Rapid detection of mycotoxins on foods and beverages with enzyme-linked immunosorbent assay

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RESEARCH ARTICLE

Abstract

Mycotoxins are toxic secondary metabolites of fungi and their presence in foods and feed is potentially hazardous to the health of both humans and animals. This study has assessed the presence of aflatoxins, ochratoxins and fumonisins in 183 samples of a variety of foods and beverages (nuts, cereals, milk, cheese, wine and beer) in the Greek market by an enzyme-linked immunosorbent assay. Overall, 42.6% of samples had detectable levels of any of the above mycotoxins and 15.3% had levels above the European Union (EU) legal limit. About 48.1% (n = 27) of nuts were found to be contaminated with aflatoxin in which 33.3% were above the EU legal limit (4 ppb), 25.9% with fumonisin and 29.6% with ochratoxin, and in 14.8% of samples co-occurrence of all three mycotoxins was observed. For cereal-based products, 15.9% (n = 38) were detected with aflatoxin and 59.3% (n = 64) of milk and cheese samples were detected with aflatoxin M_1 (AFM_1), but no sample exceeded the EU legal levels. The levels of AFM_1 were found significantly lower in ultra-high temperature pasteurised milk (long-life milk) than in pasteurised milk. Detection of ochratoxin in vine grapes and non-commercial wines produced in small-scale wineries indicated that 43.5% (n = 23) of samples contained ochratoxin above the EU limit (2 ppb). Analysis of barley malts, barley seeds and beers revealed that 29% of samples (n = 31) were contaminated with ochratoxin at a level above the EU limit (3 ppb). The results confirm the widespread and persistent presence of mycotoxins in various foods and beverages; therefore, continuous monitoring and awareness is required to safeguard public health.

Keywords: aflatoxin, aflatoxin M_1, ochratoxin, fumonisin, ELISA

1. Introduction

Aflatoxins, ochratoxins and fumonisins are among the most common mycotoxins found in foods worldwide (Creppy, 2002). The aflatoxins are known to be human carcinogens based on sufficient evidence of carcinogenicity from studies in humans (Report on Carcinogens, 2011a). They are produced from fungi of the genus *Aspergillus* (mainly *Aspergillus flavus* and *Aspergillus parasiticus*) that contaminate different agricultural commodities such as nuts, grain, several food and feedstuffs (Murphy *et al.*, 2006). Considering the risk of exposure of the general population to aflatoxins primarily by eating contaminated food, maximum levels of aflatoxins (aflatoxins B_1, B_2, G_1, G_2 and M_1) in foods and feed are established in Commission Regulation (EC) No. 1881/2006. Ochratoxins are mycotoxins produced by certain moulds of the genera *Aspergillus* and *Penicillium*, and the most toxic ochratoxin found naturally in food is ochratoxin A (OTA), classified as a group 2B carcinogen (carcinogenic to animals and possible carcinogenic to humans) which possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties (Bui-Klimke and Wu, 2015; Covarelli *et al.*, 2012; Report on Carcinogens, 2011b). The presence of OTA in

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wine is a significant problem mainly for Southern Europe where climatic conditions favour the growth of OTA-producing fungi in grapes (Battilani *et al.*, 2006; Visconti *et al.*, 2008). OTA occurrence has also been related to the contamination of malt barley with ochratoxigenic species, particularly *Penicillium verrucosum*. The occurrence of OTA in beer is a worldwide problem due to the widespread consumption of this beverage. The European Union has established maximum permitted levels for OTA at 2 μg/kg for wine, grape, juice, grape nectar and grape must and 3 μg/kg for malt (EC Regulation 1881/2006). There are currently no limits for beer because the level of OTA in beer is indirectly controlled as the OTA in beer originates from the presence of OTA in malt. Contamination of fumonisins in corn, corn-based food and feed has been steadily reported from all over the world (Castelo *et al.*, 1998). The International Agency for Research on Cancer evaluated the carcinogenic potential of fumonisins and classified them in group 2B (possibly carcinogenic to humans) (IARC, 1993). Maximum levels allowed by the European Union are 2,000 μg/kg for unprocessed maize, 1,000 μg/kg for maize flour, 400 μg/kg for maize-based foods and 200 μg/kg for maize-based baby foods (EC Regulation 1881/2006).

There is growing concern for ways in which these fungi and their mycotoxins can be prevented from entering the human and animal food chain. Strict legislation imposed worldwide and the global demand for continuous monitoring of aflatoxin levels in foods and feed have led to the development in recent years of highly specific antibody-based rapid assays that have facilitated the detection of mycotoxins even in very low concentrations (ng/kg or ppt in the case of aflatoxin M_1 [AFM_1]). These enzyme-linked immunosorbent assays (ELISAs)-based methods can be used for quick and reliable screening of samples (Shanakhat *et al.*, 2018; Turner *et al.*, 2015), while high-performance liquid chromatography with fluorescence detector (HPLC-FLD) remains the reference method used for confirmation (Cigić *et al.*, 2009; Rosi *et al.*, 2007). The aim of this study was to assess the levels of specific mycotoxins (total aflatoxins, AFM_1, ochratoxin, fumonisin) by a rapid ELISA method in a wide variety of foods and beverages classified in four categories: (1) nuts, (2) cereal-based products, (3) milk and cheese, and (4) wine and beer.

2. Materials and methods

Samples

Nuts and cereal-based products

Sixty-five samples including 27 nuts (peanuts, walnuts, pistachio and almonds) and 38 cereal-based products (25 breakfast cereals and 13 corn-based samples) were collected randomly from Greek super markets.

Milk and cheese

Sixty-four samples (57 milk and 7 cheese samples) from the Greek market (Athens area) were analysed from March to May 2014. Milk samples included branded milks from 10 different Greek dairy companies, private label milk from 5 super markets and 2 imported brands. Samples were classified according to fat content in full fat (>3.0% fat) and light fat (1.5% fat) and according to pasteurisation in fresh milk (7 days expiry date, pasteurised at 72°C for at least 15 sec) and long life (>7 days expiry date, pasteurised by ultra-high temperature processing above 135°C for 1–2 sec). Four groups of milk were tested: (1) full fat fresh, (2) full fat long life, (3) light fresh and (4) light long life. Cheese samples were traditional local varieties of cheese from the Aegean area.

Wine and beer

Wine samples (n = 23) were selected from North East Attica area during September–November 2012. Samples included 4 varieties of vine grapes (Agiorgitiko, Savatiano, Roditis and Attikis), 10 wines from small-scale wineries (non-commercial wines) and 9 brands of commercial wines. Beer-related samples (n = 31) were imported barley malts and beers available on the Greek market (6 barley, 14 barley malts and 11 beers).

ELISA method

For nuts, breakfast cereals and corn-based products, the direct competitive ELISA Veratox® HS for aflatoxin (by Neogen Corporation, Lansing, MI 48912 USA) was used for the screening of total aflatoxins with level of detection (LOD) 0.5 ppb and range of quantitation 1–8 ppb (the standard curve was constructed with concentrations of 0, 1, 2, 4 and 8 ppb of standards provided by the kit). This test is approved by Association of Official Analytical Chemists (AOAC) and is validated for the matrices used in this study, such as peanuts, tree nuts, corn-based products, barley, soy flour, oat fibre, rice bran and wheat. Breakfast cereals that were tested were a mixture of these ingredients. For nuts and corn-based products, the direct competitive ELISA Veratox® HS for fumonisin (by Neogen Corporation) was used which is a test approved by AOAC and validated for barley, corn, Dried Distillers Grains with Solubles (DDGS), milo, popcorn, rice, soybeans and wheat for the screening of total fumonisins (B1, B2, B3) with the LOD 50 ppb and range of quantitation 50–600 ppb. For testing aflatoxin and fumonisin, the samples were thoroughly mixed in a blender and then 5 g of ground sample was blended with 25 ml of 70% methanol/water solution and was shaken vigorously for 3 min. The extract was filtered through a Whatman#1 filter and at least 5 ml of the filtrate was collected as sample. Quantitation was performed by following the test procedure provided in the kit.

For milk and cheese, all samples were analysed on the same day of purchasing by the competitive ELISA method using Veratox® for AFM_1 (provided by Neogen Corporation), a kit validated for quantification of AFM_1 in liquid raw milk and cheese with a LOD of 4.3 ppt and range of quantitation 5–100 ppt. Samples of liquid milk (10 ml) were centrifuged at 3,500 rpm at 10°C and the defatted supernatant was collected and used directly in the test. Samples of cheese were prepared as follows: 2 g of finely grated cheese was placed in a 50 cc centrifuge tube, 10 ml of 100% dichloromethane was added and the mixture was vortexed for 1 min. Extraction was performed in a rotary shaker for 15 min followed by incubation at 50°C for 30 min and centrifugation at 3,500 rpm (2,740 × *g*) for 15 min at 10°C. Then 5 ml of dichloromethane supernatant was withdrawn and was placed in a glass tube in a water bath evaporator set at 60°C until evaporation to dryness. The oily residue was re-dissolved in 0.5 ml of 100% methanol and 0.5 ml of distilled water, 4 ml of 100% hexanes was added and the mixture was vortexed for 1 min. After centrifugation at 3,500 rpm (2,740 × *g*) for 10 min at 10°C, the upper layer (hexanes) was removed completely using a Pasteur pipette. A 0.5 ml aliquot of the remaining methanol–water layer was dissolved in 2 ml of the sample diluent and the mixture was vortexed for 1 min. 100 µl of this reconstituted material was used directly for the test. Each sample of either milk or cheese was transferred in the microwells coated with anti-AFM_1 monoclonal antibody and the wells were placed on an automatic plate shaker for 20 min at room temperature (RT). The contents of the antibody wells were washed with wash buffer 5 times and the wells were turned upside down and tapped on a paper towel. 100 µl of aflatoxin–enzyme (Horseradish Peroxidase) conjugate was added to the wells and a second incubation on an automatic plate shaker for 10 min at RT was performed. The washing step was repeated for 5 times and then 100 µl of the enzyme's substrate was added at the microwells which were shaken for 15 min at RT. Finally, 100 µl of the stop solution was added to stop the enzymatic reaction. The optical densities were read in a microwell reader (Epoch, Biotek, Winooski, VT, USA) at 650 nm. A six-level standard curve was constructed (with concentrations of 0, 5, 15, 30, 60 and 100 ng/L). The limit of quantitation was 5 ppt, and the lowest LOD was 4.3 ppt AFM_1. For cheese, the values obtained were multiplied by 5 because the samples were diluted 1:5 during extraction.

In wine and beer, detection of ochratoxin was performed in all samples with the competitive direct ELISA with the commercial kit Veratox® for ochratoxin (Neogen Corporation) with LOD = 1 ppb and Level of Quantitation = 2–25 ppb. A five-level standard curve was constructed with concentrations of 0, 2, 5, 10 and 25 ppb. This is a test validated for corn, barley, wheat, green coffee, oats, rice, soybeans and various dried fruits. In commodities not validated such as beer and wine, positive samples should be confirmed with an additional approved method. An amount of 10 g of samples was blended with 40 ml of 70% methanol/water solution and the mixture was shaken vigorously for 5 min. The extract was filtered through a Whatman#1 filter and at least 5 ml of the filtrate was collected as sample. Quantitation was performed by following the test procedure provided in the kit. All measurements were performed in duplicate, and ELISA spectrophotometric readings were made at 650 nm by the Epoch™ Multi-Volume (Biotek) spectrophotometer.

Statistical analysis

Data for milk samples were analysed with one-way analysis of variance (ANOVA) post hoc tests, and pairwise multiple comparisons were conducted using Duncan test. A difference was considered significant at a level of $P < 0.05$ (Statistica software).

3. Results and discussion

Mycotoxins in nuts

In this study, 48.1% (n = 27) of nuts were found to be contaminated with aflatoxin in which 33.3% were above the EU limit (4 ppb), 25.9% with fumonisin and 29.6% with ochratoxin (Table 1). A co-occurrence of aflatoxin,

Table 1. Mycotoxin levels in nuts (peanuts, walnuts, pistachio and almonds).

Type of sample	Aflatoxin[1] (total) (ppb)	Fumonisin[2] (ppb)	Ochratoxin[3] (ppb)	Co-occurrence of all three mycotoxins
Positive samples/samples analysed	13/27 (48.1%)	7/27 (25.9%)	8/27 (29.6%)	4/27 (14.8 %)
Range	1.02–10.9	121–613	13.11–19.01	
Mean ± SD	4.83 ± 3.18	408 ± 179	16.87 ± 1.9	
Samples above the EU maximum allowable level	9/27 (33.3%)	3/27 (11.1%)	8/27 (29.6%)	

[1]Maximum EU allowable levels 4 ppb apply for cereals and nuts.
[2]Maximum EU allowable levels 400 ppb apply only for maize based products.
[3]Maximum EU allowable levels 3 ppb apply only for cereals.
SD, standard deviation; EU, European Union.

fumonisin and ochratoxin was observed in 14.8% of samples. Although maximum allowable levels for nuts by the European Union are applied only for aflatoxin, 11.1 and 29.6% of nut samples were contaminated with fumonisin and ochratoxin above the EU maximum allowable levels (400 ppb for fumonisin and 3 ppb for ochratoxin); therefore, the high incidence of different mycotoxins confirms the fact that nuts are among the crops that are susceptible for contamination by mycotoxins (Battilani, 2010). Also, the mycotoxins typically co-occur in foods and feed and their occurrences vary from year to year depending on weather and other environmental conditions (Eskola *et al.*, 2018). Recent studies have revealed that contamination of nuts by aflatoxins is common. In a Portuguese study, 12 out of 16 mycotoxins were found in 37 nut samples analysed (Cunha *et al.*, 2018), and in a similar study in China, 16 mycotoxins were detected at a contamination frequency of 124 out of 253 samples of dried fruits and nuts (Wang *et al.*, 2018). In Pakistan, an analysis of 320 samples of nuts and dried fruits revealed the co-occurrence of OTA and total aflatoxins in 25% of total samples (Iqbal *et al.*, 2018). In Algeria, 70 out of 112 samples of peanuts, almonds and dried figs contained detectable levels of aflatoxins and 11 peanut samples exceeded the European maximum limits for aflatoxin B_1 (AFB_1) (Mimoune *et al.*, 2018).

Mycotoxins in cereal-based products

This study has revealed a low presence of aflatoxins in breakfast cereals (8%), with none of the samples exceeding the EU maximum allowable levels (Table 2). Other studies in Europe have shown higher incidence, such as the detection of AFB_1 in 56.3% of breakfast cereals (n = 55) in a Greek study in which seven samples were found to be contaminated at levels higher than the EU limit for AFB_1 (Villa and Markaki, 2009). In Spain, 9% of breakfast cereals samples (n = 46) were contaminated with AFB_1, although no sample exceeded the EU limit and 28% of samples showed co-occurrence of OTA and zearalenone (Ibáñez-Vea *et al.*, 2011). In Portugal, in a study of mycotoxin co-occurrence in 26 breakfast cereals, all aflatoxins except aflatoxin G_2 were detected in the samples, with higher incidence of positive samples 69% for AFB_1, and three samples exceeded the maximum limit for infants and young children of 0.100 µg/kg (Martins *et al.*, 2018). AFB_1 was found in low concentrations in 11.1% (n = 136) of the samples collected in 2015 in Serbia, mainly corn based (Torović *et al.*, 2017). In Canada, in 349 samples of breakfast and infant cereals, 50% had detectable levels (limit of detection = 0.002 ng/g) of AFB_1 but only 4% of the breakfast cereals and 1% of the infant cereals had AFB_1 levels exceeding the EU maximum limit of 0.100 µg/kg for baby food (Roscoe *et al.*, 2008). However, in a recent study in the United States, aflatoxins were not detected in 215 retail infant foods and breakfast cereals (Zhang *et al.*, 2018). In Pakistan, in 237 breakfast cereal samples analysed, 41% were found contaminated with aflatoxins, out of which 16 and 8% samples were found to be above the EU maximum limit for AFB_1 and total aflatoxins, respectively (Iqbal *et al.*, 2014).

Corn-based products were found to be contaminated with aflatoxin (30.8%, n = 13) and fumonisin (26.9%), but no sample exceeded the EU maximum allowable levels (Table 2). In a similar study in Spain, 92 corn-based food and feed samples were analysed with ELISA, and the levels of fumonisins in corn flour, corn meal and flaking grits samples exceeded the limit of 1,000 µg/kg in 98, 30 and 2% of samples, respectively, but in cornflakes the levels were lower than 400 µg/kg, the maximum tolerable limit set by the European Union (Castells *et al.*, 2008). Also in Spain, out of 41 organic and non-organic corn-based food samples, seven were contaminated with fumonisin, but only one sample exceeded the EU maximum level for fumonisin. Also, the contamination frequency of organic

Table 2. Mycotoxin levels in cereal-based products.

Type of sample	No. of samples	Aflatoxin[1] (total) ppb (samples detected with aflatoxin/samples analysed)	Fumonisin[2] (ppb) (samples detected with fumonisin/samples analysed)
Breakfast cereals (from nine different brands)	25	2/25 (8%) Range: 0.13–1.36 Mean ± SD: 0.74 ± 0.87	
Corn-based products (corn seeds and popcorn)	13	4/13 (30.8%) Range: 2–4 ppb Mean ± SD: 2.88 ± 0.85	3/13 (23.1%) Range: 79.8–281.2 Mean ± SD: 279 ± 198
Total	38	6/38 (15.9%)	3/13 (23.1%)
Samples above the EU maximum allowable level		0/38	0/13

[1]Maximum EU allowable levels 4 ppb apply for cereals and nuts.
[2]Maximum EU allowable levels 400 ppb apply only for maize-based products.
SD, standard deviation; EU, European Union.

corn samples (40%) was higher than non-organic ones (3.7%), and contained higher levels of fumonisin B_1 and B_2 (Silva *et al.*, 2009). In Korea, co-occurrence of AFB_1 and fumonisin B_1 in corn foods was detected in 4/47 (8%) and 9/47 (19%) samples (Park *et al.*, 2002). In Serbia, in 71 samples of corn flours and corn flakes marketed in Serbia, 96.4% (n = 56) of samples were positive for fumonisins B_1 and B_2, but only two samples were above the EU maximum allowed levels (Torović, 2018).

Aflatoxin M_1 in milk and cheese

AFM_1 is a major metabolic hydroxylation product of AFB_1 which is the most toxic aflatoxin. AFM_1 is formed in the liver and excreted into the milk of lactating animals following ingestion of feed contaminated with AFB_1. AFM_1 is transferred to milk and consequently milk products (human breast milk also) destined for human consumption (IARC, 2002) and therefore represents a serious health concern (Ketney *et al.*, 2017). A limit of 50 ng/kg of AFM_1 for milk and 25 ng/kg for baby milk food was set (Commission Regulation (EC) No. 1881/2006). In addition, regulatory limits for dairy products, such as cheese, have also been introduced by some European countries, for example, the Netherlands (200 ng/kg), Austria and Switzerland (250 ng/kg) and Italy (provisional limit of 450 ng/kg) (Anfossi *et al.*, 2011). Contamination of AFM_1 in milk-based products occurs in many countries all over the world, especially when climatic conditions (warm temperature and humidity) favour the growth of toxinogenic fungi and vary from year to year (Iha *et al.*, 2013). In Asian countries such as Iran, Pakistan, India, Turkey and Korea, very high incidence (70–100%) of contaminated samples and samples exceeding the EU limit of 50 ng/kg was reported in many studies as reviewed by Nuryono *et al.* (2009) and Womack *et al.* (2016).

AFM_1 in milk

A relatively high incidence of AFM_1 was observed in the milk samples analysed. Overall, 63.2% (36/57) of milk samples purchased from the Greek Market were detected positive for AFM_1 with values >4.3 ng/kg which was the LOD of the method and up to 32.75 ng/Kg, with a mean value of 9.15 ng/kg (Table 2). Regarding the differences in levels of AFM_1 in the various categories of milk tested, the highest mean concentration of AFM_1 was found in pasteurised full fat milk (full fat fresh) which was significantly higher than both types of ultra-high temperature pasteurised milk, as shown in Table 3. This finding suggests that levels of AFM_1 decrease in milk pasteurised in ultra-high temperature. However, more samples should be analysed to confirm this trend and rule out the possibility that different levels of AFM_1 in milk reflect differences in batches of milk from various farms used in the industry. Studies on thermoresistance of AFM_1 in milk during various processes have shown contrasting data (Galvano *et al.*, 1996) and it is generally believed that AFM_1 is resistant to pasteurisation, although some researchers have shown that pasteurisation process could decrease AFM_1 content (Deveci and Sezgin, 2006; Iha *et al.*, 2013; Tsakiris *et al.*, 2013). In general, the various food processes reduce mycotoxin concentrations significantly, but do not eliminate them completely (Bullerman and Bianchini, 2007).

The level of contamination of milk samples varied between types of milk with the largest percentage (56%) of total samples having a level of AFM_1 from 4.3 to 25 ng/kg and 7% (four samples) ranging between 25 and 50 ng/kg which are the EU maximum allowable levels for infant milk food and milk in general, respectively. From the four samples of this 'alarming' level range above 25 ng/kg, only one was a brand that was marketed for consumption by children >1 year of age. Overall, none of the samples

Table 3. Percentages of positive milk samples for aflatoxin M_1 (value of AFM_1 > 4.3 ppt) in various types of milk tested.

Type of milk	Samples detected with AFM_1/total samples analysed[1]	Mean value[2] (ng/kg)	Standard deviation	Range of values[3]
Full fat fresh (>3% fat, pasteurised)	80.0% (12/15)	14.03[a]	10.43	0–32.75
Full fat long life (>3% fat, Ultra High Temperature)	42.9% (6/14)	5.99[b]	7.40	0–25.82
Light fresh (1.5% fat, pasteurised)	84.6% (11/13)	10.26[ab]	7.62	1.73–27.62
Light long life (1.5% fat, Ultra High Temperature)	46.7% (7/15)	6.28[b]	7.34	0–20.09
Total	63.2% (36/57)	9.15		

[1]Positive samples: samples with detected aflatoxin M_1 > LOD (level of detection) of the method (LOD = 4.3 ppt); UHT = pasteurized in ultra high temperature.
[2]Mean values with different letters are statistically significantly different (P < 0.05).
[3]The EU maximum levels for infant milk food and milk in general for AFM_1 are 25–50 ng/kg.

exceeded the level of 50 ng/kg of AFM_1 set by the European Commission for milk.

These results are in accordance with similar studies in Greece, which have shown that although samples with AFM_1 residues were detected, very limited or zero samples were above the maximum EU limits. Markaki and Melissari (1997) detected 32 samples out of 81 with AFM_1 at levels of 2.5–5 ng/L, while none contained more than 5 ng/L and 31 contained only traces of aflatoxin (0.5–1 ng/L). Roussi *et al.* (2002) analysed 114 samples of raw and market milk in Greece and found three samples (2.6%) to be contaminated with AFM_1 > 50 ng/L. Kaniou-Grigoriadou *et al.* (2005) reported that in Thessaloniki Province, levels of AFM_1 in milk were far below the tolerance level (highest value 18.2 ng/L). More recently, Malissiova *et al.* (2013) have reported that AFM_1 contamination levels in raw milk in Greece are relatively low (18.4%) and Tsakiris *et al.* (2013) have detected AFM_1 residues in 46.5% of samples, but none exceeded the EU limits. Other European countries have also reported contamination of dairy products by AFM_1. In Italy, only 4 (2.5%) out of 161 samples of dairy products were contaminated at a level of >50 ng/kg (Galvano *et al.*, 2001). The concentration of AFM_1 exceeded the EU limit in 6.7% of cow milk samples from eastern Croatia (Bilandzic *et al.*, 2014).

AFM_1 in cheese

Traditional local varieties of soft white cheese (n = 7) from the Aegean area were analysed for the presence of AFM_1. Most of these cheeses were produced from raw or slightly thermised ewe's or goat's milk, without the addition of commercial starters but by using only the natural microbiota of the milk. These cheeses have local names such as Kopanisti, Volaki, Armeksia and so on, and their ripening period ranged from 1 month to 8 years (Table 4). The results showed that two out of the seven cheeses analysed were contaminated with AFM_1 at levels 174.73 and 113.85 ng/kg, which were below the recommended maximum allowable levels of most European countries. Both contaminated cheeses had a low ripening period (1 month). Further studies should be conducted to obtain a clear picture of the AFM_1 contamination in cheese in Greece because it varies according to the initial AFM_1 levels in milk, the cheese type and the technologies applied (Cavallarin *et al.*, 2014; Iha *et al.*, 2013; Ketney *et al.*, 2017; Virdis *et al.*, 2008).

Ochratoxin in wine

Climatic conditions in the Mediterranean basin favour the growth of ochratoxin-producing fungi (Mateo *et al.*, 2007). Several researchers have studied the occurrence of OTA in wines identifying contaminated wine in high incidence (>50%) (Markaki *et al.*, 2001; Soufleros *et al.*, 2003; Stefanaki *et al.*, 2003) and products such as grapes (Tjamos *et al.*, 2004, 2006) and dried vine fruits (Stefanaki *et al.*, 2003). Greece is a large wine-producing country, characterised by a vast range of wineries spread all over the country, ranging from some large world-class wineries to many small-scale domestic ones which were the main focus of this study. Grapes that were selected from four different farms in North East Attica and were destined for vinification were found to be naturally contaminated with high levels of ochratoxin (all >15 ppb), as shown in Table 5. Three out of four samples had a clear indication for the relevant contamination (presence of mould), suggesting an inconsistency with the rules of good agricultural practice. A random screening of local non-commercial wines (year of production 2012) in the same area indicated that 4 out of 10 samples were found to contain ochratoxin in levels ranging from 13.61 to 15.61 ppb, a level considered very high for European wines (Visconti *et al.*, 2008). In commercial wines, two out of nine samples were detected

Table 4. Detection of aflatoxin M_1 in traditional Greek cheese.

	Cheese samples	Type of milk	Characteristics	Traditional name	Region	AFM_1 ppt (ng/L)[1]
1	Soft white	Cow	Unsalted	Mizithra	Evia	<LOD
2	Soft white	Cow	Unsalted	Soft	Evia	<LOD
3	Soft white	Goat	1 month ripened	Malakto	Andros	174.73
4	Soft white	Goat	1 month ripened contains domestic starter culture	Armeksia	Andros	113.85
5	Soft white	Goat	Ripened 6 months	Volaki	Andros	<LOD
6	Soft white	Goat	Ripened 8 years	Volaki	Andros	<LOD
7	Soft white	Goat	Ripened, intense salty and peppery taste due to enzymatic activity	Kopanisti	Andros	<LOD

[1]The EU has no official regulatory limits for cheese, but some European countries have introduced maximum allowable limits: the Netherlands (200 ng/kg), Austria and Switzerland (250 ng/kg), and Italy (provisional limit of 450 ng/kg). AFM_1, aflatoxin.LOD, level of detection.

Table 5. Detection of ochratoxin in vine grapes and wines in North East Attica.

Type of sample	Samples detected with ochratoxin/ total samples analysed	ppb ochratoxin[1] Range	Mean ± SD	Samples above the EU limits (2 ppb)
Grape vines (from four different farms)	4/4	16.10–20.43	18.02 ± 2.30	4/4
Non-commercial wine	4/10	13.67–to15.61	14.95 ± 2.58	4/10
Commercial wine	2/9	3.44–3.49	3.46 ± 0.04	2/9
Total	10/23 (43.5%)			10/23 (43.5%)

[1]EU maximum allowable levels for ochratoxin A are 2 μg/kg for wine, grape, juice, grape nectar and grape must (EC Regulation 1881/2006).
SD, standard deviation; EU, European Union.

Table 6. Detection of ochratoxin in samples of barley malts, barley seeds and beers.

Types of sample	Samples detected with ochratoxin/ total samples analysed	Ochratoxin (ppb)	Mean ± SD	Samples above the EU limits (3 ppb)
Barley malts	8/14 (57.1%)	3.08–12.30	7.27 ± 3.72	8/14
Barley seeds	1/6 (16.6%)	6.24		1/6
Beers	2/11 (18.2%)	1.03 and 1.60	1.31 ± 0.4	0/11
Total	11/31 (35.5%)			9/31 (29%)

SD, standard deviation; EU, European Union.

with ochratoxin, above the EU maximum allowable limit which is 2 μg/kg (Table 5).

These results indicated that the presence of mycotoxin in grapes and their products is a potential problem especially in small-scale wine making facilities in Greece. Good agricultural practice should be embraced not only by large facilities but also by any small or domestic producer. Wine-making is an important economic sector and strategies should be developed to ensure that the wine and all products of vinification are free of mycotoxin contaminants.

Ochratoxin in beer

Analysis of barley malts, barley seeds and beers revealed that 29% of samples (n = 31) were contaminated with ochratoxin at a level above the EU limit (3 ppb) (Table 6). Barley malts were found to be the most contaminated (8 out of 14 samples of barley malts were detected with ochratoxin content > LOD of the method, ranging from 3.08 to 12.30 ppb with an average of 8.66 ppb). All contaminated samples of barley malt were above the EU limits of 3 ppb OTA. Ochratoxin was detected in only 1 sample out of 6 barley seeds (6.24 ppb) and in 2 out of 11 beers (1.03 and 1.60 ppb, levels which were below the EU limit).

In a similar study in Czech Republic, 237 samples of malting barley, malt, hop, wort and beer were analysed for OTA and 1 barley sample (0.3 μg/kg), 1 malt (0.7 μg/kg) and 1 hop sample (0.6 μg/kg) were found contaminated. OTA content was determined in 39% of beer samples, but none was above the EU limit (Běláková *et al.*, 2011).

The higher presence of ochratoxin in barley malt (57.1%) suggests that contamination of malt with ochratoxigenic fungi can easily occur. However, beer as a final product of the brewing process had a low percentage of contaminated samples in low levels probably because, as stated in previous studies, the production processes that take place (i.e. mashing, fermentation, filtration and so on) reduce ochratoxin, probably though adsorption and removal of spend grains and yeast. Good agricultural practices should be implemented to reduce fungal contamination of malt barley to avoid heavy contamination by ochratoxin that will result in contaminated beers.

4. Conclusions

This study confirmed that the presence of mycotoxins in foods and beverages is a wide spread and persistent problem in a wide range of either imported or locally produced food and beverages in Greece. Out of 183 total samples of nuts, cereal-based products, dairy, wine and beer, 42.6% were detected with mycotoxin (any of total aflatoxin, AFM_1, ochratoxin or fumonisin) and 15.3% of samples had levels of mycotoxins above the EU legal limit. The incidence of mycotoxins in foods and feed is expected to rise in the coming decades due to climate change which will impact fungal growth. Management of mycotoxin levels is vital to preserve the quality of

commercial products and good agricultural practices should be applied as a prevention strategy. Continuous monitoring of the levels of various mycotoxins is essential for ensuring food safety and compliance with EU regulation. The ELISA method can be used as an easy and rapid method to screen samples for the presence of mycotoxins at the EU maximum allowable limits and prevent the distribution of unacceptably highly contaminated foodstuffs.

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