazi 2010). Techniques such as natural convection drying, microwave and hot air drying methods are used in the drying process (Yıldız & İzli 2020). However, this technique may not be sufficient in terms of food safety. Analysis of aflatoxins is important in terms of food safety of many foods such as nuts, legumes, spices and dried fruits and vegetables that we consume dry. Aflatoxins are generally divided into six main compounds, namely B1, B2, G1, G2, M1 and M2. The dihydro derivative of aflatoxin B1 is B2, and the dihydro derivative of aflatoxin G1 is G2. Apart from these four aflatoxins, aflatoxins M1 and M2 were isolated from the milk and urine of lactating mammals fed with aflatoxin feed. Plant-based foods contain the most aflatoxin B1, B2, G1 and G2 types (CAST 2003; Oner et al., 2022; Sevdin et al., 2021). It has been determined that aflatoxin M1 and M2 species are found in directly consumed animal products such as meat, milk and eggs (Desjardins et al., 2003; Magan & Olsen 2004). Aflatoxin-containing products are of great importance not only in terms of health but also in economic terms. Turkey is one of the important countries exporting nuts, legumes, spices, fruits and vegetables. The amount of aflatoxin in these products should not exceed the desired limits. Otherwise, the rejection of the products causes economic damage. The Rapid Alert System for Food and Feed (RASFF 2022), which belongs to the member states of the European Union community, sends notifications to the commission in case any risk that threatens human and animal health is detected. RASFF has given our country a limit refund for aflatoxin in 2018, with 14.56 μg kg^{-1} for aflatoxin B1 type and 21.44 μg kg^{-1} for total aflatoxin in peanut kernels, as it exceeded the upper limit. The upper limit accepted by RASFF is 5 μg kg^{-1} for aflatoxin B1 type (RASFF 2022; FAO 1987a; FAO 1987b). Routine analysis of aflatoxin derivatives in foods is very important for both human health and national economy. Aflatoxins are present in trace amounts in real samples. Therefore, many analysis methods have been developed to obtain results with high sensitivity and precision. In the determination of aflatoxins, thin layer chromatography (Turner et al., 2009), gas chromatography (Trucksess 1984), liquid chromatography (Zhao et al., 2017), high performance liquid chromatography (Hamed et al., 2017), enzyme bound many analysis methods such as immunosorbent analysis (Li et al., 2016) and biosensor (Pagkali et al., 2018) have been used.

In this study, the extraction of aflatoxin types B1, B2, G1 and G2 in red pepper flakes products sold in Kayseri street markets was done by shaking technique. Then, the amount of aflatoxin species was determined by HPLC device. The AOAC Official Method 999.07 was used for aflatoxin analysis (AOAC 2007). Three parameters were studied from each sample.

2. Material and Method

2.1. Chemical Materials and Standard Solutions

All chemicals used are of analytical grade and supplied by Merck, Darmstadt, Germany. The aflatoxin standards used were obtained from Sigma Aldrich, Steinheim, Germany, in analytical purity suitable for HPLC equipment. The solutions in the experiment were prepared with deionized water.

2.1.1. Instruments and General Procedure

Samples were taken from 20 different Kayseri street markets, they are homestead production. All samples were preserved in their original packaging at 4°C until analysis date. The samples were pulverized with a hand blender (Arzum Ar1025 Smart Max hand blender) and homogenization of the samples was achieved. 50 g of the red pepper flakes sample was taken into 500 mL flask. 5 g of NaCl and 200 mL of methanol/water (8+2, v/v) mixture were added on samples and were added to it and placed in a shaker at room temperature (n=3), shaken at 100 rpm for 30 minutes. Samples were filtered through Whatman filter paper blue ribbon. It was taken from the extraction solution by autosampler and injected into the high performance liquid chromatography column. Agilent 1260 series HPLC device was used for the determination of aflatoxin types. The fluorescence detector was operated at excitation wavelength of 360 nm and emission wavelength of 420 nm. The run time and the retention times were 30 min. Table 1 shows the chromatographic conditions.

Table 1. The chromatographic conditions

Instrument	Agilent 1260 series HPLC
Mobile Phase	Water: acetonitrile: methanol (600 mL + 200 mL + 200 mL) 120 (mg/L mobile Phase) KBr + 100 (µL/L mobil faz) %65 HNO_3
Detector	Floresans Dedector, ex: 360 nm, em: 440 nm
Column	ODS-2 (4.6 mm x 25 cm x 5µm)
Flow rate	1 mL min^{-1}
Derivatization	with bromine produced by post-column electrochemical cell
Column Temperature	30 ^{O}C
Injection Volume	100 µL

Table 2. The concentration of standard solutions of aflatoxin B1, B2, G1 and G2

Injection	Quantities obtained for injections of 100 µL (µg L^{-1}) Official method AOAC 999.07			
	B1	B2	G1	G2
1. Standard	0.720	0.216	0.720	0.216
2. Standard	2.160	0.648	2.160	0.648
3. Standard	3.600	1.080	3.600	1.080
4. Standard	5.040	1.512	5.040	1.512
5. Standard	6.480	1.944	6.480	1.944

2.1.2. Experimental

For the determination of aflatoxin B1, B2, G1 and G2, standard solutions of analytes at known concentration were prepared according to the official method AOAC 999.07 (Table 2). The calibration line was prepared according to the standards and the results were given according to the calibration line. The detection limits (LOD) of device were determined as 0.216 µg L^{-1} for B1 and G1 and 0.0648 µg L^{-1} for B2 and G2. In Figure 1 shown The chromatogram from a standard mixture of aflatoxin B1, B2, G1 and G2 respectively.

Standard solutions of known concentrations were given to the HPLC device. The retention times of aflatoxin B1, B2, G1 and G2 were determined. Concentrations of analytes in real samples were calculated according to these retention times. The retention times were determined as 22.108 for aflatoxin B1, 18.154 for B2, 14,464 for G1, and 12,733 min for G2, respectively.

2.1.3. Calculation of accuracy of the method

Additional samples studies were carried out to determine the accuracy of the applied method. In order to calculate the accuracy of the method, the model samples were prepared at the known concentrations of aflatoxins. Samples were analyzed by the applied method. Recovery studies were carried out by adding sample 1 and sample 3. Aflatoxins were determined by HPLC. It was found that recovery is in the range of 85 - 93%. The working ranges were set in 0.720-6.480 µg L^{-1} for aflatoxin B1 and G1 and in 0.216-1.944 µg L^{-1} for aflatoxin B2 nad G2. The detection limits (DL) of device were determined as 0.216 µg L^{-1} for B1 and G1 and 0.0648 µg L^{-1} for B2 and G2. The results of the additional samples studies are shown in Table 3.

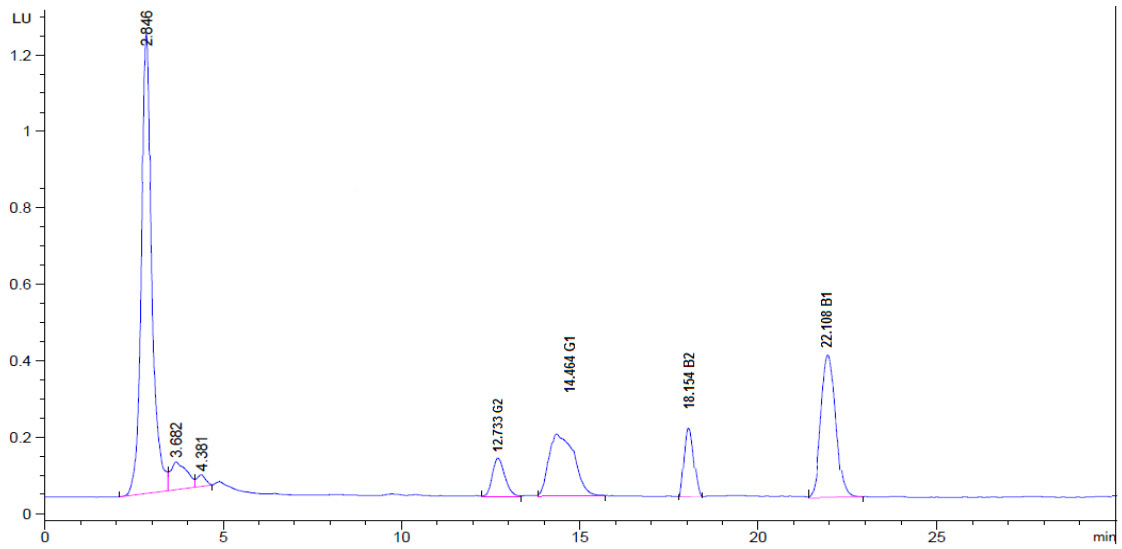

Figure 1. The chromatogram from a standard mixture of aflatoxin B1, B2, G1 and G2 respectively.

Table 3. The additional samples studies (n=3)

Red pepper flakes samples (µg L^{-1})	B1	B2	G1	G2
Samples 1	1.2416 ± 0.2[a]	0.2649 ± 0.03	-[b]	-
Added	1	1	1	1
Found	2.2238 ± 0.3	1.2740 ± 0.1	0.93 ± 0.1	1.02 ± 0.2
Recovery %	99	101	93	102
Samples 3	2.1410 ± 0.2	1.3908 ± 0.1	-	-
Added	1	1	1	1
Found	3.2842 ± 0.4	2.2716 ± 0.1	0.90 ± 0.1	98 ± 0.2
Recovery %	104	95	90	98

[a] Average ± standard deviation; [b] Below detection limit.

3. Results and Discussion

The official method AOAC 999.07 was applied to red flakes pepper samples purchased from 20 different Kayseri street market. As shown in Table 4, while the minimum value of the aflatoxin B1 amount was found as 1.242 µg kg^{-1} in the sample 1 and the maximum value of its was found as 7.320 µg kg^{-1} in the sample 5. The amount of aflatoxin B2 was calculated in the range 0.265 - 0.321 µg kg^{-1} in the samples. In addition that, the amount of aflatoxin G1 1.228 µg kg^{-1} in sample 2, 1.124 µg kg^{-1} in sample 3, 0.980 µg kg^{-1} in sample 4, 1.937 µg kg^{-1} in sample 8. As in Table 4, the highest G1 concentration is seen in sample 8. The aflatoxin G2 was not observed in any of the samples. No aflatoxin residue was found in 12 red flakes pepper samples.

Demircioğlu and Filazi (Demircioğlu & Filazi 2010), aflatoxin analysis was performed on pepper varieties grown in various regions of Turkey. Aflatoxin analyzes in red peppers were measured semi-quantitatively by Thin Layer Chromatography. Demircioğlu and Filazi found no mycotoxin residues in 7 types of red pepper, fine pepper, isot and chili pepper, while silk pepper, leaf pepper, sweet and hot ground pepper were not found. Approximately 28.6%, 57%, 28.6% and 57% of aflatoxin residues were found, respectively. They found the amount of aflatoxin B1 in leaf pepper as 3.5, B2 as 12.5 and G1 as 8 ppb. Kanbur et al. (Kandur et al., 2006), presented to consumption in Kayseri. They determined that AF B1 was detected in the range of 1.48-70.05 ppb in all red pepper samples by ELISA method, and only 3 of them were not in compliance with the Turkish Food Codex Regulation. Kanbur et al. results were lower than the results obtained from this study. Ardıc et al. (Ardıc et al. 2008) analyzed aflatoxin and aflatoxin B1 by ELISA method on 75 red pepper powder samples sold in the city of Urfa. Aflatoxin B1 was found in the range of 0.11–24.7 ppb in the samples. They determined that it was above the legal limit used in the European Union and

Table 4. Amounts of aflatoxin types (μg kg^{-1}) in the real samples taken from Kayseri street markets (n = 3).

Red pepper flakes samples, (μg kg^{-1})	B1	B2	G1	G2	Total (μg kg^{-1})
1	1.2416 ± 0.2[a]	0.2649 ± 0.03	-[b]	-	1.5065
2	4.2564 ± 0.6	2.1804 ± 0.2	1.2284 ± 0.01	-	7.6652
3	2.1410 ± 0.2	1.3908 ± 0.1	-	-	3.5318
4	5.2064 ± 0.4	2.3903 ± 0.1	1.1242 ± 0.01	-	8.7209
5	7.3200 ± 0.5	2.1898 ± 0.3	0.9803 ± 0.02	-	10.4901
6	-	-	-	-	
7	-	-	-	-	
8	-	-	-	-	
9	0.820 ± 0.02	-	-	-	0.820
10	3.0508 ± 0.1	0.3212 ± 0.01	-	-	3.372

Turkey in 11 samples. Aydın (Aydın et al., 2007) and his colleagues carried out Aflatoxin B1 analysis by ELISA method on 100 red pepper samples collected from markets in Istanbul. In 18 samples, acceptable contamination levels were determined to be above the maximum tolerable limit (5 ppb) according to the Turkish Food Codex and the European Commission.

4. Conclusions and Recommendations

In this study, aflatoxin analysis was performed and the results were evaluated, considering the importance of the contamination values of red flakes pepper, one of the most popular spices in the daily diet of Turkish people, in terms of public health, food safety and quality. While the highest concentration was analyzed for AFB1 as 7.320, for AFB2 as 2.390 and for AFG1 as 1.937 μg L^{-1}, it was observed that the concentration for AFG2 remained below the quantification limit in samples. The quantification limits were 0.720 μg L^{-1} for aflatoxins B1 and G1, and 0.216 μg L^{-1} for B2 and G2. Accordingly, we see that the amount of aflatoxin B1 in samples 4 and 5 exceeds the limit specified in the Turkish Food Codex Regulation. It is below the maximum limit in other examples. When we look at the total amount of aflatoxin, we see that only exceeds the maximum limit value in sample 5.

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