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### Jewelry Boxes Contaminated by *Aspergillus oryzae*: An Occupational Health Risk?

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# Jewelry Boxes Contaminated by *Aspergillus oryzae*: An Occupational Health Risk?

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*In 2009, 100,000 jewelry boxes, manufactured in China, were delivered to a jewelry manufacturer in Besançon, France. All the boxes were contaminated by mold. Because the workers refused to handle these jewelry boxes, the company contacted our laboratory to determine how to deal with the problem. Three choices were available: (1) decontaminate the boxes, (2) return the boxes to the Chinese manufacturer, or (3) destroy the entire shipment. Based on microscopic identification, the culture analysis was positive for *A. oryzae*. This could not be confirmed by molecular techniques because of the genetic proximity of *A. oryzae* and *A. flavus*. Because *A. flavus* can produce aflatoxins, we tested for them using mass spectrometry. Aflatoxins B1, B2, G1, G2, and M1 were not detected; however, given the specifics of this situation, we could not discard the possibility of the presence of other aflatoxins, such as P1, B3, GM2, and ethoxyaflatoxin B2. We concluded that the contamination by *A. oryzae* was probably due to food products. However, because of the possible presence of aflatoxins, occupational health risks could not be entirely ruled out. The decision was therefore taken to destroy all the jewelry boxes by incineration. To avoid a similar situation we propose: (1) to maintain conditions limiting mold contamination during production (not eating on the work site, efficient ventilation systems); (2) to desiccate the products before sending them; and (3) to closely control the levels of dampness during storage and transport.*

**Keywords** *Aspergillus oryzae*, aflatoxin, mass spectrometry, occupational health risk

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## INTRODUCTION

Fungi in the environment are a health hazard due to their capacity to induce inflammatory responses, sensitization,

and toxic reactions. Infection may also occur in subjects with a depression of the immune defense. Recent studies investigating host-fungus interactions have highlighted the role of beta-glucans, which are major fungal cell-wall polysaccharides, in the development of inflammatory responses.<sup>(1,2)</sup>

The risk of work-related exposure to fungal growth on imported objects may be growing due to the increase in international commerce; manufacturing conditions vary from one country to another and high levels of dampness may occur during storage and transport.

A number of methods (morphological, molecular, biochemical)—each with their advantages and disadvantages—are now available to identify fungal species accurately, but all aim to help differentiate between closely related species. Identification of mycotoxins and secondary metabolites produced by fungi may be of interest for assessing fungal health hazards, especially in the case of known mycotoxin producers.<sup>(3,4)</sup>

*Aspergillus oryzae*, a member of the Flavi section, is widely used in traditional Japanese fermentation industries, including soy sauce, sake, bean curd seasoning, and vinegar production.<sup>(5)</sup> This species is closely related to *A. flavus*, another member of the Flavi section. While it is well known that *A. flavus* produces carcinogenic aflatoxins (B1, G1, M1, B2, G2),<sup>(6,7)</sup> whether *A. oryzae* produces aflatoxins is unclear. Authors' arguments that support the inability of *A. oryzae* to produce aflatoxins depend mainly on the absence or mutation of aflatoxin clusters (*pksA*, *fasIA*, *aflR*, *ver1*, *vbs*).<sup>(5,8)</sup> In contrast, other authors are convinced that because its identity is almost indistinguishable from that of *A. flavus*, an efficient aflatoxin gene homolog cluster is always a possibility. Moreover, *A. oryzae* is able to produce other secondary metabolites with unknown health impacts.<sup>(9)</sup>

In 2009, 100,000 jewelry boxes, manufactured in and shipped from China, were delivered to a jewelry manufacturer in Besançon, France. Both external and internal surfaces of the boxes were contaminated by mold as evidenced by their

dirty-looking appearance and dark stains. Because the employees refused to handle the boxes, the company contacted our laboratory to determine how to deal with the problem. Three choices were available: (1) decontaminate the boxes, (2) return the boxes to the Chinese manufacturer, or (3) destroy the entire shipment.

We identified the fungal species of the contamination as *Aspergillus oryzae*. Due to the difficulties in differentiating between *A. oryzae* and *A. flavus*, and because of the potential health hazard related to these two fungi, we decided to test for the presence of mycotoxins in an organic extract of the insert tissue in the jewelry boxes. First, using mass spectrometry (MS), we tested for the presence of the more toxic aflatoxins: B1, G1, M1, B2, and G2. This was followed by a complementary identification of the extracted compounds as possible *A. oryzae* secondary metabolites.

## MATERIAL AND METHODS

### Culture Analysis

External and internal surfaces of nine jewelry boxes were sampled by swabbing. Culture was performed on DG18 medium (Oxoid, Basingstoke, Hampshire, U.K.) at 30°C and Malt agar (MEA) (Difco, Detroit, Mich.) at 20°C. Cultures were observed after 3 and 7 days of incubation. Fungal species were identified by macroscopic and microscopic examination using Klich's identification key.<sup>(10)</sup>

### Liquid Chromatography-Mass Spectrometry (LC/MS-MS)

#### Instrumentation

LC/MS-MS analyses were performed on a LC 1100 series HPLC (high performance liquid chromatography) system (Agilent Instrument, Santa Clara, Calif.), coupled with an Esquire HCT Ion Trap (Bruker Daltonics, Bath, U.K.) using an electrospray ionization (ESI) interface alternating between negative and positive modes.

Chromatographic separation was achieved with a Kromasil 5C18 column (100 × 2 mm; Interchim, Montluçon, France) fitted with an identical packing material guard column (10 × 2 mm; Interchim).

#### Conditions

The mobile phase was A (10 mM ammonium acetate in water/acetonitrile, 90/10 v/v) and B (water/acetonitrile, 10/90 v/v). The mobile phase was run as a gradient at a flow rate of 0.2 mL/min. Initially, the mobile phase consisted of 25% B. At 2 min, the gradient was increased to 90% B for 22 min, maintained at 90% B for 3 min, decreased to 25% B over 2 min, and maintained at 25% B for 5 min. Separation was performed at ambient temperature.

ESI was performed with capillary voltage at 4.5 kV, skimmer voltage at 40V and exit capillary at -117.5 V. Dry temperature was maintained at 310°C using a gas flow of 10 L/min. Collision energy was set at 0.70 V.

### Calibration Standards

Aflatoxin B + G mixture (B1, G1, B2, G2), Aflatoxin M1 solution, acetonitrile, and ammonium acetate were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Aflatoxin B+G mixture was dissolved in acetonitrile/water 50/50 v/v producing a concentration of 80 µM and 20 µM, respectively, to aflatoxins B1, G1 and B2, and G2.

### Sample Preparation

The lining (70 cm<sup>2</sup>) of one jewelry box was removed using a sterile scalpel, cut up into 1-cm<sup>2</sup> pieces, and immersed in 40 mL of a solution of water/acetonitrile/methanol (40/30/30 v/v). Glass microbeads (5 g) (0.4–0.6 mm; B. Braun, Melsungen, Germany) were added to the mixture, which was stirred in a magnetic stirrer for 48 hr. The resulting supernatant was filtered through a PVDF membrane (0.22 µM; Millipore, Billerica, Mass.) and 20 µL of the filtrate injected for HPLC. A volume of this extract was also dried in a speed-vac concentrator (Savant SC-200; Savant Instruments, Farmingdale, N.Y.) and dissolved in a volume of acetonitrile/water (50/50 v/v) to concentrate the samples prior to HPLC analysis.

## RESULTS

### Culture Analysis

Cultures were all positive, pure, and abundant for *A. oryzae*, using Klich's morphologically based system for the identification of *Aspergillus* species.<sup>(10)</sup> *A. oryzae* and *A. flavus*, both members of the *Circumdati* subgenus and classified in the Flavi section, are closely related species. Aspergilli in this section are mainly characterized by their uni- or bi-seriate vesicles, which are either spherical or pyriform; their colors range from yellow-green to olive-brown, and they have rough colorless stipes and echinulated conidia. In this study, the identification of *A. oryzae* was done easily because the strain was typical: marked brown color of colonies (on MEA medium), mostly uniseriate vesicles and large ovoid conidia, but fewer conidia chains and longer stipes than a reference strain of *A. flavus*.

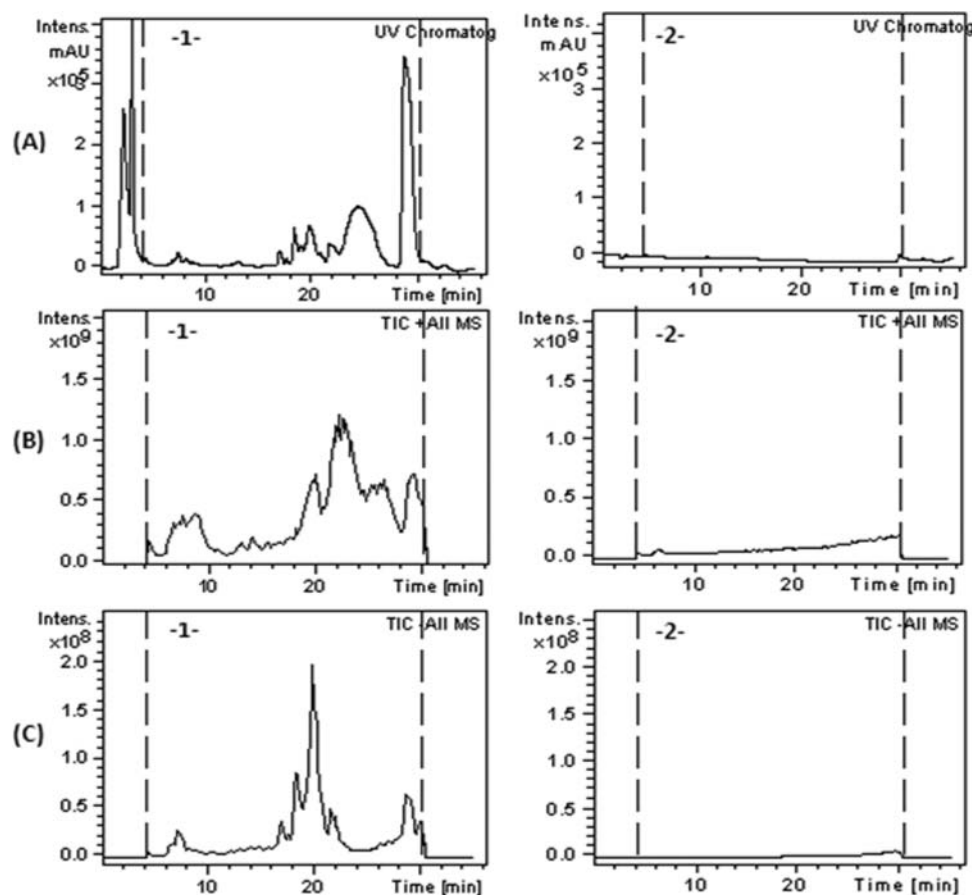
### Liquid Chromatography-Mass Spectrometry (LC/MS-MS)

#### Global Profile

Chromatograms recorded by liquid chromatography coupled to a UV detector and a mass spectrometer showed that several UV and total ion current plot (TIC) signals were present in the sample and absent in the solvent (Figure 1).

#### Search for Aflatoxins M1, B1, B2, G1, and G2

First, the extract was tested for the presence of recognized toxic aflatoxins: M1, B1, B2, G1 and G2. Results were compared to spectra of pure compound positive controls. None of the compounds present in the extract had the same retention time as the reference aflatoxins (Table I). Only precursor ions at mass to charge (m/z) 311 in negative mode and m/z 329 in positive mode gave significant MS signals. The m/z 311



**FIGURE 1.** HPLC UV and MS chromatograms of the jewelry box extract compared to those of the blank solvent. A 3.5-cm<sup>2</sup>/mL organic extract from the fungus-contaminated internal lining was analyzed (left column) by UV (A), MS total ion current in positive (B), and negative (C) modes and compared to the blank solvent (right column).

ion is theoretically that of aflatoxin B1, but the peak in the sample extract had a different retention time than that of the standard aflatoxin B1: 18.2 min versus 11.9 min (data not shown). The  $m/z$  329 peak had a retention time of 20 min, whereas aflatoxins M1 and G1 were eluted at 7.0 and 10.1 min, respectively (Figure 2). Interestingly, the ion at  $m/z$  329 is part of a MS spectrum cluster for which nominal mass is  $m/z$  327.

A cluster that exhibits a 1/1/03 ratio is characteristic of a trichlorinated compound and not of aflatoxin derivatives.

#### *Search for Other Aflatoxins and *A. oryzae* Secondary Metabolites*

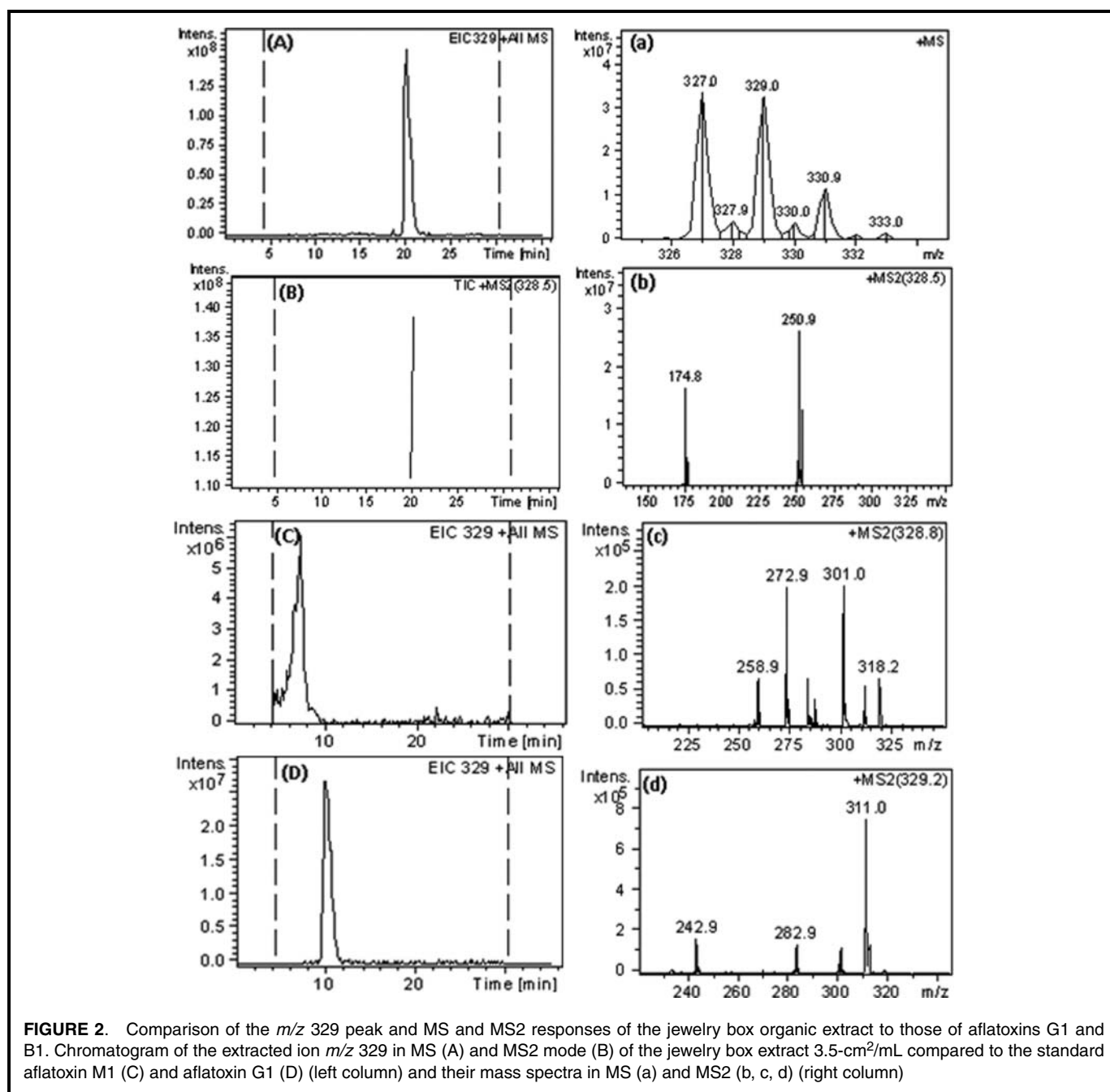
Due to the number of signals that were detected, the presence of several secondary metabolites was suspected.

**TABLE I.** Retention Time and MS Characteristics of Aflatoxin B1, B2, G1, G2, and M1 Standards

Aflatoxins	Molecular Weight	Retention Time (min)	Positive Mode (ESI+)		Negative Mode (ESI-)	
			Precursor Ion <sup>A</sup> ( $m/z$ )	Product Ion <sup>B</sup> ( $m/z$ )	Precursor Ion <sup>A</sup> ( $m/z$ )	Product Ion <sup>B</sup> ( $m/z$ )
Aflatoxin B1	312.27	11.9	313	285	311	296
Aflatoxin B2	314.29	9.5	315	387	313	296
Aflatoxin G1	328.27	10.1	329	311	327	283
Aflatoxin G2	330.29	8.5	331	313	329	285
Aflatoxin M1	328.27	7	329	301–273	327	312

<sup>A</sup>Precursor ion was obtained from the MS signal given from the mass spectrometer operating in positive or negative mode.

<sup>B</sup>The product ion obtained from induced collision of the precursor ion isolated in the trap

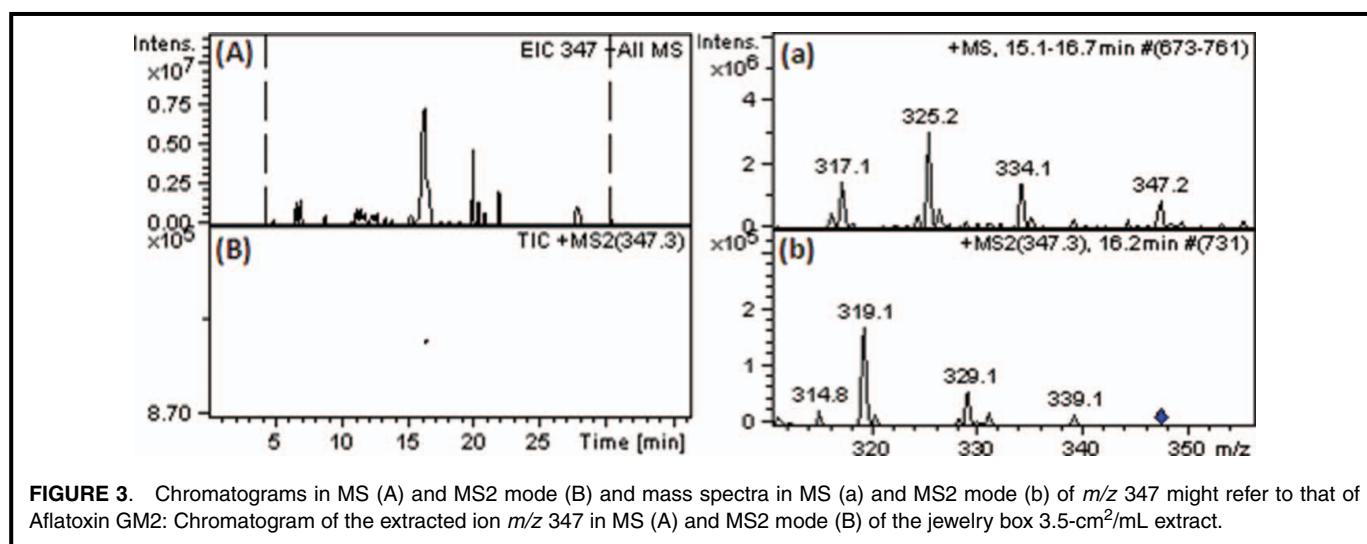


The database ANTIBASE 2008<sup>(11)</sup> was used to obtain a list of the already identified aflatoxins. Twenty-three aflatoxins were listed, and these were tested for in the sample in positive and negative modes.

Of the 23, only the  $m/z$  of aflatoxins P1, B3, GM2, and 1-ethoxy-aflatoxin B2 were compatible with the detected signals in the sample (Table II). Figure 3 shows a chromatogram of the extract ion current (EIC) at  $m/z$  347, as well as the MS and collision-induced MS2 of the 15.5-min retention peak; this might correspond to aflatoxin GM2. We also tested for 35 potent secondary metabolites from *A. oryzae*<sup>(11,12)</sup> and found the possible presence of 10 of them (Table III).

## DISCUSSION

Our laboratory was consulted about jewelry boxes contaminated by mold. Culture methods identified the mold as *A. oryzae*, but the presence of aflatoxins could not be ruled out. A decision to decontaminate or return or destroy the jewelry boxes had to be made. The company decided to destroy the entire shipment of boxes for the following reasons: (1) we could not guarantee that there were no health risks because secondary metabolites—the health effects of which were unknown—had been detected, and (2) the Chinese producer offered to send a complete, “clean” batch of 100,000 jewelry boxes to replace the moldy ones.



In the United States, *A. oryzae* is listed in the Generally Recognized as Safe (GRAS) category by the Food and Drug Administration (FDA),<sup>(13,14)</sup> and the World Health Organization (WHO) has also evaluated it as safe.<sup>(15)</sup> It is important to differentiate between *A. oryzae* and *A. flavus* because the latter systematically produces well-known carcinogenic aflatoxins. However, in some cases, neither the use of culture-based criteria<sup>(10)</sup> nor molecular biology<sup>(16,17)</sup> is sensitive enough to distinguish between the two. In our study, the *A. oryzae* strain we identified was very typical, according to the identification key. This was a strong indication that the cause of the mold was food product contamination. It is possible that the contamination issued from meals eaten at the workshop.

Mass spectrometry analysis confirmed that the jewelry boxes were not contaminated by carcinogenic aflatoxins commonly produced by *A. flavus*. However, signals were detected by mass spectrometry with  $m/z$  that were compatible with four aflatoxins (P1, B3, GM2, and 1-ethoxyaflatoxin B2) and 10 other secondary metabolites from *A. oryzae*. All these compounds generating UV or mass spectrometry signals could

also result from the materials used in the manufacture of the boxes themselves, such as glue, tissue, or dyes. Unfortunately, because no uncontaminated jewelry box was found in the shipment, this hypothesis could not be investigated.

In our analysis, the presence of aflatoxins whose health impact is unknown, could not be ruled out. *A. oryzae* has the capacity to produce aflatoxins in cases of extended fermentation.<sup>(9)</sup> The time lapse of the fungal development in the present case was not known (production, transport). Moreover, the fabric in the jewelry boxes could have facilitated the *A. oryzae* production of secondary metabolite.<sup>(18)</sup> Furthermore, while there is no evidence to support the role of *A. oryzae* in invasive fungal infections, it has already been reported to be the cause of allergic reactions such as asthma.<sup>(19–21)</sup>

Thus, several concerns remained about the occupational health hazards associated with the contamination by *A. oryzae* of these jewelry boxes. We had no knowledge about the quality and conditions of the cleaning of the boxes if they were returned to China (i.e., the use of protective devices such as masks or gloves would prevent inhalation or contact with

**TABLE II.** Possible Existence of Other Aflatoxins in the Contaminated Jewelry Box

Aflatoxins	Molecular Weight	Retention Time (min)	Positive Mode (ESI+)		Negative Mode (ESI-)	
			Precursor Ion <sup>A</sup> ( $m/z$ )	Product Ion <sup>B</sup> ( $m/z$ )	Precursor Ion <sup>A</sup> ( $m/z$ )	Product Ion <sup>B</sup> ( $m/z$ )
Aflatoxin P1 <sup>C</sup>	298.25	14.5	299	n.d.	n.d.	n.d.
		17	n.d.	n.d.	297	n.d.
Aflatoxin B3	302.28	10.8	303	n.d.	n.d.	n.d.
Aflatoxin GM2	346.29	16.2	347	319–329	345	n.d.
1-Ethoxy-aflatoxin B2	358.34	11.5	359	283	n.d.	n.d.

Note: n.d., not determined.

<sup>A</sup>Precursor ion was obtained from the MS signal in positive or negative mode.

<sup>B</sup>The product ion was obtained from induced collision of the precursor ion isolated in the trap.

<sup>C</sup>For the same  $m/z$  corresponding to the compound, several peaks were observed. The targeted compound has only one peak. A precise retention time attribution must be validated by comparison to reference compound.

**TABLE III. Possible Secondary Metabolites of *A. oryzae* Identified in Jewelry Box**

Secondary Metabolites	Molecular Weight	Retention Time <sup>A</sup> (min)	Precursor Ion <sup>B</sup> ( <i>m/z</i> )	
			Positive Mode (ESI+)	Negative Mode (ESI-)
Kojic acid dimer	282.20	11.6	283	n.d.
Dechloro-aspirochlorine	326.35	20.2	327	325
Bissecodehydrocyclopiazonic acid <sup>A</sup>	338.40	16.5	339	n.d.
		20.6		
		20.2	n.d.	337
Kojistatin A <sup>A</sup>	372.46	26.9	373	n.d.
		16.8	n.d.	371
Ergosta-5,7,24(28)-trien-3beta-ol	396.65	19.4	398	n.d.
Ergosta-5,22-dien-3beta-ol	398.66	19.7	n.d.	398
Bromo-dechloroaspirochlorine	405.24	20.8	406	n.d.
Stigmasta-7,24(28)-dien-3beta-ol	412.69	19.6	414	n.d.
6-( $\alpha$ -D-Glucosyl)-maltose	504.44	11.3	n.d.	503
9(11)Drimen-8-ol	222.37	9.1	n.d.	221
Mutaaspergillic acid <sup>A</sup>	226.27	19	227	n.d.
		23.2	n.d.	225
Hydroxyaspergillic acid	240.30	27.2	n.d.	239

<sup>A</sup>For the same *m/z* corresponding to the compound, several peaks were observed. The postulated compound can correspond to only one peak. A precise attribution must be validated by comparison to reference compound.

<sup>B</sup>Precursor ion was obtained from the MS signal in positive or negative mode.

conidia or toxins, or the use of disinfectant to permanently stop the growth of the fungus). Similar situations have been reported where mold-contaminated imported objects were destroyed after mycological analysis (e.g., footballs from Pakistan contaminated by *A. flavus* and *A. tamarii*).<sup>(22)</sup>

If the jewelry boxes had been contaminated by several different fungal strains, showing a biodiversity close to what is commonly observed in the environment, or, as in this case, highly contaminated by a single strain of fungus that was neither responsible for invasive infection nor known to produce major mycotoxins (such as *Cladosporium herbarum* spp., *Geotrichum* spp., and other fungi generally regarded as safe by the U.S. Food and Drug Administration,<sup>(23)</sup> an in situ manual decontamination would have been considered. This could have been done by brushing the 100,000 jewelry boxes with masks, glasses, gloves, and efficient ventilation.

## CONCLUSIONS

The present study illustrates the limits of comprehensive mycotoxin analysis: there is a significant risk of detecting secondary metabolites whose health effects are unknown.

It is probable that mold conidia were initially present only on some boxes, and that dampness during storage or transport encouraged the mold to spread from one box to another. To avoid this situation, we suggest: (1) maintaining conditions that limit mold contamination during production (not eating on the work site, efficient ventilation systems); (2) desiccating the products before sending them; and (3) closely controlling levels of dampness during storage and transport.

Current regulations (Registration, Evaluation, Authorization, and Restriction of Chemical substances, REACH) now prohibit the use of dimethyl fumarate (DMFu), and this could result in an increase of mold contamination of imported manufactured products. Research should be started to select alternative biocide to replace DMFu.

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