

Aflatoxin contamination in nuts marketed in Italy: preliminary results

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Parole chiave: Frutta a guscio, micotossine, aflatossina, mandorle, semi di albicocca, pistacchio

Abstract

Background. Aflatoxins (AFs) are one of the main groups of mycotoxins produced by molds. Nuts, although recognized as a food with health benefits, are frequently contaminated by AFs.

Study design. In this preliminary study we evaluated the contamination by total AFs and AFB1 in different types of nuts from different countries marketed in Apulia.

Methods. Overall, 124 samples (almonds, apricot kernels, chestnuts, hazelnuts, peanuts, pistachios, walnuts and Brazil nut) were analyzed using an High-Performance Liquid Chromatography system.

Results. Twenty samples (16.1%) were contaminated with AFs of which 55% were non-compliant, according to Reg. 165/2010. The median values ($\mu\text{g}/\text{kg}$) of total AFs and AFB1 were 16.6 and 15.1, respectively. Pistachios appeared more susceptible to AF contamination than the other nuts, with levels of total AFs ranging from 8.8 to 387.3 $\mu\text{g}/\text{kg}$ and of AFB1 from 8.2 to 354.5 $\mu\text{g}/\text{kg}$. The majority of contaminated samples came from Asia and AF contamination was different in the various Asiatic sub-regions: regardless of the type of nuts, samples from Western Asia were the least contaminated.

Conclusions. As geographical origin may influence the risk of contamination, in order to protect human health, customer countries should increase AF monitoring in nuts coming from those countries with favorable environments for the growth of aflatoxigenic molds or with less strict regulations.

Introduction

Aflatoxins (AFs) are one of the main groups of mycotoxins produced in nature (1-3). They are secondary metabolites produced by fungi belonging to the genus *Aspergillus* (3). They are able to cause several diseases with carcinogenic, mutagenic, teratogenic and immunosuppressive effects (4-6). *Aspergillus flavus* and *A. parasiticus*

are the main species responsible for food contamination. Under favorable conditions (e.g. high temperature, high relative humidity, unseasonal rains, poor harvesting practices and improper storage during transport and marketing) they produce aflatoxin B1 (AFB1), B2 and AF (B1, B2, G1, G2), respectively (7-9). In particular, AFB1 is the major AF produced by toxigenic strains and is classified by the International Agency for

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Research on Cancer as a human carcinogen (group 1) (10), in particular, it can play an important role in the etiology of liver cancer (11).

Nuts, although recognized as a food with health benefits such as the prevention of coronary heart disease and diabetes (12-14), are frequently contaminated with AFs. Many European countries have established the restriction of aflatoxins in nuts through the Commission Regulation (EU) of European Union No. 165, dated February 26, 2010 (15). This regulation establishes maximum levels for AFB1 and total AFs (B1, B2, G1 and G2). In addition, the regulation distinguishes between nuts subject to sorting or other physical treatment (e.g. bleaching) before human consumption or before their use as an ingredient in foodstuffs, and nuts intended for direct human consumption or use as an ingredient in foodstuffs.

Italy is one of the major consumers of nuts in the world, both as direct consumption and as ingredients in confectionary and baked foods (16). Given the high consumption, Italian nut production is insufficient to cover its domestic demand, therefore a significant amount is imported.

Aim of this preliminary study was to evaluate the amount of contamination by AFs and AFB1 in different types of nuts, intended for direct human consumption, from different countries.

Materials and Methods

Samples

A total of 124 nuts samples were randomly collected from January 2014 to December 2015, by local Health Authorities of Apulia, Southern Italy, in accordance with the frequency of checks established by regulations (17, 18). The samples were mostly imported from America, Africa, Asia, Oceania and Europe and included 46 almonds, 27 apricot kernels, 14 chestnuts, 14

hazelnuts, 9 peanuts, 8 pistachios, 5 walnuts and 1 Brazil nut (Table 1).

Following Reg. (EU) No. 178/2010 amending Reg. (EC) No. 401/2006 (19), the nuts, ranging from 2 to 20 kg weights, were stored in the dark before analysis, at low relative humidity and above 20°C. Nuts with shells were dehulled and pulverized using a Robot Coupe Blixer 5 Plus (Robot-Coupe, Vincennes Cedex, France).

Chemicals and reagents

Sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), NaOH, acetonitrile, and methanol were purchased from Carlo Erba (Cornaredo, Milano, Italy); potassium chloride (KCl) from VWR International Srl (Milan, Italy) and potassium dihydrogen phosphate (KH_2PO_4) from Sigma-Aldrich Srl (Milan, Italy). To prepare phosphate buffered saline (PBS) solution, 8.18 g NaCl, 1.15 g Na_2HPO_4 , 0.2 g KCl and 0.2 g KH_2PO_4 were dissolved in distilled water, adjusting the pH to 7.2–7.4 with HCl or NaOH solutions and reaching a final volume of 1 L (20). Immunoaffinity columns (IAC) were obtained from Neogen (Lansing, MI, USA). The reference standard solutions of AF mixture (AF total 5 µg/mL: B1 and G1, 2 µg/mL; B2 and G2, 0.5 µg/mL) in acetonitrile were obtained from Romer Labs Division (Getzersdorf, Austria). Stock solutions of AFs (500 ng/mL and 100 ng/mL) were prepared in acetonitrile and stored in amber glass vials, away from light, at -20°C.

Extraction and clean-up

The extraction, clean-up and detection procedures were performed according to UNIEN 14123:2008 (21). 25 g of thoroughly homogenized sample, with 5 g of NaCl, was extracted with 125 mL of a methanol/water mixture (60:40, v/v) with a blender (UltraTurraxT25, Janke & Kunkel GmbH & Co. KG, IKA, Staufen, Germany) at high speed for 1 min. The samples were left to

Table 1 - Distribution of 124 analyzed samples in relation to their origin.

Continent (No. samples)	Sub-region	No. samples	Country	No. samples
Asia (65)	Western Asia	33	Turkey	18 (1 Al, 8 Ap, 8 Ch, 1 Ha)
			Georgia	10 (10 Ha)
			Syria	3 (2 Al, 1 Ap)
			Israel	1 (1 Pe)
			Azerbaijan	1 (1 Ha)
	Southern Asia	14	Afghanistan	6 (5 Al, 1 Ap)
			Iran	8 (1 Al, 7 Pi)
Central Asia	11	Uzbekistan	6 (6 Ap)	
		Tajikistan	5 (5 Ap)	
Eastern Asia	7	China	7 (6 Ap, 1 Pe)	
America (31)	Northern America	28	USA	28 (24 Al, 1 Pi, 3 Wa)
	Southern America	3	Chile	2 (1 Al, 1 Wa)
			Bolivia	1 (1 Br)
Europe (15)	Southern Europe	14	Italy	7 (4 Al, 2 Ha, 1 Pe)
			Albania	5 (5 Ch)
			Macedonia	1 (1 Ch)
			Spain	1 (1 Al)
			Western Europe	1
Africa (7)	Northern Africa	7	Egypt	6 (6 Pe)
			Morocco	1 (1 Al)
Oceania (6)	Australia	6	Australia	6 (6 Al)

Al=Almond; Ap=Apricot kernel; Br=Brazil nut; Ch=Chestnut; Ha=Hazelnut; Pe=Peanut; Pi=Pistachio; Wa=Walnut.

sediment for 3 min, the supernatant was then filtered using Whatman filter paper No 4 (diameter 0.185 mm) (Whatman GmbH, Dassel, Germany) to efficiently remove the solid component. A volume equal to 20 mL of clear filtrate was then transferred into a graduated 50 mL DigiTUBE and 20 mL of PBS solution was added; the whole solution was mixed and further filtered by glass microfiber filter (porosity 1 µm) if the presence of precipitate was observed. A volume of 20 mL of diluted extract (equivalent to 2 g of sample) was passed through the IAC, with a flow rate of 1–2 drops per second. After washing the column with 20 mL of a methanol/water (25:75, v/v), the AFs were eluted with 2 mL of methanol

and 3 mL of distilled water. An aliquot of the extract was then transferred into an amber vial and analyzed with an High-Performance Liquid Chromatography (HPLC) system.

Chromatographic analysis

The chromatographic analysis was performed using an HPLC system (ThermoFinnigan, San Jose, CA, USA) consisting of a Thermo Surveyor Pump and Autosampler and a Thermo Spectra System FL3000 detector with post-column photochemical derivatization by UVETM-LC Tech (Dorfen, Germany). The analytical separation of mycotoxins was attained by employing a C18 analytical column (150 mm x 4.6 mm, 5 µm particle size) (Thermo

Fisher Scientific, Sunnyvale, CA, USA). The injection volume was 100 μL , injected automatically by the HPLC injection system. The chromatographic separations were achieved in isocratic elution using a water/methanol/acetonitrile mixture (64:23:13, v/v/v) as a mobile phase with the flow rate of 1.0 mL/min and in a run time of 20 minutes. After each run, the column was cleaned and re-equilibrated to normal operating conditions. The detection of the analytes was performed by fluorescence detection, setting 364 nm as the excitation wavelength and 440 nm as the emission wavelength (20).

The XCALIBUR™ software version 1.4 (Thermo Fisher Scientific, Sunnyvale, CA, USA) was used to reprocess all the chromatographic information. Samples were considered as positive (i.e. naturally contaminated) when AF concentration was higher than the Limit of Quantification (LOQ = 1.3 $\mu\text{g}/\text{kg}$ for total AFs and 0.4 $\mu\text{g}/\text{kg}$ for AFB1). The AF quantification was achieved using calibration curves; the results were provided with the relevant uncertainty, which was calculated according to the Horwitz equation applying the Thompson correction.

Method validation

Validation was carried out according to Reg. (EC) No. 401/2006 (20) and UNI EN 14123:2008 (21). Linearity was determined by injecting triplicate AF standard solutions at different concentrations (0.5, 1, 2, 5 and 10 ng/mL). The acceptance criterion considered to establish the linearity was $R^2 > 0.990$. The analysis of regression residuals was performed through using ANOVA for repeated measures and their normal distribution. Precision was calculated as relative standard deviation, verifying the corresponding HorRat Ratio ($\text{RSDR}/\text{RSDTh} < 2$) according to Annex 2 Reg. (EC) No. 401/2006 (22), and compatibility with UNI EN 14123:2008 (19). Recovery tests,

verified on fortified AF materials, satisfied the criteria of performance established in Reg. (EC) No. 401/2006 (22). Six replications were performed for all parameters.

Interpretation of analytical results and statistical analysis

Reg. (EU) No. 165/2010 was used to interpret the results (15). The levels referred to the edible part of the nut. Maximum levels are variable in relation to nut typology; in particular range from 2.0 $\mu\text{g}/\text{kg}$ to 8.0 $\mu\text{g}/\text{kg}$ for AFB1, range from 4.0 $\mu\text{g}/\text{kg}$ to 10.0 $\mu\text{g}/\text{kg}$ for total AFs (15). Nuts with AF levels above the maximum level beyond reasonable doubt, taking into account the correction for recovery and measurement uncertainty (22), were considered non-compliant.

Only positive AF samples with values higher than the LOQ were statistically analyzed. An association between the presence of AFs in nuts as a categorical dichotomous (Y/N) variable and the region of origin was assessed using a Fisher exact test. The results of the multiple comparison were corrected with Hochberg adjustment. Data analysis was performed using STATA version 12 (StataCorp LLC, College Station, TX, USA).

Results

Overall, 20 samples (20/124; 16.1%) were contaminated with AFs, of which nine were apricot kernels (9/20, 45.0%), six were almonds (6/20, 30.0%), four were pistachios (4/20, 20.0%) and one was a hazelnut sample (1/20, 5.0%). The levels of total AFs and AFB1 are shown in Table 2. The median values were 16.6 $\mu\text{g}/\text{kg}$ (range 2.5 \pm 1.1 – 387.3 \pm 170.4 $\mu\text{g}/\text{kg}$) for total AFs, and 15.1 $\mu\text{g}/\text{kg}$ (range 1.8 \pm 0.8 – 354.5 \pm 156.0 $\mu\text{g}/\text{kg}$) for AFB1. Among the different types of nuts, pistachios were the most contaminated (4/8, 50.0%) (Figure 1), with levels of total AFs ranging from 8.8 \pm 3.9 to 387.3 \pm 170.4 $\mu\text{g}/\text{kg}$

(median 33.9 $\mu\text{g}/\text{kg}$), and of AFB1 ranging from 8.2 \pm 3.6 to 354.5 \pm 156.0 $\mu\text{g}/\text{kg}$ (median 31.9 $\mu\text{g}/\text{kg}$).

The majority of contaminated samples were found in nuts coming from Asia (19/20; 95.0%); one from Australia (1/20; 5.0%). Specifically, Asian contaminated samples came from Iran (5/19; 26.3%), Afghanistan (4/19; 21.1%), Uzbekistan (4/19; 21.1%), Tajikistan (2/19; 10.5%), Turkey (2/19; 10.5%), China (1/19; 5.3%) and Georgia (1/19; 5.3%). No contaminated samples came from North and South America, Europe, and Africa (Figure 2). Eleven out of 20 (55.0%) AF contaminated samples were non-compliant for both AFB1 and total AFs, and they were from Asia. Analyzing the Asian samples, AF contamination was different in the various sub-regions ($p < 0.001$). In particular, nuts coming from Southern Asia resulted the most contaminated (9/14; 64.3%); while those coming from the Western were the least contaminated (3/33; 9.1%). In addition, a statistically significant difference was detected between Western and Southern Asia ($p < 0.001$) and the Western and the Central ($p < 0.05$).

Discussion

Nuts are an important nutritional resource for humans, consequently it is necessary to provide the consumer with an uncontaminated and safe product. Moreover, nuts are a component of many foodstuffs, such as baked products, ice cream and chocolate bars, so it is necessary to ensure the commercial product quality.

Our results show that pistachios appeared to be more susceptible to AF contamination than other analyzed nuts samples. This finding is consistent with other studies (23-25) and with the totality of Italian surveillance data, transmitted through Rapid Alert System for Food and Feed (RASFF).

Table 2 - Contaminated samples and concentrations of Aflatoxin B1 and total Aflatoxins in different types of nuts detected by HPLC analysis

	Nut samples (No.)			
	Almonds (46)	Apricot kernels (27)	Hazelnuts (14)	Pistachios (8)
No. of contaminated sample with AFB1 concentrations [$\mu\text{g}/\text{kg}$]	6 [121.2\pm53.3 ; 72.2\pm31.8 ; 50.9\pm22.4 ; 10.8 \pm 4.8; 6.6 \pm 2.9; 2.4 \pm 1.1]	9 [56.3\pm24.8 ; 23.4\pm10.3 ; 19.9\pm8.8 ; 14.3\pm6.3 ; 8.9 \pm 3.9; 5.2 \pm 2.3; 4.1 \pm 1.8; 3.9 \pm 1.7; 1.8 \pm 0.8]	1 [56.1\pm24.7]	4 [354.5\pm156.0 ; 48.0\pm21.1 ; 15.8\pm7.0 ; 8.2 \pm 3.6]
Median AFB ₁ /IQR ($\mu\text{g}/\text{kg}$)	30.9/78.9	8.9/17.7	-/-	31.9/267.8
No. of contaminated sample with total AFs concentrations [$\mu\text{g}/\text{kg}$]	6 [129.1\pm56.8 ; 77.0\pm33.9 ; 57.1\pm25.1 ; 12.1 \pm 5.3; 7.5 \pm 3.3; 4.1 \pm 1.8]	9 [62.9\pm27.7 ; 41.6\pm18.3 ; 24.5\pm10.8 ; 16.3\pm7.2 ; 9.9 \pm 4.3; 5.7 \pm 2.5; 4.6 \pm 2.0; 4.4 \pm 1.9; 2.5 \pm 1.1]	1 [124.0\pm54.6]	4 [387.3\pm170.4 ; 50.8\pm22.4 ; 16.9\pm7.4 ; 8.8 \pm 3.9]
Median total AFs/IQR ($\mu\text{g}/\text{kg}$)	34.6/83.4	9.9/28.6	-/-	33.9/292.4

AF = Aflatoxin

IQR = Inter-quartile range

Bold indicate samples with Aflatoxin B1 and total Aflatoxin contamination over maximum levels [15, 22].

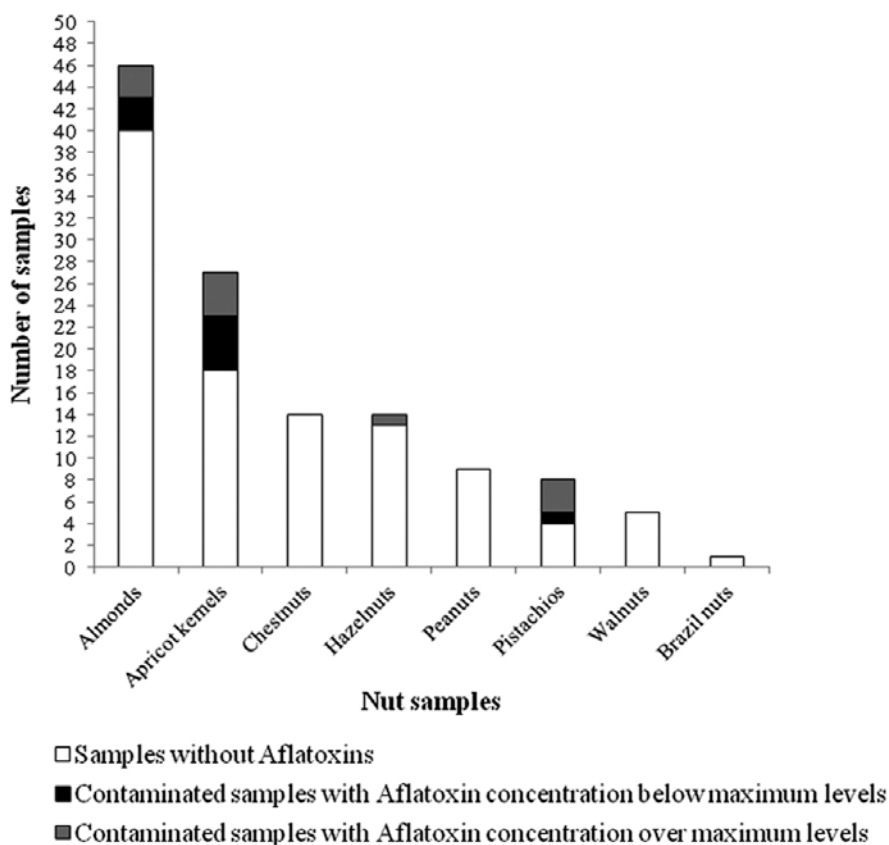


Figure 1 - Distribution of 124 analyzed samples in relation to their degree of Aflatoxin contamination.

Pistachios are widely regarded as the main contributor to dietary AF exposure from tree nuts, representing 7–45% of humans' total AF exposure from all sources (26–28). Several studies (25, 29) have shown that pistachios could be contaminated with AFs in every stage, at both pre- and post-harvest.

Several species of *Aspergillus* are able to infect and decompose kernels, while nuts are still on the tree (25, 30). High levels of AFs are also associated with hull rupture (“early split”) nuts and wounding by insect (25, 31, 32). The transport phase increases the possibility that these nuts undergo natural (insects) or man-made trauma, promoting further the mold contamination.

Iran and the United States dominate the global pistachio market; 47% of the world's pistachio exports come from Iran and 25% from the US (28). However, there is a difference in the harvest quality between countries: Iran pistachios contain an average of 54 ng/g AFs, while US pistachios contain average levels below the EU standard of 10 ng/g (27, 28). Our results are in agreement with the above-mentioned data: analyzed pistachios were mostly imported from Iran (87.5%), and 37.5% of them had exceeded maximum tolerable limit set for AFB1 and total AFs by European Union (15). These high levels of AF contamination may be due to agronomic technique: in Iran pistachios are usually dehulled very soon after harvest

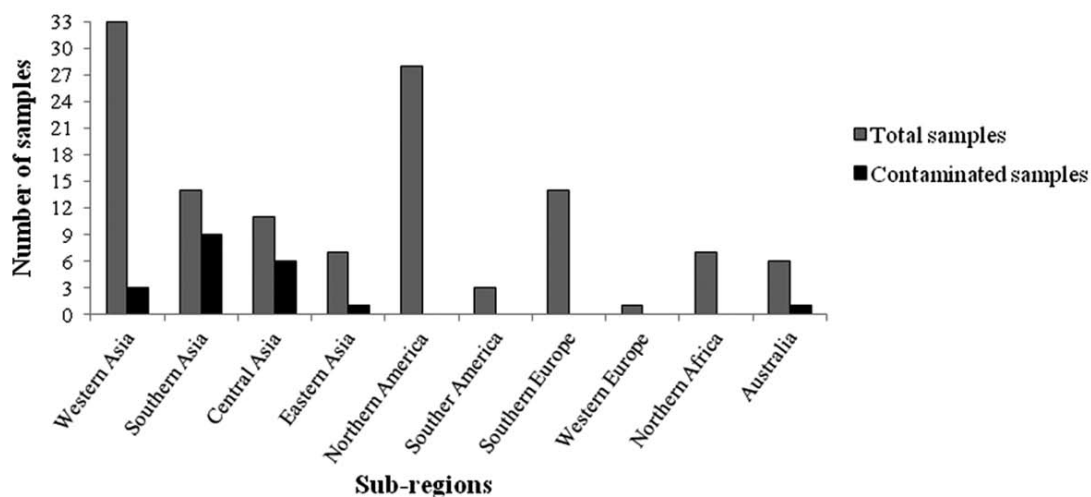


Figure 2 - Presence of Aflatoxins in 124 nuts imported from different geographical regions.

and then stored and processed, exposing the nuts at an early stage to *A. flavus* and *A. parasiticus* spores which have the potential to produce AFs (33).

Regarding to the association between the presence of AFs and geographic areas, AFs are more prevalent in Asiatic samples compared with those from other parts of the world, and could pose a great threat to health if not properly subject to official controls. In particular, Southern Asia was the region with the highest percentage of contaminated samples, probably due to local climate patterns characterized by semi-arid, warm and drought conditions, which are favorable environments for the growth of aflatoxigenic molds (29, 34). Differences between samples from various geographical origins have already been demonstrated. Prele *et al.* (35) revealed differences between hazelnuts coming from an Asian country (mean value = 0.33 $\mu\text{g}/\text{kg}$) compared with those European (0.14 $\mu\text{g}/\text{kg}$) and other countries including the USA (0.19 $\mu\text{g}/\text{kg}$). This variability of data regarding the

presence of AFs and geographic areas may reflect differences in agronomic techniques and climate among different countries, as well as the study design adopted by different authors, including differences in analyzing methods.

Conclusions

These data are preliminary, and further information will be collected in order to get statistically significant data and confirm our results. In fact, this study reveals that pistachios are the nuts with the highest frequency of occurrence for AFs and that, regardless of the type of nuts, location may influence the risk of being contaminated. Therefore, to maintain high quality standards and ensure consumer protection, nations should increase AF monitoring in nuts coming from countries with favorable environments for the growth of aflatoxigenic molds or with less strict regulations.

Riassunto

Contaminazione da Aflatossine nella frutta a guscio commercializzata in Italia: risultati preliminari

Introduzione. Le aflatossine (AF) sono uno dei principali gruppi di micotossine prodotte da muffe. La frutta a guscio, anche se ritenuta alimento benefico per la salute, è spesso contaminata da AF.

Disegno dello studio. In questo studio preliminare è stata valutata la contaminazione da AF totali e AFB1 in diversi tipi di frutta a guscio proveniente da diversi Paesi in Puglia.

Metodi. Complessivamente, 124 campioni (mandorle, noccioli di albicocca, castagne, nocciole, arachidi, pistacchi, e noce del Brasile) sono stati analizzati utilizzando un sistema di cromatografia liquida ad alta prestazione.

Risultati. Venti campioni (16,1%) sono risultati contaminati da AF di cui il 55% non era conforme, in accordo con il Reg. 165/2010. I valori medi ($\mu\text{g}/\text{kg}$) di AF totali e AFB1 erano 16,6 e 15,1, rispettivamente. I pistacchi apparivano più sensibili alla contaminazione da AF rispetto agli altri tipi di frutta a guscio, con livelli di AF totali compresi tra 8,8 e 387,3 $\mu\text{g}/\text{kg}$ e di AFB1 tra 8,2 e 354,5 $\mu\text{g}/\text{kg}$.

Conclusione. La maggior parte dei campioni contaminati proveniva dall'Asia e la contaminazione da AF risultava diversa nelle varie sub-regioni asiatiche: indipendentemente dal tipo di frutto, i campioni provenienti dall'Asia occidentale erano i meno contaminati. Poiché l'origine geografica può influenzare il rischio di contaminazione, al fine di tutelare la salute dell'uomo, le nazioni dovrebbero aumentare il monitoraggio delle AF in frutta a guscio proveniente dai Paesi con condizioni ambientali favorevoli alla crescita di muffe aflatossinogene o con regolamenti meno rigidi.

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