T.R.

YEDİTEPE UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

NUTRITION AND DIETETICS DEPARTMENT

# RESEARCH ON THE PRESENCE OF OCHRATOXIN A IN THE CORNS SOLD OUTSIDE IN İSTANBUL PROVINCE

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Master Thesis

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ONAY

Bu tez Yeditepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliğinin ilgili maddeleri uyarınca yukarıdaki jüri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu'nun 31/08/2018 tarih ve 2018/15-02 sayılı kararı ile onaylanmıştır.

İmza

Prof. Dr. Bayram YILMAZ

Sağlık Bilimleri Enstitüsü Müdürü

## DECLARATION

I declare that the whole information in this study has been obtained in accord with the academic rules and the codes of conduct. As stated in the rules and acts, I specify that I have reported all of the materials and results completely within content of the study and provided all references.

16.08.2018

NİHAN YAKUT

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## SYMBOLS AND ABBREVIATIONS

**AFB:** Aflatoxin

**FB1, FB2 :** Fumonisin

**OTA:** Ochratoxin

**ELISA**:  Enzyme-Linked Immunosorbent Assay

**RNA** :  Ribonucleic Acid

**WHO:** World Health Organization

µg: Micro Gram

µL : Micro Liter

**ABSTRACT**

**Yakut, N. (2018). Research on the Presence of Cchratoxin a in the Corns Sold Outside in İstanbul Province. Institute of Health Science, Department of Nutrition and Dietetics, M.Sc. Thesis, İstanbul**

Mycotoxins which are found in foods, can go into human body through consumption of those foods, gain more importance day by day and endanger health due to generation of toxic metabolite have become increasingly a substantial research subject. The fungi that are responsible from the generation of mycotoxins can be found everywhere, because they are conveyed by air. However, their contamination can vary by the type of the product, geographical situation, seasons and years. Mycotoxins are highly toxic secondary metabolites produced by filamentous fungi of Fusarium, Aspergillus and Penicillium. Apart from several toxins produced by limited number of species, numerous mycotoxins are generated by a great variety of species. Grain production has a place in the global food supply which represents 50 percent of total daily caloric intake of people.

Ochratoxins usually exist in barley, wheat, corn, rye, oat, fig, bean olive, raisin, nuts, spices, coffee, beer, medicinal herbs, herbal tea and grapefruit juice. Fungal injection and production of mycotoxins can start in harvest, processing and storage. In particular, situation of the cereals during storage is a basic factor for the growth of mycotoxins. Ambient conditions such as humidity and temperature are the leading factors for safety of the cereal products.

The study aimed to analyze 40 corn samples gathered from various district bazaars and markets of İstanbul province. The samples were taken from the corns which are not sold under a brand. The risk evaluation of OTA in this product group was performed.

**Key Word:** Corn, Ochratoxin A, ELISA Method, contamination

# ÖZET

**Yakut, N. (2018). İstanbul ilinde Açikta Satilan Misirlarda Okratoksin A Varliğinin Araştirilmasi, Sağlık Bilimleri Enstitüsü, Beslenme ve Diyetetik Bölümü, Yüksek Lisans Tezi, İstanbul**

Gıdalarda bulunan ve tüketilmeleriyle insan vücuduna girebilen, gün geçtikçe önemi giderek artan, toksik metabolit üretimi nedeniyle sağlığa zararlı olan mikotoksinler gün geçtikçe önemli bir araştırma konusu olmuştur. Mikotoksinlerin üretiminden sorumlu mantarlar hava yolu ile taşınarak her yerde bulunabilirler ancak kontaminasyonu, ürün cinsine, coğrafi duruma, mevsimlere ve yıllara göre değişiklik gösterebilmektedir Mikotoksinler, Fusarium, Aspergillus ve Penicillium'a ait olan filamentöz mantarlar tarafından üretilen yüksek derecede toksik sekonder metabolitlerdir. Birçok mikotoksin, sınırlı sayıda türün ürettiği bazı spesifik toksinler hariç, çok çeşitki farklı cinsler tarafından üretilir. Hububat üretimi, insanların günlük toplam kalori alımının %50'sini temsil eden küresel gıda tedariğinde önemli bir yer tutmaktadır.

Okratoksinler sıklıkla arpa, buğday, mısır, çavdar, yulaf, incir, fasulye, zeytin, kuru üzüm, kabuklu yemişler, baharatlar, kahve, bira, şifalı bitkiler, bitki çayları ve greyfurt suyunda bulunur. Mantar enfeksiyonu ve mikotoksin üretimi, hasat, işleme, depolama ve işleme alanlarında başlayabilir. Özellikle depolama sırasında tahılların durumu, mikotoksinlerin büyümesi için temel etkenlerdir. Nem ve sıcaklık gibi ortam koşulları tahıl ürünlerinin güvenliği için önemli faktörler olarak başta gelmektedir.

Bu çalışmada, İstanbul ilinin çeşitli semtlerinde pazar ve marketlerden toplanan toplam 40 adet mısır örneğinin analizinin yapılması amaçlamıştır. Örnekler açıkta markasız şekilde satılan mısırlardan alınarak OTA'nın bu ürün grubundaki risk değerlendirilmesi yapılmıştır.

**Anahtar Kelimeler:** Mısır, Okratoksin A, ELISA Yöntemi, Kontaminasyon

# 1. INTRODUCTION AND PURPOSE

Mycotoxins which are found in foods, can go into human body through consumption of those foods, gain more importance day by day and endanger health due to generation of toxic metabolite have become increasingly a substantial research subject.[1] Mycotoxins are the secondary metabolites produced during consecutive enzyme reactions of the simple intermediate products that form from the primary metabolism of acetates, mevalonates, malonits and several amino acids as a result of the various biochemical means.[5] Toxic effect of the mycotoxins, called "Mycotoxicosis" and its severity on animal and human health depend on the toxicity of mycotoxin, extent of the exposure and age and nutritional status of the person exposed.[2]

The fungi that are responsible from the generation of mycotoxins can be found everywhere, because they are conveyed by air. However, their contamination can vary by the type of the product, geographical situation, seasons and years.[1] Contamination of foods and animal feeds with mycotoxins is a world-wide problem and is one of the significant risk factors for human health.[2] The most common mycotoxin in nature which has the highest risk of causing toxicity is Aflatoxin B1. It is followed by Ochratoxins. Ochratoxins are the second common mycotoxin group in nature. They were first isolated from the culture of *Aspergillus ochraceus* isolated from sorghum grains, in 1965. The foremost two factors which determine the formation of OTA are temperature and moisture.[4] Toxins of Ochratoxin A (OTA) are found the most frequently. Toxicity degree of this type is relatively higher than that of the others. Ochratoxins usually exist in barley, wheat, corn, rye, oat, fig, bean olive, raisin, nuts, spices, coffee, beer, medicinal herbs, herbal tea and grapefruit juice. However they are found in cereal products such as wheat and corn more frequently.[6] Today, OTA is accepted to be one of the most hazardous infective substances in foodstuffs and animal feeds. Therefore numerous countries focus on screening and monitoring programs to reveal the distribution of OTA in foodstuffs while determining the legal limits for reducing recycling of the exported products with the aim of avoiding harmful effects of the foodstuffs contaminated by OTA.

According to effective "Turkish Food Codex Regulation on Contaminants" published in Official Gazette dated December 29, 2011 and numbered 28157, OTA level of corn grains is 3.0 μg/kg.[5]

The thesis study aimed to analyze 40 corn samples gathered from various district bazaars and markets of İstanbul province. The samples were taken from the corns which are not sold under a brand. It was planned to make the risk evaluation of OTA in this product group.

# 2. GENERAL INFORMATION

## 2.1. Mycotoxins

The term mycotoxin was generated with the combination of the word "myco" which means fungus, and the word "toxin" which means poison. It was specified that about 400 fungi produces mycotoxins under suitable ambient conditions.[6] Several fungi offer the foods which are frequently used in industry as well, such as cheese and antibiotic. On the other hand, some of them are responsible for the presence of toxic compounds like mycotoxins.[7]

Mycotoxins are highly toxic secondary metabolites produced by filamentous fungi of Fusarium, Aspergillus and Penicillium. Apart from several toxins produced by limited number of species, numerous mycotoxins are generated by a great variety of species. Currently, almost 300 fungal metabolites produced by more than 100 fungi were reported to have toxigenic potential. The toxigenic metabolites described can have acute and chronic impacts on both humans and animals. There are six mycotoxins that are prevalently seen in e-food systems of agriculture: Aflatoxins, Ochratoxins, Patulin, Fumonisin, Trichothecenes and Zearalenon. Each of them can be produced by a few different fungi. Contamination of food products and animal feeds is a serious problem all around the world. According to estimations of Food and Agriculture Organization, approximately 25 percent of the crops in the world can be contaminated with the mycotoxins generated by fungi.[2] Mycotoxins are categorized according to species, structure and mode of action of fungi. As is seen in Table 1, , a single fungus species can produce one or a few mycotoxins or different species of fungi can produce different mycotoxins.[6]

**Table 1 .** The Most Common Fungus Species Which Produce Mycotoxin [6]

| **Mycotoxins** | **Fungus Species** |
|---|---|
| Aflatoxin | Aspergillus Flavus, A. Parasiticus, a. Nomius, A. Argenticus |
| Ochratoxin A | Penicillium verrucosum, P. Nordicum, A. Ochraceus, A. Carbonarius, A. Niger, A. Sclerotioniger |
| Deoxynivalenol | Fusarium graminearum, F. Culmorum, F. Sporotrichioides, F.poae, F. tricinctum |
| T-2 Toxin | *F. sporotrichioides, F. poae* |
| Diacetoxyscripenol | *F. graminearum, F. semitectum, F. tricinctum, F. oxysporum* |
| Nivalenol | *Fusarium nivale, F. poae* |
| Zearalenon | *Fusarium graminearum, F. culmorum* |
| Fumonisin B1 | *Fusarium proliferatum, F. verticillioides (* syn . *F. moniliforme), A. niger, A. carbonarius* |

Grain production has a place in the global food supply which represents 50 percent of total daily caloric intake of people.[7] Fungal injection and production of mycotoxins can start in harvest, processing and storage. In particular, situation of the cereals during storage is a basic factor for the growth of mycotoxins. Ambient conditions such as humidity and temperature are the leading factors for safety of the cereal products.[8]

### 2.1.1 The Fungi Leading to Formation of Mycotoxins

Researchers noticed numerous secondary metabolites produced by fungi at the end of the 1800s and in the early 1900s. Scientists gave weight to studies on antibiotics after Alexander Fleming discovered Penicilin in 1928. Clues about the toxic effects of fungal metabolites were obtained when it was realized that they have toxic impact on animals during the researches. The species of fungi which comes to the forefront as the producers of mycotoxins are *Aspergillus, Penicillium, Fusarium* and *Alternaria*.[11]

## 1.2. The Factors Which Affect the Formation of Mycotoxins

The principal factors which affect the formation of mycotoxins are environmental factors. Moisture content of the agricultural product or nutrient (temperature and relative humidity) is the most important factor that influences the formation of toxins by allowing for the germination of the spores of fungi and the development of micelles. The other factors which affect the formation of mycotoxins are as follows;

- Type of the agricultural product and nutrient,
- Physical conditions of the environment in which the product is cultivated and stored (moisture content, temperature and pH) (moisture of more than 70 percent and temperature between 12 and 47 °C provide the optimum conditions for the production of mycotoxins),
- Maturity status, harvest processes and storage status of products,
- Presence of the other microorganisms in the environment,
- Insufficient fertilizing and drought (attenuation of the protection systems in vegetables),
- Impairment of the protective structure of vegetables caused by the mechanical process or insects.[12]

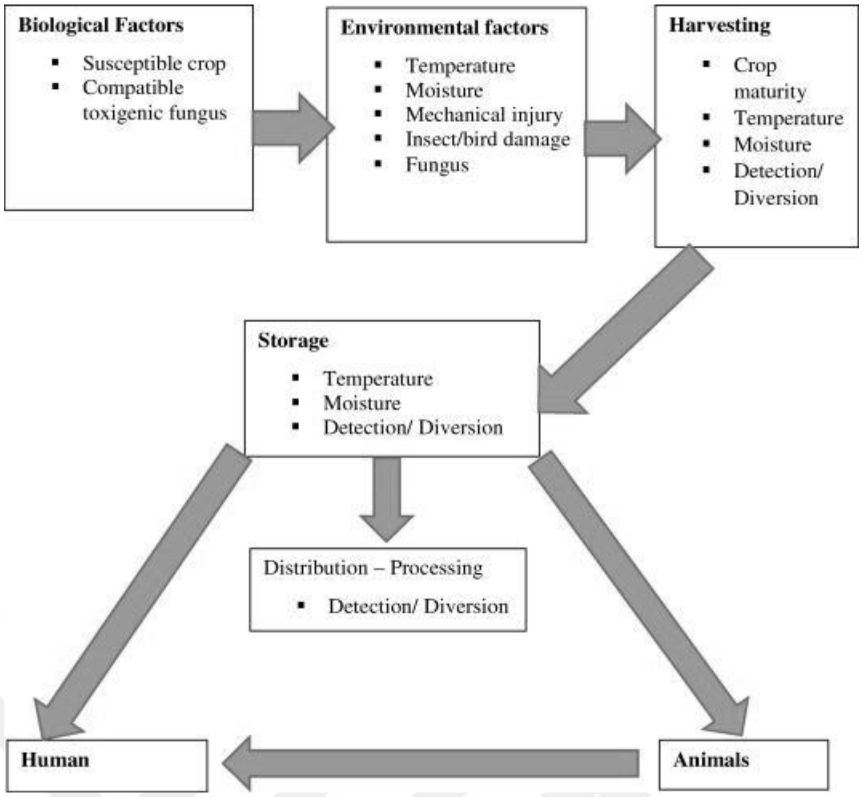


**Figure 1.** The factors which affect the formation of mycotoxins in food chains of human and chains of animal feed [10]

### 2.1.3. Exposure to Mycotoxins / Mycotoxicosis

The primary source of the human exposure to mycotoxins is nutrition. Exposure happens when the foods which were contaminated directly, or the foods attained from the animals which were fed with contaminated forages are consumed. The incidents caused by mycotoxins which humans and animals go through are intoxication of mycotoxins called "mycotoxicosis." Such situation shows up depending on the chemical structure of mycotoxin, duration of exposure to toxic effects, age and nutritional status of the person and the chemical interaction of the toxin and the other chemicals exposed by the person. [13]

Exposure to mycotoxins continues to be a significant health problem across the world. The recent studies have enabled the systemic effect of fungal toxicity on human body, including brain to be understood broadly. Exposure to mycotoxins shows positive correlation with asthma, wheezing, bronchitis, fatigue, musculoskeletal pain, headache, anxiety, mood, cognitive disorders and depression. Further studies about the impact of fungi and mycotoxins on immune and nervous systems that will clarify the molecular pathways which create the connection between exposure to mycotoxins and cognitive disorders are needed.[14]

Severity of the mycotoxicosis may vary based on the factors such as vitamin deficiency, caloric deficit, alcohol abuse and contagious disease. On the other hand, mycotoxicoses may raise vulnerability to microbial diseases, worsen the influences of malnutrition further and interact with the other toxins synergistically. Number of the people affected by mycoses and mycotoxicoses is not known. Although it is believed that number of the people affected by bacteria, protozoa and viral infections is lower, fungus diseases create a serious international health problem. The mycoses led by the opportunist pathogens are generally the diseases of the developed world and come to exist in the patients with immune system weakened due to advanced medical treatments. On the contrary, mycotoxicoses are more prevalent in undeveloped countries. One of the characteristics of mycoses and mycotoxicoses is that the disease categories usually do not transmit from human to human.[15]

International Agency for Research on Cancer (IARC) reported that 500 million people of the poorest population in Sub Saharan Africa, Latin America and Asia are exposed to mycotoxins which increase mortality and morbidity considerably. Most of the developing countries are in the tropics of the world. They experience monsoon rains, high temperature and high moisture levels which contribute to product losses after the great harvest. For this reason, influential reduction strategies to control the profound effect of mycotoxins are of vital importance for the world.[32]

### 2.2 Ochratoxins

Ochratoxins are produced primarily by *Penicillium verrucosum* and *Aspergillus ochraceus*. It was reported that low amount of *Aspergillus niger* can synthesize ochratoxins as well.[16]

*Aspergillus niger* is used commonly in the production of enzyme and citric acid for human consumption, so it is important to ensure that the industrial strains do not have productivity [15] Ochratoxins are called A, B and C according to their chemical structures. Ochratoxin A is a water soluble and colorless compound. It exhibits blue fluorescence under UV light. OTA is an acidic compound which contains an amino acid ( l phenylalanine). Structure of OTA is substantially affected by the composition of its mobile phase and its pH.[17] It forms with the combination of phenylalanine and dihydroisocoumarin through peptide bond. Figure 2 shows the chemical structure of ochratoxins.[18]

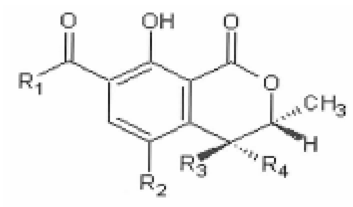


**Figure 2.** Chemical Structure of Ochratoxins[18]

The foods which contain ochratoxins are usually barley, wheat, corn, rye, oat, fig, bean olive, raisin, nuts, spices, coffee, beer, medicinal herbs, herbal tea and grapefruit juice. They are generally found in the cereal products such as wheat and corn, and in the animal products.[6]

### 2.2.1. The Factors Which Affect the Formation of Ochratoxins

There are numerous factors which affect the production of OTA in foods and animal feeds. The factors such as temperature, water activity (aw), pH, type of substrate, the presence of competitive microflora, fungus strain, integrity of the seed (degradation and damage), gas atmosphere (CO2 and O2) and protective use affect the production of OTA.[22]

The most effective way to prevent mycotoxins in musty grains is to harvest the fully-fledged grains and to keep the moisture level below 15 percent. As the moisture in storage environment of the grains rises, the number of fungi increases and they produce mycotoxins due to their metabolism. Furthermore, the grains such as groundnut should be stored well and protected from mold growth. Moisture of at least 18 percent and relative humidity of 85 percent, temperature of 30oC and pH of 3-5 in the store make up the most suitable reproduction conditions of fungi. Ochratoxin B is rarely found as a natural contaminant and is less toxic. Other ochratoxins are never found in natural products. [20]

Optimum development temperature of *asp. achraceus* is 28 °C. Temperature between 20 - 30 ºC is required for the formation of OTA. It generates maximum level of toxin at 30 ºC and 95 percent of relative humidity. In case of a fall in temperature, it requires higher relative humidity. For example, 99 percent of relative humidity is needed at 15 ºC. Species of *penicillium* can generate toxins at low temperatures. *Pen. verrucosum* produces ochratoxins between 5 and 10 ºC. The fungi which are responsible for OTA contamination in the cereals cultivated in hot climates and in cold-cool climates such as Canada and Scandinavian countries are *Asp. ochraceus* and *Pen. aurantiogriseum* and *Pen. verrucosum,* respectively. Both species can develop slowly even under -2 ºC. *Asp. ochraceus* ranks among xerophilic fungi. Min. value of water activity for germination and development of the spores is AS=0.76- 0.83. It can grow in the cereal products if their moisture content becomes 15-16 percent. OTA amount decreases and the amount of penicillic acid increases under the optimum temperature of development. Min. water activity values of *Pen. aurantıogriseum* and *Pen. verrucosum* are quite low as well (AS = 0.79; 0.80-0.81). All species require higher AS values for the formation of OTA. Particularly, *Pen. aurantiogriseum* requires min. AS =0.97-0.99 (Table 2). Ochratoxin A is a teratogen, immunosuppressive and carcinogenic compound which causes strong nephrotoxicity. It has acute and chronic toxic effect. Dose of its acute toxicity for male and female rats correspond to LD50 = 29 mg.kg-1 and LD50 = 22 mg.kg-1, respectively. Teratogenic effect causes especially the deformity in head and beak of the chicks and mice and prevents their eye growth.[21]

**Table 2**. Xerophilic fungi and their mycotoxins and minimum AS values[21]

| Fungi | Development | Formation of Toxin | Mycotoxins |
|---|---|---|---|
| *Asp. clavatus* | 0.85 | 0.99 | Patulin |
| *Asp. flavus* | 0.78-0.874 | 0.83-0.87 | AFB1, Aspergillic acid, Aspertoxin, |
| *Asp. fumigatus* | 0.82 | - | Fumagiline, Gliotoxin |
| *Asp.parasiticus* | 0.78-0.82 | 0.87 | AFB1 |
| *Asp. ochraceus* | 0.76-0.83 | 0.83-0.87<br>0.80-0.88 | Ochratoxin A,<br>Penicillic Acid |
| *Asp. versicolor* | *0.74-0.78* | - | Sterigmatocystin |
| *Emer. nidulans* | 0.78-0.82 | - | Sterigmatocystin |
| *Eurotium spp.* | 0.62-0.74 | - | Fisikon, Ekunilin, Ksantosilin |
| *Pen. aurantiogriseum* | 0.79-0.85 | 0.97-0.99<br>0.87-0.90 | Penicillic Acid, Ochratoxin A, Tremortin A and B, Cyclopiazonic Acid |
| *Pen. expensum* | 0.82-.085 | 0.99 | Patulin, Citrinin |
| *Pen. griseofulvum* | 0.81-0.85 | 0.85-0.95 | Patulin |
| *Pen. islandicum* | 0.83 | - | Islanditoxin, Cyclochlorotin, Luteocyclin |
| *Pen. patulum* | 0.81-0.85 | 0.95 | Patulin |
| *Pen. puberulum* | 0.81 | - | Penicillic Acid |
| *Pen. verrucosum* | 0.81-0.83 | 0.83-0.90 | Ochratoxin A |
| ***Pen. viridicatum*** | **0.80-0.81**<br>**0.83** | **-** | **Citrinin Ochratoxin A,**<br>**Penicillic Acid** |

### 2.2.2. Structure and Characteristics of Ochratoxin

Ochratoxin is in the form of white crystalline powder. It is recrystallized from xylene and has a crystal form which spreads blue (in alkaline solutions) and green (in acid solutions) fluorescent under ultraviolet light. Melting point of this crystal structure is 169ºC. Free acid and sodium salts of OTA are solved in solvents and water, respectively. It exhibits instability to light and air. It fragments and evaporates especially under humid conditions even if it is exposed to light for a short time. Ethanol solution can be stored in refrigerator without decomposition for more than a year. OTA has a low resistance to heat. About its 65 percent fragments when it is subject to auto-claving for 3 hours.[23]

OTA is a molecule with moderate stable structure and does not decompose until a definite degree during numerous food processes. It can be seen in food products consumed. OTA can break down to certain extent during boiling, baking, frying or fermentation depending on several parameters such as pH and temperature. OTA is degraded partially over the course of cooking and making bread. It fragments during baking and frying by approximately 20 percent. However, if the grains are exposed to physical process such as selection of the crusts of wheat during the making of wheat flour, OTA decreases by about 50 percent.

It was seen that milling does not affect OTA level or has minimum effect. [23.24]

### 2.2.3. Absorption, Distribution, Metabolism and Excretion of OTA

OTA is absorbed from the upper part of gastrointestinal system, particularly small intestine. In general, its absorption rates among pigs, rats, rabbits and chickens are 60 percent, 56 percent, 56 percent and 40 percent, respectively. It establishes bond to serum albumin in blood and to other macromolecules. Only 0.02 percent of OTA does not establish bond in humans and rats. It is distributed to primarily kidneys in pigs, rats, chickens and goats. Low concentration of OTA is found in liver, muscle and fat tissue of these species. It passes to placenta of rats and pigs. It is found in the milk of rats, rabbits and humans. It is excreted from blood more slowly than kidneys, liver and other tissues. The highest contamination is seen in the kidneys of pigs. This ratio is five times of the ratio in blood. It is subject to hydrolysis together with the bacteria and protozoa living in cardia of ruminants, so very low amount of OTA is found in tissues. [26]

Serum half life of OTA taken orally corresponds to 510 hours, 72–120 hours, 55–120 hours, 24–39 hours and 4.1 hours in pigs, rats, mice and chickens, respectively. This ratio is 840 hours (35 days) in humans. OTA is excreted through gall and urine. It was seen in all of the species analyzed that OTA turns into Ochratoxin α through hydrolysis (detoxification) primarily by means of the microflora in intestines. Hydrolysis capacity of liver and kidneys is low. However, the hydrolyzed OTA sent to gall is reabsorbed by the microflora in intestines, thereby going into enterohepatic cycle. It was observed in the in vitro studies on humans, mice, rats and pigs that OTA was oxidized to 4-OH-ochratoxin A by cytochromes P450 in microsomes of the liver. All metabolites of OTA are thought to be less toxic than itself. The most significant metabolite of radiolabeled OTA given to rats is 26 percent of ochratoxin α. Its 33 percent is excreted in stool and urine. Ochratoxin α is followed by 6 percent of OTA and 1.5 percent of 4R-OH-ochratoxin A, respectively.[27]

### 2.2.4. Toxicity of OTA

Exposure to mycotoxins in food during nutrition is associated with neurotoxicity, nephrotoxicity, hepatotoxicity and gastrointestinal disorders.[28] Genotoxic effect of OTA originates from an indirect mechanism which includes an impaired protein synthesis. [29] It affects animals by impairing the protein synthesis, influencing lipid peroxidation and leading to DNA damage and oxydoreductive stress.[33]

With regard to human foods, OTA can be transmitted particularly from the animal meat (liver, kidney and blood) and animal feeds to animal products. This situation results in risks and hazards for public health and veterinaries.[30] IARC classified OTA as possible carcinogen substances for human in group 2B.[50]

It is absorbed quickly from the stomach and small intestines of rats and mice after oral intake. Absorption percentage can vary depending on the species (66 percent, 56 percent and 40 percent in pigs, rats and rabbits, respectively). OTA is attached to blood proteins, especially albumin and other macromolecules when it passes to blood circulation. This fact may change between the species. In animals, the concentration of OTA and its metabolites in tissues and plasma may alter based on species, gender, dose, exposure route (direct or intake with food; oral or intravenous intake), duration of exposure, diet content and health status of the animal.[18]

It was indicated that OTA accumulates in different rates in kidneys, liver, muscles, lungs, heart, intestines, testicles, spleen, brain, skin and fat tissues depending on the route and frequency of exposure. Kidneys and liver are shown as the main target tissues. They are followed by muscles and the tissues of lungs and heart. It was reported that specific carriers may play role in cellular intake of OTA from kidneys.[31]

Exposure to Ochratoxin A reduces mitochondrial function and may cause apoptosis in neurons as well as cultured murine microglia and malfunction in astrocytes.

The studies on mice showed that satratoxin G results in apoptosis in sensory neurons; bulb-like encephalitis is related to continuous high levels of proinflammatory cytokines in fore brain region.[34]

According to several speculations, ochratoxins are associated with an endemic human disease called Balkan Nephropathy. This is a progressive chronic nephritis that shows up in the populations living in some regions of Romania, Bulgaria and former Yugoslavia, and in the regions on the border of Danube River. In a study conducted in Bulgaria, ochratoxin contamination of foods and the presence of ochratoxin in human serum were more prevalent in the families with Balkan endemic nephropathy and urinary tract tumors than in the unaffected families. This interesting disease is related to genetic factors, heavy metals and possible hidden infection factors as well as the intoxication of ochratoxin. Even though etiology of the Balkan endemic nephropathy is not know by the current consensus, numerous analyses on mycotoxins are listed as ochratoxicosis with no warning.[36] Besides, it was asserted that the gene for phenylketonuria may come to exist with a relatively high frequency because of a heterozygotic advantage against the intoxication of ochratoxin, and be a risk factor for testicular cancer.[37,38]

It will be a cautious act for the medical society to attach more importance to the possibility of ochratoxin toxicity among the patient with symptoms of kidney pathology. Although the role of Ochratoxin A in human diseases is still speculative, it creates worry considering its acute nephrotoxicity, immunosuppressive activities and teratogenic effects in animal models, and its capability to convey the food chain together.

### 2.2.5. Legal Regulations on Ochratoxin Levels of Food Products

OTA was known to be transmitted to only cereal products such as corn, wheat, barley, rye, oat, rice and malt until quite recently. However, it has been reported that OTA is found in dry fruits, groundnut, coffee, cacao, spices, wine, beer and some of the animal products as well. Nowadays, OTA is regarded as one of the most hazardous contagious substances found in foodstuff and animal products.

Maximum OTA levels were specified in directive of the commission published by the EU in 2002 (EC 472/2002).40 According to this directive, maximum OTA levels for cereals and cereal grains, all of the processed cereal products and dried fruits of wine are 5 μg/kg, 3 μg/kg and 10 μg/kg, respectively. Baby formulas, cereal-based foods (0,5 μg/kg) and especially the food products used medically for babies (0,5 μg/kg) were included in the directive of the commission published in 2004 (EC 683/2004), considering the excessive risk for babies and children.[41]

In directive of the commission published in 2006 (EC 1881/2006), definite limits were determined for roast and ground coffee beans (5μg/kg), instant coffee (10μg/kg), several wine types (2 μg/kg) and grape juice and grape juice concentrate (2 μg/kg). 42 This directive was replaced by another directive of the commission (EC, 105/2010) in 2010, and maximum limits up to 80 μg/kg for spices and licorice extract were included.[43]

### 2.2.6. The Studies about Ochratoxin Levels Conducted in Turkey

Of mycotoxins, limit values of only aflatoxins, patulin and ergot alkaloids were included in Turkish Food Codex Regulation which came into force in 1997 (Official Gazette no. 23172, 1997). With "The Notice on the Determination of Maximum Levels of Certain Contaminants in Food Products" (Notice No: 2002/63) published in 2002, the maximum OTA levels of three products were reported.44,45 Maximum OTA limits of food products were redetermined through the notice dated May 17, 2008 no. 2008/26 published in Official Gazette numbered 26879 as well. 46 Later, this notice was abolished and "Turkish Food Codex Regulation on Contaminants" published in Official Gazette dated December 29, 2011 and numbered 28157 began to be applied. The maximum OTA limits of the foods specified based on this notice are given in Table 3. OTA limits of spices and licorice were added to said notice in 2011.[47]

**Table 3.** Maximum OTA limits in foods determined according to Turkish Food Codex Regulation on Contaminants (Official Gazette no. 28157, 2011) [47]

| | | |
|---|---|---|
| 2.2 | OCHRATOXIN A | |
| 2.2.1 | Unprocessed cereals | 5.0 |
| 2.2.2 | All of the products made of unprocessed cereals (including the cereals directly offered to human consumption and processed cereal products) | 3.0 |
| 2.2.3 | Dried fruits of grapevine (currant, raisin and seedless grape) | 10.0 |
| 2.2.4 | Roast coffee bean and ground coffee | 5.0 |
| 2.2.5 | Coffee extract, extract of instant coffee or instant coffee | 10.0 |
| 2.2.6 | Wine and Fruit Wines<br>(including sparkling wine/champagne and excluding liquor wines and the wines with alcohol amount of at least 15 percent in volume) | 2.0 |
| 2.2.7 | Fortified wine, fortified wine-based drink and fortified wine cocktail | 2.0 |
| 2.2.8 | Grape juice made of grape juice concentrate, grape nectar, fermented grape juice and fermented grape juice made of concentrate<br>(directly offered to human consumption) | 2.0 |
| 2.2.9 | Supplementary foods for babies and kids | 0.5 |
| 2/2/2010 | Special medical foods for babies | 0.5 |
| 2/2/2011 | For the following types of spices;<br>- Red pepper *(Capsicum spp.)*<br>- Black pepper *(piper spp.)*<br>- Nutmeg *(Myristica fragrans)*<br>- Ginger *(Zingiber officinale)*<br>- Curcuma *(Curcuma longa)*<br>- Any spice which contains one or some of them | 30.0<br>(Before June 30, 2012)<br><br>15.0<br>(After July, 01, 2012) |
| 2.2.12<br>2.2.12.1<br><br>2.2.12.2 | Licorice (*Glycyrrhiza glabra,g. İnflate* and other species)<br>Licorice (used as herbal infusion compound)<br>Licorice Extract<br>(used especially in the production of soft drinks and candies) | <br>20.0<br><br>80.0 |

## 2.3. Effect of Mycotoxins on Human Health

Toxicologists tend to concentrate on the hazardous chemicals such as polyaromatic hydrocarbons, heavy metals and organic pesticides. The agriculturists, chemists, microbiologists and veterinaries which are not usually familiar with the fundamental principles of toxicology due to making less effort for natural products performed majority of the researches on mycotoxins. For example, mycotoxicoses can be categorized as acute or chronic just like the toxicological syndromes. Acute toxicity has an apparent toxic response which is generally distinguished with a quick start. Chronic toxicity is characterized by exposure to low dose for a long time, and results in cancers and other irrevocable impacts. [48]

The primary mycotoxin which poses risk for human health is aflatoxin B1 (AFB1) produced by *Aspergillus flavus.* It was revealed that AFB1 leads to liver cancer in humans. It was suggested that about 250,000 people in China and Africa died because of liver cancer which resulted from the daily intake of high AFB1 amount (1.4 μg/kg). Detection of aflatoxin in tissues of the children with Kwashiorkor and Reye's syndromes raised doubts on if AFB1 has an impact on the emergence of both diseases.[49] It was observed in epidemiological studies that the people nourished with the foods which contain aflatoxin are more likely to have primary liver cancers and liver cirrhosis, and that AFB1 has mutagen, teratogen and immunosuppressive effects as well as carcinogenic effect.

OTA is an immunosuppressive, teratogenic, genotoxic and carcinogenic substance. Target organ of ochratoxins is kidneys. Afterwards, they result in liver cancer. Protein synthesis of the organism impairs coagulation mechanism of the blood by inhibiting mitochondrial transport system and gluconeogenesis. Moreover, OTA with teratogenic efficacy leads to an increase in the possibility of secondary infection (*E. coli*) by suppressing the immune system. It raises lipid peroxidation in body as well. In addition, OTA is held responsible for the nephropathies and kidney tumors with unknown etiology which are seen in Egypt and Tunisia, particularly "Balkan Endemic Nephropathy" (BEN) observed in the population of rural regions (Bosnia, Bulgaria, Croatia, Romania and Serbia).[51]

The studies performed in South Africa and several regions of China indicated that the incidence of oesophageal cancer is high in the regions which are rich in corn with high concentrations of FB1 and FB2. It was demonstrated that equine leucoencephalomalacia (ELEM) and pulmonary edema develop in the horses and pigs fed with corn which contains fumonisin, and the feeds made of such corns.[52,53]

All of these species are the regular contaminants of cereal products across the world. A relationship between the consumption of moldy cereals and hyperestrogenism has been observed since the 1920s. A modern study shows that diet concentrations of zearalenon which are as low as 1.0 ppm may result in hyperoestrogenic syndromes in pigs.[54] When the epidemiological data of Canada and Scandinavia were extensively analyzed, it was concluded that the risk of human population is minimum. The safe zearalenon intake for human is estimated to be 0.05 µg / kg of the body weight.

Patulin may have toxic, mutagenic and cytotoxic effect on numerous living systems. It may cause symptoms of poisoning such as edema, infection of collagen tissue, rise in the blood glucose and hypertension. Patulin may lead to symptoms in skin as well. In case of oral intake, it may give rise to nausea, vomiting and complaints about stomach. It was determined that patulin inhibits synthesis of RNA and protein by reducing RNA- Polymerase activity. Patulin influences the cellular respiration, chromosomes and membrane adversely. It inhibits the synthesis of macromolecules, impairs the immune system of complex organism and results in reflex movements in muscles and extremities, shakes and uncontrolled reflexes. Occlusion in kidneys, degeneration of urinary tract and decrease in urine are observed. Quantity of the water accumulated in lungs rises and acute toxic effect ends up with death.[55]

In many countries of the world, trichothecenes are found in various cereals. The most common mycotoxin among trichothecenes is deoxynivalenol (DON). Trichothecenes have caused a lot of disease cases in humans until now. Vomiting, digestive disorders, dizziness, diarrhea and headache are substantial symptoms.[55]

**Table 4**. Important mycotoxins, the fungi producing mycotoxins, contaminated products and their toxic effects[56]

| Mycotoxin | Source | Effects | Contaminated foods |
|---|---|---|---|
| Aflatoxin B1, B2, G1,G2 | *Aspergillus flavus, Asprgillus parasiticus* | Acute Aflatoxicosis Carcinogenesis | Corn, Groundnut and others |
| Aflatoxin M1 | *AFB1 metabolite* | Hepatotoxicity | Milk |
| Fumonisin B1, B2, B3, A1, A2 | *Fusarium verticillioides* | Kidney and Liver Cancer | Corn |
| Trichothecenes (T2 toxin, Deoxynivalenol, Diacetoxyscripenol) | *Fusarium Myrothecium* | Toxic effect on blood, cerebral hemorrhage, neurotic disorder, skin necrosis, gastrointestinal bleeding, vomiting and immunosuppressant | Cereal grains, corn |
| Zearelenon | *Fusarium* | Oestrogenic effect | Corn, Cereal |
| Cyclopiazonic Acid | *Aspergillus, Penicillium* | Muscle, Liver and Spleen Toxicity | Cheese, cereals, peanuts |
| Kojic Acid | *Apergillus* | Hepatotoxic | Cereals, Animal Feeds |
| 3- Nitropropionic Acid | *Arthrinium sacchari, Arthrinium saccharicola, Arthrinium phaespermum* | Damage of central nervous system | Sugar cane |
| Citreoviridin | *Penicillium citreoviride, Penicillium Toxicarium* | Cardiac Dysrhythmias | Rice |
| Cytochalasin E,B,F,H | *Aspergillus and Penicillium* | Cytotoxicity | Corn and cereal grains |
| Sterigmatocystin | *Aspergillus Versicolor* | Carcinogenesis | Corn |
| Penicillinic Acid | *Penicillium Cyclopium* | Nephrotoxicity, miscarriage | Corn, dry bean, cereals |
| Rubratoxin A, B | *Penicillium Rubrum* | Hepatotoxicity, teratogenic effect | Corn |
| Patulin | *Penicillium Patulum* | Carcinogenesis, liver damage | Apple and Products of Apple |
| Ochratoxins | *Aspergillus oschraceus, Aspergillus carbonarius, Penicillium verrucosum* | Endemic Nephropathy, Carcinogenesis | Corn, peanut, grape, green coffee |
| Citrinin | *Aspergillus and Penicillium* | Nephrotoxicity | Cereal grains |
| Penitrem | | Shaking, ataxia, bloody diarrhea, death | Blue cream cheese, English walnut, hamburger bread, beer |
| Ergot alkaloids | *Claviceps purpurea* | Ergotism | Cereal |

# III. MATERIAL and METHOD

## III.1. Material

50 g of corn samples sold outside were gathered from various districts of İstanbul. A total of 40 samples were taken. They were brought to laboratory in sterile packages under sterile conditions and stored between 2ºC and 8 ºC until the date of study.

## III.2. Method

The study will take place in Nutrition and Dietetics Department of Yeditepe University and at the Laboratories of Medical Microbiology Department of Medical Faculty. The following tools/instruments will be used;

- Rondo: To crumble the corn samples.
- Laminar cabin: To process the samples without contamination.
- Elisa washer (Biotech 50): To wash in Elisa.
- Elisa Reader (Beckman): To read the results of Elisa.
- Fume hood: To prepare the chemicals.

### II.2. 1. Determination of Ochratoxin with Elisa Method

Quantity of ochratoxin in the samples collected was determined with Elisa method, using AgraQuant Ochratoxin (0.2 - 40 ppm) kit. This kit contains a direct competitive enzyme-linked immunosorbent assay. Ochratoxin released by means of 70 percent of methanol is detected by the wells covered with enzyme conjugate. The procedure recommended by the manufacturing firm was followed. First, the samples to be used in Elisa method were prepared in line with the following procedure.

- 20 g of samples were weighed separately for the analysis.

**Figure 3.** Weighing of Samples

Later, the mixture of methanol and distilled water was prepared with a ratio of 70/30 in fume hood. 100 ml of the mixture was added to each sample. The samples were shaken in shaker incubator for 3 min.

At the following stage, the samples were filtered, using Whatman No:1 filter paper.

**Figure 4.** Filtering of samples

50 µl of the elution obtained at the last stage was used in Elisa test.

Components of the kit were primarily brought to room temperature to make Elisa test work. No dilution process was performed, since the standards inside the kit are ready for use.


**Figure 5.** Preparation of Elisa kits

The plate was washed for five times in Elisa automatic washer following the completion of incubation (10 min.). Thanks to this device, contamination between the samples and false positivities based on manual washing were minimalized.


**Figure 6.** Washing Samples with Elisa Washer

Later, 100 μL substrate was added to all wells, using an automatic pipet. It was incubated for 5 min. at room temperature and in the dark. Lastly, 100 μL of stop solution was included and Elisa test was completed. Elisa plate was read in 450 nm filter (with 630 reference filter) by Elisa reader and the absorbance values were taken. Furthermore, ochratoxin values of the samples were detected quantitatively by drawing a standard curve chart with the program of the device.

## IV. RESULTS

In this study, the corn samples sold outside in İstanbul were analyzed with respect to presence of ochratoxin. Calibration curve (Figure 3) was attained according to absorbance values of the standards used to determine ochratoxin levels in this study. Concentrations of ochratoxin were evaluated as ng / kg based on the form obtained from the calibration curve, considering the absorbance values read at 450 nm with 620 nm reference filter of the corn samples analyzed (Table 5).

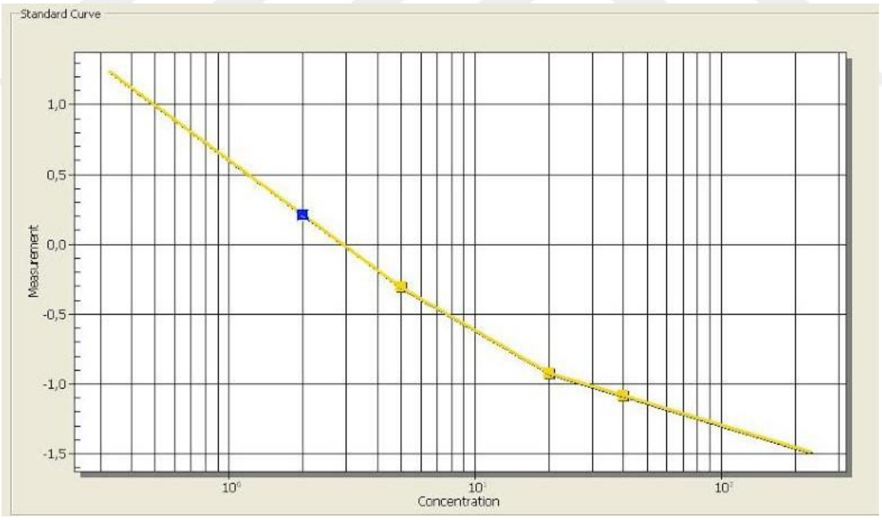


**Figure 7.** Standard Curve Analyses

According to results of the analysis on corn samples, the minimum and maximum quantities of ochratoxin were determined to be 0.59 μg and 1.19 μg, respectively. Harvest period of corn contains July and August, so the products were likely to be fresh and the drying process was likely to be performed completely. This point supports that the limit values in results of the analysis and formation of fungi did not increase.

**Table 5**. Ochratoxin Values of the Samples

| Sample No | Results (µg/20 gr) | Sample No | Results (µg/20 gr) |
|---|---|---|---|
| **1** | 1.103 | **21** | 1.019 |
| **2** | 1.184 | **22** | 0.897 |
| **3** | 1.103 | **23** | 0.749 |
| **4** | 1.074 | **24** | 0.678 |
| **5** | 1.133 | **25** | 1.111 |
| **6** | 0.907 | **26** | 1.048 |
| **7** | 0.813 | **27** | 1.067 |
| **8** | 0.79 | **28** | 0.926 |
| **9** | 1.196 | **29** | 1.059 |
| **10** | 1.145 | **30** | 0.851 |
| **11** | 0.977 | **31** | 0.706 |
| **12** | 0.904 | **32** | 0.634 |
| **13** | 0.92 | **33** | 0.923 |
| **14** | 0.92 | **34** | 1.076 |
| **15** | 0.841 | **35** | 1.08 |
| **16** | 0.841 | **36** | 0.905 |
| **17** | 1.015 | **37** | 0.963 |
| **18** | 1.048 | **38** | 0.722 |
| **19** | 1.125 | **39** | 0.59 |
| **20** | 1.019 | **40** | 0.644 |

As part of the research, OTA levels of the corn samples sold outside in İstanbul province differ. It is thought that the difference results from the type and supply location of corn. In the study performed with a total of 40 samples, the limit values (5 µg/kg for unprocessed cereals) given by "Turkish Food Codex Regulation on Contaminants" (published in Official Gazette dated December 29, 2011 and numbered 28157) were not exceeded.

## V. RESULT AND DISCUSSION

Cereals and cereal products make up a significant food group which are frequently used in our country and in the world, and even listed in staple foods. Cereal products that can be used as the raw material of many products are always in the risky group with regard to contamination of mycotoxin. However, the increasing awareness in food safety and public health raised the worries for preventing, minimizing and controlling the mycotoxins in foods and animal feeds. To obtain safe corn, mycotoxins should be prevented and their control strategies should depend on the principles of food safety.

The corn samples analyzed in this study did not exceed the limit values of ochratoxin (5 µg/kg) determined for food safety by Turkish Food Codex and the European Union in 2002. If corn samples does not exceed limit values, this situation annihilates their association with any disease factor.

The results of the analysis which do not exceed the limit values make us think that freshness of corn products results from the plantation and harvest periods of corn which are May, and June & July, respectively rather than proper storage conditions. Fresh products did not pend and were not exposed to varying balance of moisture. Moreover, reproduction does not occur mainly due to the drying processes.

İstanbul province is in the category of 2nd Degree Mesothermal subhumid climate (water shortage is experienced in summer) when Turkey is analyzed by the moisture and precipitation based on seasons (Figure 3).[67]

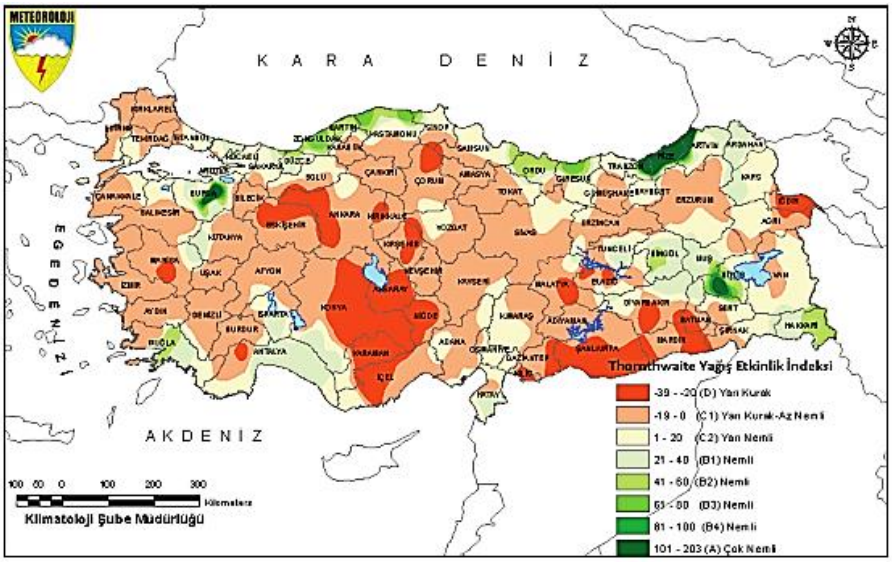


**Figure 8**. Thornthwaite Precipitation Effectiveness Index and Climatic Characteristics[67]

Ochratoxin was not detected even though moisture rate rose by seasons. This fact shows that the products were kept through a proper storage method and the air conditioners and ventilation systems in the location of the supplier eased the control of reproduction. However, it is seen in the other studies that there are still cases associated with exposure to mycotoxin in many countries today.

In a study on Green coffee beans conducted in the U.S. in 1995, OTA was found in 13 of 25 samples (detection limit, 0.5 microgram OA / kg). It was clarified that the people who consume coffee regularly can be exposed to OTA at high doses.[57]

Barley, wheat, green coffee and roast coffee imported from the U.S. were analyzed in another study. 56 of 383 wheat samples, 11 of 103 barley samples, 19 of 19 green coffee samples and 9 of 13 roast coffee samples were detected to have high concentrations of OTA.[58]

In a study conducted in Morocco in 2010, barley, wheat and corn each quantity of which corresponded to 60 were analyzed. In consequence of the study, 4-8 ng/g concentrations of OTA were found in 40 samples of barley, 40 samples of wheat and 55 samples of corn.[59]

Another study performed in Korea in 2005 evaluated rice with respect to exposure to mycotoxin. Species of Fumonisin, Aflatoxin, Trichothecenes and Zearalenon were sought in the study. Although Ochratoxin A is the most common mycotoxin species analyzed in the current study, the samples were reported to be close to tolerance limits of the EU (3 ng/g).[60]

In a study made in 2007, 37 grain samples collected randomly were subjected to mycotoxin tests. It was seen that 16 percent of the samples contained high doses of OTA (9.8 microg/kg).[61]

In South, Middle and North Italy, 300 cacao and chocolate-based product samples were analyzed in 2011. Positive results withe regard to OTA were attained from 179 samples (60 percent of the total samples). However, maximum dose of the samples was found to be 0.39 ng/g. Although the cacao and chocolate products analyzed did not exceed definite limits of the EU, the limit values were characterized to be high, considering their consumption by children.[62]

In 2010, OTA of 99 percent was found in red peppers and peppers collected randomly in Spain. The results attained were between 0.62 and 44.6 ng/g. It was thought that the cause of high contamination was being sold outside.[63]

OTA milk concentrations in several countries such as Egypt, Turkey and Sierra Leone were found to be 100 times higher than those in Europe. OTA concentrations of human milk are much lower than those of milk. However, it was concluded that OTA contamination of human milk poses a serious health risk potentially.[66]

In a study performed at the end of 2006 in Adana, Turkey, sultana samples supplied from 22 different locations were analyzed. According to analysis, OTA values varied between 5.1 and 18.2 µg/kg (ppb). It is seen that most of our findings in this research exceed maximum OTA limit specified in Turkish Food Codex (10 µg/kg).[65]

In a declaration of Food and Feed Safety Alerts (RASFF) of the EU published between 2000 and 2015, the risky OTA levels of the imported products were designated in 2015 and the products were reported by their countries of origin (Table 6). [64]

**Table 6.** OTA and warning statements in the EU[64]

| | | | |
|---|---|---|---|
| 16/01/2015 | Finland | Pumpkin seed from China | 19 |
| 22/01/2015 | Germany | Dry fig from Spain | 124 |
| 2015/03/03 | Belgium | Wheat from Canada | 17 |
| 13/03/2015 | The Netherlands | Pumpkin seed from China | 29 |
| 13/03/2015 | France | Dry fig from Spain | 183 |
| 24/03/2015 | France | Wheat from Canada | 18 |
| 2015/04/06 | Ireland | Licorice from Turkey | 433.5 |
| 2015/10/06 | Poland | Raisin from Turkey | 19.3 |
| 15/07/2015 | Slovak republic | Raisin from Chili | 11.8 |
| 2015/10/08 | France | Rye flour from France | 12.9 |
| 2015/12/08 | Finland | Pumpkin seed from China | 20000 |
| 13/08/2015 | Luxembourg | Dried red pepper from Thailand | 30.8 |
| 2015/01/09 | Romania | Sultanas from Turkey | 15.6 |
| 25/09/2015 | Croatia | Black pepper from Vietnam | 155 |
| 2015/02/12 | Belgium | Dry fig from Turkey | 14.4 |
| 2015/08/12 | Latvia | Pepper from China | 40 |
| 2015/11/12 | Cyprus | Raisin from Greece | 18.5 |
| 23/12/2015 | Belgium | Dry fig from Turkey | 27.8 |
| 16/01/2015 | Finland | Pumpkin seed from China | 19 |

(*Alert statements are sent when there is food product which poses serious health risk for humans and the authorized bodies in domestic market need to act quickly*.)

Broad effect of mycotoxins requires protection of corn and corn products from toxic fungi along the food chain. The procedures for controlling mycotoxins are preventive to a great extent. They are good agricultural practices and drying of the harvested products at sufficient level. Effective drying and storage techniques were developed for corn. Drying technologies such as sun drying, column dryers and shallow-bed dryers are recommended for the period from production to storage. Several researchers on the methods to prevent the products from contamination before harvest have been conducted for a long time. Such approaches contain the development of host resistance through the development of antifungal genes via plant breeding, the use of biocontrol agents and targeting of the regulatory genes in the development of mycotoxins.

Comprehensive studies are of importance for correct diagnosis of the results obtained. Conducting studies with more samples and evaluating the studies by months and seasons will help more accurate results to be attained to determine the reproduction status.

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