DETERMINATION OF THE PRESENCE OF MYCOTOXINS IN NUTS IN STAGES OF POST-HARVEST HANDLING AND STORAGE

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The potential sources of mycotoxin contamination of nuts and peanuts are microscopic fungi, which are an inseparable part of our life and environment. The *Aspergillus* toxinogenic species whose mycotoxins (aflatoxins) contaminate a wide spectrum of food and cause various toxigenic syndromes called mycotoxicosis represent an eminent problem worldwide. In the period from 2010 to 2015, 368 nut and peanut lots were sampled for aflatoxin determination by HPLC at different stages of post-harvest handling and storage, i. e. placement on the market and during import from Third Countries. The presence of aflatoxins in hazelnuts, peanuts, cashews and almonds did not exceed maximum levels set by the European Commission. The aflatoxin concentration in Brazil nuts and pistachios exceeded the set maximum levels in 16 samples. These results may contribute to the implementation of effective measures in order to eliminate risk connected with aflatoxin formation and food safety.
Keywords: aflatoxins, *Aspergillus Flavus, Aspergillus Parasiticus*, HPLC

Bestimmung des Vorhandenseins von Mykotoxinen in Nüssen nach der Ernte und Lagerung. Die potenziellen Quellen für eine Mykotoxinkontamination von Nüssen und Erdnüssen sind mikroskopisch kleine Pilze, die untrennbar Bestandteil unseres Lebens und unserer Umwelt sind. Die toxinogenen Arten von *Aspergillus* deren Mykotoxine (Aflatoxine) ein breites Spektrum von Nahrungsmitteln kontaminieren und verschiedene toxigene Syndrome verursachen, die als Mykotoxikose bezeichnet werden, stellen weltweit ein bedeutendes Problem dar. Im Zeitraum von 2010 bis 2015 wurden 368 Nuss- und Erdnuss-Chargen zur Bestimmung von Aflatoxin durch HPLC in verschiedenen Stadien nach der Ernte und Lagerung untersucht, d. h. bei Inverkehrbringung und beim Import aus Drittländern. Der Gehalt an Aflatoxinen in Haselnüssen, Erdnüssen, Cashewnüssen und Mandeln überschritt die von der Europäischen Kommission festgelegten Höchstgehalte nicht, lag aber bei Paranüssen und Pistazien in 16 Proben über dem festgelegten Höchstwert. Diese Ergebnisse können zur Einführung wirksamer Maßnahmen beitragen, um Risiken für die Lebensmittelsicherheit im Zusammenhang mit der Bildung von Aflatoxin zu beseitigen.
Schlagwörter: Aflatoxine, *Aspergillus Flavus, Aspergillus Parasiticus*, HPLC

The objectives of the presented study are to determine the source of the nut contamination by mycotoxins (aflatoxins), to analyse the factors which have an effect on the production of mycotoxins, to monitor and to analytically determine aflatoxin presence in nuts and peanuts at the different stages of the food chain. Mycotoxins are toxigenic secondary metabolites of filamentous fungi, which can contaminate a wide spectrum of food. The mycotoxins are regarded as high risk contaminants with acute or chronic effects on humans and animals and a food safety issue globally. The filamentous fungi cause the decomposition and degradation of agricultural products and food, development of diverse diseases of plants, infectious illnesses of people, allergy or poisoning including food destruction, which is a serious problem worldwide. According to Halász et al. (2009), it is essential to eliminate mycotoxin contamination and prevent hazards related to toxins. The main fungal aflatoxins producers are *Aspergillus flavus* and *Aspergillus parasiticus.* The tropical and sub-tropical areas are the most convenient environment for the *Aspergillus* species development with subsequent aflatoxin formation particularly in damaged or stressed crops (Battilani, 2010). Especially the climatic conditions in planting areas, the crop genotype and daily temperatures in connection with net evaporation play a significant role (Ono et al., 1999; Wilson and Payne, 1994; Brown et al., 2001; Bankole and Mabekoje, 2004). The products damaged by insects, birds, hailstones, long-lasting droughts during growing, inadequate agrotechnical measures, fungicidal doses, mineral and nutrient deficiency and careless harvest and post-harvest manipulation are a suitable environment for fungi development with a subsequent mycotoxin formation. The aflatoxins B1, B2, G1 and G2 belong to the naturally occurring aflatoxins of the B and G range. The aflatoxins B1, G1 are classified as carcinogens owing to the double bond in the terminal furan ring, which is under the influence of oxidation by hepatic enzymes to an epoxide that can intercalate into DNA as published by (Eaton and Gallagher, 1994). The aflatoxins produced by filamentous micromycetes cause aflatoxicosis and have a cumulative effect with a risk of liver cancer. The aflatoxicoses are characterized by severe bleeding, acute kidney damage and failure, edema and death (Liu and Wu, 2010). From the point of view of Brown et al. (1999), the set and enforcement of aflatoxin maximal levels by the European Commission and the implementation of good manufacturing practices have mostly decreased harmful exposures in developing countries. Nevertheless, the application of mentioned strategies in these countries is difficult due to the financial aspect and farming subsistence, impossible screening and testing of all food and feed lots (Abbas et al., 2004; Strosnider et al., 2006).

MATERIAL AND METHODS

MATERIAL

The nuts and peanuts for aflatoxin determination were sampled in the period from 2010 to 2015 at stages of import from Third Countries and placing on the market (processing, distribution and selling). The peanuts, cashews, hazelnuts, almonds, Brazil nuts and pistachios were sampled at producers (in shipping room or storeroom), in warehouses and retails. Apart from pistachios and cashews, the peanuts and nuts were also sampled during import from the Third Countries in Designated Points of Import (DPI). The presence of aflatoxins B1, B2, G1 and G2 in μg/kg in peanuts and nuts was monitored, analysed and determined in 368 samples in hazelnuts from Turkey and Georgia (84), in peanuts from Argentina and China (60), in cashews from India (33), in Brazil nuts from Bolivia (17), in almonds from the United States (107) and in pistachios from the United States and Iran (67).

SAMPLE PREPARATION

The nut and peanut samples (ranging between 1 to 10 kg according to lot amount) were homogenized including nutshells in a large capacity homogenizer (Silverson type L4RT, Silverson Machines Ltd, Chesham, UK) after the addition of 1 to 2 multiples of water at 5000 revs/min for about 15 min, subsequently together with extracting the solvent – water/methanol mixture. In case of nuts and peanuts in shells, the coefficient for

recalculation of the result on the edible quotient was determined by shucking and weighing 100 nuts and peanut pieces. The filtered extract was diluted with a phosphate buffered saline, subsequently cleaned and concentrated by extraction on solid phase using immunoaffinity bases founded in the interaction antibody-antigen.

METHODS

The laboratory analysis for aflatoxin determination in nuts and peanuts was carried out by the accredited HPLC method according to ČSN EN 14123 (56 0069), Foodstuffs – Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in nuts, peanuts, pistachios, figs and powdered pepper – High-performance liquid chromatographic method.

CHEMICALS

Phosphate buffered saline, pH = 7.4 (8 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate dissolved in 1 litre of water), potassium bromide, acetonitrile, methanol, toluene, nitric acid); sodium chloride (Ing. Petr Švec - PENTA s.r.o., Prague, CZ); potassium bromide, acetonitrile, methanol, toluene, nitric acid (Sigma-Aldrich s.r.o., Prague, CZ); potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate (Erba Lachema s.r.o., Brno, CZ).

HPLC – AFLATOXIN ANALYSIS

The aflatoxins were divided (separated) by isocratic HPLC (Agilent type 1100 Series, Agilent Technologies International, Santa Clara, USA) in a reverse phase (RP-HPLC – chromatography column C18, length 25 cm, internal mean 4.6 mm, particles size 5 μm), at laboratory temperature, with a mobile phase (water:methanol:acetonitrile 56:22:22) + 119 mg/l KBr + 350 μl/l 4M-HNO3) and detected after post-column derivatization on a fluorescence detector at wavelengths Ex = 365 nm and Em = 435 nm. The flow rate of the mobile phase was 1 ml/min. The aflatoxins were eluted from the chromatography column in sequence (order) G2, G1, B2, B1 in 12 minutes.

STATISTICAL ANALYSIS

The results were statistically evaluated by a non-parametric Kruskal-Wallis test.

RESULTS AND DISCUSSION

The presence of aflatoxins was analysed in 368 samples of peanuts and nuts. The measured values were compared to the set maximum levels for aflatoxins in the Commission Regulation (EC) No 1881/2006. The concentration of aflatoxins in hazelnuts, peanuts, cashews and almonds did not exceed the set maximum levels for aflatoxin B1 and the total content of aflatoxins B1, B2, G1 and G2 (μg/kg). According to the study by Abdulkadar et al. (2004) in Qatar 23.4 % of nut samples contained a total content of aflatoxins B1, B2, G1, G2 ranging from 0.53 to 289 μg/kg. Aflatoxin presence was detected in pistachios and peanuts. Almonds, cashews, walnuts and hazelnuts were not contaminated with aflatoxins. In comparison with the research by Bhat et al. (1996), 21 % of peanut lots sampled in 11 states of India exceeded the Indian regulatory limits for aflatoxins of 30 μg/kg, especially the peanut lots from the states Andhra Pradesh, Haryana and Gujarat. In the state of Gujarat the maximum level of aflatoxin contamination of 833 μg/kg was determined. Additionally, according to Ostadrahimi et al. (2014), a survey in Zanjan (Iran) proved that 60 % of salted peanuts and 93.7 % of pure samples were contaminated with AFs (aflatoxins). In the study by Alwakeel (2011) in Saudi Arabia, the aflatoxin concentration in peanuts was 28 μg/kg. The aflatoxin formation in nuts and peanuts is not homogenous. The growth of microscopic fungi and the formation of mycotoxins are especially higher in damaged products. Furthermore, within the exploration of total aflatoxin levels in some dried nuts and spices in markets and spice shops in Istanbul by Yilmaz and Garipoğlu (2014), aflatoxins were determined in 156 (30 %) of 513 hazelnut samples, in 93 (51.96 %) of 179 pistachio samples, in 107 (43.85 %) of 244 almond samples and in 23 (44.23 %) of 52 peanut samples. High temperatures, relative humidity, low light intensity and long-term storage can significantly influence aflatoxin formation.

By monitoring aflatoxins in Brazil nuts during import

from Third Countries it was discovered that Brazil nuts from Bolivia exceeded the set maximum levels of aflatoxin B1 5.0 µg/kg and the total content of aflatoxins B1, B2, G1, G2 10.0 µg/kg in two samples in 2010 and in one sample in 2014 (Fig. 1; Table 1.).

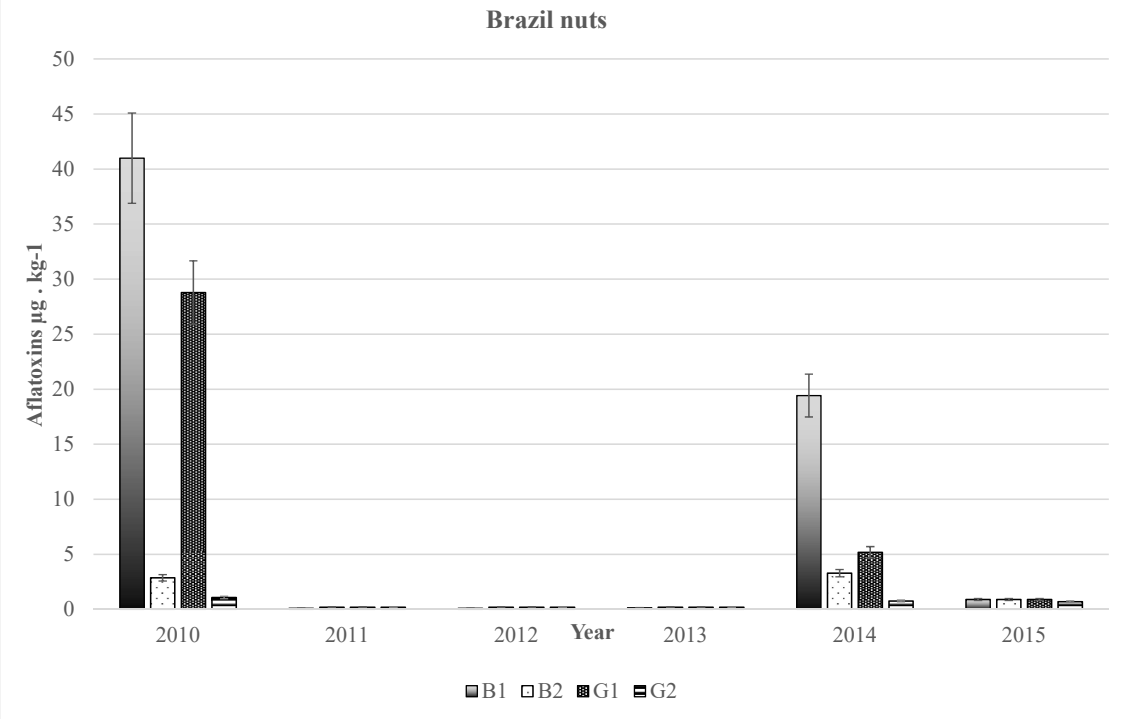

Fig. 1: Aflatoxins (in µg/kg) in Brazil nuts from Bolivia; maximum level for aflatoxin B1 = 5 µg/kg, maximum level for total contents of aflatoxins B1, B2, G1, G2 = 10 µg/kg

Table 1: The concentration of aflatoxins (µg/kg) in Brazil nut samples analysed in 2010 and 2014 exceeding set maximum levels

Brazil nuts (Bolivia)	AF B1 µg/kg	AF B2 µg/kg	AF G1 µg/kg	AF G2 µg/kg
2010	46.37 ± 0.45	3.07 ± 0.06	33.30 ± 1.00	1.30 ± 0.00
	35.60 ± 0.20	2.67 ± 0.06	24.27 ± 0.06	0.87 ± 0.06
2014	56.47 ± 0.30	8.07 ± 0.09	13.75 ± 0.16	0.89 ± 0.00

In 2010, the Rapid Alert System for Food and Feed (RASFF) reported a total of 427 notifications for mycotoxins in nuts, nut products and seed, with two consignments of Brazil nut kernels from Bolivia (first consignment with a content of aflatoxin B1 = 46.4 µg/kg, the second one B1 = 35.6 µg/kg; Tot. = 63.5 µg/kg). In 2014, the RASFF reported one consignment of Brazil nuts from Bolivia with exceeding aflatoxin limits, from a total of 220 notifications for mycotoxins in nuts, nut products and seed, the consignment was rejected at the border with a content of aflatoxin B1 = 56.47; Tot. = 79.18 µg/kg (European Commission, 2010 - 2014). Brazil nuts are planted in the Amazon rainforest located in Brazil, Bolivia, and Peru with minimal agrotechnical treatment and pest control. The aflatoxin contamination of Brazil nuts exceeding limits set by the European Commission has led to extensive export reduction as is published by Andrade et al. (2012). The cause is direct contact of fruit pods with the soil before harvest and their invasion by the *Aspergillus* species (Pacheco and Scussel, 2009). On the basis of statistical analysis, the contamination of pods by A. flavus lying on the soil can reach 43 % per day (Arrus et al., 2005). By analysing pistachio samples, it was proved that the concentration of aflatoxins in pistachios from Iran exceeded the set maximum levels of aflatoxin B1 and the total content of aflatoxins B1, B2, G1 and G2 (µg/kg) in two samples in 2010, in nine samples in 2011, in one sample in 2012 and in one sample in 2013. (Fig. 2). All non-conforming samples came from Iran, the country with high risk as for aflatoxin contamination of pistachios. In 2013 the Iranian pistachio sample was contaminated with a level of 516.47 µg aflatoxins B1/kg, with a level of 575.24 µg for the total content of aflatoxins B1, B2, G1 and G2/kg. (Table 2.) From Table 3, the summary of RASFF notifications in the period from 2010 to 2013, is apparent, how risky pistachios are regarding aflatoxins. According to Cheraghali et al. (2007) pistachios are highly contaminated by aflatoxins. Yilmaz and Garipoğlu (2014) published that 367 samples of pistachios produced in the period from 2002 to 2004 were checked. 33 samples exceeded the maximum levels set by Turkish and European legislation. According to Doster and Michailides (1995) and Battilani (2010) the early split of pistachio hulls before harvest can provoke infection by the Aspergillus species and subsequent AF formation. Lynch and Wilson (1991) reported that insect Navel Orange worm larvae (*Amyelois transitella*) damage tree nuts enabling spore dissemination afterwards. The correlation between

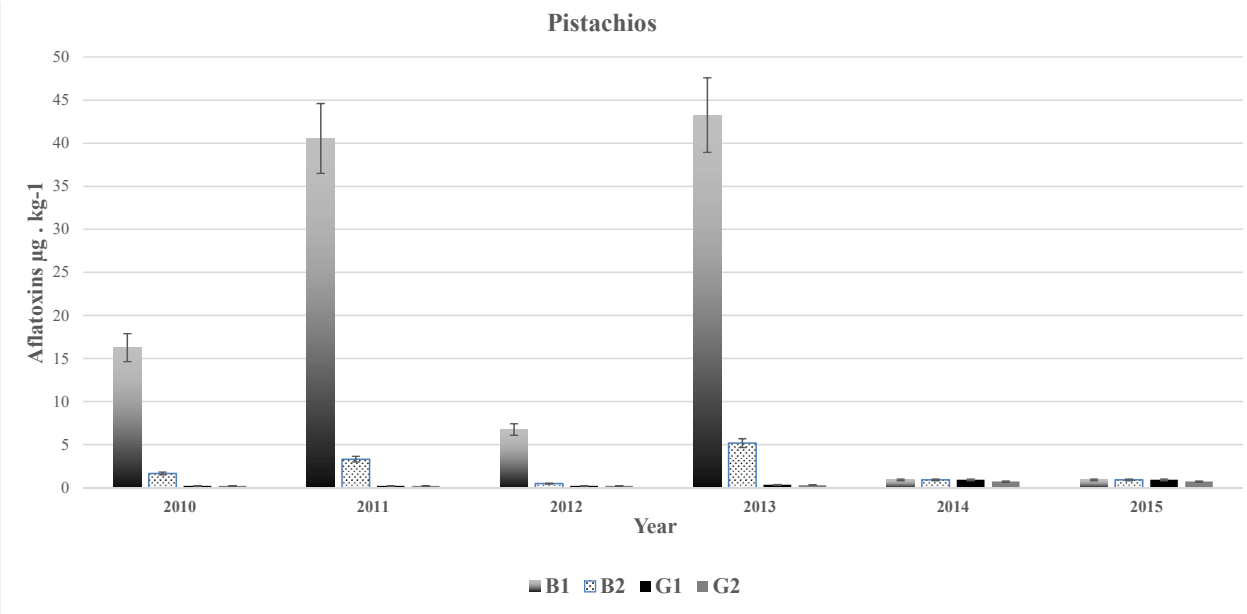

Fig. 2: Aflatoxins in pistachios from Iran and USA (in µg/kg); maximum level for aflatoxin B1 = 8 µg/kg, maximum level for total contents of aflatoxins B1, B2, G1, G2 = 10 µg/kg

Table 2: The concentration of aflatoxins (µg/kg) in pistachio samples analysed in 2010, 2011, 2012 and 2013 exceeding set maximum levels

Pistachios (Iran)	AF B1 µg/kg	AF B2 µg/kg	AF G1 µg/kg	AF G2 µg/kg
2010	40.4 ± 0.1	1.8 ± 0.00	< 0.2	< 0.2
	179.5 ± 0.00	18.77 ± 0.06	< 0.2	< 0.2
2011	92.27 ± 2.4	7.03 ± 0.12	< 0.2	< 0.2
	26.07 ± 0.15	2.7 ± 0.10	< 0.2	< 0.2
	263.9 ± 4.15	21.1 ± 0.2	< 0.2	< 0.2
	61.7 ± 0.1	4.37 ± 0.06	< 0.2	< 0.2
	307.67 ± 2.52	23.3 ± 0.3	< 0.2	< 0.2
	22.99 ± 0.06	1.19 ± 0.01	< 0.2	< 0.2
	40.8 ± 0.00	5.2 ± 0.00	< 0.2	< 0.2
	20.37 ± 0.75	2.0 ± 0.3	< 0.2	< 0.2
	55.17 ± 1.95	3.3 ± 0.00	< 0.2	< 0.2
2012	51.17 ± 0.55	2.6 ± 0.00	< 0.2	< 0.2
2013	516.47 ± 0.15	58.37 ± 0.85	< 0.2	< 0.2

high temperature and A. flavus invasion is significant. The aflatoxin contamination depending on early split can be decreased by the irrigation of pistachios in the orchards (Battilani, 2010). Furthermore, high aflatoxin concentration in nuts can be elicited by obsolete or inappropriate technological procedures, storage, manipulation and distribution. Surprisingly, the results of Ostadrahimi et al. (2014) showed that walnuts (90 %) and pure pistachios (2.3 %) were the most and the least contaminated samples, respectively. The mean of aflatoxin concentration in salt-roasted samples (19.88 ± 19.41 µg/kg) was substantially higher than in the pure ones (6.51 ± 9.4 µg/kg). In particular, 58.6 % of salt-roasted pistachios, 48.4 % of salt-roasted peanuts and 47.6 % of walnut samples had a content higher than the maximum tolerated level of aflatoxins in Iran (MTL 15 µg/kg). In addition, the roasting procedure can destroy micromycetes, nevertheless it is not effective for aflatoxin reduction. As published by Basaran and Ozcan (2009), the risk of aflatoxin contamination is expected in nuts which are transported for long distances and stored under unhygienic, unventilated, inappropriate temperature and humid conditions for a long time.

CONCLUSION

The level of aflatoxin contamination depends on the range of factors which have effect at various stages of the food chain. The nut species, country of origin and year or period of growth and harvesting also play a significant role. According to the results obtained by the laboratory analyses, Brazil nuts and pistachios were the most dangerous nut species imported from Third Countries and placed on the market in the period from 2010 to 2015. It is necessary to eliminate risks connected with mycotoxin formation and set effective measures for food safety production. The implementation of preventive measures is the most effective strategy for the obviation of filamentous microscopic fungi, their spreading and secondary metabolite formation. It is the safest way to protect consumer health.

Table 3: Rapid Alert System for Food and Feed (RASFF) notifications on mycotoxins in nuts, nut products and seed in the period from 2010 to 2013

RASFF	Notifications on mycotoxins	Iranian pistachio lots with AFs
2010	427	56
2011	310	38
2012	204	20
2013	215	14

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