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Survey of aflatoxins and ochratoxin a contamination in food products imported in Italy

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ABSTRACT

In this study the levels of aflatoxins (AF B_1, B_2, G_1 and G_2) and ochratoxin A (OTA) were monitored in several food products imported in Italy with a high contamination risk. A total of 345 samples were collected from the Maritime Authority of Salerno Customs Port during the period from January 2008 to December 2009 and analyzed by immunoaffinity chromatography as clean-up, high performance liquid chromatography with fluorescence detection for quantification and tandem mass spectrometry for confirmation. The analytical methods were validated on different food matrices and meet the performance criteria set by EC Regulation No. 401/2006 for mycotoxin analysis. The results obtained in this survey showed that 7% of the total samples contained detectable levels of AFs and OTA, and 1.2% had AFs concentrations exceeding the maximum limits set by EU regulation. OTA was the most prevalent mycotoxin, with an incidence of 17.6% of samples analyzed for OTA. The highest detected levels were 23.70 μg kg^{-1} of OTA in a green coffee sample and 70.69 μg kg^{-1} of AFs in an apricot kernels sample. Among the food products analyzed, hazelnuts paste and dried vine fruits were the commodities mainly contaminated with AFs and OTA, respectively.

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1. Introduction

Mycotoxins are natural foodstuff contaminants mainly produced by fungi of genera *Aspergillus*, *Penicillium* and *Fusarium*. These mycotoxin-producing mould species can grow on a wide range of agricultural commodities in the field, but also during post-harvest operations and storage conditions (Zinedine & Mañes, 2009). The degree of contamination depends on several factors such as temperature, humidity and substrate. Mycotoxin contamination is a major problem in the tropics and sub-tropics areas, where climatic conditions and storage practices are favorable to fungal growth and toxin production (Kumar, Basu & Rajendran, 2008). Major food commodities affected are cereals, nuts, dried fruits, coffee, cocoa, spices, oil seed, dried peas, beans and fruits. Mycotoxins are presently considered as the most important chronic dietary risk factor, higher than synthetic contaminants, food additives, or pesticide residues (Van Egmond, Schothorst & Jonker, 2007). Among the known mycotoxins, ochratoxin A (OTA) and aflatoxins (AFs) are of greatest concern due to their frequent occurrence in foods and their severe effects on animal and human health.

AFs are difuranocoumarin derivatives produced primarily by two species of *Aspergillus (A. flavus* and *A. parasiticus*). AFs naturally occurring in foodstuff are AFB_1, AFB_2, AFG_1 and AFG_2 and they are most likely to contaminate nuts, figs and other dried fruits, spices, crude vegetable oils, cocoa beans and maize. Exposure to AFs is generally considered to occur mainly from imported materials from countries well known for their warm and humid climates. AFs have several toxic effects against animals and humans, including carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Eaton & Gallagher, 1994). AFB_1, the major AF produced by toxigenic strains, is the most potent hepatocarcinogen known in mammals and is classified by the International Agency of Research on Cancer (IARC) as human carcinogen (group 1) (IARC, 1993a) with a role in etiology of liver cancer, notably among subjects who are carriers of hepatitis B virus surface antigens (IARC, 2002).

OTA is an ubiquitous mycotoxin produced by the genera *Aspergillus* and *Penicillium*. Invasion with ochratoxin-producing fungal species has been reported worldwide: cereals and cereal products, dried fruits, coffee and wine are the most important sources of intake (Jørgensen, 2005). Exposure to OTA has been associated with a human disease of kidney referred to as Balkan endemic nephropathy and with high incidence of kidney, pelvis, ureter and urinary tumors (EFSA, 2006). The IARC classified OTA as a possible human carcinogen (group 2B) (IARC, 1993b) and the European Food

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Safety Authority (EFSA) has established for OTA a Tolerable Weekly Intake of 120 ng/kg bw (EFSA, 2006).

National and international institutions and organizations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), have recognized the potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication and the economic consequences of mycotoxin contamination were well demonstrated. Regulations have been established in many countries to protect consumers from the harmful effects of these natural toxins, as well as to ensure fair practices in food trade. The European Union (EU) has established with the Commission Regulation No. 1881/2006 severe limits for major mycotoxin classes in many products at high risk of contamination. As regards the AFs, the maximum levels (MLs) set by the EC in food for direct human consumption are 2 $\mu g\ kg^{-1}$ for AFB_1 and 4 $\mu g\ kg^{-1}$ for the sum of AFs, whereas for OTA, the MLs adopted ranging from 2 to 10 $\mu g\ kg^{-1}$ (European Commission, 2006a). This regulatory limits force all Member States to monitor and control mycotoxin levels in foodstuffs, which pass through the customs, in order to reduce the intake of this toxic metabolites.

The growing concern over food safety has led to the development of several methodologies for mycotoxins determination. Analytical procedures based on extraction with organic solvents, clean-up by immunoaffinity chromatography (IAC) and analysis by HPLC coupled to fluorescence detection (FLD), were widely used for the official control of the levels of AFs and OTA determination in many food commodities (Krska, Schubert-Ullrich, Molinelli, Sulyok, MacDonald & Crews, 2008). Methods of analysis used for food control purposes shall comply with the performance criteria set by European Regulation No. 401/2006 (European Commission, 2006b), to show that they produce accurate and reproducible results in order to monitoring and risk-assessment studies.

In this study, a large number of different food products imported from "high-risk" countries into Italy were analyzed to evaluate the occurrence of AFs and OTA. Analytical methods based on IAC followed by HPLC-FLD analysis, were used to determine the levels of mycotoxins in imported samples and the positive samples were confirmed by HPLC-tandem mass spectrometry methods highly selective for AFs and OTA. The procedures used for analysis of AFs and OTA in different food matrices were validated according to European Regulation No. 401/2006.

2. Materials and methods

2.1. Chemicals and reagents

Sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), sodium hydrogen carbonate ($NaHCO_3$), acetic acid and formic acid were provided by Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and water were purchased from Romil (Cambridge, UK). Analytical-grade dichloromethane, hexane and methanol were supplied by Carlo Erba reagents (Milan, Italy). Phosphate buffered saline (PBS) solution was prepared by dissolving 8 g NaCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.20 g KCl in 900 mL of distilled water. The pH of the solution was adjusted to 7.4 with HCl or NaOH. AFs mixture (B_1 and G_1, 2 $\mu g\ mL^{-1}$; B_2 and G_2, 0.5 $\mu g\ mL^{-1}$) and OTA (10 $\mu g\ mL^{-1}$) reference standard solutions in acetonitrile were obtained from LGC Promochem GmbH (Wesel, Germany). Stock solutions of AFs (B_1 and G_1, 20 $ng\ mL^{-1}$; B_2 and G_2, 5 $ng\ mL^{-1}$) and OTA (100 $ng\ mL^{-1}$) were prepared in acetonitrile and stored in amber glass vials at −20°C and held for at least three months. Certified reference materials (RM) of almond T0482 (AFs assigned values (satisfactory range): B_1 4.87 (2.73–7.01), B_2 1.84 (1.03–2.64), G_1 2.83 (1.58–4.07), G_2 0.71 (0.40–1.02) $\mu g\ kg^{-1}$), dried figs T04100 (AFs assigned values (satisfactory range): B_1 1.20 (0.67–1.73), B_2 1.10 (0.61–1.58), G_1 1.64 (0.92–2.36), G_2 0.94 (0.53–1.36) $\mu g\ kg^{-1}$) and cereal T1756 (OTA assigned value (satisfactory range): 2.99 (1.67–4.30) $\mu g\ kg^{-1}$) were purchased from FAPAS (The Food and Environment Research Agency, York, UK). As safety notes, all used laboratory glassware were treated with an aqueous solution of sodium hypochlorite (5%) before the discarding to minimize health risks due to AFs contamination.

2.2. Samples

A total of 345 samples (nuts, nut products, dried fruits, cereals, cereal products, pulses, dried vine fruits, coffee) imported from several countries (Algeria, Argentina, Australia, Bolivia, Bulgaria Brazil, Canada, Chile, China, Colombia, Costa Rica, Egypt, Ghana, India, Indonesia, Israel, Lebanon, Peru, Syria, Sri Lanka, Switzerland, Tunisia, Turkey, Uganda, USA, Vietnam) to Italy, were collected from the Maritime Authority of Salerno Customs Port (Italian Department of Health) during the period from January 2008 to December 2009, according to the methods set out in the Regulation (EC) 401/2006. All collected samples were stored under cool conditions and out of direct sunlight until the analysis and were processed for mycotoxin analysis within 36 hours. Samples, ranging from 5 to 30 kg, were ground and homogenized to achieve a representative sample and then dispensed into plastic bags. All samples, except dried figs and dried vine fruits, were milled and mixed thoroughly using a cutting mill SM 2000 (Retsch, Haan, Germany) with a sieve range of 0.5 mesh. For dried figs, the slurried samples were prepared by blending the sample with water (1:1) in a knife mill Grindomix GM 200 (Retsch), whereas for dried vine fruits samples the slurry was prepared using five parts of sample and four parts of water. Finally, aliquots of the primary samples were accurately weigh and analyzed within 24 h.

2.3. Sample preparation procedure

Samples preparation was conducted in according with instruction of immunoaffinity columns (IAC) manufacturing (LCTech GmbH, Dorfen, Germany) and are carefully described in the following sections.

2.3.1. Extraction and clean-up for AFs

Twenty g of thoroughly homogenized fatty matrix samples (nuts and nut products, apricot kernels, seeds), added to 2 g of NaCl, were extracted with 100 mL of a mixture of methanol/water (8:2, v/v) and 50 mL of *n*-hexane with a blender (Ultra–Turrax T25, Ika®–Werke GMBH & CO.KG, Staufen, Germany) at high speed for 1 min. After separation of the two phases, *n*-hexane was eliminated. The non-fatty matrix samples (chestnuts, cereals and cereal products, pulses) were extracted only with 100 mL of methanol/water (8:2, v/v). For the slurried samples (dried figs), 80 mL of methanol were used to extract 40 g of slurried figs. In this way, the water contained in the test portion of slurried samples (20 mL) was taken into account. Then, the extracts were filtered on a filter paper (Whatman No 44) and 10 mL were diluted with 40 mL of PBS solution. The diluted extract (50 mL, equivalent to 2 g of sample) was passed through the IAC (AflaCLEAN™, 3 ml, LCTech GmbH), previously equilibrated with 20 mL of PBS solution, at flow rate of 1–2 drops per second. The column was washed with 20 mL of distilled water and subsequently AFs were eluted with 1.5 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen and reconstituted in 1 mL of methanol/water (1:1, v/v) for HPLC analysis.

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