

Aflatoxins in Walnut (*Juglans regia* L.), Pecan (*Carya illinoensis* (Wangenh.) K. Koch) and Cashew (*Anacardium occidentale* L.) Nuts of Mexico

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Abstract

Aflatoxins (AF) are toxic and carcinogenic secondary metabolites produced by *Aspergillus* molds, in oilseeds.

Objective: To identify and quantify AF (AFB₁, AFB₂, AFG₁ and AFG₂) in walnuts, pecans and cashews consumed in Mexico using a validated method.

Methods: The nut sampling was conducted in the three main markets of the 16 boroughs of Mexico City. The samples were homogenized, the extraction method was validated, and the concentrations of the 4 AF were determined by immunoaffinity columns. The identification and quantification of the AF was carried out by high performance liquid chromatography. A statistical analysis included the Wilcoxon/Kruskal-Wallis test to compare the variation of the origin of samples, types of AF and nut.

Results: The recovery percentages of the AF ranged from 75% to 95%. The limits of detection (LOD) of the AF (ng/g), based on the calibration curves, were: 0.1 (AFB₁), 0.01 (AFB₂), 0.01 (AFG₁) and 0.05 (AFG₂). Of the 50 samples analyzed, 22% were contaminated with AFB₁, and 100% were contaminated with AFT. The average concentrations of AF in the walnut were 0.05 ng/g of AFB₁ and 2.10 ng/g of AFT. For the pecan, the concentrations were 0.09 ng/g of AFB₁ and 0.44 ng/g AFT, and for the cashew, 0.02 ng/g of AFB₁ and 1.36 ng/g AFT. The walnut was the most significantly (p<0.05) contaminated by AFB₁, AFB₂, AFG₁, AFG₂ and AFT, and the most contaminated boroughs with AFB₁ were Tlalpan (0.23 ng/g) and Coyoacan (0.26 ng/g). For the pecan and cashew, no significant difference was found between the boroughs in AFB₁ and AFT contamination.

Conclusion: Aflatoxins are potent mutagens and proven carcinogens, Type I for humans that should be prevented to warranty the quality of oilseeds, nuts are a source of carcinogen ingestion and their consumption can be a risk to human health.

Keywords: Aflatoxins; Nuts; Carcinogens; Mutagens; Food contamination; Mycotoxins.

Introduction

The world's production of unshelled nuts in 2010 was 2,545,388 tons, and the main nut production countries were China (1,060,600 tons), the United States of America (458,000 tons), Iran (270,300 tons), Turkey (178,142 tons), Ukraine (87,400 tons) and Mexico (76,627 tons). The countries with the highest nut consumption by inhabitant during 2009 were Lebanon (15.9 kg), Maldives (13.7 kg), Greece (11.1 kg), Iran (10.2 kg), Syria (10.1 kg), The Netherlands (8.7 kg), Spain (8.6 kg), Switzerland (8.4 kg), Italy (7.4 kg) and Austria (7 kg). Mexico had a 1.8-kg consumption [1].

The estimated current population of Mexico was approximately 123,278,559 in March of 2014 [2]. Mexico City includes the capital city called Federal District, which is home to 9 million inhabitants, while the entire metropolitan area had a population of 21.2 million people in 2013 [2]. This metropolitan area has 60 municipalities from the surrounding State of Mexico and one from the State of Hidalgo. Mexico City has 20% of Mexico's entire population, making it the most populous metropolitan area in the Western Hemisphere, four times the population of Norway or Denmark, and twice the population of Sweden. Mexico City is the third most densely populated city in the world after Tokyo and Delhi [3]. The Federal District is divided into 16 boroughs, which had the high populations in 2010 [4]. The Federal

District receives food from the entire country and is a reliable sampling place to gain an understanding of the nuts consumed in Mexico.

Aflatoxins (AF) are toxic secondary metabolites that correspond to bis-dihydrofurane coumarins [5-7] and are produced by the molds *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* [8] and *A. bombycis* [9] that can grow on the field, soil, debris, straw, or in storage warehouses [10] with low humidity and high osmotic pressure [11].

During 2011, Jalisco was the main production state for the walnut (*Juglans regia* L.), and in total, Mexico sowed a total of 511.75 hectares (Ha) of walnuts, yielding 2.78 tons/Ha with a crop value of 2,044,840 USD. Chihuahua was the main production state for the pecan (*Carya*

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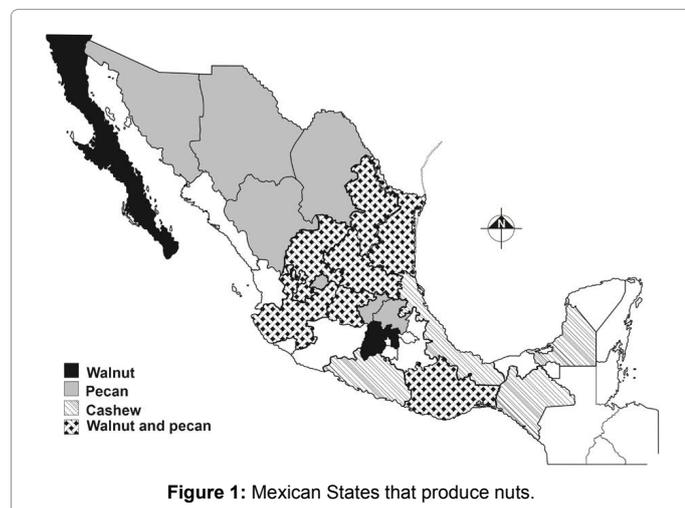
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illinoensis (Wangenh.) K. Koch), and the country sowed 95,668 Ha, with a yield of 1.41 ton /Ha and a crop value of 457,977,252 USD. Finally, Chiapas was the main production State for the cashew (*Anacardium occidentale L.*), with the entire country sowing 1,232 Ha, and yielding 3.53 tons/Ha and a crop value of 1,311,031 USD, considering a money conversion of 13.10 Mexican pesos per USD on August 26, 2014 [12] (Figure 1). The nutrimental value of the three types of nuts has been reported [13].

Oilseeds, such as nuts [8,14], cereals [15] and spices are susceptible crops to molds and their toxins, including AF [16]. Although 18 AF have been identified, only four, Aflatoxin B₁ (AFB₁), Aflatoxin B₂ (AFB₂), Aflatoxin G₁ (AFG₁) and Aflatoxin G₂ (AFG₂), are common in oilseeds, many foods and feed [17] and are, thus, a risk for animal and human health due to their worldwide frequency [18]. Other AF (M₁, M₂, P₁, Q₁, G_{2a}, B_{2a}, aflatoxicol, etc.) are products of microbial or animal metabolism [19].

AFB₁ is the most potent carcinogen that affects the human liver [20] and has been classified as a Group 1 proven carcinogen for humans [21], based on epidemiological studies that associated it with liver cancer and acute hepatitis [22]. AFB₁ is also teratogenic, abortive [23], mutagenic [24], and immunosuppressive [25]. It weakens veins and artery walls, breaks platelets and causes internal hemorrhages [26].

Acute intoxications or aflatoxicoses cause vomiting, abdominal pain, lung edema, fat infiltration with fat and necrotic liver [27,28] and can even lead to fast death. The most important chronic AF diseases are hepatic diseases and cancers in humans [18,29,30], with 600,000 deaths per year [31,32] and survival of less than a year after diagnosis [33]. Mexico has the most liver diseases (e.g., cirrhosis, hepatitis and cancer) of all countries on the American continent [34], and Mexicans eat more maize and spices, such as chili, but the AF contamination of nuts have not been reported, and all the AF sources have to be described to prevent this problem. Although nuts are well known as susceptible to AF, there are no AF contamination in walnut, pecan or cashew reported for Mexico. Of the 64,000 cancer deaths in México, liver cancer accounts for 13.5% of the deaths [35]. AF are also related to the human papilloma virus in human cervical cancer [36], as well as colorectal, pancreas, and lung cancer [37]. Other chronic effects of AF are Reye syndrome [38], biliary duct proliferation [39], marasmus and kwashiorkor [40], which are associated with malnutrition, hepatitis, cirrhosis [41] and growth failure in children [42].



In West India in 1970, several persons died from AF poisoning when they consumed AF-contaminated maize [41]. In 1995, the consumption of rice spaghetti contaminated with AF caused brain damage, encephalopathy, and acute damage in the livers of children from Malaysia [43]. Several human deaths were reported due to the consumption of AF-contaminated foods in Kenya in 1980 [44]. In rural Kenya in 2005, there were 317 aflatoxicosis cases that led to 125 deaths from eating AF-contaminated maize [30].

The production or inadequate storage conditions of food favors AF production in oilseeds, such as nuts [45]. AF have been detected in walnuts in Turkey [46], Morocco [47], and Pakistan [48] and in pecans that contain *Aspergillus flavus* and *A. parasiticus*, which are molds that produce AFB₁ and AFG₁ [49], cashews [50], almonds, pistachios, and hazelnuts when the nut is still in the tree with or without damage [49,51,52].

AF levels do not diminish with domestic cooking in either a microwave [53] or gas oven [54]. High concentrations of AFB₁ were detected under storage conditions of 97% relative humidity (RH) and temperatures of 25 to 30°C. Stored nuts were protected from AF for two months at a RH of 75 to 97% and a temperature of 10 to 30°C [55].

Although there are reports detailing AF contamination in nuts of many countries, there are no reports on those of Mexico. The aims of the present study were to purify, identify and quantify the AF (AFB₁, AFB₂, AFG₁ and AFG₂) in three types of nuts: the walnut, pecan and cashew from Mexico. Additionally, we aimed to: a) validate the AF HPLC quantification method for the three nuts, including defining the linearity, selectivity, recovery percentage, limit of detection, and limit of quantification; b) extract and purify the AF present in the three nuts; c) compare the AF content in the three nuts statistically; and d) determine whether the AF content of the three nuts is under the tolerable limit defined in Mexican legislation for AF in food.

Materials and Methods

AF standards

The AF standards (AFB₁, AFB₂, AFG₁ and AFG₂) from Sigma-Aldrich (St. Louis MO, USA) were prepared as 1 µg/mL solutions [56], dissolved in a benzene: acetonitrile mixture (98:2 v/v) and stored in amber vials at 4°C. The UV-Vis spectrophotometer (Genesys 10 UV-Vis, Thermo Electron Corporation, Madison, Wisconsin, USA) was calibrated for the correction factor (method 971.22B) [56], the absorbance was adjusted to 0 with a blank of benzene: acetonitrile (98:2% v/v) and the wavelength of maximum absorbance for the four AF was determined.

Sampling

Fifty grams of each of the three types of nuts were sampled in the three biggest and most important markets of each one of the 16 boroughs of Mexico City. The samples of the same kind of nut from the three markets of each borough were mixed to make a compound sample that was ground and homogenized to obtain 51 g for the AF analysis (Table 1). There were two 50-g samples (walnut and cashew) purchased in 2012 from the Spices Market in Istanbul, Turkey.

Chemical analysis

A stock solution of 1 µg/mL each of AFB₁, B₂, G₁ and G₂ was used to make 16 dilutions for the linearity validation assay.

One milliliter of benzene: acetonitrile (98:2% v/v) was injected in a new AF standard vial. Afterwards, an unknown amount of AF standard

Date in 2012	Boroughs/inhabitants in 2010	Markets	Nut type
30/ago	Álvaro Obregón/ 727,034	Corpus Christi	Walnut, pecan and cashew
		Olivar del Conde	
		Melchor Múzquiz	
19/sep	Azcapotzalco/ 414,711	Azcapotzalco.	Pecan and cashew
		Prohogar.	
		Nueva Santa María.	
30/ago	Benito Juárez/ 385,439	Portales.	Walnut, pecan and cashew
		Mixcoac.	
		Independencia.	
29/ago	Coyoacán/ 620,416	Coyoacán.	Walnut, pecan and cashew
		Ajusco-Moctezuma.	
		Santo Domingo "Las Rosas".	
13/sep	Cuajimalpa/186,391	Rosas Torres.	Pecan.
		Cuajimalpa.	
		Contadero.	
6/sep	Cuauhtémoc/ 531,831	Arcos de Belén.	Walnut, pecan and cashew
		San Juan.	Pecan and cashew.
		Hidalgo.	
19/sep	Gustavo A. Madero/ 1,185,772	Río Blanco.	Pecan
		La Estrella.	Walnut, pecan and cashew
		Martín Carrera.	Pecan and cashew
San Miguel Iztacalco.			
26/sep	Iztacalco/ 384,326	Santa Anita.	Walnut, pecan and cashew
		Tlacotal	
		Central de Abasto.	
26/sep	Iztapalapa/1,815,786	Santa Cruz Meyehualco.	Walnut, pecan and cashew
		Jacarandas.	
		Cerro del Judío.	
12/sep	La Magdalena Contreras/ 239,086	La Loma.	Pecan
		Turístico La Magdalena.	
		Tacubaya.	
12/sep	Miguel Hidalgo/ 372,889	Plutarco Elías Calles, "El Chorrillo".	Walnut, pecan and cashew
		Argentina.	
		San Antonio Tecómitl.	
5/sep	Milpa Alta/ 130,582	Benito Juárez.	Pecan and cashew
		San Pedro Atocpan.	
		Colonia del Mar.	
27/sep	Tláhuac/ 360,265	La Nopalera.	Walnut, pecan and cashew
		Tláhuac.	
		Torres de Padierna.	
6/sep	Tlalpan/ 650,567	Tlalcoligía.	Walnut, pecan and cashew
		De la Luz	
		San Ciprián.	
20/sep	Venustiano Carranza/ 430,978	Jamaica.	Walnut, pecan and cashew
		La Merced.	
		Xochimilco.	
5/sep	Xochimilco/ 415,007	San Gregorio Atlapulco.	Walnut and cashew
		Tulyehualco	
		Market of Spices Istanbul, Turkey	
5/nov	Turkey	Market of Spices Istanbul, Turkey	Walnut and cashew

Table 1: The samples of the same kind of nut from the three markets of each borough were mixed to make a compound sample that was ground and homogenized to obtain 51 g for the AF analysis

was placed in the vial, and it was diluted to one mL with HPLC-grade methanol (MeOH); later, an unknown amount was placed in a quartz cell for the absorbance measurement in the spectrophotometer (Thermo Modelo Genesys 10 UV) at a wavelength of 362 nm, and the concentration was calculated with the following formula [56]:

$$\text{Conc } (\mu\text{g/mL}) = \text{Abs at } 362 \text{ nm} \times \text{Mw} \times 1000 / \text{Extinction coefficient}$$

where Conc = concentration, Abs = Absorbance, Mw = molecular weight and Ec = extinction coefficient

Depending on the AF, the molecular weight (mw) and the extinction coefficient (ec) were: AFB₁ (mw=312; ec=21,800); AFB₂ (mw=314; ec=24,000); AFG₁ (mw=328 and ec=17,700) and AFG₂ (mw=330; ec=17,100) [56].

Derivatization

The dry AF standards were resuspended with 200 µl acetonitrile (ACN) and 800 µl of derivatizing solution to increase fluorescence. The derivatizing solution consisted of 5 mL of trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis MO, USA), with 2.5 mL of glacial acetic acid (Merck, Naucalpan, Edo. Mex., México) and 17.5 mL of deionized water to obtain a final concentration of 20% TFA (v/v); the mixture was vortexed (Vortex G-560, Bohemia, N.Y., EEUU) for 30 seconds.

The vials were placed in a water bath (Aparatos de Laboratorio BG Mod. BM 40T, Mexico) at 65°C for 10 min [57,58]. Following this period, they were injected into the HPLC for chemical quantification.

AF extraction of the samples

The walnut, pecan and cashew nuts were independently ground (Black and Decker Crush Master, Mod. V2350BP), and 50 g of each type of nut from the three markets in each borough were added together to make a 51-g compound sample. The compound samples were blended with 100 mL of a solution of methanol:distilled water (H₂O_d) (80:20 v/v) and 2 g NaCl for 2 min.

The samples were filtered through a Büchner funnel adapted to a vacuum pump, and 2 mL of the filtrate were diluted with 14 mL of phosphate-buffered saline (PBS) at pH 7.4. The diluted filtrate was passed through an immunoaffinity column (Easi-Extract Aflatoxin R-Biopharm Rhône, Ltd.) previously balanced with 20 mL of PBS [59,60] for the detection of total AF (AFt). After receiving the sample filtrate, the immunoaffinity column was washed with 20 mL of H₂O_d at a flux of 5 mL per min. Air was passed through the agarose gel of the immunoaffinity column to dry it, and it received 1.5 mL of HPLC-grade MeOH by gravity at one drop per second. Afterwards, 1.5 mL of H₂O_d was applied to denature the gel with AFt antibodies and release the pure AF in the 3-mL eluate. Finally, the eluate was dried at 40°C, and it was derivatized as mentioned before.

AF quantification by HPLC

The standard AF HPLC analysis [61] and the eluting of samples were performed with an Agilent Technologies HPLC (Series 1200) with an isocratic pump (G1310A Series DE62957044), fluorescence detector (G1321A Series DE60456380) and autosampler (G1329A Series DE64761666), a chromatographic Agilent Eclipse XDS-C18 column (4.6 × 250 mm, 5 µm of particle size) and the HPLC program Chem Station 32. The analytical conditions were: H₂O/ACN/MeOH (65:15:20 v/v/v) mobile phase, 60-µL injection volume, 1 mL/min fluid speed for 20 min, excitation at 362 nm and emission at 425 for AFB₁ and AFB₂ and 450 nm for AFG₁ and AFG₂.

Validation of the HPLC quantification method for AF in nuts

Validation is the confirmation, with objective evidence, that the requirements for a certain application of the method have been realized [62]. The validation of the method was done in agreement with the EC Regulatory Commission 2004/882 that considers the following steps:

Selectivity: Selectivity is the degree to which the method can determine the analyte (AF) without matrix interference. A mixture of the 4 AF standards was analyzed (blank), as well as the three matrices (walnut, pecan and cashew) independently enriched with the four AF. The blank and the three nut sources with AFs were extracted, purified with immunoaffinity total anti-aflatoxin columns, derivatized and the resulting chromatograms were compared.

Lineality: Lineality is the ability to obtain proportional results to the analyte (AF) concentration. Sixteen standard dilutions (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 200, 600, 800 and 1000 ng/mL) of each of the four AF were tested separately to obtain the calibration curves and the limits of detection (LOD) and quantification (LOQ).

Limits of detection (LOD) and quantification (LOQ): The LOD was determined by observing the minimal concentration that displayed the chromatographic peak signal by HPLC. The LOQ was calculated taking into account the sample results, and it was calculated as the LOD per five.

Recovery percentage (R%): The recovery percentage is the efficiency of the method to detect all of the analyte (AFs) present in a sample. Each matrix (walnut, pecan and cashew) was fortified with 100 ng/g of each one of the four AF independently before the analysis. Later, the extraction and derivatization were performed, and the recovery percentage allowed for the adjustment of the AF concentrations in the samples. This validation method parameter is important to obtain the real results.

Statistical analysis: A non-parametric Kruskal-Wallis test, followed by a Wilcoxon range test, was conducted to determine which groups had the significant differences in quantity and nut type of the samples composed from nuts from three markets in each borough of Mexico City and the Turkey samples.

Results and Discussion

Validation of the method to quantify AF in nuts

Selectivity: The chromatograms after the AF addition the three matrices showed that the elution order of the analytes and the retention times (rt) were not modified by the different nut matrices, and the four AF analytes did not overlap (Figure 2).

Lineality and limits of detection (LOD) and quantification (LOQ): The standard solutions that had positive results were: AFB₁ (0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng/mL), AFB₂ (0.01, 0.05, 1, 5, 10, 20, 40, 70, 100 and 200 ng/mL), AFG₁ (0.01, 0.05, 0.1, 0.5, 1, 4, 16, 100 and 128 ng/mL) and AFG₂ (0.5, 1, 2, 4, 8, 16, 32, 64, 100, 200, 600, 800 and 1000 ng/mL). The parameters of the AF calibration curves (LOD and LOQ) were: for AFB₁, slope = 2.8299, correlation coefficient (R²) of 0.9973, n=28, LOD=0.1 ng/mL, and LOQ=1 ng/mL; AFB₂, slope=1.7786, R² =0.9908, n=38, LOD=0.01 ng/mL and LOQ=0.1 ng/mL; AFG₁, slope=1.7607, R² =0.9969, n=26, LOD=0.01 and LOQ=0.1 ng/mL and AFG₂, slope=1.247, R²=0.9908, n=38, LOD=0.5 ng/mL and LOQ=5 ng/mL. The LOD results were very good, the equipment detected low levels of AF that made the quantification accurate.

Retention times (RT) and recovery percentage (R%)

The retention times were adjusted according to the selectivity, lineality and recovery percentage for each type of nut.

For the walnut, the following were obtained: AFB₁, RT=7.709-9.478 min and R%=88.12±2.31; AFB₂, RT=17.590 - 19.804 min and R%=95.16±0.59; AFG₁, RT=5.642-6.447 min and R%=82.92±2.58; AFG₂, RT=11.319-13.247 min and R%=82.01±3.13.

For the pecan, the following were obtained: AFB₁, RT=7.709 - 9.478 min and R%=85.17±1.85; AFB₂, RT=17.590-19.804 min and R%=86.89±13.66; AFG₁, RT=5.642 - 6.447 min and R%=75.24±9.35; AFG₂, RT=11.319 - 13.247 min and R%=85.92±3.49.

For the cashew, the following were obtained: AFB₁, RT=7.709 - 9.478 min and R%=84.15±0.9; AFB₂, RT=17.590 - 19.804 min and R% 87.31±6.03; AFG₁, RT=5.642 - 6.447 min and R%=86.11±3.55 and AFG₂, RT=11.319-13.247 min and R%=86.33±4.75 (Figure 3A).

The three nut recovery percentages were from 75.24 % to 95.16% that are excellent recoveries from natural products. These results validated the AF percentage of recovery of the three nuts.

AF quantification of nut samples by HPLC

A total of 50 samples were analyzed, of which 22% (11/50) were contaminated with AFB₁, and 100%, with AFt. Of the walnut samples, 35.30% (6/17) had AFB₁ contamination, while 17.65% of the cashew samples (3/17) and 12.50% of the pecan samples (2/16) were contaminated with AFB₁. Four of the five most contaminated samples were walnuts from the center-south boroughs, such as Coyoacan (0.16 ng/g), Tlalpan (0.23 ng/g), Tlahuac (0.10 ng/g) and Xochimilco (0.15 ng/g), as shown in Table 2.

AFB₁ contamination was present in pecan samples from the Álvaro Obregón (0.08 ng/g) and La Magdalena Contreras (0.06 ng/g) boroughs, which are geographically close; walnut and cashew samples from this area were not contaminated. The three AFB₁ contaminated cashew samples had no geographical relation (Tláhuac, 0.17 ng/g, Tlalpan, 0.07 ng/g and Venustiano Carranza, 0.07 ng/g), and these boroughs also had high AFB₁ in the walnut (Figure 3).

All of the boroughs had AFt contamination, and the walnut was the most affected type of nut, with the Turkey samples (12.10 ng/g) and those from Iztacalco (7.92 ng/g) being the most contaminated ones, followed by cashew samples from Milpa Alta (6.89 ng/g). The boroughs with AFB₁ contamination were not the same as the ones with high AFt, due to the AFt values that were heavily affected by the high AFG₂ values (Figure 3).

The percentages of AFB₁ and AFt in the samples from Mexico City were 35.30% for the walnut, 17.65% for the cashew, and 12.50% for the pecan. In comparison the AFt contamination percentage in the City of Mekkah was higher, 50% in walnuts and 15% in cashews [63], and in Malaysia a lower frequency was found, 16.3% AFt contamination (17.2 to 350 µg/kg) [64]. Poland had walnuts that were 38% contaminated, with 5.50 µg/kg AFt and 4.04 µg/kg AFB₁, and cashews with 0.35 µg/kg AFB₁ [50]. In Morocco, walnuts had 30% AF contamination with a sample that included 2500 g/kg of AFB₁ [47], and in Pakistan, the walnut contamination was 40% with the shell intact and 70% in a shelled nut [48].

Toxigenic *A. flavus* strains produce only AFB₁ and AFB₂, and *A. parasiticus* produce the four AF, AFB₁, AFB₂, AFG₁ and AFG₂ [65,66].

In the analyzed samples of Mexico City, all four AF were found, so the conclusion is that the nuts were invaded by both *A. flavus* and *A. parasiticus*, and the pH conditions determined the type of metabolites synthesized by the mold: pH values below 6 favored AF type B, and pH values above 6, in the case of nuts, stimulate the synthesis of AF from group G [67], which would explain the high values of AFG₂ found in the samples (Figure 3).

Although AFB₁ and AFG₁ are more toxic than AFB₂ and AFG₂ [68], the latter two can oxidize *in vivo* to AFB₁ and AFG₁, respectively [69]. The obtained Aft averages for the walnut (2.7 µg/kg), pecan (0.4 µg/kg) and cashew (1.4 µg/kg) are in agreement with the European Commission Rules and the NMX-FF-093-SCFI-2011 Rule that establish a legal limit of 10 µg/kg and are below the tolerance limit and acceptable for exportation [70].

AF were analyzed at a ng/g level, but the amount that a person consumes in cooked food and desserts exceeds tolerance limits, and depending on the frequency, can be considered a risk for chronic health effects. The total AF calculation from nut ingestion in different commercial presentations exceeds 10 µg/kg, the amount needed to produce a mutation in cells; therefore, a carcinogenic risk persists. Taking into account the Aft (ng/g) average of the walnut (2.7), the calculation in cooked food per consumption was: 1) of 46.25 g of walnut sauce (“nogada”) for stuffed chiles would be 125 ng Aft; and 2) one kg of walnuts=2700 ng Aft. With respect to the cashew, the average was 1.4 ng/g Aft, so a package of 40 g would be 56 ng, and a package of 250 g would be 350 ng Aft. Finally, the Aft average for the pecan was 0.4 ng/g, so a package of 100 g would equal 44 ng/g Aft and a package of 250 g would equal 110 ng/g Aft. Two hundred and fifty grams of pecan cookies would equal 110 ng/g, a 375-g slice of pecan cake would equal

150 ng/g Aft, and 50 g of pecan ice cream would equal 20 ng/g Aft.

Nuts are part of a healthy diet against diabetes [13] because they contain antioxidants, unsaturated fatty acids, and vitamins, according to reports on the walnut [71]. Additionally, pecans contain compounds that have protective effects on the heart [72], and cashews contain cardol, which has antimicrobial activities [73].

The walnut is an important crop for the food industry, as a nutriment, and for the health benefit it provides due to its oils, proteins, minerals, flavonoids, phenolic acids (antioxidants), polyphenols and vitamins [71]. Nut antioxidants help protect against fat nodules (atheroma plaques) in the arteries, are anti-inflammatory and anti-mutagenic [74].

The pecan is a source of monounsaturated fatty acids and heart-protective compounds, including vegetable sterols, vitamins A, B and E, folic acid, calcium, magnesium, phosphorus, zinc and fiber [72]. It is a food that is free of cholesterol with a high protein content, and its oleic acid is similar to that in olives [75].

The cashew has more ascorbic acid than oranges, vitamins B₁, B₂, pantothenic acid, and minerals, such as magnesium [76], and it is rich in unsaturated fatty acids, such as oleic and linoleic acids. The cashew has raw protein and lysine comparable to peanuts [77] and the soybean, and it has higher sulfur amino acids, and oils and gums with cardol, which is a caustic and poisonous substance that evaporates when the nuts are heated [78]. The cashew contains a group of phenolic compounds known as “cashew nut shell liquid” (CNSL) [79,80] that includes anacardic acid, cardanol and cardol, this last of which has been reported to have antimicrobial activities [73], possibly giving resistance to *Aspergillus* invasion.

All of the samples contained the AF carcinogens. In the millions of years of co-existing together, it seems that as a reaction to mold and its aflatoxins, nut trees have developed high amounts of resistance [81,82] and antioxidant phytochemicals [83] such as caffeic acid, which reduces the AFs by 99.5%, quinic acid (90.2%) and chlorogenic acid (88.5%) [84]. Therefore, beneficial components, such as antioxidants, and dangerous toxins appear together.

Statistical Analysis

The Kruskal-Wallis test with the Statistical Program R showed that the walnut had significant differences among the boroughs for AFB₁, AFB₂ and AFG₁. For the pecan and cashew, significant differences were only found among the boroughs for AFB₂ and AFG₁ content; no differences for AFG₂ and Aft were observed (Table 3).

With respect to the type of nut as a variation source, there were significant differences for the four AF and Aft. The results of the Kruskal-Wallis statistical values were: AFB₁=9.24, AFB₂=8.03, AFG₁=62.57; AFG₂=17.24 and Aft=40.31, all displaying significance levels <0.05.

Figure 4 gives the AFB₁ results from the Wilcoxon test, which was used to find significant differences among the groups. The walnut samples from the Tlalpan and Coyoacan boroughs were the most contaminated with AFB₁ and were significantly different (p<0.05) from the other boroughs, while there were no significant differences between the source (or location) for AFB₁ in pecans and cashews. In the case of Aft, there was not a significant difference when considering borough as the variation factor.

The walnut was the most contaminated nut for AFB₁ (0.04 ng/g,

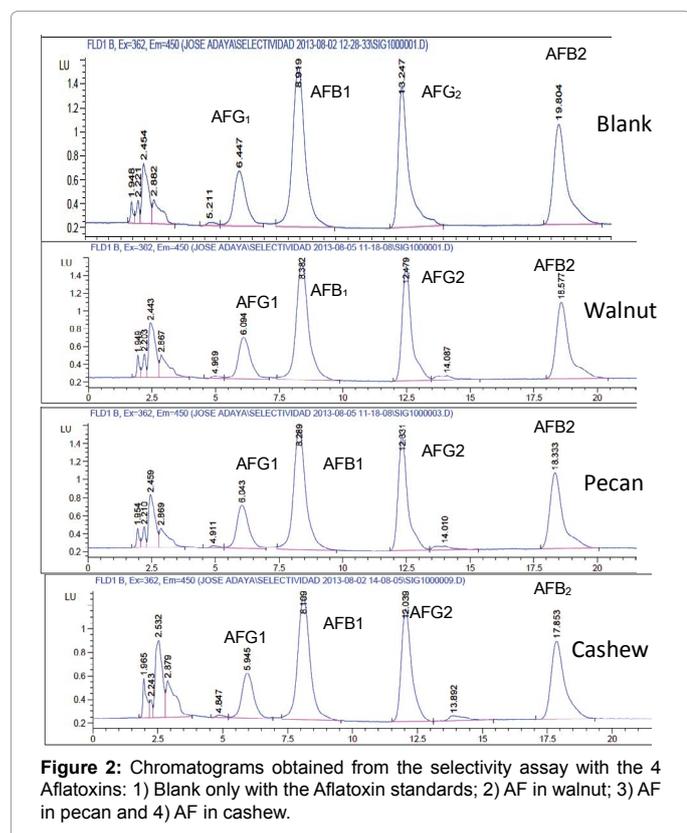


Figure 2: Chromatograms obtained from the selectivity assay with the 4 Aflatoxins: 1) Blank only with the Aflatoxin standards; 2) AF in walnut; 3) AF in pecan and 4) AF in cashew.

Nut type	Borough	AFB ₁		AFB ₂		AFG ₁		AFG ₂		AF _T	
		Ø	SD	Ø	SD	Ø	SD	Ø	SD	Ø	SD
Walnut	Álvaro Obregón	<LOD	0.00	0.04	0.01	0.14	0.05	<LOD	0.00	0.18	0.04
	Azcapotzalco	<LOD	0.00	0.03	0.02	0.26	0.17	2.04	1.77	2.33	1.65
	Benito Juárez	<LOD	0.00	0.16	0.06	0.10	0.02	1.75	1.01	2.01	0.98
	Coyoacán	0.16	0.02	0.62	0.79	0.29	0.26	1.08	1.05	2.15	1.05
	Cuajimalpa	<LOD	0.00	0.07	0.07	0.19	0.10	0.63	0.05	0.89	0.13
	Cuauhtémoc	<LOD	0.00	0.04	0.01	0.05	0.03	0.70	0.61	0.80	0.62
	G.A. Madero	<LOD	0.00	0.04	0.02	0.32	0.07	1.31	1.15	1.67	1.20
	Iztacalco	<LOD	0.00	0.05	0.01	0.22	0.05	7.65	6.63	7.92	6.67
	Iztapalapa	<LOD	0.00	0.04	0.01	0.26	0.01	2.13	1.92	2.43	1.92
	La M. Contreras	<LOD	0.00	0.03	0.00	0.28	0.07	0.36	0.63	0.67	0.58
	Miguel Hidalgo	0.03	0.06	0.04	0.01	0.38	0.03	0.86	0.99	1.32	0.94
	Milpa Alta	<LOD	0.00	0.28	0.36	0.13	0.02	<LOD	0.00	0.41	0.37
	Tláhuac	0.10	0.18	0.07	0.00	0.61	0.12	0.79	0.68	1.46	0.57
	Tlalpan	0.23	0.25	0.06	0.02	2.20	0.67	<LOD	0.00	2.50	0.80
	V. Carranza	0.07	0.06	0.08	0.04	0.35	0.05	0.84	0.73	1.34	0.79
	Xochimilco	0.15	0.05	0.55	0.05	0.51	0.11	4.29	2.56	5.50	2.55
Turkey	<LOD	0.00	0.04	0.01	0.37	0.06	11.69	10.29	12.10	10.36	
Pecan	Álvaro Obregón	0.08	0.07	0.16	0.19	0.92	0.04	0.13	0.12	1.30	0.32
	Azcapotzalco	<LOD	0.00	0.03	0.01	0.04	0.00	0.23	0.20	0.30	0.20
	Benito Juárez	<LOD	0.00	0.05	0.02	0.05	0.02	0.17	0.17	0.27	0.17
	Coyoacán	<LOD	0.00	0.03	0.00	0.03	0.01	0.17	0.14	0.23	0.15
	Cuajimalpa	<LOD	0.00	0.18	0.14	0.02	0.00	0.12	0.12	0.32	0.19
	Cuauhtémoc	<LOD	0.00	0.06	0.02	0.03	0.01	0.23	0.21	0.32	0.22
	G.A. Madero	<LOD	0.00	0.09	0.04	0.06	0.02	0.18	0.15	0.33	0.13
	Iztacalco	<LOD	0.00	0.12	0.04	0.04	0.01	0.72	0.16	0.88	0.19
	Iztapalapa	<LOD	0.00	0.04	0.01	0.02	0.00	0.25	0.22	0.31	0.22
	La M. Contreras	0.06	0.10	0.12	0.16	0.18	0.25	0.28	0.36	0.64	0.87
	Miguel Hidalgo	<LOD	0.00	0.07	0.03	0.07	0.03	0.17	0.17	0.32	0.18
	Milpa Alta	<LOD	0.00	0.02	0.00	0.13	0.03	0.22	0.19	0.37	0.17
	Tláhuac	<LOD	0.00	0.09	0.04	0.07	0.02	0.20	0.17	0.35	0.20
	Tlalpan	<LOD	0.00	0.08	0.02	0.09	0.01	0.20	0.18	0.37	0.16
	V. Carranza	<LOD	0.00	0.12	0.02	0.10	0.02	0.16	0.14	0.38	0.11
	Xochimilco	<LOD	0.00	0.11	0.05	0.12	0.01	0.19	0.16	0.42	0.21
Cashew	Álvaro Obregón	<LOD	0.00	0.05	0.02	0.03	0.01	0.16	0.28	0.23	0.27
	Azcapotzalco	<LOD	0.00	0.02	0.00	0.04	0.01	1.50	2.60	1.56	2.59
	Benito Juárez	<LOD	0.00	0.03	0.02	0.05	0.02	1.51	2.62	1.59	2.60
	Coyoacán	<LOD	0.00	0.03	0.01	0.02	0.01	<LOD	0.00	0.05	0.01
	Cuajimalpa	<LOD	0.00	0.06	0.02	0.06	0.01	0.46	0.80	0.58	0.81
	Cuauhtémoc	<LOD	0.00	0.05	0.02	0.02	0.00	0.72	1.25	0.79	1.23
	G.A. Madero	<LOD	0.00	0.05	0.01	0.03	0.02	3.99	6.92	4.08	6.93
	Iztacalco	<LOD	0.00	0.04	0.00	0.04	0.01	<LOD	0.00	0.08	0.01
	Iztapalapa	<LOD	0.00	0.03	0.00	0.05	0.01	0.28	0.49	0.36	0.49
	La M. Contreras	<LOD	0.00	0.04	0.02	0.09	0.02	1.18	2.04	1.31	2.05
	Miguel Hidalgo	<LOD	0.00	0.08	0.10	0.08	0.03	<LOD	0.00	0.16	0.07
	Milpa Alta	<LOD	0.00	0.03	0.01	0.11	0.02	6.75	11.69	6.89	11.71
	Tláhuac	0.17	0.30	0.08	0.03	0.10	0.01	<LOD	0.00	0.35	0.29
	Tlalpan	0.07	0.12	0.04	0.00	0.09	0.02	<LOD	0.00	0.20	0.12
	V. Carranza	0.07	0.11	0.09	0.03	0.06	0.01	3.30	5.72	3.51	5.82
	Xochimilco	<LOD	0.00	0.09	0.04	0.06	0.00	1.10	1.91	1.25	1.88
Turkey	<LOD	0.00	0.02	0.01	0.12	0.02	<LOD	0.00	0.14	0.03	

Table 2. Aflatoxin concentration in samples, adjusted with the recovery percentage (ng/g). AF = Aflatoxins, Aflatoxin B1 = AFB1, Aflatoxin B2 = AFB2, Aflatoxin G1 = AFG1, Aflatoxin G2 = AFG2 and Total Aflatoxins = AFT

Figure 5), followed by the cashew (0.02 ng/g) and the pecan (0.01 ng/g), with no significant difference between them, but with a direct relation with tannin content that are also a protection for the plant and have an indirect proportion with AF amount [85]. With respect to Aft, there was a significant difference among the three types of nuts, with the walnut being the most affected (2.10 ng/g), followed by the cashew (1.36 ng/g)

and the pecan (0.44 ng/g). These results are in agreement with other reports [50,63,86,87] of the walnut being more contaminated by AFB₁ and AFT than the cashew.

The average AFB₁ contamination in the three nuts was 0.024 ng/g, and the average AFT was 1.30 ng/g. Taking into account that 1.8 kg is the annual consumption by a person in Mexico City, Mexicans living

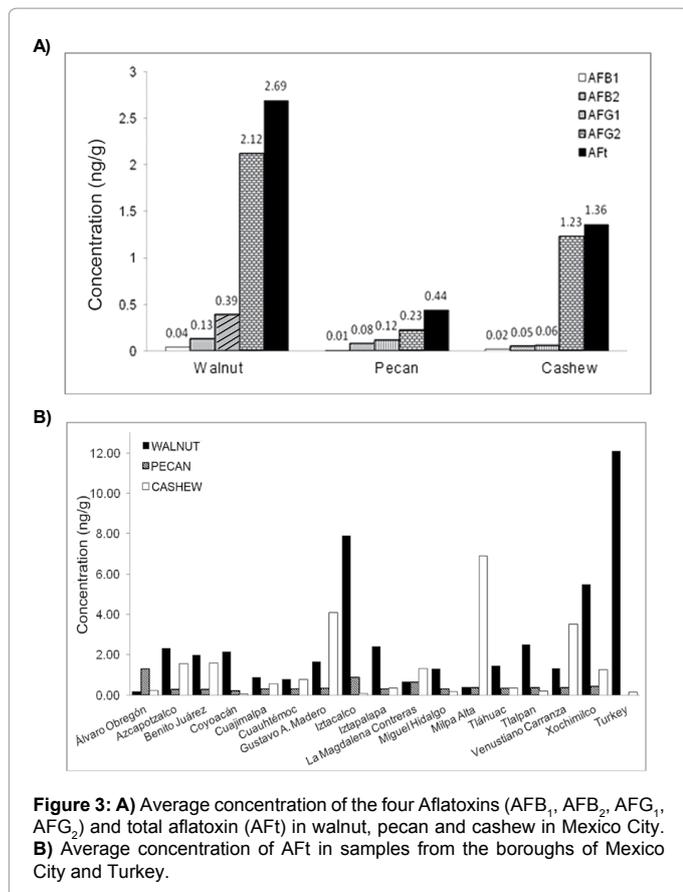


Figure 3: A) Average concentration of the four Aflatoxins (AFB₁, AFB₂, AFG₁, AFG₂) and total aflatoxin (AFt) in walnut, pecan and cashew in Mexico City. B) Average concentration of Aft in samples from the boroughs of Mexico City and Turkey.

Nut type	Aflatoxin	Kruskal-Wallis statistical value	Significance	Significant difference
Walnut	AFB ₁	34.90	<0.05	Yes
	AFB ₂	27.41	<0.05	Yes
	AFG ₁	39.34	<0.05	Yes
	AFG ₂	21.50	0.16	No
	AFt	24.35	0.08	No
Pecan	AFB ₁	24.24	0.06	No
	AFB ₂	31.84	<0.05	Yes
	AFG ₁	38.48	<0.05	Yes
	AFG ₂	12.61	0.63	No
Cashew	AFB ₁	14.59	0.56	No
	AFB ₂	31.38	<0.05	Yes
	AFG ₁	42.80	<0.05	Yes
	AFG ₂	7.68	0.96	No
	AFt	17.97	0.33	No

Table 3. Kruskal-Wallis test applied to find significant differences between source boroughs of the samples.

in Mexico City consume 24 ng of AFB₁ and 1,300 ng of AFt only by nut ingestion per year, and this amount of carcinogens increases with the AF contribution of the other foods.

AF are regulated in more than 75 countries [88]. The world tolerance limits for AFB₁ are 1 to 20 µg/kg and 0 to 35 µg/kg for AFt [89].

The World Health Organization (WHO) established a maximum tolerance limit of 5 µg/kg for AFB₁ and 10 µg/kg for AFt in several foods [90]. The European Commission established an AFB₁ limit of 2

µg/kg and an AFt limit of 4 µg/kg for nuts, dry fruits and cereals in 1998 [70,91]. In Mexico, the Official Mexican Law regulates AF only in maize (20 µg/kg of AFt). The NMX-FF-093-SCFI-2011 law is not compulsory and establishes a maximum tolerance limit of 10 µg/kg [92].

About the lethal dose we can take into account an outbreak in humans occurred in India in 1974, when almost 400 people became ill with fever and jaundice after eating maize contaminated with between 0.25 and 15 mg/kg aflatoxin and more than 100 died [93]. The median

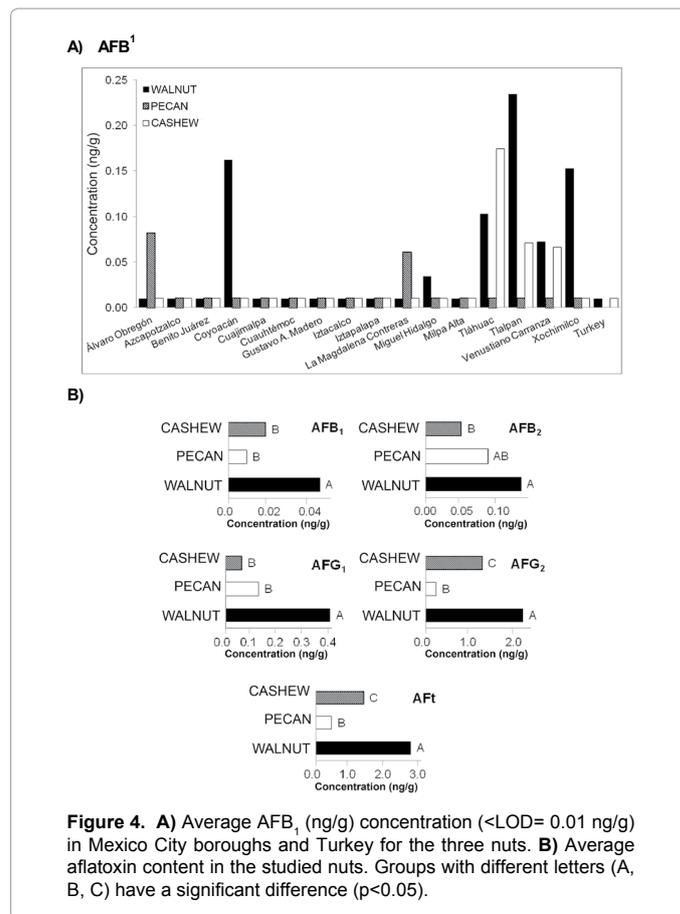
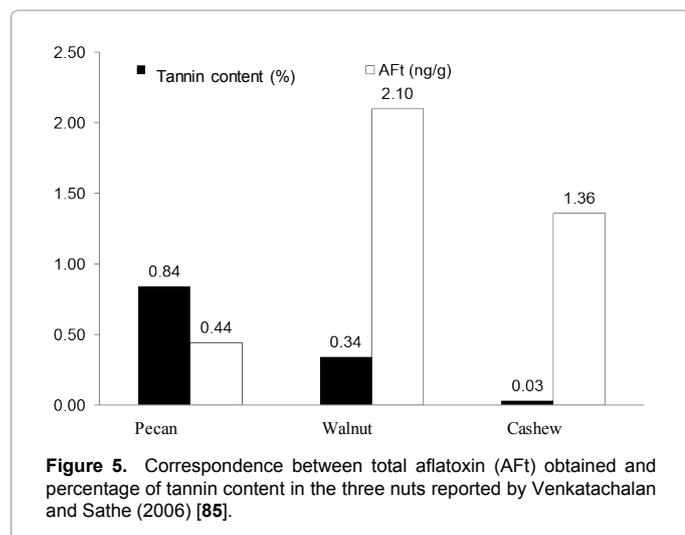


Figure 4. A) Average AFB₁ (ng/g) concentration (<LOD= 0.01 ng/g) in Mexico City boroughs and Turkey for the three nuts. B) Average aflatoxin content in the studied nuts. Groups with different letters (A, B, C) have a significant difference (p<0.05).

lethal dose of AFB₁ 0.36 mg/kg body weight is a special range of highly toxic poison (aflatoxin animal half of the lethal dose is found in the strongest carcinogens). Its carcinogenicity is 900 times more than dimethylnitrosamine induced liver cancer in the large capacity 75 times higher than the 3,4-benzopyrene, a large 4000-fold. It is mainly to induce liver cancer in animals, can also induce cancer, renal cancer, colorectal cancer and breast, ovary, small intestine and other sites of cancer.

The aflatoxicoses was characterized by high fever, high colored urine, vomiting, and edema of feet, jaundice, rapidly developing ascitis, portal hypertension and a high mortality rate. The disease was confirmed to the very poor, who were forced by economic circumstances to consume badly molded corn containing AF between 6.25 -15.6 ppm, an average daily intake per person of 2-6 mg of aflatoxins [94].

In conclusion, the method was carefully validated, and no nut sample exceeded the limits established by Mexican legislation for AFt (20 µg/kg) in food for humans and 10 µg/kg AFt for the pecan. The walnut samples from the Tlalpan (0.23 ng/g) and Coyoacán (0.16



ng/g) boroughs were the most contaminated by AFB₁. The pecan and cashew were not significantly different based on sample origin for AFB₁ content. There was no significant difference in AFt content with respect to sample origin.

The average contamination of the walnut for AFB₁ (0.05 ng/g) and AFt (2.10 ng/g) was significantly ($p < 0.05$) higher than that for the cashew (AFB₁, 0.02 ng/g and AFt, 1.36 ng/g) and the pecan (AFB₁, 0.01 ng/g and AFt, 0.44 ng/g).

The yearly contribution of AFB₁ (24 ng/g) and AFt (1300 ng/g) from nuts to the diet of an inhabitant of Mexico City is under the tolerable limit.

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