

## Identification and HPLC Quantification of Aflatoxins in Dried Chili Peppers (*Capsicum annum L.*) in Mexico and Other Countries

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### Abstract

In this study, we aimed to identify and quantify aflatoxins (AFs) (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>) by liquid chromatography and to validate the methodology in 3 types of chili peppers, “Ancho”, “Guajillo” and “Piquín” which are the most frequently consumed chili peppers in the 16 boroughs of Mexico City. As a separate aim, we analyzed the AFs in some chili pepper samples from India, Turkey and South Africa to determine whether the amount of AF contamination in chili peppers represents a health risk. Sixty-four compound samples of the three types of chili peppers in 48 markets of Mexico City and nine foreign samples from India, Turkey and South Africa were analyzed. The validation of the method for analyzing AFs included selectivity, linearity, recovery percentage and limits of detection and quantification. The average AF concentrations (µg kg<sup>-1</sup>) for “Ancho” chili pepper were AFB<sub>1</sub> (1.46), AFB<sub>2</sub> (0.15), AFG<sub>1</sub> (1.28), AFG<sub>2</sub> (2.08) and total aflatoxins (AFt) (3.49), which exhibited the highest contamination. The average AF concentrations (µg kg<sup>-1</sup>) for “Guajillo” hot pepper were AFB<sub>1</sub> (0.53), AFB<sub>2</sub> (0.08), AFG<sub>1</sub> (0.40), AFG<sub>2</sub> (0.85), and AFt (0.92). The average AF concentrations (µg kg<sup>-1</sup>) for “Piquín” chili pepper were AFB<sub>1</sub> (1.44), AFB<sub>2</sub> (0.10), AFG<sub>1</sub> (1.57), AFG<sub>2</sub> (1.09) and AFt (3.14). The 8 samples from foreign countries had average AF concentrations (µg kg<sup>-1</sup>) of AFB<sub>1</sub> (0.7), AFB<sub>2</sub> (0.2), AFG<sub>1</sub> (0.7), AFG<sub>2</sub> (1.1), and AFt (1.8 µg kg<sup>-1</sup>). Most of the chili peppers exhibited a significant difference in relation to their origin in Mexico City, with the exception of AFG<sub>2</sub> in “Guajillo” chili pepper. The Mexican chili peppers had more complete sampling for the AFs than the few samples analyzed from Turkey, India and South Africa, which did not represent the whole country.

**Keywords:** Aflatoxins; Chili peppers; Food contamination; Spices

**Abbreviations:** ACN: Acetonitrile; AFs: Aflatoxins; AFB<sub>1</sub>: Aflatoxin B<sub>1</sub>; AFB<sub>2</sub>: Aflatoxin B<sub>2</sub>; AFG<sub>1</sub>: Aflatoxin G<sub>1</sub>; AFG<sub>2</sub>: Aflatoxin G<sub>2</sub>; AFM<sub>1</sub>: Aflatoxin M<sub>1</sub>; AFM<sub>2</sub>: Aflatoxin M<sub>2</sub>; AFP<sub>1</sub>: Aflatoxin P<sub>1</sub>; AFQ<sub>1</sub>: Aflatoxin Q<sub>1</sub>; AFL: Aflatoxicol; AFt: Total aflatoxin; IARC: Agency for the Research of Cancer; NaN<sub>3</sub>: Azide; R<sup>2</sup>: Correlation Coefficient; DNA: Deoxyribonucleic Acid; Na<sub>2</sub>HPO<sub>4</sub>: Dibasic Sodium Phosphate; Dist: Distilled; g: gram(s); Ha: Hectares; HPLC: High Performance Liquid Chromatography; LOD: Limits of Detection; LOQ: Limits of Quantification; MeOH: Methanol; µg kg<sup>-1</sup>: micrograms per kilogram; µL: microliter; mL: milliliter; min: minute(s); MW: Molecular Weight; ng: nanogram; PBS: Phosphate Buffer Saline; KCl: Potassium Chloride; KH<sub>2</sub>PO<sub>4</sub>: Potassium Dihydrogen Orthophosphate; RT: Retention Time; RNA: Ribonucleic Acid; 1000 kilograms: Tons; ATF: Trifluoroacetic Acid

### Objectives

- To identify and quantify the AFs (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) in 3 types of chili peppers (“Ancho”, “Guajillo” and “Piquín”) that are consumed at the highest levels in the 16 boroughs of Mexico City, using liquid chromatography.
- To identify and quantify the AFs of chili pepper samples from India, Turkey and South Africa.
- To determine if the AF contamination level in chili peppers represents a health risk.
- To validate the methodology.

### Introduction

The Mexican chili pepper was called chilli in Náhuatl, the Aztec

Mexican language, and was taken to European herbaries in the sixteenth century and named genus *Capsicum* from the Solanaceae family. In western languages, the name is related to the pepper; thus, it is chili pepper in English, pimient enragé or poivre rouge in French, pepperone in Italian, and pimentao in Portuguese [1,2]. The scientific classification has been reported [2,3].

The chili pepper (*Capsicum annum L.*) is a staple food in Mesoamerican cultures and is called cococ, cocopatic and cocopalatic to differentiate the varieties according to their degree of pungency [1,2]. The production characteristics of chili pepper have been described [3,4]. There are five commercial varieties produced: *Capsicum annum*, *C. frutescens*, *C. baccatum*, *C. pubescens* and *C. chivense* [5,6].

Dry chili peppers can be stored for months without deterioration; they are lighter in weight during transportation and their price is more stable [2,7]. These chili peppers can be dehydrated by sun drying, with a water reduction of 80% [2,8], in drying chambers to obtain seeds [9], or in ovens [2,10].

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**Received** December 25, 2015; **Accepted** January 11, 2016; **Published** January 21, 2016

**Citation:** Rosas-Contreras C, Carvajal-Moreno M, Rojo-Callejas F, Ruiz-Velasco S et al. (2016) Identification and HPLC Quantification of Aflatoxins in Dried Chili Peppers (*Capsicum annum L.*) in Mexico and Other Countries. J Drug Metab Toxicol 7: 198. doi:10.4172/2157-7609.1000198

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In 2010, the fresh chili pepper production from 68 countries was approximately 28,405.27 million tons, with China producing 54%, followed by Mexico with 6.5% [11]. The global production of dry chili peppers is 3,071 million tons, with India producing 32%, China 11%, Bangladesh and Perú 7%. Mexico occupies the 10th place, with 60,000 tons in 37,000 Ha, representing the 2.6% of the global production [12].

Mexico is the primary exporter of fresh green chili pepper and the sixth for dry chili pepper [13]. Between 2000 and 2009, the average consumption per inhabitant was 15 kg of chili peppers per year [14].

The largest number of varieties, more than 100, are produced with Mexico as the country of origin, and the dry chili peppers that are most frequently produced and consumed in Mexico are “Chile Ancho” (20,000 ton), “Piquín” (8500 ton) and “Guajillo” (8400 ton) [13,15,16]. In Mexico, dry chili peppers are produced in the States of Chihuahua (520,000 tons), Sinaloa (460,000 tons), Zacatecas (288,000 tons) and San Luis Potosí (158,000 tons) [14] (Figure 1).

Chili peppers are a favorite spice and give flavor to foods, but they can be contaminated by fungi and their mycotoxins due to high temperatures and humidity during storage [17]. Aflatoxins (AFs) are trace secondary, toxic metabolites (MW ~ 700) that are mainly produced by the fungi *Aspergillus flavus* [18-20], *A. parasiticus* [21-23] and *A. nomius* [24,25], although other fungi also produce them [26]. *Aspergillus bombycis*, *A. ochraceoroseus*, and *A. pseudotamarii* are also aflatoxicogenic fungi, but are less frequent [27-29]. Only half of the *A. flavus* strains produce AFs [30].

Spices are very susceptible to AF contamination [31-33]. Other frequently contaminated crops include cereals [19], oilseeds [34], dry fruits [35,36] and chili peppers [37-40].

The structure and properties of AFs have been described [41,42]. AFs include approximately 20 secondary metabolites, and only the aflatoxins B<sub>1</sub> (AFB<sub>1</sub>), B<sub>2</sub> (AFB<sub>2</sub>), G<sub>1</sub> (AFG<sub>1</sub>) and G<sub>2</sub> (AFG<sub>2</sub>) are in foods of vegetable origin [43]. The microbial or animal metabolism protects the organism and produces biotransformation products that incorporates an OH<sup>-</sup> group to produce hydroxylates that are soluble in water and can be distributed as detoxification metabolites, such as AFM<sub>1</sub>, AFM<sub>2</sub>, AFP<sub>1</sub>, AFQ<sub>1</sub>, AFB<sub>2a</sub>, AFG<sub>2a</sub> and Aflatoxicol (AFL) [44]. So when domestic animals eat AF-contaminated feed, the AFs are passed to meats, eggs, milk, and dairy products as hydroxylates.

AFB<sub>1</sub> is the most frequently studied biological toxin, as it often produces a carcinogenic effect [45]. The International Agency for the Research of Cancer (IARC) classified AFB<sub>1</sub> as a Group I carcinogen in humans [46].

AFB<sub>1</sub> is a potent genotoxic and carcinogenic agent in many animal species and causes hepatocellular carcinoma [47]. AFs damage all living creatures, including poultry, fish, rodents, primates, and viruses, with differences depending on age, sex, weight, diet, exposure to infections and pharmacological drugs [39]. The consumption of high concentrations of AFs cause aflatoxicosis in animals and man [48-50]. Other acute symptoms include diarrhea, vomiting, internal bleeding, and death [51]. Chronic ingestion of small quantities of AFs for large periods causes immunodepression [52], miscarriages, teratogenicity [53], cirrhosis, hepatitis [54,55], Reye syndrome [56], marasmo, kwashiorkor [57], and different types of cancers [34,58,59].

The cytochrome P450 enzymes change AFs into the reactive AFB<sub>1</sub>-8,9-epoxide that interacts with DNA, RNA, and proteins [48]. The lethal dose for adult humans is approximately 10–20 mg of AF [60].

Chili peppers are susceptible to AF contamination [61-64]. The tolerance limits for AFB<sub>1</sub> and total AF (AFt) are 5 and 10 µg kg<sup>-1</sup>, respectively [65].

AF contamination occurs in stored fruits, deteriorating their quality and affecting exportations [66]. Mexico has no legislation to control AFs in chili peppers, representing a health risk for those who consume significant amounts of this vegetable.

The maximum tolerance levels for AFB<sub>1</sub> in foods vary from 1 to 5 µg kg<sup>-1</sup> used by many countries worldwide. The USA and Canada do not have a unique limit for AFB<sub>1</sub> [67]. Another important AFt limit is 20 µg kg<sup>-1</sup> and is applied in 76 countries, half of which are from Latin America and Africa; the USA also uses this limit.

## Materials and Methods

### Sampling

A total of 56 samples were obtained from the three types of chili peppers (“Ancho”, “Guajillo” and “Piquín”) that are consumed at the highest levels in Mexico City. The 48 Mexican samples of each type of chili peppers were obtained from the three most important markets in each of the 16 boroughs of Mexico City from August 30 to Sept 27, 2012. Three samples from India, five samples from Turkey and one sample from South Africa were also obtained. The three samples of the same type of chili pepper from the three markets comprised a compound sample that represented that type of chili pepper in each borough. The samples were placed in labeled plastic bags and were processed in the lab.

The three markets from each of the 16 boroughs were:

**I) Álvaro Obregón** (1. Melchor Múzquiz; 2. Olivar del Conde; and 3. Corpus Christi),

**Azcapotzalco** (1. Azcapotzalco; 2. Prohogar; and 3. Nueva Santa María),

**II) Benito Juárez** (1. Mixcoac; 2. Portales; and 3. Independencia),

**III) Coyoacán** (1. Ajusco-Moctezuma; 2. Santo Domingo Las Rosas; and 3. De Coyoacán),

**IV) Cuajimalpa** (1. Rosas Torres; 2. Cuajimalpa; and 3. Contadero),

**V) Cuauhtémoc** (1. Hidalgo; 2. Arcos de Belén; and 3. San Juan),

**VI) Gustavo A. Madero** (1. Río Blanco; 2. De la Estrella; and 3. Martín Carrera),

**VII) Iztacalco** (1. San Miguel Iztacalco; 2. Tlacotal; and 3. Santa Anita),

**VIII) Iztapalapa** (1. Central de Abastos; 2. Santa Cruz Meyehualco; and 3. Jacarandas),

**IX) La Magdalena Contreras** (1. Cerro del Judío; 2. La Loma; and 3. Turístico La Magdalena),

**X) Miguel Hidalgo** (1. Tacubaya; 2. Plutarco Elías Calles, “El Chorrillo”; and 3. Argentina),

**XI) Milpa Alta** (1. San Antonio Tecómitl; 2. Villa Milpa Alta; and 3. San Pedro Actopan),

**XII) Tláhuac** (1. Colonia del Mar; 2. La Nopalera; and 3. Tláhuac),

**XIII) Tlalpan** (1. Tlalcoligia; 2. Torres de Padierna; and 3. De la Luz),

**XIV) Venustiano Carranza** (1. La Merced; 2. Jamaica; and 3. San Ciprián), and

**XV) Xochimilco** (1. De Xochimilco; 2. San Gregorio Atlapulco; and 3. Tulyehualco).

The nine foreign samples were: three from India (Bophal Market), five from Turkey (Istanbul Market), and one from South Africa (Pretoria Market).

### Ground and weight

The sample size was 50 g of dry weight from each of the 48 markets. Each sample was labelled and blended (Oster Mod. 465-43, USA) to homogenize them and generate a representative AF concentration.

The samples from foreign countries were purchased in the Istanbul and Bhopal markets, dried in an oven (Novatech BTC-9100, Houston Texas, USA), and weighed on a balance (Ohaus 700 Series, Parsippany, NJ, USA). Later, they were ground into a fine powder.

### AF extraction and purification

The 50 g sample of dry weight was enriched with 100 mL methanol (JT Baker, Center Valley, PA, USA) /water<sub>dist</sub> (80/20 v/v) and 2 g NaCl; each sample was individually blended for 1 minute. The mixture was filtered and the filtrate was collected. Two mL of filtrate were diluted in 14 mL phosphate buffer saline (PBS). PBS was composed of 10.0 g KCl, 10.0 g KH<sub>2</sub>PO<sub>4</sub>, 58.0 g of Na<sub>2</sub>HPO<sub>4</sub>, 400.0 g of NaCl, 2.5 g of NaN<sub>3</sub>, all from JT Baker. The buffer was brought to 5 L with water<sub>dist</sub>, and the pH was adjusted to 7.4 [68].

The immunoaffinity column for Aft (Easi-Extract R-Biopharm Rhône LTD, UK) was equilibrated with 20 mL of PBS and then received 16 mL of diluted filtrate, which was equivalent to one gram, at a speed of one drop by second. The column was then washed with 20 mL of water<sub>dist</sub>. The AFs from the sample were eluted and separated from their antibodies in the agarose gel with 1.5 mL of MeOH, HPLC purity, by gravity and 1.5 mL of water<sub>dist</sub> with reflux. The eluate was collected in a labelled amber vial. The eluates were dried in a 40°C oven.

### Derivatization

For the AF standards for the calibration curves, the derivatization consisted of adding 200 µL of acetonitrile (ACN), 800 µL of derivatizing solution (5 mL of trifluoroacetic acid (ATF) (Sigma-Aldrich, St. Louis MO, USA), 2.5 mL of glacial acetic acid (Merck, Naucalpan, Edo. Mex., México) and 17.5 mL of deionized water to the reactive compounds, followed by homogenization in a vortex (Vortex G-560, Bohemia, NY USA) for 30 seconds. The vials were then placed in a 65°C water bath for 10 min. The dried samples were dissolved in 100 µL of ACN and 400 µL of derivatizing solution to increase the fluorescence, as previously reported [69,70].

### AF quantification and identification by HPLC

The AF standards and derivatized samples were brought to room temperature and 60 µL were injected into the high-performance liquid chromatograph (HPLC) with a 20-µL loop for analysis in triplicate. The HPLC (Series 1200) had an isocratic pump (G1310A Series DE62957044), a fluorescence detector (G1321A Series DE60456380) and an autosampler (G1329A Series DE64761666), all from Agilent Technologies, as well as a chromatographic C18 column from Agilent Eclipse (XDS-C18, 4.6 × 250 mm, 5 µm particle size) and the ChemStation 32 software. The mobile phase or isocratic solution for HPLC contained H<sub>2</sub>O/ACN/MeOH (65:15:20 v/v/v) and was filtered

under a vacuum for degasification. The solvents were: methanol (CH<sub>3</sub>OH) (JT Baker, USA), acetonitrile (CH<sub>3</sub>CN) (JT Baker, USA) and distilled water (H<sub>2</sub>O<sub>dist</sub>). All solvents were of HPLC purity.

### Validation of the method

Validation consisted of the following parameters:

1. Selectivity 2. Lineality (calibration curves) 3. Recovery percentage and 4. Limits of detection (LOD) and Limits of quantification (LOQ).

1. Selectivity: Selectivity is the parameter that determines whether the analyte (100 ng of the four AFs) and the matrixes (1. "Ancho", 2. "Piquín", and 3. "Guajillo") interfere with each other.

Three samples of each matrix without AF contamination were measured:

1) "Ancho" chili pepper from Tacubaya market, Miguel Hidalgo borough,

2) "Piquín" chili pepper from the Turistic market, La Magdalena Contreras borough, and

3) "Guajillo" chili pepper from the Mixcoac market, Benito Juárez borough.

One gram was weighed and enriched with a combination of 100 ng of each of the AFs (AFB<sub>1</sub> + AFB<sub>2</sub> + AFG<sub>1</sub> + AFG<sub>2</sub>). They were analyzed by HPLC.

2. Lineality: Lineality is the ability to obtain results that are in proportion to the analyte concentration. An individual stock solution of one µg mL<sup>-1</sup> of each AF (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>), as determined by spectrometry, was prepared as previously described [71]. The calibration curves were constructed by plotting the peak areas against the concentrations of each AF standard solution. The AF standards were prepared in 1 mL of benzene/ACN (980:20 v/v) and homogenized as previously described [71]. The absorbance of the AF standards was measured at 362 nm in a spectrophotometer (Genesys 10 UV Model Thermo Electron Corp., Massachusetts, USA). Additionally, the molecular weight (MW) and the extinction coefficient (CE) were calculated as previously described [71]. The following molecular weights (MW) and extinction coefficients (EC) were calculated for each AF standard solution: AFB<sub>1</sub> (MW 312, EC 21,800), AFB<sub>2</sub> (MW 314, EC 24,000), AFG<sub>1</sub> (MW 328, EC 17,700), and AFG<sub>2</sub> (MW 330, EC 17,100).

The calibration curves included the following concentrations of the standards: AFB<sub>1</sub> (0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng mL<sup>-1</sup>), AFB<sub>2</sub> (0.01, 0.05, 1, 5, 10, 20, 40, 70, 100 and 200 ng mL<sup>-1</sup>), AFG<sub>1</sub> (0.01, 0.05, 0.1, 0.5, 1, 4, 16, 100 and 128 ng mL<sup>-1</sup>) and AFG<sub>2</sub> (0.5, 1, 2, 4, 8, 16, 32, 64, 100, 200, 600, 800 and 1000 ng mL<sup>-1</sup>). The linear regression for each AF was obtained using the Excel program.

3. Recovery percentage: The recovery percentage determines the efficiency of the method for detecting all of the studied analyte in a sample. One gram of each chili pepper type with no AF contamination, "Ancho", "Guajillo" and "Piquín", was placed in a 50-mL centrifuge tube (Falcon) and separately fortified with 100 ng g<sup>-1</sup> of each of the 4 AFs. To this, 3 mL MeOH was added, along with 2 mL water<sub>dist</sub> and 1 g NaCl; the sample was then centrifuged (ALC 4235 with cooling system CWS) at 4000 rpm for 15 minutes. The supernatant was diluted with PBS, pH 7.4, and applied to the immunoaffinity column that had previously been equilibrated with 20 mL of PBS. The columns were washed with water and eluted with 1.5 mL of HPLC MeOH, followed by 1.5 mL of water with reflux to break the agarose gel and recover the

AFs.

The eluates were dried in a 40°C oven, derivatized, and 60 µL were injected into the HPLC in triplicate. The amount of each recovered AF was calculated to adjust each AF concentration in the samples.

#### 4. Limits of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is the smallest reliable quantity of AF detected by the HPLC and reproduced in the matrix. The LOD was calculated with a regression analysis of the calibration curve for each AF. The LOQ is considered to be 5 times the LOD.

### Statistical method

The Kruskal-Wallis test was applied to the nonparametric data and uses ranges of sampling data from three or more independent populations. It is used to prove the null hypothesis that the independent samples come from populations with the same medians. The alternative hypothesis is the statement that populations have different medians.

## Results and Discussion

### Validation of the method

**Selectivity:** The selectivity chromatograms for each chili pepper type were Blank (Figure 2A), “Ancho” (Figure 2B), “Guajillo” (Figure 2C) and “Piquín” chili pepper (Figure 2D) fortified with 100 ng g<sup>-1</sup> AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, respectively. There was no interference between the retention times of the different AFs and the matrixes; therefore, the separation was correct (Figure 2).

**Linearity:** The concentrations for each AF curve produced the following linear equations and correlation coefficients: AFB<sub>1</sub>: y=2.8299x and R<sup>2</sup>=0.9973; AFB<sub>2</sub>: y=1.7786x, R<sup>2</sup>=0.9892; AFG: y=1.7607x, R<sup>2</sup>=0.9969; and AFG<sub>2</sub>: y=0 1.2411x, R<sup>2</sup>=0.9986. All the curves were correct, with a regression coefficient of R<sup>2</sup>>0.9892.

**Recovery percentage:** The ranges of the recovery percentage were AFB<sub>1</sub> (83%), AFB<sub>2</sub>, 75%, AFG<sub>1</sub> (96%), and AFG<sub>2</sub> (81%). These data were considered in the sample concentration calculations.

**Limits of detection (LOD) and quantification (LOQ):** The LOD (ng) were AFB<sub>1</sub> (0.1 ng), AFB<sub>2</sub> (0.01 ng), AFG<sub>1</sub> (0.01 ng), and AFG<sub>2</sub> (0.5 ng). The LOQ depends on the LOD and is multiplied by a factor of 5; therefore, the LOQ were AFB<sub>1</sub> (0.5 ng), AFB<sub>2</sub> (0.05 ng), AFG<sub>1</sub> (0.05 ng), and AFG<sub>2</sub> (2.5 ng).

### Aflatoxins in chili pepper

The identification of the AFs was based on the retention times (RT) in minutes (min) of the peak areas (ng g<sup>-1</sup>) in the chromatographs that were calculated from the calibration curve. The ranges of the RT times were: AFB<sub>1</sub> 7.504 to 8.158 min; AFB<sub>2</sub> 15.119 to 18.260 min; AFG<sub>1</sub> 5.570 to 5.930 min; and AFG<sub>2</sub> 10.269 to 11.568 min. The AF concentrations obtained in the chili peppers are presented in Tables 1 and 2.

The three types of Mexican chili peppers were contaminated with traces of the 4 AF types. AFB<sub>1</sub> is the most potent genotoxic and carcinogenic compound that is frequently detected in agricultural products [72]. Approximately 49%, 34%, 41%, and 24% of the “Ancho”, “Guajillo”, “Piquín”, and chili peppers from the other countries were contaminated with AFB<sub>1</sub>. Additionally, 44% and 56% of Mexican chili peppers were contaminated with Aft B, and Aft G, respectively, and 40% and 60% of foreign chili peppers were contaminated with Aft B and Aft G (Figure 3).

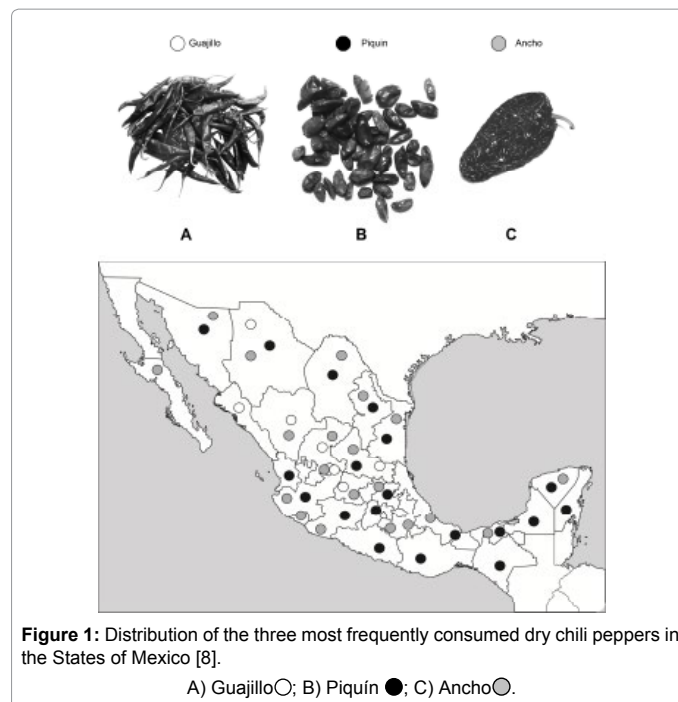


Figure 1: Distribution of the three most frequently consumed dry chili peppers in the States of Mexico [8].

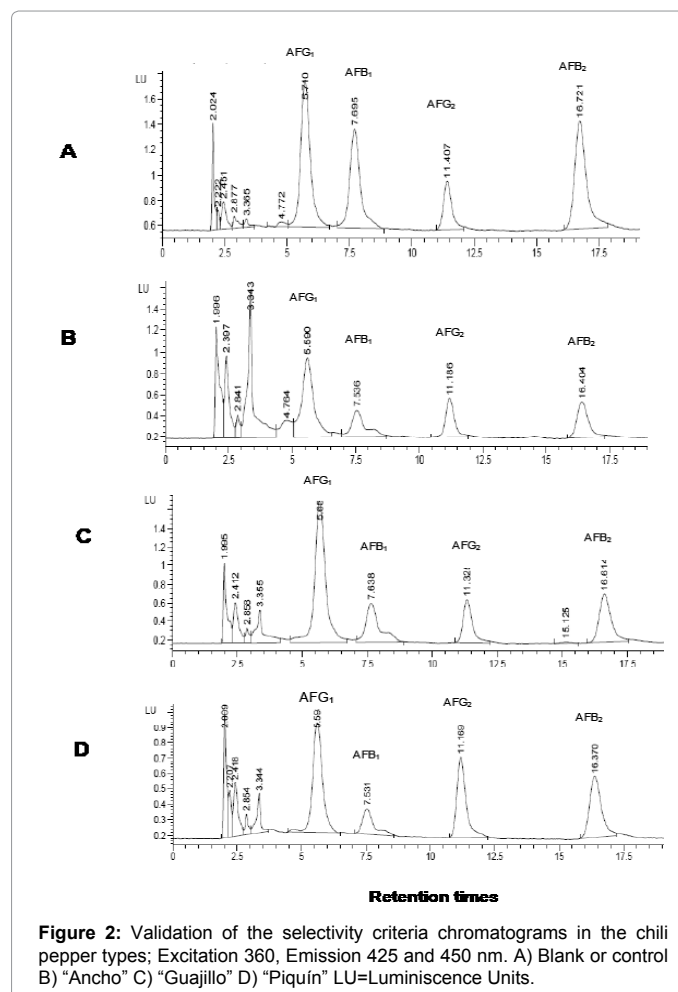


Figure 2: Validation of the selectivity criteria chromatograms in the chili pepper types; Excitation 360, Emission 425 and 450 nm. A) Blank or control B) “Ancho” C) “Guajillo” D) “Piquín” LU=Luminiscence Units.



For AFB<sub>1</sub> (μg kg<sup>-1</sup>) in the Mexican samples, the boroughs with highest AF concentrations were Cuauhtémoc for “Ancho” (10.62), Iztapalapa for “Guajillo” (1.83), and Cuauhtémoc for “Piquín” (6.82). For AFB<sub>2</sub> (μg kg<sup>-1</sup>) contamination in the Mexican chili pepper samples, the boroughs with the highest concentrations were: Cuajimalpa for “Ancho” (1.04), Miguel Hidalgo for “Guajillo” (0.49), and Benito Juárez for “Piquín” (0.79). The highest concentrations of AFG<sub>1</sub> (μg kg<sup>-1</sup>) were present in the samples from the Mexican boroughs of Coyoacán for “Ancho” (5.97), Gustavo A. Madero for “Guajillo” (1.99) and Tláhuac for “Piquín” (5.23). The Mexican boroughs with the highest levels of AFG<sub>2</sub> contamination (μg kg<sup>-1</sup>) were: Cuauhtémoc for “Ancho” (2.76), Iztacalco for “Guajillo” (1.62) and Gustavo A. Madero for “Piquín” (1.26). Trace contamination of AFG<sub>2</sub> (μg kg<sup>-1</sup>) was more abundant than AFB<sub>1</sub>, AFB<sub>2</sub> and AFG<sub>1</sub>. The boroughs with the highest levels of AFt contamination were Coyoacán for “Ancho” (14 μg kg<sup>-1</sup>), Iztacalco for “Guajillo” (2.96 μg kg<sup>-1</sup>), and Tláhuac for “Piquín” (9.5 μg kg<sup>-1</sup>) (Figure 4).

None of the three analyzed chili peppers surpassed the tolerance limit for AFt of 20 μg kg<sup>-1</sup> [68].

The average AF (μg kg<sup>-1</sup>) (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>, and AFt) concentrations in the dry chili peppers from India, Turkey and South Africa were also reported. The peppers with the most AFB<sub>1</sub> (μg kg<sup>-1</sup>) contamination were from Turkey b (1.21); Turkey c had only traces (<0.1). The samples with the most AFB<sub>2</sub> (2.32), AFG<sub>1</sub> (3.00 μg kg<sup>-1</sup>) and AFt (6.84 μg kg<sup>-1</sup>) contamination were from Turkey d, and the samples with the highest levels of AFG<sub>2</sub> (2.42) were from Turkey e, as shown in Figure 5.

A comparison of the average AFt in the chili peppers from Mexico and these other countries was also provided in Figure 6.

The “Ancho” and “Piquín” chili pepper samples with the highest levels of contamination were statistically significantly different. The samples with the highest levels of AFB<sub>2</sub> contamination were from the chili peppers from the foreign countries (0.4 μg kg<sup>-1</sup>), followed by “Ancho” (0.15 μg kg<sup>-1</sup>), “Guajillo” (0.08 μg kg<sup>-1</sup>) and “Piquín” (0.09 μg kg<sup>-1</sup>), as shown in Figure 7. The contaminated samples were not significantly different. The sample with the highest levels of AFG<sub>1</sub> contamination was “Piquín” (1.57 μg kg<sup>-1</sup>), which was significantly different from “Guajillo” (0.4 μg kg<sup>-1</sup>) and the samples from other countries (0.79 μg kg<sup>-1</sup>); however, the latter samples were not significantly different, as shown in Figure 7. There was a significant difference between the AFs in the samples from different boroughs, with the exception of AFG<sub>2</sub> in “Guajillo” chili pepper. For AFt, the “Ancho” chili pepper (3.49 μg kg<sup>-1</sup>) was the most contaminated, followed by “Piquín” (3.14 μg kg<sup>-1</sup>), those from the foreign countries (2.50 μg kg<sup>-1</sup>) and “Guajillo” (0.92 μg kg<sup>-1</sup>). The latter was the only one to exhibit significant differences with the other three types, as shown in Figure 7.

The weather of the tropical regions is humid, dry, and semiarid with a minimum temperature of 25°C, which influences the growth of toxicogenic fungi and the AF production [73]. The AF producer fungi are common in air, soil and crops and the AFs are not produced or are produced at low levels in areas with cold weather below 20°C [74].

Twelve industrialized spices in Portugal were analyzed [62], and 43% of the samples, including chili pepper powder, contained AFB<sub>1</sub> (1.9-2.5 μg kg<sup>-1</sup>) at similar amounts as those recorded in the present study.

Our analysis of the few samples from India detected only traces of AF (0.13-0.62 μg kg<sup>-1</sup>) and our results were not conclusive, but other

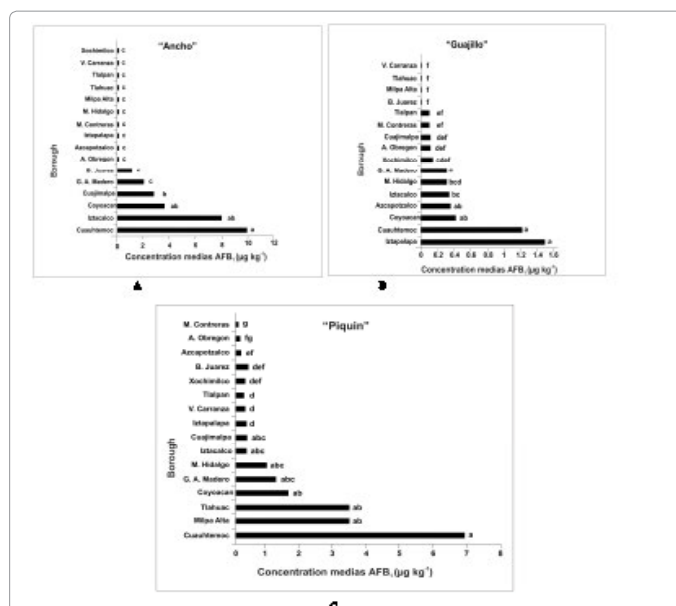


Figure 3: Average concentration of AFB<sub>1</sub> (μg kg<sup>-1</sup>) in chili pepper in each borough of Mexico City. A) “Ancho” B) “Guajillo” and C) “Piquín”. Groups with common letters (ab, abc, def, efg and fg) were not significantly different, while groups with different letters (a, d, and g) were significantly different.

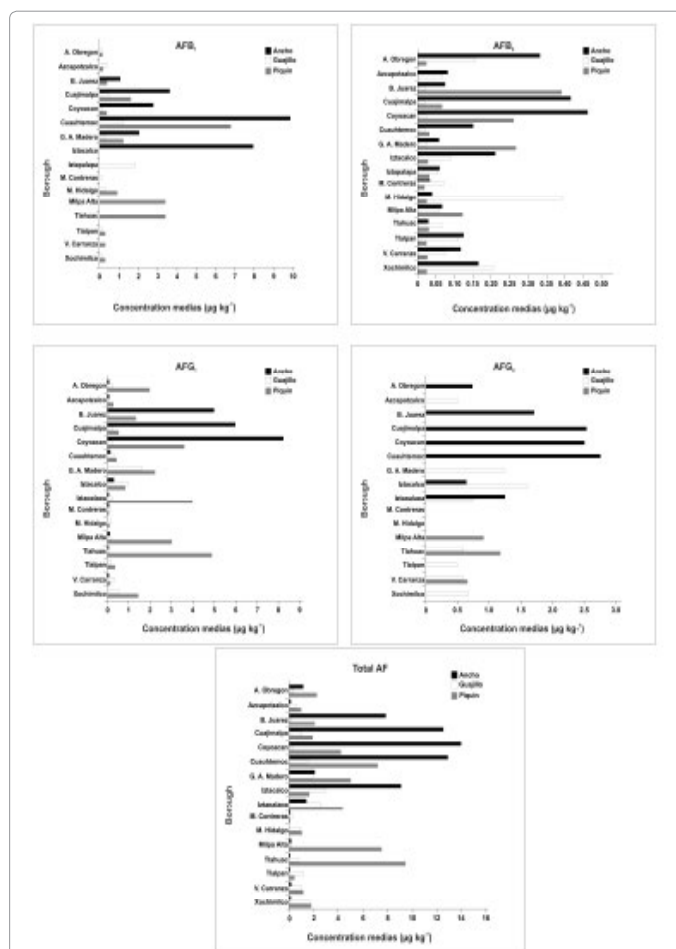


Figure 4: AF type and concentration in each borough of Mexico city.

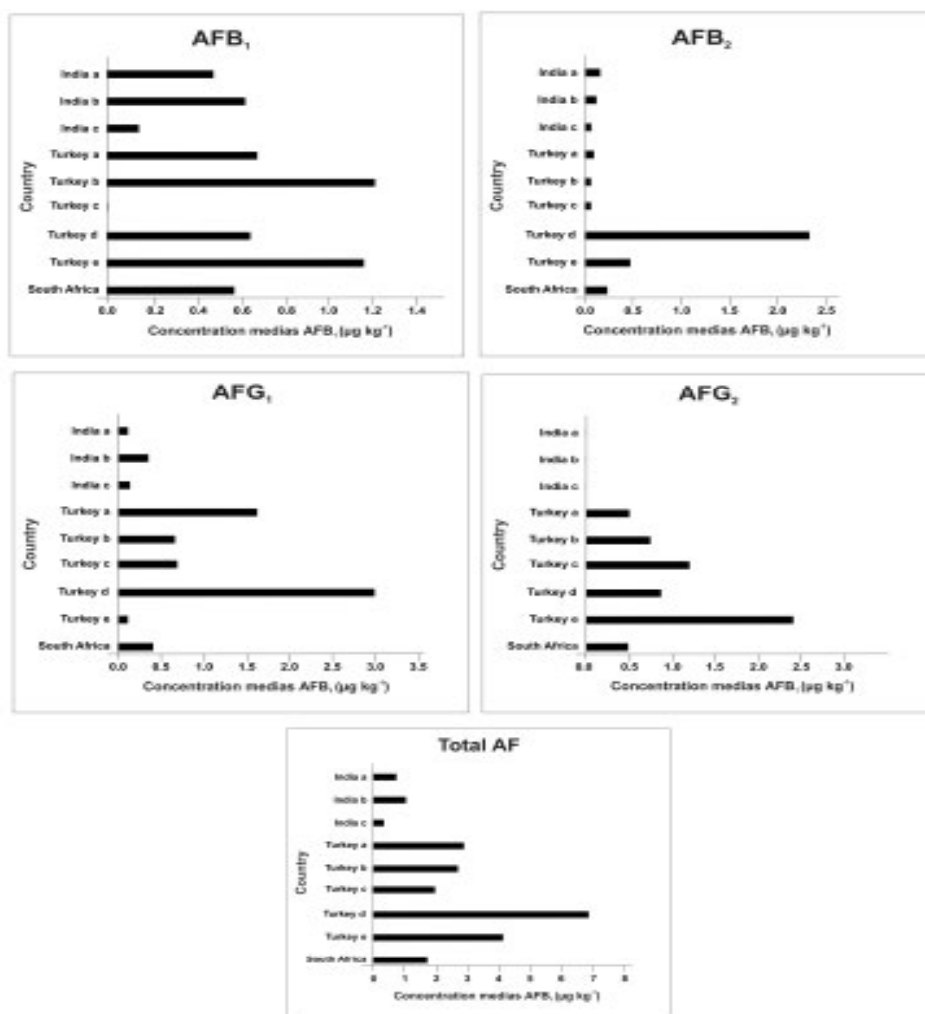


Figure 5: Average AF (µg kg<sup>-1</sup>) concentration in dry chili pepper from India, Turkey and South Africa. A) AFB<sub>1</sub> B) AFB<sub>2</sub> C) AFG<sub>1</sub> D) AFG<sub>2</sub> and D) Total AF.

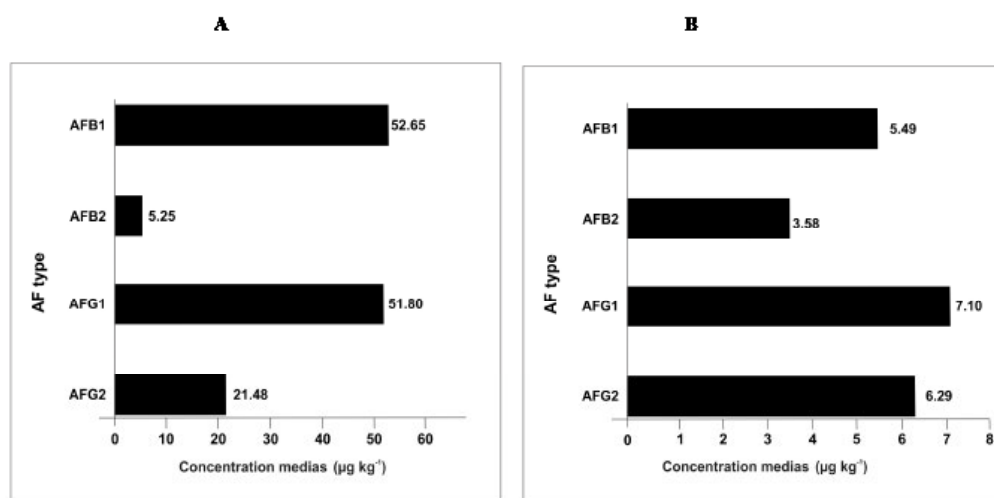


Figure 6: Average total aflatoxin (AFt) concentration in chili peppers in: A) Mexico and B) Other countries (India, Turkey and South Africa).

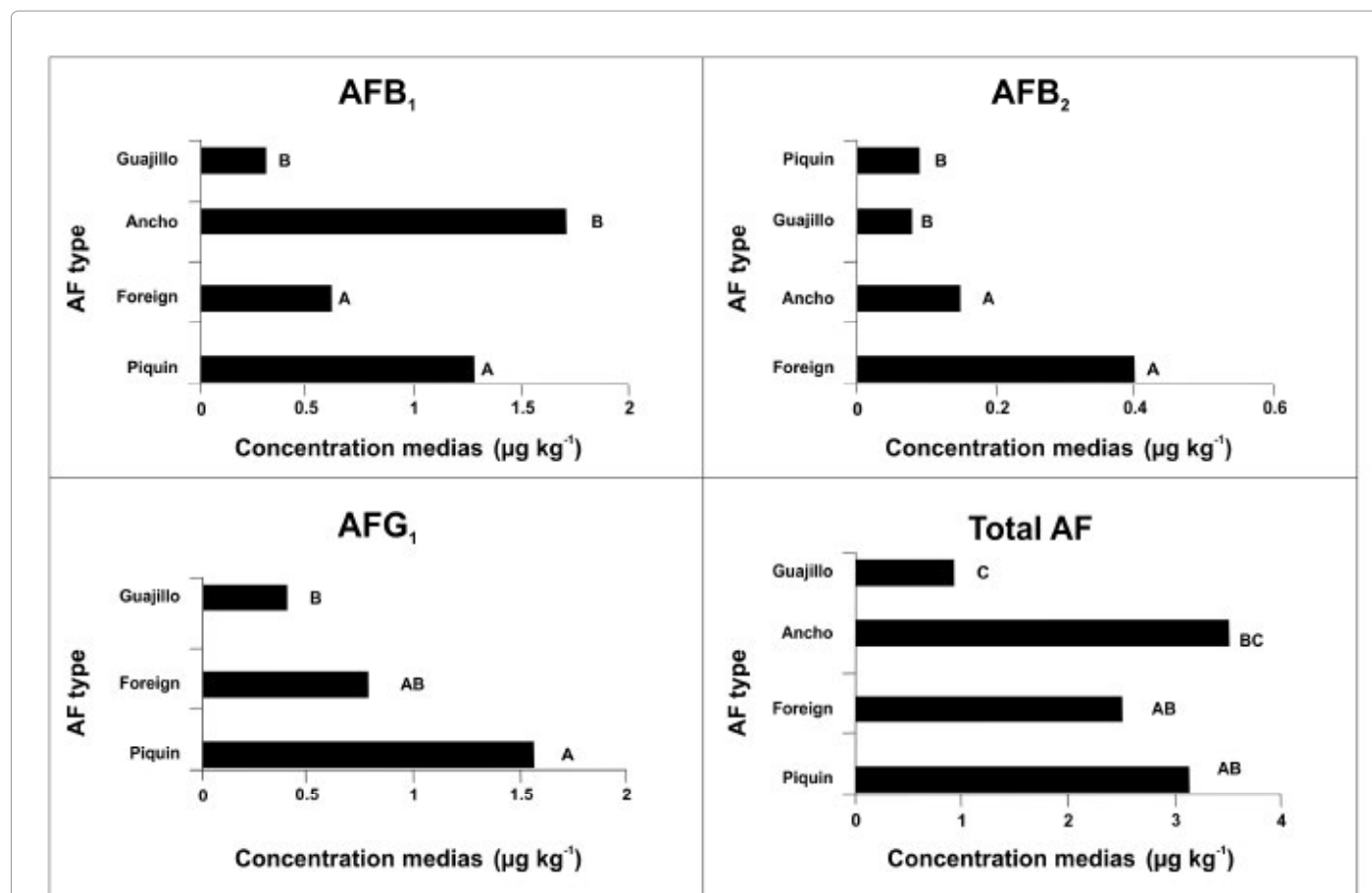


Figure 7: Average concentration of AF (µg kg<sup>-1</sup>) in three different chili peppers. The groups with common letters (AB, BC) were not significantly different, while the ones with different letters (A, B, C) were significantly different.

Borough	Aflatoxin concentration (µg kg <sup>-1</sup> ) in chili peppers														
	"Ancho"					"Guajillo"					"Piquin"				
	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	AFt	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	AFt	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	AFt
Alvaro	0.00	0.23	0.08	LOD	<b>0.31</b>	0.11	0.01	0.52	LOD	<b>0.64</b>	0.15	0.08	0.93	LOD	<b>1.16</b>
	0.00	0.50	0.12	0.73	<b>1.35</b>	LOD	0.02	0.14	LOD	<b>0.16</b>	0.13	0.16	2.73	LOD	<b>3.01</b>
Obregon	0.00	0.27	0.11	LOD	<b>0.38</b>	LOD	0.04	0.17	LOD	<b>0.21</b>	LOD	0.24	2.32	LOD	<b>2.56</b>
	0.00	0.02	0.09	LOD	<b>0.11</b>	0.34	0.06	0.12	LOD	<b>0.52</b>	0.21	LOD	0.23	LOD	<b>0.44</b>
Azcapotzalco	0.00	0.05	0.09	LOD	<b>0.14</b>	0.39	0.11	0.15	LOD	<b>0.65</b>	0.17	LOD	0.20	LOD	<b>0.37</b>
	0.00	0.18	0.08	LOD	<b>0.25</b>	0.32	0.03	0.17	LOD	<b>0.52</b>	0.14	LOD	0.46	0.52	<b>1.13</b>
	0.23	0.05	3.31	LOD	<b>3.59</b>	LOD	0.02	0.08	LOD	<b>0.10</b>	LOD	0.16	0.93	LOD	<b>1.09</b>
Benito Juarez	0.45	0.02	9.21	LOD	<b>9.68</b>	LOD	0.02	0.13	LOD	<b>0.16</b>	0.10	<b>0.79</b>	1.28	LOD	<b>2.17</b>
	2.49	0.15	2.50	1.71	<b>6.85</b>	LOD	0.02	0.04	LOD	<b>0.07</b>	0.67	0.24	1.84	LOD	<b>2.75</b>
	3.63	0.53	6.85	2.83	<b>13.85</b>	0.58	0.02	1.03	LOD	<b>1.63</b>	1.55	0.16	0.29	LOD	<b>2.00</b>
Coyoacan	3.65	0.21	4.09	2.20	<b>10.14</b>	0.43	0.02	0.52	LOD	<b>0.98</b>	1.84	0.02	0.10	LOD	<b>1.96</b>
	3.52	0.51	6.98	2.62	<b>13.64</b>	0.26	0.02	0.15	LOD	<b>0.42</b>	1.44	0.02	0.41	LOD	<b>1.86</b>
	2.72	<b>1.04</b>	8.29	3.04	<b>15.09</b>	0.11	0.04	0.05	LOD	<b>0.19</b>	0.33	0.39	3.49	2.26	<b>6.47</b>
Cuajimalpa	2.77	0.21	8.11	2.52	<b>13.61</b>	LOD	0.02	0.55	LOD	<b>0.57</b>	0.33	0.24	3.78	1.01	<b>5.35</b>
	2.89	0.15	8.29	1.99	<b>13.32</b>	LOD	0.01	0.12	LOD	<b>0.13</b>	0.38	0.16	3.60	LOD	<b>4.14</b>
	9.36	0.10	0.01	1.68	<b>11.15</b>	1.28	0.01	0.41	LOD	<b>1.70</b>	<b>6.82</b>	0.02	0.35	LOD	<b>7.19</b>
	9.58	0.20	0.43	3.25	<b>13.45</b>	1.33	0.02	0.47	LOD	<b>1.82</b>	<b>6.82</b>	0.02	0.76	LOD	<b>7.59</b>
Cuauhtemoc	<b>10.62</b>	0.15	0.02	3.35	<b>14.15</b>	1.04	0.02	0.03	LOD	<b>1.09</b>	6.70	0.06	0.29	LOD	<b>7.05</b>
	2.05	0.11	0.02	LOD	<b>2.19</b>	LOD	0.03	<b>1.99</b>	LOD	<b>2.02</b>	1.38	0.31	2.56	LOD	<b>4.25</b>
	0.00	0.04	0.04	LOD	<b>0.07</b>	0.20	0.01	1.51	LOD	<b>1.73</b>	1.42	0.25	2.21	LOD	<b>3.88</b>
Gustavo A. Madero	0.00	0.03	0.05	LOD	<b>0.08</b>	0.43	0.03	1.42	LOD	<b>1.87</b>	0.84	0.24	2.03	1.26	<b>4.36</b>

Iztacalco	6.16	LOD	0.85	0.65	<b>7.67</b>	0.34	0.04	0.87	1.80	<b>3.05</b>	0.88	0.04	1.05	LOD	<b>1.96</b>
	9.75	0.38	0.04	LOD	<b>10.17</b>	0.23	0.09	1.51	1.44	<b>3.27</b>	0.88	LOD	0.81	LOD	<b>1.69</b>
	0.00	0.04	0.06	LOD	<b>0.10</b>	0.21	0.15	0.58	LOD	<b>0.95</b>	0.63	0.02	0.70	LOD	<b>1.34</b>
Iztapalapa	0.00	0.05	0.09	1.41	<b>1.56</b>	<b>1.83</b>	0.07	0.29	LOD	<b>2.20</b>	0.46	LOD	3.26	LOD	<b>3.72</b>
	0.00	0.08	0.10	LOD	<b>0.19</b>	1.66	0.03	0.16	LOD	<b>1.85</b>	0.36	0.03	4.53	LOD	<b>4.93</b>
	0.00	0.05	0.07	1.10	<b>1.21</b>	0.99	0.06	0.22	0.77	<b>2.03</b>	0.21	0.03	4.18	LOD	<b>4.42</b>
Magdalena Contreras	0.00	0.04	0.10	LOD	<b>0.14</b>	LOD	0.04	0.13	LOD	<b>0.18</b>	LOD	0.02	0.08	LOD	<b>0.10</b>
	0.00	0.04	0.07	LOD	<b>0.11</b>	0.10	0.07	0.17	LOD	<b>0.35</b>	LOD	0.02	0.12	LOD	<b>0.14</b>
	0.00	0.02	0.09	LOD	<b>0.11</b>	LOD	0.10	0.19	LOD	<b>0.29</b>	LOD	0.02	0.12	LOD	<b>0.13</b>
Miguel Hidalgo	0.00	0.05	0.04	LOD	<b>0.09</b>	0.36	0.42	0.24	LOD	<b>1.01</b>	0.88	0.02	0.12	LOD	<b>1.02</b>
	0.00	0.04	0.05	LOD	<b>0.08</b>	0.26	<b>0.49</b>	0.16	LOD	<b>0.90</b>	0.96	0.02	0.15	LOD	<b>1.13</b>
	0.00	0.03	0.04	LOD	<b>0.07</b>	LOD	0.27	0.16	LOD	<b>0.43</b>	0.96	0.03	0.10	LOD	<b>1.09</b>
Milpa Alta	0.00	0.08	0.09	LOD	<b>0.17</b>	LOD	0.03	0.16	LOD	<b>0.19</b>	3.68	0.18	4.88	LOD	<b>8.74</b>
	0.00	0.10	0.21	LOD	<b>0.31</b>	LOD	0.07	0.19	LOD	<b>0.26</b>	3.39	0.14	1.86	LOD	<b>5.39</b>
	0.00	0.02	0.12	LOD	<b>0.14</b>	LOD	0.06	0.15	LOD	<b>0.21</b>	3.15	0.05	2.32	0.92	<b>6.44</b>
Tlahuac	0.00	0.03	0.11	LOD	<b>0.14</b>	LOD	0.03	0.12	0.60	<b>0.75</b>	3.31	0.04	<b>5.23</b>	1.86	<b>10.43</b>
	0.00	0.05	0.09	LOD	<b>0.14</b>	LOD	0.10	0.13	LOD	<b>0.23</b>	3.31	0.02	4.88	0.91	<b>9.11</b>
	0.00	0.02	0.06	LOD	<b>0.08</b>	LOD	0.06	0.06	LOD	<b>0.13</b>	3.61	0.04	4.51	0.81	<b>8.97</b>
Tlalpan	0.00	0.08	0.07	LOD	<b>0.15</b>	LOD	0.07	0.39	LOD	<b>0.46</b>	0.33	0.05	0.46	LOD	<b>0.85</b>
	0.00	0.11	0.07	LOD	<b>0.18</b>	LOD	0.14	0.42	LOD	<b>0.57</b>	0.26	0.01	0.05	LOD	<b>0.32</b>
	0.00	0.19	0.05	LOD	<b>0.24</b>	0.10	0.12	0.35	0.50	<b>1.07</b>	0.21	0.01	0.04	LOD	<b>0.26</b>
Venustiano Carranza	0.00	0.15	0.09	LOD	<b>0.24</b>	LOD	0.09	0.41	LOD	<b>0.49</b>	0.23	0.03	0.12	0.66	<b>1.04</b>
	0.00	0.14	0.09	LOD	<b>0.23</b>	LOD	0.12	0.38	0.62	<b>1.13</b>	0.42	0.03	0.13	LOD	<b>0.58</b>
	0.00	0.06	0.09	LOD	<b>0.15</b>	LOD	0.01	0.21	0.54	<b>0.76</b>	0.25	0.02	0.23	0.67	<b>1.18</b>
Xochimilco	0.00	0.17	0.07	LOD	<b>0.23</b>	0.14	0.18	0.66	0.53	<b>1.51</b>	LOD	0.02	0.64	LOD	<b>0.65</b>
	0.00	0.20	0.05	LOD	<b>0.25</b>	0.13	0.30	0.70	LOD	<b>1.12</b>	0.42	0.02	1.86	LOD	<b>2.30</b>
	0.00	0.13	0.05	LOD	<b>0.18</b>	LOD	0.14	0.39	0.84	<b>1.37</b>	0.19	0.04	1.98	LOD	<b>2.20</b>
<b>Average</b>	<b>1.46</b>	<b>0.15</b>	<b>1.28</b>	<b>2.08</b>	<b>3.49</b>	<b>0.53</b>	<b>0.08</b>	<b>0.40</b>	<b>0.85</b>	<b>0.92</b>	<b>1.44</b>	<b>0.10</b>	<b>1.57</b>	<b>1.09</b>	<b>3.14</b>

Table 1: Quantification of AFs ( $\mu\text{g kg}^{-1}$ ) in chili peppers.

Sample/Average	AF concentration ( $\mu\text{g kg}^{-1}$ )				
	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	Aft
India a	<LOD	0.11	0.12	<LOD	<b>0.23</b>
	0.43	0.22	0.10	<LOD	<b>0.75</b>
	0.51	0.14	0.11	<LOD	<b>0.76</b>
India b	0.70	0.11	0.44	<LOD	<b>1.25</b>
	0.70	0.15	0.28	<LOD	<b>1.13</b>
	0.44	0.11	0.35	<LOD	<b>0.90</b>
India c	0.10	0.05	0.12	<LOD	<b>0.27</b>
	<LOD	0.08	0.18	<LOD	<b>0.26</b>
	0.16	0.07	0.12	<LOD	<b>0.35</b>
<b>Average of India</b>	<b>0.4</b>	<b>0.1</b>	<b>0.2</b>	<b>0</b>	<b>0.7</b>
Turkey a	1.11	0.09	1.77	<LOD	<b>2.97</b>
	0.68	0.08	1.44	0.51	<b>2.71</b>
	0.25	0.10	1.65	<LOD	<b>2.00</b>
Turkey b	1.20	0.11	1.24	0.82	<b>3.37</b>
	1.16	0.03	0.34	<LOD	<b>1.53</b>
	1.28	0.06	0.41	0.69	<b>2.44</b>
Turkey c	<LOD	0.06	0.67	0.61	<b>1.34</b>
	<LOD	0.06	0.68	1.53	<b>2.27</b>
	<LOD	0.05	0.71	1.50	<b>2.26</b>
Turkey d	0.60	2.32	3.00	0.90	<b>6.82</b>
Turkey e	1.18	<b>0.48</b>	0.12	2.42	<b>4.20</b>
<b>Average of Turkey</b>	<b>1</b>	<b>0.3</b>	<b>1</b>	<b>1</b>	<b>2.9</b>
South Africa	0.65	0.38	0.65	0.51	<b>2.19</b>
	0.65	0.23	0.28	<LOD	<b>1.16</b>
	0.43	0.10	0.30	<LOD	<b>0.83</b>
<b>Average of South Africa</b>	<b>0.6</b>	<b>0.2</b>	<b>0.4</b>	<b>0.5</b>	<b>1.5</b>
<b>Average of foreign countries</b>	<b>0.7</b>	<b>0.2</b>	<b>0.7</b>	<b>1.1</b>	<b>1.8</b>

Table 2: Quantitation of AFs in dry chili peppers samples from other countries.



reports from this same country [63] showed that 59% of 182 samples of chili pepper contained AFB<sub>1</sub> (2-283 µg kg<sup>-1</sup>). India has warm weather and AFs are frequently produced.

The ingestion of large amounts of chili pepper was associated with a higher risk of gall bladder cancer in Chilean women [75].

The species that frequently produce AFs are *A. flavus* and *A. parasiticus*, and those that do not frequently produce AFs are *A. bombycis*, *A. ochraceoseus*, *A. nomius* and *A. pseudotamarii* [27-29]. The fungal strains have different AF production capacities, and only half of the *A. flavus* strains produce approximately 106 µg kg<sup>-1</sup> AF [30]. Inadequate cleaning or inappropriate drying of the harvested chili pepper increases the risk of fungal contamination and AF production [76]; therefore, the harvested fruits must be cleaned and the rotten ones discarded [77].

Capsaicin is the most important compound of chili (*Capsicum* spp.) pungency [78], and it is reported to decrease AFB<sub>1</sub> linkage to DNA and adduct formation. In the presence of 25 µM capsaicin, there was a decrease in the number of adducts (AFB<sub>1</sub>-N<sup>7</sup>-Gua), and with 100 µM, the inhibition was 71-75% when it was incubated with an S9 microsomal concentrate of rat liver to activate the animal's metabolism. It appears that the chili pepper has developed defenses against AFB<sub>1</sub> through capsaicin formation; thus, more pungent chili peppers, which have more capsaicin, will inhibit adduct (AFB<sub>1</sub>-N<sup>7</sup>-Gua) formation, which are active carcinogens in animals and man. Therefore, the chili peppers that are not pungent are the most dangerous because they have less capsaicin and they can increase AF-DNA adduct formation. Capsaicin reduced the AFB<sub>1</sub> metabolism that depends on the S9 microsomal concentrate, and it also altered the formation of the water-soluble AFB<sub>1</sub> hydroxylate. The activity of the organ-soluble fractions and the AFB<sub>1</sub>-glutathione conjugates were reduced, as analyzed by HPLC. Capsaicin inhibits the AFB<sub>1</sub> biotransformation because it modifies Phase I of the enzymatic activity in the liver.

Several mycotoxins can be present simultaneously in chili peppers, and their consumption can increase the risk of damages to health

[79]. Due to frequent AF contamination, the European Union has established a tolerance limit for spices of 5 µg kg<sup>-1</sup> for AFB<sub>1</sub> and 10 µg kg<sup>-1</sup> for AFt (B<sub>1</sub>+B<sub>2</sub>+G<sub>1</sub>+G<sub>2</sub>) [65]. Taking into account this limit for AFt, the "Ancho" chili pepper of the Coyoacan borough (14 µg kg<sup>-1</sup>) surpasses this limit, while "Piquín" (9.5 µg kg<sup>-1</sup>) of Tláhuac borough is on the border. The samples from India, Turkey and South Africa were not over this limit; the sample with the highest level of contamination was from Turkey (6.84 µg kg<sup>-1</sup>). There is no specific legislation for spices in Mexico; the AF legislation for cereals of human consumption established a limit of 20 µg kg<sup>-1</sup> AFt [68], and no chili pepper surpassed this amount. However, when considering the amount of AFt and the weight of the chili pepper that is eaten, the limits would be surpassed. Table 3 shows the amount of AFs consumed in dishes, sauces, and Mexican food.

### Statistical analysis

The results of the Kruskal-Wallis test are shown in Tables 4 and 5 and were obtained with the statistical program R. For the "Ancho" and "Piquín" chili peppers, there were significant differences in the AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> contents in the boroughs. In "Guajillo" chili pepper, the AFG<sub>2</sub> was not significantly different (Tables 4 and 5).

There was a significant difference for the 4 AFs and AFt according to the type of chili pepper, as shown in Tables 4 and 5.

The Wilcoxon test was performed to detect significant differences between samples. For AFB<sub>1</sub>, only the "Ancho" and "Piquín" samples were considered because they had a higher than average AF concentration.

In conclusion, the result of this study indicates that the analytical method was validated, chili pepper is almost always contaminated with all AFs. Until now, there has been an indirect relationship between the pungency and the amount of AF. In this study, we showed that the "Ancho" chili pepper is not pungent and exhibited the highest levels of AF contamination. Chili peppers are daily ingested in Mexico and they are frequently contaminated with aflatoxins so they can be considered a risk for health, because they greatly contribute to increase the carcinogen amount in the staple diet. The AF legislation for foods

Chili pepper type	Detected AFt (ng g <sup>-1</sup> )	Amount of chili pepper (g) by food	AFt (µg kg <sup>-1</sup> ) ingested in 25 g
"Ancho"	3.49	25	87.3
"Guajillo"	0.92	25	23.0
"Piquín"	3.14	5	15.7

Table 3: Calculated AFt (µg kg<sup>-1</sup>) concentration in ingested chili pepper by meal or dish.

Type of chili	AF	Test value	Significance	Significant difference
"Ancho"	AFB <sub>1</sub>	40.95	<0.05	Yes
	AFB <sub>2</sub>	31.26		
	AFG <sub>1</sub>	35.25		
	AFG <sub>2</sub>	38.27		
	AFT	37.08		
"Piquín"	AFB <sub>1</sub>	42.83		
	AFB <sub>2</sub>	35.55		
	AFG <sub>1</sub>	42.99		
	AFG <sub>2</sub>	27.55		
	AFT	43.61		
"Guajillo"	AFB <sub>1</sub>	38.09	0.11	No
	AFB <sub>2</sub>	35.16	<0.05	Yes
	AFG <sub>1</sub>	31.97		
	AFG <sub>2</sub>	21.96		
	AFT	38.93		

Table 4: Kruskal-Wallis test for the three chili pepper types.

AF	Value of the test	Significance
AFB <sub>1</sub>	21.89836	<0.05 *
AFB <sub>2</sub>	16.20834	<0.05 *
AFG <sub>1</sub>	31.83879	<0.05 *
AFG <sub>2</sub>	3.831674	0.28
AFT	21.13252	<0.05*

\*=Significant Difference

Table 5: Kruskal-Wallis test by source.

of human consumption established a limit of 20 µg kg<sup>-1</sup> Aft [68] and spices as chili peppers can be included in this category. Although no chili pepper surpassed this tolerance limit, when considering the amount and weight of chili peppers that are eaten, the limits would be surpassed.

### Acknowledgements

The authors thank the following IBUNAM personnel: Noemí Chávez, from the Technical Secretary (Secretaría Técnica); Joel Villavicencio, Jorge López, Alfredo Wong, Jimena Gutiérrez for computer assistance; Georgina Ortega Leite and Gerardo Arévalo for library information; Diana Martínez and Julio César Montero for design.

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