Detection of Aflatoxins, Mutagens and Carcinogens in Black, White and Green Peppers (*Piper Nigrum* L.)

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**Abstract**

Aflatoxins, bis-dihydro-furancoumarins, are secondary metabolites that are produced by molds of *Aspergillus* sp. with adverse effects in humans and animals. The International Agency for Research on Cancer classifies aflatoxins in Group 1 of proven human carcinogens. Thus, aflatoxins in foods are highly regulated throughout the world. The purpose of this research was to identify and quantify aflatoxins in 54 pepper samples (19 black, 19 white and 16 green peppers) from markets in Egypt, India, Turkey and the 16 boroughs of Mexico City, as well as to validate the experimental method used. All samples were contaminated with at least one aflatoxin: 95% (51/54) were contaminated with aflatoxin B1 (0.1 to 218 µg kg⁻¹); 80% (43/54) with aflatoxin B2 (0.4 to 382 µg kg⁻¹); 67% (36/54) with aflatoxin G1 (0.4 to 612 µg kg⁻¹); and 93% (50/54) with aflatoxin G2 (1.37 to 494 µg kg⁻¹). Only 9.26% of the samples (5/54) were under the Mexican legislation limit, whereas all foreign samples surpassed the limits established for their respective countries.

Although the aflatoxin concentrations in peppers are high, their ingestion is minimal because peppers are used in only small quantities as a flavor-enhancing product. Therefore, the contribution of aflatoxins from a pepper to an organism is relatively low in comparison to other agricultural products, such as maize, pistachio, peanuts and dairy products. Green pepper was the most contaminated with aflatoxins, white pepper was the least contaminated and black pepper had an intermediate level of contamination. This study describes a detailed analysis of aflatoxin contamination in pepper in three different ripening stages: green, black and white. The lack of normativity in countries on this subject prevents the reduction of AF concentrations in the diet.

**Keywords:** Pepper; Carcinogens; Aflatoxins; Spice contamination

**Introduction**

Pepper (*Piper nigrum* L.) is the most important spice with economic value and is used as an ingredient in many dishes to give flavor to foods. *Piper nigrum* L. is also a tropical arbus that forms clusters or racemes in warm climates of 25 to 30°C and 60 to 93% humidity [1,2]. The different types of peppers are due to the different ripening stages of the grains. Green peppers are unripe grains that are dried or preserved in vinegar or citrus acid. Black peppers are harvested halfway through the maturation period when they are green-yellowish, and their berries are submerged in boiling water for 10 min. This treatment favors fermentation, which produces the black color and disinfects the surface. Black berries are sun-dried for 2 weeks to reach 12% humidity; this type of pepper has been the most commonly used since ancient times. White peppers are mature, peeled grains without a husk; they are harvested when they are red or orange in color, soaked in water for one week to peel them and are later dried until they have a white-brownish color. The flavor of white pepper is milder than that of black pepper [1].

Pepper is found in five continents, and its economic value is more than 1,000,000,000 US dollars [3]. The countries that produce the most pepper are Vietnam (146,000 tons), Indonesia (65,000 tons), Brazil (44,610 tons) and China (31,963 tons) [4]. Mexico is not a sufficient producer of pepper (6,335 tons); therefore, it imports black pepper [2]. The main production states in Mexico are Veracruz (5,053.7 tons), Tabasco (900 tons), Chiapas (174 tons), Puebla (138.5 tons) and Oaxaca (2.8 tons) [2]. In 2009, Veracruz was the most productive state, contributing 53.5% of the sowen surface, 80.6% of the volume production and 59.2% of the generated value [2] (Figure 1).

Between 2000 and 2008, world-wide pepper production increased by 31.8% (414,849 tons) [2]. The world’s pepper consumption is approximately 350,000 tons [5].

In spices, fungal growth occurs in warm and humid conditions [6]. Chemically, aflatoxins (AFs) are bis-dihydrofururan coumarins, fluorescent compounds with chemical structures and physicochemical properties that are well-described [7]. They are secondary toxic metabolites produced between 25 and 35°C by *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and *A. pseudotamarii* and they can affect human health [6].

In *A. flavus* and *A. parasiticus*, growth occurs at a relative humidity ranging from 88 to 95%, a pH between 3.5 and 5.5 and at a high water activity (wa). Other factors that are important for fungal growth and AF synthesis are the environmental gaseous composition and light. Some aerobic fungi grow well at a concentration of 20% CO₂; however, at 10% CO₂, they cease AF production [8].

The main types of AFs in pepper are aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂). AF toxicity in decreasing order is AFB₁>AFG₁>AFB₂>AFG₂ [9].

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The ingestion of foods contaminated with AFs predisposes humans and animals to disease and ultimately death [10]. The most common cause of chronic intoxication (for weeks, months and years) is the ingestion of small quantities of AFs in foods. AF consumption results in damaging effects, such as immunosuppression, hemorrhages, malformations, abortions, fetal diarrhea, vomiting, growth deficiency, certain types of cancer and death, depending on the time of consumption and the quantities ingested.

In general, the effects of AFs in humans are limited due to the number of cases [11]. Acute AF exposure has been associated with hepatitis B epidemics in China and Africa, with death rates ranging from 10 to 60% [12]. AF exposure is also associated with human hepatocellular cancer that worsens in the presence of hepatitis B virus [13]. There is also a synergistic effect between exposure to AFs and some diseases, such as malaria, Kwashiorkor, Rey’s syndrome and AIDS in children [14]. One case from Senegal showed that the daily consumption of 5 to 20 µg kg⁻¹ AFs in body weight caused abnormalities in the livers of children with Kwashiorkor within 10 months due to the ingestion of an AF-contaminated protein supplement that is used to treat this disease [15].

AFs are potent carcinogens and classified by the International Agency for the Research of Cancer as Group 1 therefore, AF levels in foods are regulated throughout the world [16]. In the European Union and Turkey, the maximum tolerance level for AFs in spices, such as pepper (Piper spp.), is 5 µg kg⁻¹ for AFB, and 10 µg kg⁻¹ for total aflatoxins (AFt). CODEX Alimentarius has established maximum tolerance limits of 15 µg kg⁻¹ AFB for some processed nuts and 10 µg kg⁻¹ for foods that are ready for consumption based on JECFA [17]. In Mexico, the legal accepted limit is up to 20 µg kg⁻¹ AFt for cereals, but there is no legislation for spices; therefore, peppers are not regulated.

The decreased susceptibility of animals to AFs ranges from poultry (ducks<turkey<chickens) to mammals (dogs<pigs<veal<sheep<cattle). AFs cause non-specific symptoms in animals, including a reduction in weight, a decrease in egg and milk production, and an increased susceptibility to infections, mutations and cancer in rats [18]. Thymus depression and a decrease in T cell function and cellular immunity are the observed effects of AFs in bovines, sheep and pigs [19-21].

Ruminants are more resistant to the effects of AFs because the microbiota can degrade AFs within the rumen [22,23]. It is likely that the sheep’s rumen can detoxify AFs and make them resistant to up to 500 mg kg⁻¹ AFs [24]. Macaque monkeys have a DL₅₀ value of 7.8 mg kg⁻¹ for females and 2.2 mg kg⁻¹ for males; the DL₅₀ in small ducks (0.4 mg kg⁻¹), rats (1.0 mg kg⁻¹), sheep (500 mg kg⁻¹), and pigs varies from 0.3 to 0.6 mg kg⁻¹ AFB [15,25]. Oral ingestion of 4 mg kg⁻¹ AFs kills bovines within 15 h due to acute liver failure [24]. AFB, is the most toxic and well-studied AF with respect to its carcinogenic and cytotoxic effects. A single dose of 5 mg kg⁻¹ AFB₁ in rat feed for 6 weeks inhibits DNA and RNA synthesis [26].

Studies on the ingestion of pure AFs in suicide attempts have demonstrated that unusually high dosages (5.5 mg for two days and 53 mg for 2 weeks) cause transitory skin eruptions, nausea and headaches up to 6 months later and are not as effective as long-term doses. A woman who attempted suicide completely recovered without liver lesions when examined 14 years later [27]. Therefore, it was concluded that subacute prolong dosages are necessary to induce toxic lethal effects (i.e., with pepper, the ingestion of small dosages for a long time) [27]. The purpose of this study was to determine the contribution of pepper to AF contamination in food.

Figure 1: The five Mexican States that produce pepper are Veracruz, Tabasco, Chiapas, Puebla and Oaxaca [2].
Methods and Materials

Sampling

The estimated current population of Mexico is approximately 130,139,368 inhabitants. Mexico City is the capital city of Mexico, with a population of 21.3 million people [28]. Mexico City contributes 20% of Mexico's entire population, making it the most populous metropolitan area in the Western Hemisphere and one of the most densely populated cities in the world [29]. Mexico City is divided into 16 boroughs and receives food from the entire country. Therefore, it is a reliable sampling site to gain an understanding of pepper consumption in Mexico. 50 g samples of the three types of peppers (green, black and white) were obtained from the three most important markets from each of the 16 boroughs of Mexico City (Figure 2).

Green peppers from foreign countries were not analyzed. Pepper grinding (Moulinex® Model AR6838C6, Mexico City, Mexico) was performed with the entire grain of each pepper. In the case of the boroughs of Mexico City, 17 g of pepper from each of the three markets per borough were mixed to obtain a compound sample of 51 g. For foreign peppers, a 51 g weight was applied directly to each sample.

Method validation

Validation is the process of establishing, through laboratory studies, a satisfactory chemical method that is suitable to analyze samples [30]. Method validation was based on Rule 401/2006 of the European Commission and on the criteria for the physicochemical method of the Ministry of Health of Mexico according to the following parameters [30,31]:

- **Selectivity**: Selectivity is the ability of a chemical process to differentiate analytes (in this case the four AFs) from other compounds of a complex matrix. We used three matrices of pepper (black, white and green) in an independent manner, with a mixture of the four AF standards (100 ng each). As a control, we used a mixture of the four AF (100 ng) standards alone. The chromatograms were compared to determine whether the AF peaks of the three matrices overlapped with the control. The retention times were consistent.

- **Lineality**: Lineality is the capacity of an analytical method to obtain calibration curves that are directly proportional to the concentration of the analyte. A stock solution of 1 μg mL⁻¹ (=1000 ng) of each AF (Sigma-Aldrich, St. Louis MO, USA) was prepared following the AOAC methodology [32]. Standards were diluted independently with benzene/acetonitrile (98:2 v/v) and homogenized, and their absorbance was measured on a UV-visible spectrophotometer (Genesys 10 UV, Thermo Electron Corporation, Madison, WI, USA) and adjusted to zero using pure HPLC methanol as a blank control [32]. The following equation was applied to determine the amount of AF and methanol needed to obtain 1 mL of an AF concentration of 1 μg mL⁻¹ (=1000 ng):

  \[
  \text{Absorbance} \times \text{AF molecular weight} = x
  \]

  Extinction coefficient

  \[
  1/x = \mu\text{L of AF in unknown solution}
  \]

  1000 μL MeOH - μL AF of problem solution = μL of MeOH to add

  The molecular weight (MW) and extinction coefficient (EC) at absorbances of 360 to 362 nm were: AFB₁ (MW 314; EC 21,800), AFB₂ (MW 328; EC 24,000), AFG₁ (MW 312; EC 21,800), and AFG₂ (MW 330; EC 17,700).

  The 16 AF concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 200, 600, 800 and 1000 ng mL⁻¹) were made using the stock solution (1000 ng) from each of the four AFs. Calibration curves were generated by Microsoft Excel.

- **Limit of detection (LOD)** and **limit of quantification (LOQ)**

  The LOD of the equipment was established in relation to the noise in the chromatogram. The LOD equals the concentration of the AF that yields a signal that is three times greater than that of the noise. The LOQ equals the concentration of the AF that is 10 times greater than that of the noise [33].

- **Recovery percentages**

  The results of this experiment complied with the acceptance criteria of the European Community (EC) and the criteria of the physicochemical methods of the Ministry of Health of Mexico (SSA) [30,31].

  The recovery percentage is a measure of the accuracy of a method and represents the proximity between the theoretical and experimental values. The recovery percentage in this study is the amount of recovered AF from a spiked sample. To determine the recovery percentage, 1 g aliquots of dried, ground pepper were individually spiked with three different concentrations (5, 20 and 40 μg kg⁻¹) of each AF (AFB₁, AFB₂, AFG₁, and AFG₂) and each spiked aliquot was subjected to the complete analytical method. The arithmetic average, standard deviation, percentage of variation coefficient and confidence interval were calculated. One aliquot without AF was used as a control, which represented the basal level of contamination. The samples were...
individually processed according to the R-Biopharm extraction method [34]. AFs were purified and concentrated using an immunoaffinity column, derivatized, and quantified by HPLC to obtain the percentage of recovery of each AF. When the derivatized mixture cooled to room temperature, 20 µL of each sample was injected for HPLC analysis. Each sample was run in triplicate. For more accurate results, the concentrations of AFs were adjusted once the recovery percentages were obtained.

Chemical extraction

Each representative sample of 51 g of pepper was blended (Black & Decker “Crush Master”) with 100 mL of a solution of acetonitrile (ACN) HPLC (JT Baker, Xalostoc, México)/distilled water (60:40 v/v) and 2 g of sodium chloride (JT Baker, Xalostoc, México) for 1 min to clarify the extract. The extracts were filtered and 2 mL (equivalent to 1 g) of the extracts were dissolved in 48 mL of phosphate-buffered saline (PBS, pH 7.4) and vortexed. The mixture was then applied to an immunoaffinity column (Easi-Extract Aflatoxin, Biopharm Rhône Ltd., Glasgow, Scotland) that was previously balanced with 20 mL of PBS and washed with 20 mL of distilled water. Air was passed through the column and AFs were eluted with 1.5 mL of methanol (MeOH) HPLC (J.T. Baker, Xalostoc, México). Distilled water (1.5 mL) was refluxed to separate the antibodies in the agarose gel and to recover pure AFs in the eluate. The eluates were collected in labeled amber vials, dried at 40°C in an oven (Novatech BTC-9100) and stored in a refrigerator.

Derivatization

The AFs have different fluorescent properties; therefore, a derivatization reaction that consists of acid hydrolysis of the double bonds of the dihydrofurane ring was applied to produce the AF B1 and G1 types, the fluorescence of which is comparable to that of AFB1 and AFG1, in an aqueous solution [35].

The AF standards were dried and resuspended in 200 µL of ACN. To enhance fluorescence, 800 µL of a derivatizing solution was added. The derivatizing solution consisted of 5 mL of trifluoroacetic acid (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. The mixture was vortexed (Vortex G-560, Bohemia, NY, USA) for 30 s. The vials were placed in a water bath (Aparatos de Laboratorio BG, Mod. BM 40T) at 60°C for 10 min [35,36]. The vials were cooled to room temperature and 20 µL were injected into the HPLC for AF quantification.

AF quantification by liquid chromatography

AF standards and samples were analyzed on an HPLC Agilent Series 1200 with an isocratic pump (G1310A Serie DE62957044), fluorescence detector (G1321A Serie DE60456380) and autosampler (G1329A Series DE64761666) using a chromatographic column (Agilent Eclipse XDS-C18, 4.6 × 250 mm) with a particle size of 5 µm. The program used for HPLC was ChemStation 32. The analysis conditions were: mobile phase H2O/ACN/Methanol (65:15:20 v/v/v); injection volume of 20 µL; flux of 1 µL min⁻¹; analysis time of 25 min; and excitation wavelength of 362 nm. Two different emission wavelengths were used: 425 nm for AFB1 and AFB2, and 450 nm for AFG1 and AFG2.

Statistical analysis

The sample-adjusted results of the three peppers were compared by borough for Mexico City, as well as for Egypt, India and Turkey. To identify potential differences between the place of origin of the sample and the three stages of maturation of the pepper, a non-parametric Kruskal-Wallis test and a Wilcoxon signed-rank test were performed to determine the differences between each group.

AF contamination in spices is frequent and at high amounts that surpass that values set by international legislation. Therefore, the purpose of this research was to identify and quantify AFs (AFB1, AFB2, AFG1, and AFG2) in green, black and white peppers consumed in Mexico, Egypt, Turkey and India and to determine whether AF contamination contributes to food carcinogens in the human diet.

Results and Discussion

Method validation

The validation parameters for each aflatoxin obtained in the experiments of linearity, selectivity and recovery percentage is presented.

Selectivity

After analyzing the three types of peppers spiked with 100 ng g⁻¹ of each of the four AFs and the blank or the control, the chromatograms obtained showed signals for the four AFs. The first chromatographic peak (~6 min) corresponded to AFG1, the second to AFB1 (~8.5 min), the third to AFB2 (~12.5 min) and the fourth to AFB2 (~19 min). The order of the four analytes did not change due to the effect of the different matrices (i.e., there was no overlap among the signals and there was no interference with any compound of the matrix) (Figure 3).

Linearity

The calibration curves were constructed with the different concentrations of the four AFs. The aflatoxin concentrations (ng mL⁻¹) for the calibration curves were as follows: AFB1 (0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128) with R²=0.9973, AFB2 (0.01, 0.05, 1, 5, 10, 20, 40, 70, 100 and 200) with R²=0.9908, AFG1 (0.01, 0.05, 0.1, 0.5, 1, 4, 16, 100 and 128) with R²=0.9969 and AFG2 (0.5, 1, 2, 4, 8, 16, 32, 64, 100, 200, 600, 800 and 1000) with R²=0.9988. The R² obtained in the calibration curves showed precision and confiability (Table 1).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were in ng mL⁻¹: AFB1, LOD=0.1; LOQ=0.5, AFB2, LOD=0.01; LOQ=0.05, AFG1, LOD=0.01; LOQ=0.05) and AFG2, LOD=0.5; LOQ=2.5 (Table 1).

Recovery percentage

The acceptance criteria to recover residues and contaminants from foods and water from both institutions (SSA from Mexico and the EC) were taken into consideration [30,31]. The analyte concentration <1 µg kg⁻¹ had an acceptance range of 50-120% from both institutions; therefore, this range was chosen because 1 g of the three types of peppers was fortified with 100 ng of each AF (Table 2).

AF concentrations in the samples

To detect the presence of AFs in the samples, the retention times (RTs) obtained by the experiments with the three peppers during method validation were taken into account (Table 3). To quantify the AFs, the recovery percentage was considered to adjust the AF quantification, as shown in Table 3. All 54 analyzed samples (19 black, 19 white and 16 green peppers) were contaminated with AFs. In total, 95% (51/54) contained AFB1, 80% (43/54) contained AFB2, 67% (36/54) contained AFG1, and 93% contained AFG2 (50/54) (Figure 4).

The incidence of AF contamination depending on the purchasing

place is presented in Figure 4 that show the increased susceptibility of AF contamination of the green pepper, which may be due to the degree of ripening; it is also less commercial and is stored for a longer period of time, thus increasing the risk of mycotoxigenic fungal growth. The pepper with the least contamination was the white pepper, which may be due to the polish treatment to the hull. Only 9.26% of samples (5/54) complied with the established limit of AFT given by NOM-188-SSA1-2002 [37] (i.e., 20 μg kg$^{-1}$). The samples from Mexico City that were under the legal limit were black peppers from the boroughs of Azcapotzalco and Iztacalco (16.75 and 2.56 μg kg$^{-1}$, respectively) and white peppers from Coyoacán, Tláhuac and Xochimilco (15.52, 7.23 and 13.4 μg kg$^{-1}$, respectively). The AFT concentrations in pepper according to sampling location are shown in Table 3.

All foreign samples surpassed the AFT limit of the corresponding regulations of the respective country (India 30 μg kg$^{-1}$, Egypt and Turkey, 10 μg kg$^{-1}$) (Table 4) [38]. Only Egypt and Turkey established
a limit of 5 μg kg⁻¹ for AFB₁ and in both cases, the samples surpassed that limit [38].

The Mexican regulations with respect to AFB₁ and AFt were not applied because commercial deals must not be regulated, and therefore, AF contamination in spices was not considered. Thus, the health of Mexicans is affected because there is no control on ingested carcinogens in foods [37].

The obtained AF concentrations varied, ranging from 0.11 to 217.50 μg kg⁻¹ for AFB₁, 0.39 to 381.87 μg kg⁻¹ for AFB₂, 0.4 to 611.88 μg kg⁻¹ for AFG₁, and 1.37 to 494.44 μg kg⁻¹ for AFG₂ (Table 5). The most contaminated samples are listed in Table 5.

**Table 3:** Average of Aflatoxins (μg kg⁻¹) in one gram of pepper from three markets of each borough in Mexico City.

<table>
<thead>
<tr>
<th>Mexico City boroughs</th>
<th>Pepper sample</th>
<th>AFB₁</th>
<th>AFB₂</th>
<th>AFG₁</th>
<th>AFG₂</th>
<th>AFt</th>
</tr>
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<td>Alvaro Obregón</td>
<td>Black</td>
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<td>60.22</td>
<td>144.57</td>
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<td>710.29</td>
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<td>Miguel Hidalgo</td>
<td>Black</td>
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<td>&lt;LOD</td>
<td>97.07</td>
<td>64.31</td>
<td>161.38</td>
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<td>White</td>
<td>4.19</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>21.34</td>
<td>25.53</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>118.22</td>
<td>79.24</td>
<td>93.74</td>
<td>197.35</td>
<td>486.55</td>
</tr>
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<td>Milpa Alta</td>
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<td>16.71</td>
<td>&lt;LOD</td>
<td>52.83</td>
<td>69.65</td>
</tr>
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<td>White</td>
<td>8.16</td>
<td>42.36</td>
<td>64.30</td>
<td>157.26</td>
<td>272.08</td>
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<td>Green</td>
<td>59.35</td>
<td>243.05</td>
<td>92.71</td>
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<td>544.34</td>
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<td>Tlahuac</td>
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<td>52.77</td>
<td>237.17</td>
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<td>Tlalpan</td>
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<td>247.27</td>
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<td>27.53</td>
<td>20.17</td>
<td>17.40</td>
<td>44.89</td>
<td>110.00</td>
</tr>
<tr>
<td>Venustiano Carranza</td>
<td>Black</td>
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<td>75.47</td>
<td>&lt;LOD</td>
<td>166.00</td>
<td>249.81</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>31.17</td>
<td>108.13</td>
<td>20.52</td>
<td>68.47</td>
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</tr>
<tr>
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<td>44.22</td>
<td>56.71</td>
<td>474.56</td>
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<td>28.52</td>
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<td>57.11</td>
<td>168.72</td>
</tr>
<tr>
<td></td>
<td>White</td>
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<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>13.40</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>19.52</td>
<td>14.05</td>
<td>57.70</td>
<td>17.49</td>
<td>108.76</td>
</tr>
</tbody>
</table>

<LOD=Less than the limit of detection
Figure 4: Average concentration of Aflatoxins in green, black, and white peppers.

Figure 5: Types of peppers.

Table 4: Aflatoxins (µg kg\(^{-1}\)) in 1 g of pepper in Egypt, India, and Turkey.

<table>
<thead>
<tr>
<th>Country</th>
<th>Pepper type</th>
<th>AFB(_1)</th>
<th>AFB(_2)</th>
<th>AFG(_1)</th>
<th>AFG(_2)</th>
<th>AFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egypt</td>
<td>Black</td>
<td>23.92</td>
<td>381.87</td>
<td>36.51</td>
<td>104.42</td>
<td>546.73</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>12.52</td>
<td>21.15</td>
<td>53.13</td>
<td>205.19</td>
<td>291.98</td>
</tr>
<tr>
<td>India</td>
<td>Black</td>
<td>8.40</td>
<td>27.12</td>
<td>35.30</td>
<td>118.01</td>
<td>188.83</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>13.72</td>
<td>&lt;LOD</td>
<td>9.51</td>
<td>14.37</td>
<td>37.60</td>
</tr>
<tr>
<td>Turkey</td>
<td>Black</td>
<td>7.57</td>
<td>30.63</td>
<td>1.11</td>
<td>185.92</td>
<td>225.53</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>27.10</td>
<td>&lt;LOD</td>
<td>18.57</td>
<td>35.15</td>
<td>80.81</td>
</tr>
</tbody>
</table>

Table 5: The most contaminated pepper samples according to the purchasing place.

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Pepper type</th>
<th>Purchasing place: Mexico City boroughs or country</th>
<th>Concentración de AF (µg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(_1)</td>
<td>Black</td>
<td>Cuauhtémoc</td>
<td>217.50</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>Iztapalapa</td>
<td>48.78</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Cuauhtémoc</td>
<td>169.59</td>
</tr>
<tr>
<td>B(_2)</td>
<td>Black</td>
<td>Egypt</td>
<td>381.87</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>V. Carranza</td>
<td>108.13</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Milpa Alta</td>
<td>243.05</td>
</tr>
<tr>
<td>G(_1)</td>
<td>Black</td>
<td>Benito Juárez</td>
<td>253.48</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>Milpa Alta</td>
<td>64.30</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Iztacalco</td>
<td>611.88</td>
</tr>
<tr>
<td>G(_2)</td>
<td>Black</td>
<td>G. A. Madero</td>
<td>193.46</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>Egypt</td>
<td>205.19</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>La M. Contreras</td>
<td>494.44</td>
</tr>
<tr>
<td>AFT</td>
<td>Black</td>
<td>Egypt</td>
<td>546.73</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>Egypt</td>
<td>291.98</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Iztacalco</td>
<td>791.13</td>
</tr>
</tbody>
</table>

Table 6: Kruskal-Wallis test for aflatoxins in peppers.

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Kruskal-Wallis test</th>
<th>Significance</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB(_1)</td>
<td>4.41</td>
<td>0.11</td>
<td>No</td>
</tr>
<tr>
<td>AFB(_2)</td>
<td>7.11</td>
<td>&lt;0.05</td>
<td>Yes</td>
</tr>
<tr>
<td>AFG(_1)</td>
<td>7.05</td>
<td>&lt;0.05</td>
<td>Yes</td>
</tr>
<tr>
<td>AFG(_2)</td>
<td>0.93</td>
<td>0.63</td>
<td>No</td>
</tr>
<tr>
<td>AFT</td>
<td>7.49</td>
<td>&lt;0.05</td>
<td>Yes</td>
</tr>
</tbody>
</table>

There were no significant differences between the three types of peppers studied with respect to AFB, and AFG\(_2\), but significant differences in AFB\(_2\), AFG\(_1\) and AFT were observed.

The Wilcoxon signed-rank test was performed to detect significant differences among the groups. There were no significant differences in green pepper, although this pepper is more susceptible to AF contamination. AFG\(_1\) contamination was the highest, with an average value of 35.88 µg kg\(^{-1}\).

White pepper was the least contaminated, but was more susceptible to AFG\(_1\), reaching an average value of 25.13 µg kg\(^{-1}\). An intermediate incidence of contamination was observed in black pepper, which reached a maximum average value of 27.53 µg kg\(^{-1}\) for AFG\(_2\).

In Mexico, the highest average concentration (27.53 µg kg\(^{-1}\)) was detected in black pepper. There were significant differences among the groups. There were no significant differences in AFB\(_2\) concentration in green pepper (p=0.011), the concentration of AFG\(_1\) in white pepper is statistically different than the concentration in green pepper (p=0.011), the concentration of AFG\(_2\) in white pepper is statistically different than the concentration in green pepper (p=0.011).

The lack of pepper contamination could be due to essential oils that contain substances such as piperin, which is important in pepper composition [39]. The levels of AF contamination in peppers from Mexico surpassed the tolerance limits set by other countries. Turkey had AFB\(_1\) concentrations ranging from 0.3 to 1.2 µg kg\(^{-1}\) and AFT concentrations ranging from 0.3 to 2.3 µg kg\(^{-1}\) in black pepper [40]. Ranges of AFT from 1.1 to 97.5 µg kg\(^{-1}\) in red pepper have been reported [41]. In Korea, ground red pepper samples had AFT levels ranging from 0.08 to 4.66 µg kg\(^{-1}\), whereas black pepper had only trace amounts below the LOD [42]. In Italy, whole pepper and ground black pepper also had only trace amounts below the LOD [43].

Pepper is a good substrate for the growth of Aspergillus spp., aflatoxigenic fungi and thus AF metabolic production. High levels of AFS indicate improper handling at some stages of the production chain and in some supplies, including bad practices during harvest, inappropriate storage or a lack of good conditions during transportation, marketing and/or processing [10].

Essential oils from some spices inhibit fungal growth and mycotoxin production [44]. The essential oils of clover, cumin and black pepper can inhibit the fungi that produce AFS. Oils, such as eugenol, eugenol acetate, β cariophylene and piperin, also inhibit AFS [39]. Matrices such as black pepper and cumin are not good substrates for AF biosynthesis due to their essential oils, although they allow fungal growth [40]. In the case of A. parasiticus, AF production is inhibited in black and white peppers by the actions of piperin and other volatile essential oils [39]. Some scientists did not detect AFS in black or white ground peppers, suggesting that these peppers are not an appropriate substrate for AF biosynthesis [45]. The antifungal power of the dioic pepper has been proven in vitro against molds, such as Aspergillus candidus, A. versicolor, Penicillium citrinum, P. aurantiogriseum, P. brevicompactum and P. griseofulvum, as well as in situ against the post-harvest-contaminating molds of oat grains, namely, Fusarium spp., Alternaria spp. and Cladosporium spp., which extracts of dioic pepper inhibit fungal growth in vitro [46].
There are several protective mechanisms in the human body against AFs. In the case of AFB$_1$, the liver biotransforms xenobiotics by the action of phase I and II enzymes and can cause AFs to be excreted in the bile or kidney. However, some phase I metabolites can react with different biomolecules, rendering them unstable [51].

AFB$_1$-8,9-epoxide is the active metabolite of AFB$_1$. With the aid of the liver microsomal enzyme CYP450, a covalent linkage with nitrogen 7 (N’$^7$) of guanine occurs and AFB$_1$-N’$^7$-guanine (AFB$_1$-N’$^7$-Gua) adducts are formed in target cells [52,53]. The adduct produces an apurinic site in the guanine imidazole ring that, when it opens, becomes the highly stable mutagenic adduct AFB$_1$-formamidopyrimidin (AFB$_1$-FAPY). The activation and reaction of AFB$_1$, with ADN has been studied [54]. This results in a guanine-thymine (G $\rightarrow$ T) transversion in codon 249 of the p53 tumor suppressor gene and to DNA lesions, mutations and the beginning of cancer with tumor formation [55,56].

The reactive epoxide can be hydrolyzed to AFB$_1$-8,9-dihydrodiol, which is ionized to form a Schiff base with amino primary groups in proteins [57]. The epoxide has a short life and has been associated with blood coagulation, decreased synthesis of vitamin K and other clot factors as a result of sub-lethal intoxication [58].

With respect to its cytotoxic effects, AFB$_1$ induces lipid peroxidation in the rat liver, resulting in oxidative damage to hepatocytes [59].

AF contamination of spices is a serious problem worldwide that can affect international trade. Black pepper is a valuable spice that is usually contaminated microbiologically, as well as by mycotoxins during harvest and processing; black pepper has large drying periods and requires sunlight [60,61]. Pepper grows in tropical, humid countries that promote the growth of fungi and the production of mycotoxins [62]. Spices, including pepper, are frequently added to foods, although they contribute to many health problems because they are highly contaminated with AFs.

Spices with AFs over the tolerated limit have been reported in the United Kingdom [63]. In fact, 43% of packed spices in Portuguese markets are reportedly contaminated with AFB$_1$ [62]. In Qatar, a mixture of spices and chili peppers had AFs ranging from 0.16 to 69.28 μg kg$^{-1}$ [64].

Analysis of spices is not simple due to the interference of colored materials that are extracted with AFs. Selective extraction and specific purification of AFs before quantification is recommended. Immunoaffinity columns with specific antibodies against AFs are efficient for their purification and concentration [42]. The analytical methods used for AF identification and quantification include thin layer chromatography (TLC) and liquid (HPLC) chromatography with fluorescence detectors as well as immunosorbent assays that involve enzyme bonding (ELISA) [65,66].

Due to the high toxicity of AFs, their reduction in foods is a worldwide goal. Adequate humidity, weed control and crop rotation can help to reduce the amount of AFs in foods. Extra irrigation, fast mechanical drying and an early harvest can also reduce AF contamination levels [67].

There are several biological detoxification methods that could be applied to reduce non-toxic strains of Aspergillus flavus and other molds [68]. The physical methods include extraction, heating and absorption with adsorbent agents, and radiation [69]. The chemical methods include treatment with ammonia, sodium bisulphite, calcium hydroxide, formaldehyde, antioxidants or other chemicals [70].

Insect infestation in agricultural products promotes fungal inoculation and subsequent AF contamination. Insect damage to the fruit surface creates infectious routes for the dispersion of fungal pathogens; therefore, pest control is important for AF control [71]. The Bt toxin produced by Bacillus thuringiensis is an efficient control that has been used in France since 1938. It is safe to use in foods for humans or in feed. However, more than 220 Bt toxin strains against different insects have been identified [72].

Our study suggests that pepper is the most AF-contaminated matrix; the AF amounts contained in pepper surpass the AF contamination of peanuts. Based on our experience, we conclude that the amounts of AFs in pepper are among the highest found in foods. Although the AF concentrations in pepper are high, their ingestion in different dishes is minimal because they are used in small quantities as a flavor-enhancing product. Therefore, the contribution of AFs from peppers to an organism can be considered to be relatively low in comparison to other agricultural products, such as maize, pistachio, peanuts or dairy products.

This study presents a detailed analysis of AF contamination in pepper in three different ripening stages (green, black and white). The lack of normativity in countries on this subject prevents the reduction of AF concentrations in the diet.

Conclusion

The extraction and purification methods of AFs in pepper were validated. The recovery values were > 80%, indicating good recovery of the four AFs. Four types of AFs were identified and quantified in the three types of pepper. All the analyzed samples were contaminated with at least one AF. The high AF content in pepper could be due to inadequate handling and storage conditions. Only 9.26% of the samples from Mexico City complied with the 20 μg kg$^{-1}$ limit established by the NOM-188-SSA1-2002 for AFs. All foreign samples surpassed the AF limits established by their respective countries (India 30 μg kg$^{-1}$, Egypt and Turkey, 10 μg kg$^{-1}$). Samples from Egypt and Turkey also surpassed the AFB$_1$ limit (5 μg kg$^{-1}$).

The levels of AFB$_1$, AFG$_1$, and AFT contamination were significantly different among the three types of pepper. The amount of AFB$_1$ and AFG$_1$ did not differ significantly. Green pepper was the most contaminated with AFs, white pepper was the least contaminated and black pepper had an intermediate level of contamination. Drying by sunlight is not efficient for AF degradation. Based on our results, ripening plays a major role in AF levels because green pepper was the most highly contaminated and the least ripened.

Acknowledgement

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