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Assessment of aflatoxin exposure of laboratory worker during food contamination analyses. Assessment of the procedures adopted by an A.R.P.A.L. laboratory (Liguria Region Environmental Protection Agency)

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Key words
Aflatoxins; food; laboratory workers

Summary
Background: *Aflatoxins are mycotoxins derived from foodstuffs colonized by fungal species of the genus* Aspergillus*; they are common food contaminants with immunosuppressive, mutagenic and carcinogenic activity. Aflatoxins are heat-resistant and are thus easily transmitted along the food chain. They are hepatotoxic and have the potential to induce hepatocellular carcinoma. Agri-food industry workers are thus at risk of ingestion as well as transmucosal absorption or inhalation of toxins released during product preparation or processing.* **Objectives:** *To measure the levels of airborne mycotoxins, particularly aflatoxins, in a laboratory analysing imported foodstuffs for mycotoxin contamination.* **Methods:** *The protocol used to analyse a batch of shelled peanuts from Vietnam, especially the grinding phase, which is held to be at the highest risk of generating airborne toxins, was assessed at the A.R.P.A.L. laboratory (Liguria Region Environmental Protection Agency) of Genoa, Italy, which participates in a European aflatoxin monitoring project.* **Results:** *Wet grinding was performed to avoid production of large amounts of dust. Comparison of airborne concentrations before and after grinding with legal thresholds disclosed that the analytical procedures involved negligible aflatoxin levels for operators (environmental burden 0,11 pg/ m^3).* **Conclusions:** *Given the toxicity of aflatoxins, worker protection measures should be consistently adopted and enforced. Threshold limit values for working environments should be introduced besides the existing ones for public health.*

Riassunto
«Valutazione dell'esposizione ad aflatossine nelle attività di ricerca di contaminazione delle derrate alimentari. Analisi delle procedure di un laboratorio A.R.P.A.L. (Agenzia Regionale per la Protezione dell'Ambiente Ligure)». *Le aflatossine, sostanze derivate dal metabolismo di alimenti colonizzati da specie fungine del genere* Aspergillus*, appartengono alla famiglia delle micotossine e sono frequenti contaminanti alimentari, in grado di esplicare azione immunosoppressiva, mutagena e cancerogena. Le aflatossine sono termoresistenti e si trasmettono attraverso la*

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catena alimentare e la loro tossicità interessa soprattutto il fegato, ove può indurre epatocarcinoma. Data la loro presenza nelle derrate alimentari i lavoratori dell'industria agroalimentare possono risultare esposti non solo all'ingestione ma anche all'assorbimento per via transmucosa o inalatoria di tossine liberate nella fase di preparazione o lavorazione del prodotto. Lo scopo di questo studio è stato analizzare l'eventuale aerodispersione di micotossine, in particolare di aflatossine, durante l'attività di laboratorio volta alla ricerca di micotossine contaminanti merci di importazione. Lo studio è stato eseguito nel laboratorio del Dipartimento A.R.P.A.L (Agenzia Regionale per la Protezione dell'Ambiente Ligure) di Genova, inserito in un progetto europeo di monitoraggio delle aflatossine, e si è concentrato sulle procedure di analisi di una partita di arachidi sgusciate provenienti dal Vietnam, in particolare sulle fasi di macinazione delle rinfuse di arachidi, ritenute il momento più delicato per una possibile aerodispersione. La macinatura è stata eseguita in umido, al fine di evitare la produzione di quantità rilevanti di polvere, e i dati raccolti prima e dopo la macinatura sono stati messi a confronto con i limiti di legge. A fronte della concentrazione presente nel campione analizzato l'esame dell'aerodisperso ha dato un valore assolutamente irrilevante per l'operatore grazie alla tipologia delle procedure di lavoro utilizzate (concentrazione ambientale di 0,11 pg/ m^3). Considerata la tossicità delle aflatossine si sottolinea l'importanza di adozione di misure di prevenzione degli operatori e si auspica l'introduzione di limiti di riferimento dedicati anche al mondo del lavoro e non solo alla salute pubblica.

Introduction

Foodborne illnesses ascribable to mycotoxicoses came to public attention in the 1960's, when an epidemic called turkey X disease, caused by peanuts contaminated with a toxin produced by *Aspergillus flavus*, affected thousands of turkeys in the UK.

Mycotoxins are a heterogeneous group of prevalently low molecular weight chemical substances produced by the secondary metabolism of some fungal species, mainly of the genera *Aspergillus*, *Penicillium* and *Fusarium*.

Of the more than 300 mycotoxins identified to date, some adversely affect cell functions, causing severe damage to human health and economic liabilities to breeders and farmers. Foodstuffs, cereals and feeds are ideal substrates for their growth.

The mycotoxins most consistently monitored by health authorities are those of the aflatoxin (AF) family (produced by some *Aspergillus* strains), the ochratoxin family (produced mainly by fungi of the genera *Aspergillus* and *Penicillium*), patulin (produced by a large number of *Aspergillus* and *Penicillium* fungi), and zearalenone, fumonisins and trichothecenes (produced especially by fungi of the genus *Fusarium*).

In suitable conditions, mycotoxins can arise at any stage of the production or processing cycle, either through direct food infestation or during processing, transport, or stocking of the food product.

For instance, formation of toxigenic moulds during culture is favoured both by climate (humidity and temperature) and by pests. Contamination after culture is affected by time of harvest, level of maturation and humidity, and even physical operations. Storage temperature and humidity, besides the duration of storage in closed environments such as silos, critically influence mould formation (6).

AFs are widely investigated cytotoxic mycotoxins and are those raising the greatest concern, both as contaminants of the food consumed by a large part of the world's population and in relation to the need to protect those who are at greatest risk of occupational exposure.

Chemically AFs are coumarin derivatives, designated as B_1, B_2 (respectively methoxy-difuro-coumarone and methoxy-difuro-coumaro-lactone), G_1, G_2 (their dihydro derivatives), and M_1 and M_2 (hydroxylic metabolites of B_1 and B_2 respectively, found in the milk of cows receiving feeds contaminated with B_1 and B_2 AFs). The letters B and G indicate the (blue or green) fluorescence emitted upon 360 nm UV irradiation; M is the initial of the hydroxylated product that is found in peanut milk. G aflatoxins contain a lactone ring, and B AFs a cyclopentenoic ring that underpins their greater toxicity.

AFs are preferably produced on carbohydrate-rich substrates. B_1 and B_2 AFs are produced by *A. flavus* and *Aspergillus parasiticus*, while G_1 and G_2 are produced only by the latter. They are crystalline structures soluble in moderately polar organic solvents such as chloroform, methanol (useful for compound extraction), and dimethyl sulfoxide; they are not easily soluble in water (10-30 μg/ml) and are insoluble in non-polar organic solvents. AFs are produced by fungal species in a wide variety of conditions, including free water values (aw) in the range of 0.82-0.87 and temperatures of 6-46°C for growth and 8-42°C for synthesis. Pure AFs are stable in the absence of light and are degraded by UV radiation, they are unstable at pH < 3 and > 10 and in the presence of oxidants. Some are endowed with native fluorescence, which is used for the detection and sorting of contaminated items (9).

Their wide-ranging activity stems from an ability to react with nucleic acids and cellular nucleoproteins, exerting adverse effects on protein synthesis and cell integrity. The main effects of mycotoxin exposure on human health are reported in table 1. Acute aflatoxin B_1 (AFB_1) administration is highly toxic in all species studied, with LD_{50} 0.5 mg/kg in ducklings, 60 mg/kg in mice, and 0.6 to 10 parts per million (ppm=mg/kg) in man.

AFs are absorbed in the GI tract, where they are metabolically activated or detoxified in the intestinal mucosa and liver. AFB_1 biotransformation varies widely between species and is greatly influenced by endogenous and exogenous factors. It takes place through epoxidation, hydroxylation, O-demethylation, conjugation and non-enzymatic processes. In particular, AFB_1 undergoes cytochrome P-450-dependent oxidation, with formation of various hydroxylated metabolites and 8,9-epoxy, an unstable, highly biologically reactive electrophile that can bind to proteins, affecting cell function, and/or nucleic acids (especially AFB-N7-guanine), inducing DNA damage. The genotoxic effect of AFs, particularly B1, was seen in *in vitro* and *in vivo* experimental systems. AFB1-N7 guanine adducts were detected in the blood and urine of individuals exposed via dietary and respiratory modes to AF (8, 17). Given its genotoxicity, a dietary consumption threshold cannot be established; the aim must thus be for exposures as low as reasonably achievable (ALARA).

Table 1 - Main effects of mycotoxin exposure on human health

Mycotoxin	Effects
Aflatoxin B_1	Carcinogenic, hepatotoxic, immunosuppressive, genotoxic
Ochratoxin A	Carcinogenic, nephrotoxic, immunosuppressive, teratogenic
Fumonisins	Carcinogenic, neurotoxic, cytotoxic
Trichothecenes	Dermatotoxic, immunosupressive, haemorrhagic
Zearalenone	Oestrogen-like
Patulin	Cytotoxic, immunosuppressive

Over the last few years *in vitro* and *in vivo* studies have documented that AFs alter macrophage and NK cell activity, inhibiting the production of interleukins involved in immune and anti-inflammatory responses. Their immunosuppressive activity could contribute to the increased incidence of lethal respiratory diseases recorded in the populations eating food contaminated with these mycotoxins.

The carcinogenic effects of AFs have been investigated in experimental models and epidemiological studies (10, 12, 13, 16). The most active AF *in vivo* was AFB1; the activity of the other AFs is lower, and has been estimated to be B1>M1>G1> B2>G2, in descending order.

Liver tumour is the main AFB1-induced neoplasm in various animal models (rat, mouse, monkey), but stomach, pancreas, lung, colon and rectum can also be affected (4, 7). A significant correlation was found in epidemiological studies between the incidence of hepatocellular carcinoma and consumption of foodstuffs containing AFs in populations inhabiting the geographical areas where climate and sanitary conditions promote *A. parasiticus* and *A. flavus* food contamination.

Epidemiological and carcinogenetic evidence has led AFB1 to be classified by the International Agency for Research on Cancer (IARC) as group 1, whereas demonstration of carcinogenic effects

Table 2 - IARC classification of carcinogenicity

Group	Carcinogenicity
1	Carcinogenic to humans; sufficient evidence of carcinogenicity in human from adequate epidemiological studies excluding random, confounding and distortion effects
2A	Probably carcinogenic to humans on limited evidence from epidemiological studies and sufficient evidence in small rodents
2B	Possibly carcinogenic to humans on limited evidence in man and non-sufficient evidence in animals, or on inadequate evidence in man
3	Non classifiable as to carcinogenicity (inadequate evidence)
4	Probably not carcinogenic to humans based on evidence suggesting lack of carcinogenicity in rodents and man, in some cases lack of carcinogenicity in rodents, and inadequacy or lack of data on man, in presence of other relevant data

only in some animal models (rat and trout) has led to inclusion of AFM1 into group 2B (possibly carcinogenic to humans; table 2).

Cohort and case control studies of hepatitis B virus (HVB) patients performed in South-East Asia (Taiwan, Shanghai and the Quidong region of China) towards the end of the last century highlighted an association between liver tumour and urinary AF metabolites. Epidemiological data indicated that AFB1 has a synergistic role with HVB in inducing hepatocellular carcinoma. Although positivity for either AFB1 or HBV increases the risk of liver tumour, the presence of both factors is associated with a 60-fold greater likelihood of developing it. HBV can increase AF metabolism, whereas AF accompanied by chronic HBV infection would be the trigger converting the HBV-related liver injury to hepatocellular carcinoma (15).

As regards AF exposure, a paper published in the AHIA journal in 1998 documented that ATB1 adheres to airborne dust dispersed in the farm environment, and that samples collected during wheat harvesting and loading into silos contained 0.04-92 ng/m^3 concentrations. Higher levels (5-421 ng/m^3) were measured in closed buildings where animals are reared and fed, and during silos cleaning (124-4849 ng/m^3) (14). Several studies investigated wheat milling. In particular, Dacarro et al. (3), who devised a microbial contamination detection method for all stages of production, documented that the phase with the highest concentrations is wheat cleaning.

In 2003 Desai and Ghosh published a study where they sampled airborne fungi in a rice mill using two multilayer samplers, an *Impinger* and a high volume sampler. The most represented fungus was *Aspergillus* (in particular *A. flavus*), irrespective of the collection method used. The authors established that inhaled dust was mainly produced by grinding, and that 8 h exposure of workers to such dust was associated with a risk of inhalation of spores or AF-containing mycelial fragments (5).

Experimental rat and hamster studies showed that after ATB1 tracheal instillation (comparable to inhalation), the lung was the main site of molecule uptake, and lung epithelium the site of molecular epoxide activation with binding to lung cell DNA (2).

In a later study AFB1 instilled in pig trachea (2000) was absorbed by the trachea, enhancing the activity of prostaglandins (PGE1 and PGE2), which at lung level have smooth muscle cell (SMC) relaxing properties; the bronchodilation effect was not affected by beta blockers. Bronchial SMC exhibit increased cyclic AMP, conceivably due to inhibition of phosphodiesterase activity (1).

Earlier studies (1989) had already examined the possibility of transcutaneous absorption, and found that it was enhanced in presence of 1) high concentrations of airborne mycotoxins, 2) occlusive bandages, and 3) conditions capable of promoting absorption (irritative dermatitis) (11).

MATERIALS AND METHODS

Laboratory worker exposure to airborne AFs was assessed during grinding of foodstuffs at the Liguria Region Protection Agency (A.R.P.A.L.). Samples were collected in the A.R.P.A.L. Departmental Laboratory (Genoa, Italy), which analyses foodstuffs imported from EU and non-EU countries according to European directives No. 1881/2006 and No. 1126/2007. One task of A.R.P.A.L. is the search for contaminants such as mycotoxins.

Airborne mycotoxins, particularly AFs (B1, B2, G1, G2) were assessed in collaboration with *Line IC/HPLC Instrumental Analysis Section* during routine grinding of a batch of shelled peanuts from Vietnam. Overall, 8.8 kg of shelled peanuts were examined using the standard protocol. Peanuts were placed in a grinder to obtain slurry; about 9 l of water were added to reduce pollution and achieve compound homogeneity. Environmental air was continuously sampled using an impinger with 50 ml of 80% methanol solution, 1 l/min velocity (aerosol) and a cyclone sampler with a fibreglass filter (diameter 7 mm) set at a velocity of 20 l/min for 20 min (total airborne dust).

The mycotoxin protocol envisages collection of ground material and the addition of 5 g NaCl and 200 ml extraction solution (80% methanol) to slurry extract (50 g) under the hood. Extraction is then performed in a horizontal mechanical shaker for 30 min. After filtration 5 ml is collected and brought to a volume of 100. The solution is concentrated and purified in an immunoaffinity column containing monoclonal antibodies. The elute is collected in vials and further concentrated to 0.5 ml for HPLC analysis.

The environmental samples were analysed in succession.

The fibreglass filter was removed from the cyclone and treated as a sample by extraction with 80% methanol solution for 30 min, followed by the procedure described above for the peanut sample.

For the impinger, 50 ml of product was first diluted 1:10 to avoid column overloading with methanol.

RESULTS AND CONCLUSIONS

Filter analysis revealed AFB1 in the airborne dust at a concentration of 0.11 pg/m^3 air and lower concentrations of AFB2. The foodstuff concentration before processing was 6.18 mg/kg, which exceeded the legal maximum as per EC regulation no. 466/2001 (table 3). The amount of aflatoxin B1 found (0.11 pg/m^3) is related to the presence of aflatoxin in the particulate which originates during sample grinding with the slurry technique, and it is a small amount compared with the amount of aflatoxin B1 in the sample. This demonstrates the effectiveness of wet grinding. It was not possible to find a correlation between the two values through the calculation of the amount of liquid vaporized: the value found in the atmosphere was not produced by the aerosol of the liquid during grinding because the determination of AFB1 in the aerosol, made with an impinger containing a methanolic solution, gave a negative result.

The airborne concentrations yielded a negligible value for worker exposure. Indeed, the worker protection measures included in the standard protocol envisage wet grinding to avoid production of large amounts of dust. The data demonstrate that myco-

Table 3 - Maximum limits of some contaminants found in foodstuffs as per EC Regulation No. 466/2001

Aflatoxins			
Product	Aflatoxin Maximum limits (ppb)		
	B_1	B_{1+} B_{2+} G_{1+} G_2	M_1
Peanuts, shell fruits and dried fruits for direct consumption or as food ingredients*	2.0	4.0	--

* The maximum limits apply to the edible part of groundnuts, nuts and dried fruits. If nuts "in shell" are analysed it is assumed when calculating the aflatoxins content that all contamination is on the edible part

toxin exposure in agri-food industry workers can occur, given their presence in a wide range of food products. With reference to the actual occupational *risk* it should be noted that in the case of mycotoxins: 1) the *likelihood* of absorption via inhalation is concrete and varies with the type of task performed and the observance of procedures; 2) the *severity of the risk* is clearly considerable, since the epidemiological and carcinogenetic data have led the IARC to include *AFB1* under Group 1. The potential presence of mycotoxins as carcinogenic contaminants in foodstuffs is a crucial element in the adoption of protective measures for agri-food worker safety, especially those employed in processing. The present study highlights the effectiveness of the wet grinding procedure in reducing contaminant concentrations in the working environment. As regards the regulations in force, a threshold is specifically set only for food poisoning. According to the evaluation of both toxic and carcinogenic effects of aflatoxins as a specific pollutant, observing standard operating procedures seems to be an evident top priority in managing healthcare of agrifood sector workers.

NO POTENTIAL CONFLICT OF INTEREST RELEVANT TO THIS ARTICLE WAS REPORTED

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