Food Research International 106 (2018) 136–140

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Aflatoxigenic and ochratoxigenic fungi and their mycotoxins in spices marketed in Brazil

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ARTICLE INFO

Keywords:
Aflatoxins
Aspergillus spp.
Fungi
Mycotoxin
Contamination
Spice

ABSTRACT

During their processing, spices usually remain close to the ground for drying, a fact that disposes to fungal contamination, as well as moisture transferred from the tropical environment can allow their multiplication and synthesis of mycotoxins. The objective of this study was to evaluate the presence of potentially toxigenic fungi and mycotoxins in spices marketed in Brazil. The fungal contamination was evaluated by direct plating for samples of clove, black and white peppers. Spread plate was used for the samples of rosemary, cinnamon, fennel, pepperoni pepper and oregano. Analyses were performed in triplicate in DG18 media with incubation at 25 °C for 7 days. The isolation and identification of fungi followed specific recommendations of culture media and incubation period for each genus. The presence of mycotoxins in spices was verified by high-performance liquid chromatography (HPLC) coupled to fluorescence. The frequency of species potentially toxigenic was high in white and black peppers with presence of both aflatoxigenic and ochratoxigenic fungi. Only rosemary and fennel showed contamination with aflatoxin B1 and there was a positive correlation ($\rho < 0.01$) between the rosemary contamination with the presence of AFB1 and *A. flavus*. Even in the presence of ochratoxigenic fungi, ochratoxin A was not detected in the samples. The presence of natural components with antimicrobial activity could justify the low presence of mycotoxins, even in the presence of known toxigenic fungi in the samples. Mycotoxins were not detected in spices covered by Brazilian regulation of mycotoxins. On the other hand, these contaminants were present in other spices consumed by population and not mentioned in the regulation, which could be considered a cause to concern.

1. Introduction

Spices are considered non-perishable products under normal conditions of storage and some of those have antimicrobial and antioxidant properties that allows them to assist in food and self-stability (Ascenção & Filho, 2013). On the other hand, most of these products can act as a major source of microbial contamination, as well as are susceptible to fungal spoilage, mainly by the genera *Aspergillus* and *Penicillium,* if any failures occurs during their processing (El Mahgubi et al., 2013; Pitt & Hocking, 2009; Ruiz-Moyano et al., 2009; Teixeira-loyola, Siqueira, & Schreiber, 2014).

Growth of fungi in spices can promote changes in sensory properties of spices, leading to the depreciation of its market value, and also the concern related to the possibility of mycotoxin formation, due the toxic, carcinogenic, mutagenic and immunosuppressive properties of these fungal compounds (IARC, 1993; Pitt & Hocking, 2009).

The mycotoxins generally associated with spices are aflatoxins produced by species of *Aspergillus* (mainly *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus*) and also ochratoxin A (OTA) (mostly by *Aspergillus carbonarius*, *Aspergillus niger and Aspergillus ochraceus*) (Pitt & Hocking, 2009). In Brazil, the regulation set maximum of 20 μg/kg for aflatoxins and 30 μg/kg for OTA in spices (Brasil, 2011). However, this resolution only mentions the following spices: *Capsicum* (Calabrese pepper, green pepper), *Piper* spp. (white and black peppers), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger) and *Curcuma longa* (turmeric). In countries such as the United States of America, the limits are 20 μg/kg for total aflatoxins while in the European Union is 5 μg/kg for aflatoxin B1, 10 μg/kg for total aflatoxins, 20 μg/kg (for dried fruits of *Capsicum* sp.) and 15 μg/kg (for spices in general) for OTA (FAO, 2017). In other countries like Hungary, Spain and Portugal, the contamination by aflatoxins and OTA in spices have been studied. In general, the values founded was higher than the estipulated for the legislation for mycotoxins in these matrices (Fazekas, Tar, & Kovács, 2005; Hierro, Garcia-Villanova, Torrero, & Fonseca, 2008; Martins, Martins, & Bernardo, 2001).

To minimize the problem of contamination and economic losses

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https://doi.org/10.1016/j.foodres.2017.12.061
Received 19 October 2017; Received in revised form 6 December 2017; Accepted 23 December 2017
Available online 26 December 2017

related to spices, in 2013 the *Codex Alimentarius* created a specific committee for spices and culinary herbs. The main objectives to be reached was to establish international standards and drawing up a code of practice for the safe production of spices and herbs. In addition, the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) made an international call for data of any microbiological risk associated with these foods in order to identify the agents that represent a hazard to consumers associated with spices and dried herbs (FAO, 2013).

The objective of this study was to verify the presence of fungi potentially producers of aflatoxins and OTA, and to investigate the presence of these mycotoxins in spices commonly marketed in Brazil.

2. Methods

2.1. Samples of spices

A total of 112 samples of the following spices were analyzed: Rosemary leafs (*Rosmarinus officinalis* L.) (n = 15), cinnamon powder (*Cinnamomum zeylanicum* Ness) (n = 13), clove flowers (*Sygygium aromaticum* L.) (n = 14), fennel seeds (*Pimpinella anisum* L.) (n = 15), black (n = 15) and white pepper corns (*Piper nigrum* L.) (n = 15), pepperoni pepper powder (*Capsicum baccatum* L.) (n = 15), oregano leafs (*Origanum vulgare* L.) (n = 12). All the evaluated samples were available in the market.

2.2. Water activity (a_w) analyses

The water activity was determined, in triplicate, directly on a water activity meter (Aqualab Series 4 TE, USA). The analyses were performed at 25 °C ± 1.

2.3. Isolation and identification of aflatoxigenic and ochratoxigenic fungi from spices

Determination of fungi followed methodology recommended Pitt and Hocking (2009).

Dilution plate was selected for evaluation of rosemary, cinnamon, fennel, pepperoni pepper and oregano samples. Briefly, 25 g of each spice was aseptically added to 225 mL of peptone water (0.1%). Serial dilutions were carried out with subsequent inoculation in Dichloran Glycerol Agar 18% with chloramphenicol (DG18). The plates were incubated at 25 °C for 7 days and the results were expressed in colony forming units per gram of product (CFU/g).

Samples of clove, black and white pepper were analyzed by direct plating method with previously disinfection in a 0.4% sodium hypochlorite solution for 1 min. Then, 11 particles were arranged in three Petri dishes containing DG18, totaling 33 particles analyzed per sample. The plates were incubated at 25 °C for 7 days and the results expressed as percentage of infected particles.

After incubation period, the colonies were isolated into Czapeck Yeast Extract Agar (CYA) and were subsequently identified according to recommendations for each genus. The identification of *Aspergillus* spp. followed Klich and Pitt (1988) and *Aspergillus* spp. from section *Circumdati* were identified following Frisvad, Frank, Houbraken, Kuijpers, and Samson (2004). For identification of *Penicillium* spp., the keys proposed by Pitt (2000) and Frisvad and Samson (2004) were used. Briefly, fungi were three point inoculated in different culture media and, after a period of cultivation in different temperatures (5, 25 and 37 °C), had their diameters measured and macro and microscopic characteristics like color of verse and reverse of colony, exudate production, shape and size of conidia and conidiophore *etc.*, characteristics observed.

2.4. Toxigenic capacity of isolates

Fungi identified as potential aflatoxins and OTA producers were inoculated on to Yeast Extract Sucrose agar for 7 days at 25 °C and then the agar plug technique (Filtenborg, Frisvad, & Svendsen, 1983) was chosen to evaluate the capability of these isolates to produce aflatoxins and OTA, respectively.

A drop of a methanol: chloroform solution (1:1, v/v) was added to fungal plugs taken with a cork borer and placed on TLC plates. The chromatography was developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualized under UV light at 365 nm. Aflatoxins B1, B2, G1 and G2 and OTA standards were used for comparison.

2.5. Determination of aflatoxins and ochratoxin A

2.5.1. Aflatoxins B1, B2, G1 and G2

2.5.1.1. Sample preparation and extraction. Ten grams of each sample, ground to a particle size of < 2 mm, was mixed with 100 mL of ACN (acetonitrile): H_2O solution (84:16, v/v) in a blender. The extract obtained was filtered and 10 mL were evaporated under a nitrogen flow at 65 °C. After drying the filtrate, it was suspended in ACN: H_2O: acetic acid (840: 160: 5, v/v/v) solution and an aliquot of the extract was derivatized with trifluoroacetic acid solution for 10 min at 65 °C.

2.5.1.2. HPLC parameters. An Agilent Series 1100 Chromatograph was used, consisting of a degasser, quaternary pump, automatic sampler, thermostatic column compartment and fluorescence detector. The chromatographic separation was performed through C_{18} monolithic columns, 100 × 4.6 mm. Reagents and solvents used as the mobile phase were: methanol, acetonitrile, acetic acid, all HPLC grade, ultrapure water (obtained from a Milli-Q purification system, resistivity around 18.2 MW cm). The standards were purchased from Sigma Aldrich, all with purity > 98%. For preparation of working solutions they were diluted in HPLC grade acetonitrile.

2.5.2. OTA

2.5.2.1. Sample preparation and extraction. In 10 g of sample was added chloroform and phosphoric acid solution (92.3: 7.7, v/v), followed by stirring shaker for 30 min at 60 rpm. After filtration, 10 mL of the extract was evaporated on a heating plate at the maximum temperature of 65 °C. The extract was suspended with ACN: H_2O: acetic acid (40: 55: 5, v/v/v) solution and 1 mL hexane was added. The suspended sample was centrifuged for 10 min at 2000 rpm and the lower phase was collected. 10 mL of this filtrate was piped into amber bottle and allowed to dry on 65 °C plate and, after drying, the solution was suspended in 600 µL of ACN: H_2O: acetic acid (40: 55: 5, v/v/v) and 1 mL of hexane. This new filtrate was centrifuged for 10 min at speed of 2000 rpm and the lower phase was collected and placed in a vial for subsequent analysis.

2.5.2.2. HPLC parameters. The determination of OTA was performed in the equipment specified above. The chromatographic separation was performed in isocratic flow at 1 mL/min with 0.2% acetic acid: acetonitrile: methanol (40: 30: 30, v/v/v) solution *via* Lichrocart RP 18 column (250 × 4 mm, 5 µm).

2.6. Statistical analyzes

Statistical analyzes (correlation analysis) were carried out using the R environment, version 3.2.4 (R Development Core Team, 2016). Correlation coefficients (r) were calculated to identify possible associations between the incidence of toxigenic fungi, a_w and the presence of mycotoxins in the evaluated samples.