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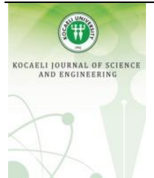

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Investigation of Mycotoxins In Packed Gluten Free Foods

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Abstract

The number of people with celiac disease and gluten intolerance is increasing day by day. This increases the variety, number and consumption of gluten-free products. However, this study was the first in our country to determine whether the packaged gluten-free products contain mycotoxins. Aflatoxin (AF), ochratoxin (OTA), zearalenone (ZON), and deoxynivalenol (DON) were analyzed in 95 imported and domestic packaged gluten-free foods of different types and brands. Analyses were made with HPLC with fluorescent detector and HPLC with diode array detector. In addition, validation studies were completed with the addition of blank samples. OTA was detected in 9 food samples. In the OTA analysis, toxins were detected in 1 buckwheat flour, 3 pasta, 3 crackers or biscuits, 2 corn flours, but these values are below the values determined by the Turkish Food Codex (TFC). DON was found to be suitable for TFC in 7 types of pasta, 1 type of bread, and 3 types of crackers or biscuits.

1. Introduction

Mycotoxins are toxic metabolic products produced by some types of molds. These mycotoxins are low molecular- weight natural toxins with a wide range of chemical structures [1,2]. Today, it is known that more than 350 mold species produce mycotoxins. Molds that produce mycotoxins can be found everywhere and are carried by wind and air currents. Many kinds of molds need certain conditions for growth, development, and mycotoxin production. These conditions; can be summarized as humidity, temperature, substrate type, nutritional factors, oxygen and carbon dioxide levels in the atmosphere, the presence of other mold species, geographical location, and genetic conditions [3,4]. The most important molds that produce mycotoxins are Aspergillus, Penicillium, Fusarium, Alternaria species [1]. These molds produce mycotoxins, that affect humans and animals seriously, such as aflatoxin, ochratoxin A, deoxynivalenol, zearalenone, and fumonisin.

The year 1960 was a turning point in understanding mycotoxins. Until that time, the molding of agricultural products was only an economic problem, and it was the center of attention due to the diseases it caused in living creatures after 1960. The cause of the disease, which resulted in the death of many poultry in England in 1960, was found to be the toxin in Brazilian peanuts used in animal feed. Since aflatoxin was first detected in Aspergillus flavus on this date, the first letters of these molds were used, and this mycotoxin was called aflatoxin [5, 6].

Molds can grow on countless food items, such as cereals, dried fruits, nuts, and spices, that can produce mycotoxins. Diseases caused by mycotoxins are called "mycotoxicoses" [7]. When mycotoxins are ingested, inhaled, or absorbed through the skin, they can cause many diseases or even death in human beings [8].

People with celiac disease and gluten intolerance have to consume gluten-free foods for their health. Especially celiac patients should definitely consume gluten-free products. Gluten is a high-molecular-weight, the main storage protein in cereals. It is a composite protein composed of glutenin and prolamines [9, 10]. Gluten is the substance that gives dough resilience and flexibility, which is why it is a common component of

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many food products.

The gluten-free product diet is characterized by more corn, rice, and other gluten-free grain consumption [11, 12]. In the past decade, some reports have focused on corn-based products, with some cases showing severe mycotoxin contamination. More recently, specific research on mycotoxin contamination of gluten-free diet products has been published with conflicting results [13–15].

Foods that mainly contribute to mycotoxin intake through a gluten-free diet are cereals such as rice, corn, buckwheat, etc., and leguminosae such as quinoa, soybean [11,12]. The increase in corn-based product consumption is the riskiest commodity due to the potential coexistence of more than one mycotoxin. This may be of particular concern for the vulnerable population group, such as celiac patients. To summarize briefly, mycotoxins are toxic compounds produced by certain fungal species that can contaminate food crops. Gluten-free foods, typically made from alternative grains such as rice, corn, and quinoa, can also be contaminated with mycotoxins. There are several types of research found in literature based on analyzed the mycotoxins in gluten-free foods [13-18]. Corn-based gluten-free products are a major essential ingredient for celiac patients. These products contain high levels of corn mold toxin. This poses a problem for those trying to improve their health after years of gluten-induced damage. A study on the presence of different mycotoxins in gluten-free products collected from the Italian market showed that FB and ZON were present in all product categories considered except pasta. Specifically, 29% of analyzed samples had FBs and 11% ZON, while few samples had low DON levels [18].

Positive values were obtained for DON in corn-based gluten-free products collected from the markets in Valencia [16]. However, urine biomarkers of mycotoxin exposure were monitored in the urine analyses of a group of celiac patients and individuals from the healthy control group with deoxynivalenol (DON), zearalenone (ZON), and fumonisin B1 (FB B1) [17]. Brera et al. (2014) detected the zearalenone contamination of gluten-free products. It has been evaluated whether those celiac patients were exposed to fumonisins (FB) and zearalenone (ZON) [15]. In the literature, the presence of mycotoxins in packaged gluten-free foods produced in Turkey was absent. It is estimated that between 250 and 750 thousand people in Turkey have celiac disease [19]. There is a group of people without celiac disease who also consume gluten-free foods because they find them healthier. Of course, they all carry the risk of consuming products containing mycotoxins. In this context, it is of great importance to investigate and report the presence of mycotoxin in gluten-free packaged foods. In this study, the presence of aflatoxin (AF), ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZON) was determined in 95 different products, and verification studies have been carried out. These products have been purchased from 2019 until 2021: pasta (16 packages), noodles (6 packages), corn flakes (6 packages), tarhana (4 packages), rice flour (5 packages), cornflour (8 packages), biscuit and cracker varieties (20 packages), bread (7 packages), cake mix (6 packages), buckwheat flour (3 packages), white mulberry flour (3 packages), carob flour (3 packages), chickpea flour (2 packages), chestnut flour (3 packages), and coconut flour (3 packages), which were collected from the Turkish hypermarkets and internet stores. Bought with the expiration date in mind.

2. Materials and Methods

2.1. Chemicals

The chemicals and reagents used during the studies were of analytical reagent grade and suitable for HPLC devices. The deionized water was obtained from the Millipore Q purification system (Millipore, Merck, Darmstadt, Germany). Methanol (CH_3OH) and acetonitrile (C_2H_3N) (both HPLC grade) were purchased from Merck (Darmstadt, Germany). Nitric acid (HNO_3), sodium chloride (NaCl), potassium bromide (KBr), acetic acid (CH_3COOH), disodium phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) potassium chloride (KCl) were all analytical reagent bought from Merck (Darmstadt, Germany). Aflatoxin mix standards were obtained from Supelco, Ochratoxin, Zearalenone, and Deoxynivalenol Standards were obtained from Trilogy Analytical Laboratory (Washington, MO, USA). The immunoaffinity columns (IACs), AFTest® (product code: 12022), OchraTest™ (product code:13012), Deoksinivalenol TEST-P, Zeralenon TEST-P were from R Biopharm Rhöne Ltd (Darmstadt, Germany). GF/A glass microfiber filters (125 mm) were from Sartorius (Göttingen, Germany).

2.2. Instruments

In weighing, analytical balances (Sartorius CP 3202S, Goettingen, Germany) were used for all experiments. Blender (Waring, USA) and grinder (Yazıcılar G2, Turkey) were used to grind and homogenize samples. Mobile phase degassed in an ultrasonic cleaner (Intersonic, Istanbul, Turkey)

Chromatographic analyses for ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZON) were carried out using an Agilent 1260 Infinity LC (Agilent Technologies, CA, USA). This HPLC is equipped with a binary pump, an online degasser, an autosampler, two

detectors (DAD and FLD), and a column thermostat. An Agilent HPLC 1100 (Agilent Technologies, CA, USA) device, which was equipped with an FLD detector (Agilent, Santa Clara, CA, USA), was used for aflatoxin analysis. A Kobra-cell-celld post-column derivative electrochemical cell was used for the complete separation of aflatoxins (B1, B2, G1, and G2) in the analytical column and to strengthen the fluorescence intensity of aflatoxin B1 and aflatoxin G1. The signals obtained as a result of the separations in the analytical column were calculated automatically by Chem Stations software (Agilent, Santa Clara, CA, USA). The data obtained during the analyses were stored on the hard disk of the computer. A centrifuge, Hettich 32A (Hettich Zentrifugen, Tuttlingen, Germany), a nitrogen generator (Parker Balston Nitro, NY, USA), and a vortex, DLAB MX-S (DLAB Scientific Inc., China), were also used during analysis.

2.3. Sample Preparation

95 different brands and types of gluten-free packaged food samples (such as biscuits, crackers, cakes, pasta, etc.) were purchased randomly from internet stores and health food departments of hypermarkets in Turkey. Some of those samples were imported from other countries. The samples were collected in Izmit (Turkey) during 2019. The duration of product collection is 2 years. During this time, the expiration dates of the products were monitored, and the products were kept in their original packaging at room temperature until they were analyzed. Blender and grinder were used to grind and homogenize samples. Homogenized samples were kept in appropriate conditions until analysis. In parallel with 95 samples and each sample, AF, OTA, DON, and ZON analyses were performed on products.

2.4. Homogenization

Mycotoxins have an irregular (not homogeneous) distribution in food and feed. Therefore, homogenization is very important in mycotoxin analysis. During this process, samples must be taken from different points to represent the lot. It is very important to homogenize the aggregate sample so that the analysis result from the laboratory is accurate and reproducible. Homogenization is a principle that reduces particle size and ensures a uniform distribution of contaminated particles in the crushed sample. For this, 10 analyses were made in parallel with the 8-numbered corn flour sample [20], and the graphic of homogenization of corn flour was obtained in Fig. 1. The blue and red colors in Fig. 1 show the results of parallel samples. They are not results before or after homogenization. And the products were homogenized and analyzed after that.

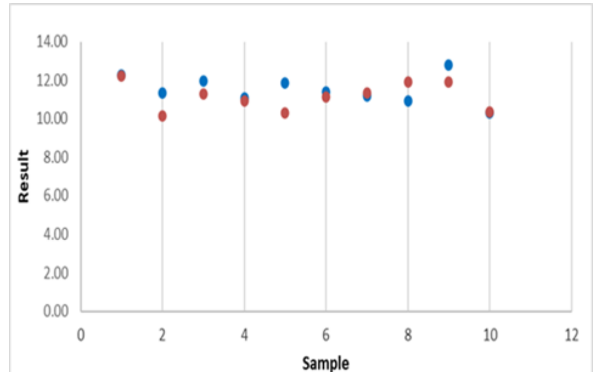

Figure 1. Homogenization of corn flour.

2.5. Analytical Methods for Mycotoxins

After all samples were homogenized by grinding, aflatoxin (AF), ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZON) analyses were performed. Aflatoxin and ochratoxin analyses were done according to AOAC Official Methods 991.31 [21] and 2000.03 [22], respectively. Zearalenone analysis was done as mentioned in the literature [23]. Deoxynivalenol analysis was done with DONPREP, which was supplied from R-Biopharm Rhöne Ltd., and deoxynivalenol analysis in cereal was done using water extraction. AF was extracted with CH_3OH:H_2O (70:30), DON with 200 mL of ultrapure water, ZON with CH_3OH:H_2O (80:20), and OTA with AcCN:H2O (60:40). The extracts were filtered through Whatman No. 4 filter paper. AF and ZON were filtered through microfiber filter paper once and passed through the IAK column. Reading was done on HPLC. LOD/LOQ, repeatability/reproducibility, recovery/accuracy, and linear measurement range (linearity) studies of all methods were performed with appropriate matrices. For this, a spike was applied to the blind sample. Measurements of aflatoxin, zeralenone, and ochratoxin were done with an HPLC/FLD device; HPLC/DAD was used for deoxynivalenol.

Aflatoxin analysis was carried out using an Agilent HPLC (1100) device equipped with an FLD detector. In the HPLC determination of aflatoxins B1, B2, G1, and G2, using fluorescence detection, excitation and emission wavelengths were set at 360 nm and 430 nm, respectively. Chromatographic separations were conducted on a column (ACE 5 C18–250 mm x 4.6 mm (Aberdeen, Scotland)), and the column temperature was 25 °C. The flow rate was 1.0 mL/min, and the injection volume was 100 µL. Isocratic mobile phase composition was 545 mL H_2O (ultrapure water), 182 mL AcCN, 273 mL CH_3OH, 120 mg KBr, and 350 µL 4M HNO_3. A post-column derivative electrochemical cell was used for the complete separation

of aflatoxins (B1, B2, G1, and G2) in the analytical column.

Ochratoxin analysis was carried out using an Agilent HPLC (1260) device. In the HPLC determination of ochratoxin by using fluorescence (FLD) detection, excitation and emission wavelengths were set at 330 nm and 460 nm, respectively. Chromatographic separations were conducted on a column (ACE 5 C18-250 mm x 4.6 mm / ODS-1 C18 5 µm 250 x 4.6 mm), and the column temperature was 40 °C. The flow rate was 1.0 mL/min, and the injection volume was 100 µL. The isocratic mobile phase composition was ultrapure water, AcCN-CH_3COOH (51:48:1) (v/v/v). Zearalenone analysis was carried out using an Agilent HPLC (1260) device. In the HPLC determination of ZON by using fluorescence (FLD) detection, excitation and emission wavelengths were set at 232 nm and 440 nm, respectively. Chromatographic separations were conducted on a column (ACE 5 C18-250 mm x 4.6 mm), and the column temperature was 40 °C. The flow rate was 1.0 mL/min, and the injection volume was 100 µL. The isocratic mobile phase composition was ultrapure water, AcCN (1/1 V/V).

Deoxynivalenol analysis was carried out using an Agilent HPLC (1260) device. In the HPLC determination of DON by using diode array (DAD) detection. The wavelength was set at 218 nm. Chromatographic separations were conducted on a column (ODS-EP 150 mm x 4.6 mm), and the column temperature was 40 °C. The flow rate was 1.0 mL/min, and the injection volume was 100 µL. The isocratic mobile phase composition was ultrapure water: AcCN-CH_3OH (94/3/3%).

2.6. Verification Studies

For method verification, detection (LOD) and quantification (LOQ) limits, precision, and reality parameters were studied. Analyses were performed by adding mycotoxin standards to homogenized samples for LOD/LOQ, precision (repeatability, reproducibility),) and reality (recovery) studies. Measurements were performed using spiked AF, OTA, DON, and ZON standards at the concentrations given in Table 1. For LOD/LOQ, 20 parallel studies were performed at the following concentrations: These concentrations in Table 1 for repeatability, recovery, and reproducibility were spiked. Six parallel studies on the same day for repeatability and recovery analysis were done. Six parallel studies were done on different days for reproducibility as well [24, 25].

Table 1. Spike concentrations of method verification.

	LOD/LOQ (µg/kg)	*Repeatability/Recovery (µg/kg)*	*Reproducibility (µg/kg)*
AF B_1	1.00	1.00 / 5.00	1.00 / 5.00
AF B_2	0.30	0.30/1.50	0.30/1.50
AF G_1	1.00	1.00 / 5.00	1.00 / 5.00
AF G_2	0.30	0.30/1.50	0.30/1.50
OTA	0.50	0.50/2.00/5.00	0.50/2.00/5.00
DON	250.00	250.00/1000.00	250.00/1000.00
ZON	10.00	10.00/50.00	10.00/50.00

LOD: limit of detection, LOQ: limit of quantification.

3. Results and Discussion

Celiac patients must eat gluten-free foods. For this reason, they cannot consume gluten-containing foods such as wheat, barley, and rye. Rice and corn or natural gluten-free products such as buckwheat, carob flour, and chickpea flour are mostly used in the products of patients with celiac disease.

Celiac disease is both a genetic disease and a lifelong food allergy. It is accepted as the most common genetic disease in men today. According to data obtained from the Health Information System, the number of diagnoses of celiac disease in Turkey is 68123 as of May 2019 [19]. Gluten-related diseases are primarily celiac disease, non-celiac gluten sensitivity, wheat allergy, and gluten ataxia [26]. The increase in celiac disease in the last 20 years may be related to, apart from changes in eating habits, the increase in awareness of the disease, the easy application of antibody screening tests, and the recognition of atypical or silent cases [19]. This situation rapidly increases the gluten-free product market in parallel with the increasing

demand for gluten-free products. The global market for gluten-free products has reached high numbers [27–30]. Today, foods that do not contain more than 20 mg of gluten per kilogram are considered gluten-free foods [31]. Therefore, it is important to determine the possible amounts of mycotoxins that may be exposed to people who eat gluten-free foods. Verification of analytical methods is found in Table 1.

3.1. Validation of analytical methods

As seen in Table 1, the concentrations in the table were spiked into the blank solution for validation studies. According to the obtained results, the study was subjected to appropriate statistical tests. For each method, measurement uncertainty parameters, which are included with the measurement result, characterize the distribution of values corresponding to the measured size, and show the quality of the measurement result, were calculated [24, 25]. The calculated results are given in Table 2.

Table 2. Validation results of methods for mycotoxins

	LOD (µg/kg)	*LOQ (µg/kg)*	*Mean (µg/kg)*	*STD*	*Recovery %*	*measurement uncertainty*
Total AF	0.40	1.32	2.47	0.75	93.47	X ± 0.13X
AF B_1	0.21	0.69	0.97	0.07	96.47	X ± 0.17X
OTA	0.09	0.29	0.24	0.02	92.01	X ± 0.14X
ZON	2.25	7.5	9.63	0.75	92.10	X ± 0.18X
DON	40.45	134.83	200.46	6.19	91.85	X ± 0.18X

According to Table 2 recovery is over 90% for all methods, and it has been found suitable according to the TFC.

After the validation processes of the methods were completed in the laboratory, 95 gluten-free foods purchased randomly were homogenized, and AF, OTA, ZON, and DON analyses were performed. Gluten-free foods with detectable levels of mycotoxins are given in Table 3 below.

In the DON analysis, results were obtained for 11 out of 95 products. The lineer range of DON analysis was 20–2000 µg/kg. The maximum limit allowed for pasta in TFC [32] is 750 µg/kg. The results were below and appropriate to this value, but the pasta5 and pasta7 results were found to be close to this value. It is thought that attention should be paid to the consumption of these products. It has been determined that these products were made from corn, which was very quickly contaminated. The maximum limit for bread is 500 µg/kg. 420 µg/kg was found to be suitable for bread. The maximum value for a biscuit or cracker is 500 µg/kg. Results were found below 500 µg/kg and are suitable. According to Tolosa J. et al. (2021), FB1, ZON, and DON had the highest incidence rates, with total incidences of 90.5%, 71.4%, and 66.7%, respectively. On the other hand, in terms of mycotoxin levels, DON > NIV > FB1 > FB2 > ZON > HT-2 were in the top five. The substance with the highest content was DON (377.4 g/kg) [33]. Despite the significant amounts of mycotoxin found, particularly for DON, NIV, and FBs, none of the samples exceeded the maximum values (MLs) stipulated by the European Regulation [34].

Table 3. Mycotoxins detected in gluten-free products

Gluten-free food samples	*OTA (µg/kg)*	*DON (µg/kg)*
Buckwheat flour[3]	1.99	
Pasta[1]	2.52	
Pasta[3]	1.36	
Pasta[5]	1.41	725
Pasta[2]		480
Pasta[7]		740
Pasta[9]		650
Pasta[11]		550
Pasta[12]		386
Pasta[13]		410
Bread[5]		420
Crackers /biscuits[4]	2.34	
Crackers /biscuits[8]	1.14	
Crackers /biscuits[10]		480
Crackers /biscuits[11]	1.33	
Crackers /biscuits[18]		430
Crackers/biscuits[19]		495
Cornflakes[4]	1.94	
Cornflakes[6]	2.48	

Superscript numbers are shown the different brand of products

As a result of the mycotoxin analysis, OTA results were found in 9 of the 95 different packaged products studied. The maximum allowable limit for processed grains and grain products (offered for direct human consumption) in the Turkish Food Codex Contaminants Regulation (TFC) [32] is 3 µg/kg. Since the results found in the studied products are below 3 µg/kg, the results are appropriate.

In the study of Giannioti Z et al. (2023, a total of 28 organic whole grain oat flours, conventional whole grain oat flour, organic rice flour, and conventional rice flour were used for the detection of three mycotoxins (DON,

ZEN, and AFB1) occurring in gluten-free flours analyzed. They used the approved extraction method, LC-MS/MS, for analysis. They stated that there was multiple mycotoxin contamination in all flour types, especially in traditional whole-grain oat flour. In rice flour, one sample was found to contain zearalenone at a concentration of 83.2 μg/kg, higher than the level set by the European Commission for cereal flours [35].

According to the data in Table 3, the population most exposed to mycotoxins found in corn and rice foodstuffs (GF products) corresponds to those with celiac disease and gluten intolerance, and the highest exposure level among these groups is children.

When we look at the table, it is thought that the gluten-free foods in separate stands in the markets prevent the contamination of mycotoxins, and the storage conditions are better than for other products. It can be said that these foods are more reliable for consumers based on the findings and the interpretation of significant data. When we look at the table, it is thought that the gluten-free foods in separate stands in the markets prevent the contamination of mycotoxins, and the storage conditions are better than for other products. It can be said that these foods are more reliable for consumers based on the findings and the interpretation of significant data.

4. Conclusions

Four mycotoxins were analyzed for 95 different brands and types of gluten-free packaged foods. The results showed that the analytical procedure performed was accurate (recovery range of 90% to 97% for the majority of analytes), precise (RSDs < 18%), and sensitive (LODs 0.09 to 40.5 μg/kg). Many of these methods fall short of the limit that can be determined. The mycotoxin values in a few of them were determined according to the Turkish food codex, but the values found do not exceed the upper limit. The values of OTA and DON mycotoxins in gluten-free food samples were found in the range of 1.14–2.52 μg/kg for OTA and 38–740 μg/kg for DON. Both mycotoxin values were found in only one pasta sample of all samples. As a result of all analysis and validation studies, it is concluded that the consumption of gluten-free foods is not harmful in terms of mycotoxins in those products. It has been determined that 95 different gluten-free products are safe in terms of mycotoxins. This shows that the products analyzed are produced, stored, and transferred to the consumer under appropriate conditions.

Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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