

Determination of aflatoxins in spices, ingredients and spice mixtures used in the formulation of meat products marketed in Mexico City



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Abstract:

Aflatoxins are toxic substances produced by some species of fungi that pose a serious danger to human health, especially aflatoxin B₁, which is one of the main analytes found in foods and is classified as carcinogenic. The objective of this study was to provide information on the presence of total aflatoxins in spices, ingredients and spice mixtures used for the formulation of meat products and meat products marketed in Mexico City, using an enzyme-linked immunosorbent assay (ELISA) method. Fifty samples were analyzed for total aflatoxins. Sixty-one percent of spices and ingredients were positive for total aflatoxins in concentrations of 0.07 to 4.24 µg/kg; 75 % of spice mixtures were positive, in quantities of 0.6 to 1.9 µg/kg and only 3.5 % of meat products were positive for total aflatoxins. The samples with the highest prevalence of total aflatoxins were chili and paprika. All results

showed concentrations below the maximum limit set by the European Union of 10 to 20 µg/kg for total aflatoxins, which does not constitute a current public health problem under the conditions analyzed. It is recommended to use the ELISA system as a screening method and subsequent confirmation by liquid chromatography with fluorescence detection, as well as to have a monitoring program to evaluate the presence of aflatoxins and other mycotoxins; there is also a need for an official Mexican regulation for mycotoxins in spices considering the high consumption of chili in the Mexican population.

Key words: Aflatoxins, Spices, Meat products, ELISA.

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According to the Food and Agriculture Organization of the United Nations (FAO), meat is a product of animal origin that provides nutrients of great value to the diet, among which are proteins, essential amino acids, fats, vitamins and minerals⁽¹⁾. With the production of meat products, the meat and the by-products of the slaughter are used to the maximum because the meat trimmings, with a lower quality, mixed with non-meat ingredients generate an important source of proteins of animal origin for the human diet. In the formulation of meat products, a wide variety of non-meat products such as spices are used, which are vegetable derivatives (dried seeds, fruits, roots, tree bark) used for the preparation of food due to their gastronomic properties such as flavor, color or aroma and even for their medicinal properties and some with antimicrobial properties^(2,3,4).

FAO documented that 25 % of crops are contaminated with at least some mycotoxin⁽⁵⁾. Spices are not exempt from this problem, and can be contaminated within the production chain (pre-harvest, harvest, processing, storage, drying or transport) due to poor handling practices^(6,7,8) and when environmental conditions such as temperature and humidity are favorable for fungal growth^(6,9).

Mycotoxins are toxic secondary metabolites of certain genera of fungi, such as *Aspergillus*, *Fusarium* and *Penicillium* and more than 400 mycotoxins are known. Aflatoxins are the most commonly found in foods, in concentrations that exceed the maximum levels (NM) for human and animal consumption, in addition, aflatoxin B1 is carcinogenic, teratogenic, immunosuppressive, hepatotoxic and mutagenic^(3,6,8,10,11), so the International Agency for Research on Cancer (IARC) classifies it in group 1 of carcinogenic to humans⁽²⁾.

The Codex Alimentarius Commission, the body that regulates food safety, has reported that some of its member countries have established NM for mycotoxins in spices between 5 and 30 µg/kg. Mexico has not set the NM for total aflatoxins (AFT) in spices, although it has set the NM for cereals.

Among the laboratory techniques used for the analysis of aflatoxins are: immunoassays^(6,8,12), high-performance liquid chromatography (HPLC) with ultraviolet and fluorescence detectors⁽⁹⁻¹⁴⁾ and thin layer chromatography (TLC)^(4,15), being the immunoassays of the most used and reported methods in the scientific literature.

The use of spice mixtures and other condiments in meat derivatives has been increasing and they run the risk of being contaminated, which poses a potential risk to public health⁽¹⁰⁾. People need safe and good quality food.

The objective of this study was to provide information on the presence of AFT in spices, ingredients and spice mixtures used for the formulation of meat products and meat products marketed in Mexico City.

A total of 50 samples were randomly collected^(2,12,14) in Mexico City, between December 2015 and January 2017, from the following matrices:

Spices and ingredients commonly used for the formulation of meat products (n= 18): onion powder, garlic powder, paprika powder, guajillo chili powder, potato starch, corn starch, texturized soybean and peanuts.

Spice mixtures for the formulation of meat products (n= 4): salami, peperoni, Argentinian chorizo, smoked chistorra.

Meat products (n= 28): pork ham, turkey ham, turkey sausage, hamburger meat, skirt steak, chicken wings, chicken nuggets and enchilada meat.

The samples of spices and texturized soybean came from two specialized companies with the highest sales volume in Mexico City, sold in sealed packaging and stored in refrigeration at 4 °C, protected from light until analysis. The sample of peanuts was taken from a place of sale in bulk, and the branded meat products packaged and with information on the labeling were obtained in supermarkets, such as hams (pork and turkey), sausages and meat for hamburger, skirt steak, chicken wings and nuggets, in addition to unbranded bulk enchilada meat.

The samples were dried in an oven (Felisa) at 50 °C for 72 h, then ground and passed through a 0.1 mm sieve. All samples were placed in sealed bags in refrigeration at 4 °C, protected from light until analysis.

Samples were analyzed with quantitative tests based on an enzyme-linked immunosorbent assay (ELISA), using Neogen® Veratox® reagent set (No 8031). For which 5 g of each sample were weighed; the extraction of aflatoxins was performed with 70% methanol. The sample mixture with methanol was stirred vigorously for 3 min in a vortex (Velp Scientifica) and 5 ml of the extract was filtered on No. 4 filter paper (Whatman). In mixture wells, 100 µl of the conjugate, used to compete with aflatoxins or controls for antibody binding sites, was added; subsequently, 100 µl of the controls (0, 1, 2, 4 and 8 µl) and samples were placed, the contents were mixed by suctioning and releasing it three times. 100 µl was transferred to the wells covered with aflatoxin-specific antibodies, mixed for 20 sec and incubated at ambient temperature in the wells for 10 min. After that period, the Stop solution was poured, 100 µl into each well and the absorbance was measured at 450 nm in an ELISA (Biorad) reader. The results were analyzed using Neogen® Veratox® Software V3.6.

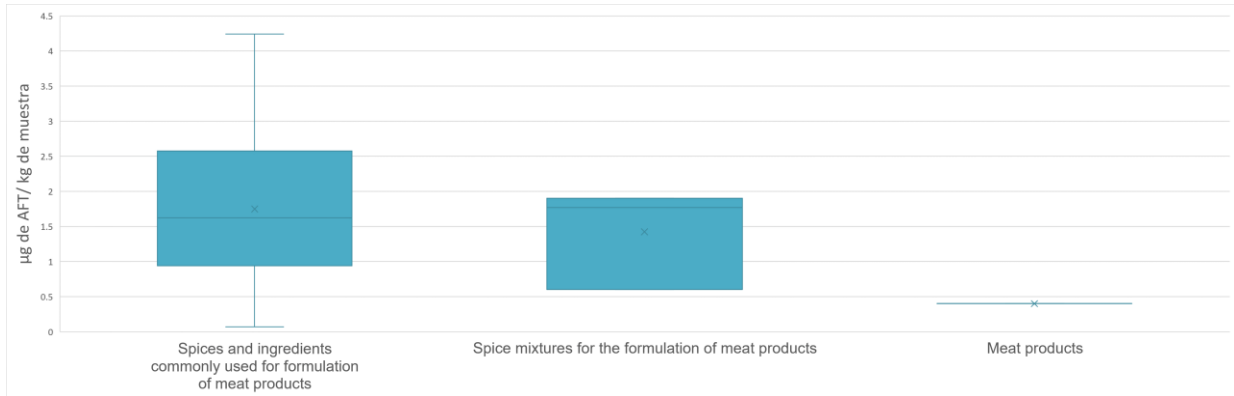
The Veratox® Neogen® Reagent Set (No 8031) presented reactivity for AFT (B1, B2, G1, G2) and a working range between 1 to 8 µg/kg. The specificity of the test was evaluated through the study with different matrices (paprika, garlic, corn starch, spice mixture for the formulation of salami and turkey sausage) corresponding to the sample groups (spices and ingredients commonly used for the formulation of meat products, spice mixtures for the formulation of meat products and meat products). The extracts obtained from each matrix were contaminated with the points of the calibration curve mentioned above. The curves of the different matrices showed a correlation above 0.98 and the error at each point of the curve, considering the matrices studied, ranged between 7 and 11%. The recovery in the matrices studied ranged between 72 and 94 %. The limit of detection and the limit of quantification are 0.5 µg/kg and 1 µg/kg respectively, reported by the manufacturer when the same curve is used and there are no differences between the matrix curve ($P= 0.909$).

The total number of positive samples was 40 %. The highest percentage of AFT was found in spices and ingredients commonly used for the formulation of meat products and in spice mixtures for the formulation of meat products with 61 and 75 % respectively, while meat products only had 3.5 % of AFT-positive samples.

Figure 1 shows the average concentration of AFT for the three groups analyzed, the average value does not exceed the value of 2 µg/kg, lower than the maximum level established by other countries with national standards for spices and their mixtures, which range from 5 to 30 µg/kg⁽¹⁶⁾. Mexico has not established a specific standard for the presence of aflatoxins in spices, so in this work for the comparison of results, the European standard was used, which

is the most exigent and proposes maximum limits for the sum of aflatoxins in spices of 10 $\mu\text{g}/\text{kg}$ ⁽¹⁷⁾.

Figure 1: Average concentration of total aflatoxins ($\mu\text{g}/\text{kg}$) in the groups of samples analyzed



As for the spices and ingredients commonly used in the formulation of meat products, guajillo chili was the one with the highest total aflatoxin content, 4.24 $\mu\text{g}/\text{kg}$ (Table 1). Similar results were reported in a study carried out in the market of Doha, in Qatar, where 14 samples of spices were analyzed, the presence of aflatoxins was detected in five spices and their mixtures (black pepper, chili, tandoori, masala, turmeric and garam masala), finding the highest value of aflatoxin B1 in chili, with a concentration of $69.28 \pm 1.08 \mu\text{g}/\text{kg}$ ⁽⁹⁾. In another study carried out in Pakistan, 170 samples of chili in different presentations (chili sauce, crushed chili and chili powder) were analyzed, the presence of aflatoxins was detected in an interval of 39 to 59 % of the samples analyzed, with a maximum value of 27.5 and 21.1 $\mu\text{g}/\text{kg}$, in samples from the market and restaurants respectively⁽¹⁸⁾. The aforementioned results corroborate that dried chili is a spice susceptible to fungal development and formation of aflatoxins if the humidity and temperature conditions are conducive during its production and storage.

Table 1: Concentration of total aflatoxins (AFT) in spices and ingredients commonly used in the formulation of meat products

Spices and ingredients	No. of samples analyzed	Contamination interval (µg/kg)
Onion	3	Nd – 1.61
Paprika	2	2 – 3.05
Garlic	3	1 – 2.05
Guajillo chili	1	4.24
Texturized soybean	2	Nd - 0.07
Peanut	1	1.57
Corn starch	2	Nd
Potato starch	4	Nd – 2.75

Nd= Not detected.

Paprika samples are the second with the highest concentration of AFT, from 2.0 to 3.05 µg/kg in 100 % of the samples analyzed. In a study conducted on seventy paprika samples collected in the city of São Paulo, Brazil, from January to April 2006, aflatoxins were detected in 82.9 % of the samples and aflatoxin B1 in 61.4 %, in a concentration range of 0.5 to 7.3 µg/kg, with an average concentration of 3.4 µg/kg⁽¹⁹⁾. Another study on 130 spice preparations that were obtained at various points of sale in Ireland (including supermarkets, shops and market stalls), found that 20 % of the samples were contaminated with aflatoxin B1, in an interval of 0.40 to 6.40 µg/kg⁽¹⁰⁾. On the other hand, in a study where the presence of aflatoxins and ochratoxin A in red paprika for retail sale in Spain was evaluated, it was found that the samples that presented aflatoxins were well below the two legal limits, of 5 µg/kg for aflatoxin B1 and 10 µg/kg for total aflatoxins, established by the European Union⁽¹⁷⁾; not so ochratoxin A, which was found more frequently, with an average of 11.8 µg/kg and in a more varied interval (SD 18.9 µ/kg)⁽²⁰⁾.

As for onion, the presence of AFT of 1.61 µg/kg was found in this, lower than that obtained in a study carried out in agricultural products in Nigeria, where the presence of AFT in onion was 3.14 µg/kg⁽²¹⁾. Another study found aflatoxin-producing fungi in garlic powder from China, onion powder and granules from France⁽²²⁾.

Regarding garlic samples, maximum values of 2.05 µg/kg were detected, a concentration much lower than that reported in a study carried out in Egypt, where values of 224.4 µg/kg were determined in whole garlic, while in peeled garlic no aflatoxins were detected⁽²³⁾. On the other hand, Sahar *et al*⁽¹⁵⁾ analyzed three garlic samples where they did not detect the presence of aflatoxins. The high levels found in whole garlic by Refai *et al*⁽²³⁾ may be debatable, the antifungal effect of garlic has been demonstrated in *in vitro* studies, where it decreases the production of aflatoxins from 5.94 to 0.15 µg/kg⁽²⁴⁾. While another study shows that there is an inhibition of mycelial growth of 61.94 % in a liquid SMKY medium and in

corn kernels inoculated with *Aspergillus flavus*⁽²⁵⁾, in another *in vitro* study using YES medium inoculated with *Aspergillus flavus*, the mycelial growth, sporulation and production of aflatoxins were evaluated with different concentrations of garlic and onion and a moderate effect of inhibition and production of aflatoxins was demonstrated⁽²⁶⁾. The aforementioned studies could explain the low values of aflatoxins found in garlic and onions in this work.

The aflatoxin content in peanuts was similar to that reported in other studies in Mexico, where AFT values range from 0.11 to 79.69 µg/kg⁽²⁷⁾.

In this work, the presence of aflatoxin in corn starch was not detected, which may be due in the first place to the process of obtaining it, where the husk is separated from the rest of the grain. In a study where the quality of starch was evaluated during the spontaneous fermentation process (21 d), no aflatoxins above 5 µg/kg were found⁽²⁸⁾ and in another study where the fate of aflatoxins during the wet grinding process and their distribution in products and by-products were analyzed, only 8.7 % of the total aflatoxins in the initial corn was found in the starch fraction, which represents 61 % of the ground corn, and it is concluded then that the aflatoxins were destroyed during the conversion to starch⁽²⁹⁾. A second aspect may be that the corn starch analyzed does not present aflatoxins or presents them in quantities below the maximum permissible limit for consumption due to the existence of good production practices⁽³⁰⁾.

Fifty percent of the potato starch samples registered positive results to AFT with concentrations of 0.92 to 2.75 µg/kg, this result is debatable since the presence of aflatoxins in harvested potato has not been reported; however, root crops are susceptible to *Aspergillus* growth and therefore to possible aflatoxin contamination⁽³¹⁾. The presence of aflatoxins has been reported in potatoes inoculated with an average of 8 µg/kg of total aflatoxins (mainly B1 and G1) at 27 °C and 95 to 97 % relative humidity in 20 d⁽³²⁾. In sweet potato, aflatoxin concentrations in the order of 0.01-0.18 µg/kg⁽³³⁾ have been reported. In starchy, raw and cooked foods, after storage aflatoxin levels in the order of 3-25 µg/kg were found in *Ipomoea batatas*⁽³¹⁾. Although there are no studies in harvested potatoes, studies in sweet potato and its starch corroborate in some way the results of this study, and that unsuitable conditions during storage can also contribute to the presence of aflatoxins, considering that potato starch is used in different culture media for the production of aflatoxins.

In the sample of texturized soybean analyzed, AFT was found with a value of 0.07 µg/kg, the amount of AFT is minimal, this is because it has been reported by various authors that soybean contains substances that inhibit fungal growth and the synthesis of aflatoxins^(34,35). In a study conducted in Serbia, 63 soybean samples were analyzed and no aflatoxins were found⁽³⁶⁾, corroborating that soybean is less susceptible to fungal infection and mycotoxin formation than larger grains such as corn. In all cases, the value of 10 µg/kg established by the European Union⁽¹⁷⁾ was not exceeded.

In meat products, of the 28 samples analyzed, only one sample of enchilada meat was positive, representing 3.5 % (Table 2). The presence of aflatoxins in meat products has been reported by several authors, for example, Markov *et al*⁽³⁷⁾ conducted a study in 90 meat products (sausages, dry products) evaluating three mycotoxins (aflatoxins, ochratoxin and citrinin), the first two by ELISA and the last by HPLC, the results showed the presence of aflatoxins in 10 % of the samples analyzed, with an average concentration of 3.0 µg/kg, however, the highest incidence in the samples was ochratoxin with 64.44 %. Similar results in relation to the abundance of ochratoxin and aflatoxins were reported in a study conducted in Croatia, where 410 samples were analyzed: hams (n= 105), dried fermented sausages (n= 208), bacon (n= 62) and cooked sausages (n= 35)⁽³⁸⁾.

Table 2: Concentration of total aflatoxins (AFT) in meat products

Meat product	No. of samples analyzed	Contamination interval (µg/kg)
Enchilada meat	2	Nd- 0.4
Pork ham	6	Nd
Turkey ham	6	Nd
Turkey sausage	3	Nd
Chicken nuggets	4	Nd
Chorizo	4	Nd
Chicken wings	1	Nd
Skirt steak	1	Nd
Hamburger meat	1	Nd

Nd= not detected.

The presence of mycotoxins in meat products can occur at different points in the production chain, in the field, where the animal is exposed to contaminated food⁽³⁹⁾, in the process of making the product, using contaminated spices or spice mixtures or during final storage⁽⁴⁰⁾. In a study conducted in Spain during the maturation of cured hams, the effect of temperature and water activity on fungal growth and aflatoxin production was evaluated, where a water activity greater than 0.9 and a temperature greater than 15 °C produce aflatoxins⁽⁴¹⁾.

Unlike spices, for which there are maximum permissible limits for aflatoxins, in meat products there are no regulations in this regard, only in Italy, the Ministry of Health has recommended, since 1999, the maximum value of 1 µg/kg of ochratoxin A in meat or meat products⁽⁴²⁾, considering that this appears more frequently and in concentrations of 1 to 10 µg/kg, while aflatoxins appear below 1 µg/kg⁽³⁸⁾.

For spice mixtures used for the formulation of meat products, 75 % of the samples were positive (Table 3). Some studies conducted in Qatar and Turkey, in spice mixtures from markets, report AFT in an interval of 0.16 to 5.12 µg/kg and 0.1-0.9 µg/kg respectively^(43,44).

These results are similar to what was found in this paper. The results are as expected if it is considered that, in the composition of the mixtures, spices such as paprika are present, which is one of the spices most susceptible to contamination⁽¹⁹⁾.

Table 3: Concentration of total aflatoxins (AFT) in spice mixtures for the formulation of meat products

Spice mixture for the formulation of:	AFT concentration ($\mu\text{g}/\text{kg}$)
Peperoni	1.9
Argentinian chorizo	0.6
Smoked chistorra	Nd
Salami	1.77

Nd= not detected.

In the case of the mixture of spices for chistorra, it gave a negative result to the presence of aflatoxins and paprika is present in the mixture, the result may be contradictory, however, within this mixture garlic is also present, which has been shown to be an inhibitor of fungal contamination and the synthesis of aflatoxins⁽²⁴⁾, so it can be inferred that there was no fungal growth or production of toxins.

As in spices and ingredients commonly used for the formulation of meat products, the values found do not exceed the permissible limit of the EU, so it can be inferred that their use in the formulation of meat products does not constitute a risk to public health⁽¹⁷⁾.

Immunoenzymatic systems in the determination of mycotoxins in different matrices have been widely used as a screening tool for subsequent confirmation by chromatographic techniques⁽³⁸⁾. The results obtained in this study in different matrices (spices, ingredients and mixtures of spices used for the formulation of meat products and meat products), applying the benefits of ELISA for the determination of AFT constitute the first report in Mexico and guide subsequent studies in those spices, ingredients or mixtures of species that presented a higher prevalence of AFT, in such a way that a complete validation of the technique is carried out in the matrix to be studied. On the other hand, those samples that exceed the NM of AFT in spices must be confirmed by chromatographic techniques.

In conclusion, the presence of total aflatoxins in spices, ingredients and spice mixtures used for the formulation of meat products and meat products is reported for the first time in Mexico, being found in concentrations ranging between 0.4 and 4.24 $\mu\text{g}/\text{kg}$. The spices of highest prevalence to AFTs are chili and paprika of high consumption in the Mexican population. The ELISA methodology used to detect AFT in spices, ingredients, spice mixtures used for the formulation of meat products and meat products makes it possible to use it as a screening test; those with concentrations above the maximum level must be

confirmed by chromatographic techniques. The results obtained in this research alert national regulatory bodies of the need to implement a standard for the presence of aflatoxins in spices, considering the high national consumption of many of the spices studied.

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