Toxicology Reports 6 (2019) 1135–1142

Contents lists available at ScienceDirect

Toxicology Reports

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

# Aflatoxin B1 in nixtamalized maize in Mexico; occurrence and accompanying risk assessment

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ARTICLE INFO

*Keywords:*
Aflatoxin B1
Maize
Mexico
Risk assessment
Margin of exposure (MOE)
Liver cancer

ABSTRACT

Maize is a staple food in Mexico that might contain Aflatoxin B1 (AFB1). Nonetheless, data on the exposure and risk assessment of AFB1 from maize for the Mexican population are limited. The aim of the present study was to analyse the occurrence of AFB1 in Mexican nixtamalized maize samples, and to assess the accompanying exposure and risk. Four out of 88 samples contained AFB1 at levels above the limit of detection (1 ng/g). AFB1 occurrence values obtained in this study and additional occurrence values from literature were combined with available literature data for mean and P95 consumption of maize based products. For a 70 kg body weight person, lower bound and upper bound exposure assessments resulted in estimated daily intakes (EDI) of 0.7–8.5 ng/kg bw/day, based on a mean maize consumption. Based on the P95 maize consumption these EDI values amounted to 3.3–11.7 ng/kg bw/day. The corresponding Margin of Exposure (MOE) values amounted to 257-20 for the mean and 50-15 for the P95 consumers. The estimated increased cancer risks were 9-320 and 43-439 cases/$10^6$ individuals/lifetime of 75 years for the mean and P95 consumers, respectively. Altogether, the assessment reveals the need for continued risk management of AFB1 in Mexico.

## 1. Introduction

Aflatoxins are mycotoxins produced by different fungal species belonging to the Aspergillus genus. These mycotoxins have been extensively studied after their first discovery in 1960 when their presence in feed caused the death of turkeys and other poultry [1]. At present, aflatoxins are regarded as important mycotoxins because of their common occurrence in food crops and because one of them, aflatoxin B1 (AFB1), is the most potent liver carcinogen known, acting by a genotoxic mode of action [2,3]. Evidence from experimental animal studies and data on the increased risk for hepatocellular carcinoma (HCC) in a cohort of individuals exposed to aflatoxins made the International Agency for Research on Cancer (IARC) to classify aflatoxins as a Group 1 agent, carcinogenic to humans [4].

The link between AFB1 exposure and HCC is well established, as well as the role of biotransformation of AFB1 in the underlying mode of action [2]. AFB1 monooxygenation by cytochromes P450 leads to a reactive AFB1-8,9-epoxide, which can either be cleared in conjugation reactions or bind to macromolecules such as DNA [5]. The covalent binding of electrophilic metabolites with the nucleophilic sites of DNA results in DNA adducts, which are considered key intermediates in the transformation of a normal cell into a malignant tumor cell [6].

AFB1 is typically produced by *A. flavus*, an ubiquitous fungus in tropical and subtropical areas, which usually affects peanuts and maize [7,4,8,3]. The production of AFB1 by the fungus is associated with harsh pre-harvest conditions of the crops or with inadequate transport, storage or manufacturing conditions [9]. In order to diminish the fungal growth and subsequent AFB1 production resulting in human exposure, intervention approaches are established at different levels. However, climate change and deficiencies in intervention practices (due to for example financial limitations) can potentially result in AFB1 related health issues [10–12].

Together with some African countries, Mexico and Guatemala are among the countries with the highest consumption of maize [13–15]. In Mexico, maize has been a traditional staple food [16]. The estimated consumption of unprocessed maize for Mexico is 267 g/person/day based on the food supply obtained in 2009 from the Food Balance Sheet of the Food and Agriculture Organization of the United Nations (FAO) [14]. However, in Mexico this cereal is mainly consumed as *nixtamalized* derived products in the form of *tortillas* (118.4 g) while a small proportion is consumed as non-nixtamalized maize (2.8 g corn/day) [17]. In brief, *nixtamalization* is a process in which maize grains are milled after being cooked in a calcium hydroxide solution to produce a fresh dough or an industrial flour [18].

Even though *nixtamalization* can reduce the levels of aflatoxins in maize products [19,20], uncertainty remains about the prevalence of

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https://doi.org/10.1016/j.toxrep.2019.10.008
Received 2 August 2019; Received in revised form 26 September 2019; Accepted 7 October 2019
Available online 15 October 2019

aflatoxins in food and the exposure of Mexican people [15,21,22]. As aflatoxins are in many cases unavoidable food contaminants, regulatory limits have been set worldwide to minimize human exposure from maize consumption at concentrations as low as reasonable achievable (ALARA). The Mexican government has set a maximum level of 12 μg/kg product for total aflatoxins in nixtamalized maize products [23], a limit slightly lower than the limit of the United States for total aflatoxins of 20 μg/kg for maize [24]. In contrast, the European Union adopted stricter levels of 2 μg/kg for AFB1 and of 4 μg/kg for total aflatoxins in ready to eat maize [25].

Despite the importance and high consumption of maize in Mexico, exposure and risk assessments of AFB1 for the Mexican population are limited [26]. In Mexico, the age-standardized HCC incidence rate in 2018 was 5.6 per 100,000 persons [27], and the main aetiology is considered to be alcoholic cirrhosis and infection with hepatitis C virus (HVC), and in a lesser extent infection with hepatitis B virus (HBV) [28]. However, the role of AFB1 exposure in the HCC aetiology in Mexico is unknown. Previous studies have suggested a possible contribution of AFB1 exposure to HCC formation in alcoholic cirrhosis and HVC [29,30]. Therefore, the purpose of this paper was to provide insight in AFB1 levels present in locally collected nixtamalized maize samples in Mexico City and to perform an accompanying exposure and risk assessment. To this end, nixtamalized maize samples were collected from Mexico City and AFB1 was extracted and quantified by LC–MS/MS. The AFB1 occurrence levels obtained for the collected samples together with those found in literature were combined with consumption data of nixtamalized maize products obtained from the literature to obtain estimated daily exposure (EDI) values. To assess the risk of these exposure levels, the Margin of exposure (MOE) approach as proposed by EFSA for compounds that are both genotoxic and carcinogenic [31] and a quantitative liver cancer risk approach proposed by JECFA [3] were used.

## 2. Material and methods

### 2.1. Determination of AFB1 in nixtamalized maize

#### 2.1.1. Chemicals and reagents

AFB1 (> 98% purity) was purchased from Sigma-Aldrich (Zwijdrecht, Netherland). Acetonitrile (ACN, ULC/MS grade) was purchased from Biosolve (Valkenswaard, Netherlands), and formic acid (FA, > 99-100%, AnalaR NORMAPUR grade), anhydrous $MgSO_4$ and acetic acid were bought from VWR International (Darmstadt, Germany). Dimethyl sulfoxide (DMSO, > 99.9%) was obtained from Acros Organics (Geel, Belgium).

#### 2.1.2. Collection of maize samples and storage conditions

Eighty-eight nixtamalized maize samples were collected from different areas in Mexico City. The sample collection took place during April 2017 and May 2019, considered a dry season period. The maize samples were either obtained from supermarkets as packaged flour or as fresh dough from local dough millers and *tortillerías* (tortilla shops). A total of 22 flour packages and 66 fresh dough samples were collected. An overview of the samples collected is provided in the supplementary material (Table S1). To keep and store the dough, the samples were vacuum-sealed packed, and were frozen in a freezer until transport to The Netherlands. During the transport, the samples were placed inside cooler bags containing gel packs to keep them frozen. Once in The Netherlands, the samples were kept at -80°C until freeze-drying and subsequent grinding with a mortar and pestle. All dry samples were kept closed and dry at room temperature (18°C).

#### 2.1.3. Extraction procedure

Aflatoxin B1 extraction was based on the multi-targeted method based on QuEChERs extraction described by Lopez et al. [32] with minor modifications. Briefly, 2.5 g dry sample were mixed with 7.5 ml ultra-pure water (Arium pro, Sartorius, Göttingen, Germany). After manual shaking, 10 ml of extraction solvent (ACN with 1% (v/v) acetic acid) were added. The extraction process consisted of 30 min shaking in a platform shaker (Innova 2300, New Brunswick Scientific, Nijmegen, Netherlands). Afterwards, the sample was cleaned-up by vortexing with 4 g of anhydrous $MgSO_4$ for 1 min, followed by centrifugation at 1822 g for 10 min. Subsequently, 6 ml of the supernatant were further cleaned with a 15 ml dSPE tube (DisQuE 186008080, Waters, Dublin, Ireland), followed by centrifugation at 2200 g for 5 min. An aliquot of 500 μL was diluted to 1 ml with 50 μL extraction solvent and 450 μL water. After vortexing the sample, 400 μL were filtrated with a syringeless 0.45 μm filter (Whatman Mini-UniPrep, GE, Buckinghamshire, United Kingdom) before LC–MS/MS analysis. The extractions were performed in triplicate, with the exception of six samples that were analysed in an additional independent fourth experiment for secondary confirmation of the analytical method used by Wageningen Food Safety Research (WFSR) (former RIKILT - Institute of Food Safety) (Wageningen, Netherlands) also using the multi-targeted method based on QuEChERs extraction described by Lopez et al. [32].

#### 2.1.4. Instrumentation and MS/MS conditions

AFB1 was quantified by triple quadruple LC–MS/MS using a Shimadzu LC/MS-8040 mass spectrometer operated in the positive electrospray ionization mode with MRM (multiple reaction monitoring). The mass spectrometer was coupled with a U(H)PLC system (Shimadzu Nexera XR LC-20AD XR). The LC analytical column used was a Kinetex C18 column (50 x 2.1 mm, 1.7 μM particle) (Phenomenex). The main MS parameters were set as follows: column temperature 40°C, desolvation temperature 400°C, source temperature 250°C, nebulation gas 2 L/min and drying gas flow 15 L/min. Elution was performed with a flow rate of 0.3 mL/min. The mobile phase consisted of a time-programmed gradient using nanopure water with 0.1% (v/v) FA (solvent A) and ACN with 0.1% (v/v) FA (solvent B). The percentage of solvent B was changed linearly as follows: 0 min, 10%; 1 min, 10%; 8 min, 100%; 10 min, 100%; then the percentage of solvent B dropped to 0% in 2 min, returned to the initial conditions (10% solvent B) in 2 min and was kept at these conditions for 4 min. The total run time was 16 min. The first minute of the U(H)PLC run was set to waste to wash away salt. The injection volume was 5 μL for the calibration curve and for the maize samples. The conditions for ESI were set at Nebuliser gas: 2.0 L/min, drying gas: 15.0 L/min, Desolvation Line temperature: 250°C, Heat Block temperature: 400 °C and Ion Spray voltage (IS): 4500 V. The MRM positive mode was performed under the following settings: precursor to product 313.05 → 285.10 (Q1 = -30V, Q3 = -30V, CE = 26kV), 313.05 → 241.05 (Q1 = -20V, Q3 = -26V, CE = 37kV), 313.05 → 269.05 (Q1 = -14V, Q3 = -26V, CE = 33kV). The retention time of AFB1 was 5.78 min. The quantification was carried out with a matrix matched calibration curve of AFB1 from 1.75 to 14 ng/g. The total area (TIC) of all three MRM of AFB1 was used and the linear coefficient of the calibration curve was $R^2 = 0.9980114$. The conditions for the samples analysed for secondary confirmation by WFSR were the same as the ones described for the multi-targeted method based on QuEChERs extraction by Lopez et al. [32].

#### 2.1.5. Extraction efficiency, limit of detection and limit of quantification

The efficiency of the method was assessed by spiking in triplicate, 5 ng/g AFB1 to a maize sample showing no detectable analyte (sample #7). The mean recovery obtained was 96.3 ± 3.0% hence the data were not adjusted for recovery. Precision was deemed adequate in terms of repeatability (r-RSD) and reproducibility within our laboratory (R-RSD) (Table 1). The limit of detection of the method (LOD) for AFB1 was 1 ng/g, equal to 3.3*Standard deviation of the residuals ($S_{y/x}$) divided by the slope (m), while the limit of quantification of the method (LOQ) corresponded to 3 ng/g, equal to 10*($S_{y/x}$/m).

**Table 1**
Recovery and precision of the analytical method for AFB1 in maize (n = 3).

| Spiked level (ng/g) | Recovery | r-RSD[a] % | R-RSD[b] % |
|---|---|---|---|
| 5 | 96.3% ± 3.0% | 3.0% | 3.6% |

[a] r-RSD: repeatability – Relative Standard Deviation.
[b] R-RSD: reproducibility within our laboratory – Relative Standard Deviation.

### 2.2. Estimated daily intakes (EDI) of AFB1

In the present study, the estimated daily intake (EDI) for AFB1 is obtained by multiplying the average concentration of AFB1 levels in the nixtamalized maize (ng/g) with the estimated daily consumption of maize from food products derived from nixtamalized maize flour and dough (g/day), divided by an average body weight (bw). Hence, the EDI is expressed in ng/kg bw/day. A conservative scenario was applied in which the thermal treatment (e.g. baking or frying) was assumed to not influence the AFB1 occurrence.

#### 2.2.1. Consumption data

The concentration levels used to determine the EDI were obtained from the occurrence data detected in this study. Additional EDI values were derived from AFB1 levels reported in the literature [21]. Meanwhile, the estimated daily consumption of maize was obtained from the study by Wall-Martínez et al. [17] who reported the food products that provide the largest amount of maize per portion in the population of Veracruz City in Mexico, which are products derived from nixtamalized maize flour and dough. Wall-Martínez et al. [17] reported that the largest average maize consumption per person per day (expressed as dry base in grams) is related to the following nixtamalized maize derived products: *tortillas* (118.4 g), *antojitos* (38.08 g), *tacos* (19.40 g) and *chilaquiles* (17.95 g). For the risk assessment, the sum of these four estimates, 193.8 g/person/day, was used as the estimated mean daily nixtamalized maize consumption. The 95$^{th}$ percentile (P95) was obtained likewise using the 95$^{th}$ percentile consumption estimates for the same food products: *tortillas* (290.1 g/day), *antojitos* (96.63 g/day), *tacos* (58.83 g/day) and *chilaquiles* (42.98 g/day), resulting in an overall P95 intake 488.5 g/person/day. Concerning the body weight, a Mexican adult was assumed to weight 70 kg, an average obtained by the average body weights of Mexican men (74 kg) and women (66.7 kg) [17]. For this study, the consumption of maize for an average Mexican person was assumed to be similar to that reported for the population of Veracruz City in Mexico.

#### 2.2.2. Management of non-detects in the average concentration of AFB1 levels

As non-detects (left censored data) could imply a true zero or a non-detect value, the non-detects were treated by a substitution method, following the options for managing left censored data by WHO/FAO [33]. The substitution method consisted of replacing the results below the analytical LOD by zero for a lower bound (LB) estimate, or by the numerical value of the LOD for an upper bound (UB) estimate. The EDI values thus obtained consisted of the average AFB1 occurrence values as LB-UB estimates for both the mean and P95 consumption of nixtamalized maize.

#### 2.2.3. Estimated daily intakes (EDI) comparison

To evaluate the EDI values obtained in this study, we compared them to EDI values we calculated using data from one other study found reporting the occurrence of AFB1 in a Mexican nixtamalized product (*tortillas*). The study of Castillo-Urueta et al. [21] reported the occurrence of aflatoxins, including AFB1, in 396 nixtamalized maize tortillas bought either from supermarkets or from traditional tortilla shops (Supplementary Table S4).

We also compared the EDI values obtained in this study to the mean international dietary exposure to AFB1 obtained by JECFA from the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets and the GEMS/Food contaminants database [3]. The GEMS/Food databases cluster countries with similar patterns of consumption to perform basic dietary exposure assessments [33,34]. Based on the GEMS/Food cluster diets, Mexico is assigned to the cluster G05 (Supplementary Figs. S1 and S2). Therefore, the following three dietary exposure scenarios were added to the exposure and risk characterization: i) the dietary exposure to AFB1 from maize for the cluster G05, ii) the total dietary exposure to AFB1 for the cluster G05 and iii) the international dietary exposure range to AFB1 [3]. When no separate high percentiles were reported they were considered to amount to twice the value of the mean dietary exposure [3]. To allow comparison all the estimates were recalculated for a body weight of 70 instead of 60 kg (Supplementary Table S5).

### 2.3. Risk characterization

Due to the genotoxicity and carcinogenicity of AFB1, the margin of exposure (MOE) approach proposed by EFSA [31] and also the quantitative liver cancer risk approach proposed by JECFA [35] were used to assess the risk of AFB1 exposure via nixtamalized maize in Mexico.

#### 2.3.1. Margin of exposure (MOE) approach

The MOE approach makes no implicit assumptions of a 'safe' intake, it indicates a level of concern and whether there is a priority for risk management [31]. The MOE is calculated as the ratio between a reference point from the dose-response curve which causes a low but measurable increase in tumor formation above a background level and the EDI. In EFSA's proposal the preferred reference point used for calculating the MOE is the benchmark dose lower confidence limit for a 10% extra incidence in tumor formation ($BMDL_{10}$). Thus, the MOE is defined as the ratio between the $BMDL_{10}$ and the EDI. When based on the $BMDL_{10}$ from an animal study, MOE values lower than 10,000 are considered of concern for public health and indicate a priority for risk management [31]. For calculation of the MOE values, we used the $BMDL_{10}$ values reported by EFSA for AFB1 [36], including the $BMDL_{10}$ derived from rat data [37] of 170 ng/kg bw/day, and the $BMDL_{10}$ of 870 ng/kg bw/day derived from epidemiological data [36].

#### 2.3.2. Quantitative liver cancer risk approach

In 1998, JECFA presented a formula to quantitatively estimate a population HCC risk associated with AFB1 exposure. The formula uses cancer potency estimates derived from a model with epidemiological data of individuals exposed to AFB1 testing positive for the hepatitis B surface antigen (HBsAg+) and testing negative for the hepatitis B surface antigen (HBsAg-) [35]. These estimates are expressed in terms of the increment in the incidence of HCC per 100,000 individuals per year from the exposure to AFB1 expressed in ng AFB1/kg bw/day. After a revision by JECFA in 2016, the cancer potency estimates were redefined by new central and upper bound estimates, and these are the values that were used in the present study (Table 2) [3]. The formula proposed by JECFA to quantitatively estimate the population cancer potency is as follows:

$$\textit{Population cancer potency}\ (\Sigma) = (\text{HBsAg-})\ (1 - p)\ +\ (\text{HBsAg+})\ (p) \quad (1)$$

Where $p$ is the proportion of HBsAg + individuals in the population. For this study, it was assumed that 0.2% of the population in Mexico is HBsAg+ (p = 0.002) [38].

To assess the AFB1-related HCC risk, the population cancer potency (Σ) is multiplied by the EDI expressed in:

$$\textit{Population cancer risk} = \textit{Population cancer potency}\ (\Sigma) * \text{EDI} \quad (2)$$

**Table 2**
Population cancer potency for the exposure of 1 ng/kg bw per day of AFB1 exposure per 100,000 individuals per year for the central and upper bound [3], and the Mexican population cancer potency (Σ) obtained by Formula 1 using p = 0.002.

| Bounds | Cancer Potency (JECFA, [3]) (*cases per 100,000 individuals/year for AFB1 exposure at 1 ng/kg bw/day)* | | Population cancer potency (Σ) in Mexico (*cases per 100,000 individuals/year for AFB1 exposure at 1 ng/kg bw/day)* |
|---|---|---|---|
| | *HBsAg –* | *HBsAg* $^{+}$ | *Σ* |
| Central | 0.017 | 0.269 | 0.018 |
| Upper bound | 0.049 | 0.562 | 0.050 |

The formula considers the estimated number of liver cancer cases per 100,000 *per year*, while in risk assessment of lifetime exposure to genotoxic carcinogens in the diet, cancer risks are often expressed in extra cases per million individuals upon lifetime exposure. To enable this comparison population cancer risk values obtained were also multiplied by a lifetime exposure of 75 years, which is the mean life expectancy for Mexicans [39], and a factor of 10 to obtain a lifetime exposure risk per million. Thus, for comparison purposes, the cancer potency estimates are expressed in extra HCC cases both, per 100,000 individuals per year, and per million individuals for a lifetime of 75 years. Assessment of the quantitative cancer risk estimate was based on the indicative tolerable cancer risk for the general population of one in a million extra risk upon lifetime exposure [40]. Thus, the margin considered in this study to judge the quantitative cancer risk estimate is 1 cancer case/million individuals/75 years, equivalent to 0.00133 cases per 100,000 per year.

## 3. Results

### 3.1. AFB1 levels in collected maize samples

Four out of 88 samples (4.5%) were above the LOD for AFB1 (Table 3); sample #S1 and #S47 were between the LOD and LOQ, with detectable AFB1 values of 1 ± 0.4 and 2 ± 0.3 ng/g, respectively. The samples above the LOQ for AFB1, sample #3 and #11, contained levels that amounted in triplicate analysis to 7 ± 4 and 10 ± 7 ng/g, respectively (Supplementary Table S2). Further reanalysis by WFSR (former RIKILT) for six selected samples, including the two samples above the LOQ, confirmed our results, with values of 6 and 12 ng/g, respectively (Supplementary Table S3). Therefore, the average AFB1 concentration considered in this study, and shown in Table 3, came from the quadruple analysis of the samples above the LOQ, amounting to 7 ± 3 ng/g and 11 ± 5 ng/g, respectively. Table 3 also presents the average AFB1 occurrence data in *tortillas* from Castillo-Urueta et al. [21]; comparison of the two data sets, the one obtained in this study and the one from Castillo-Urueta et al. [21] reveals that both provide comparable results.

**Table 4**
Mean and P95 estimated daily intakes (EDI) to AFB1 from nixtamalized maize and from the GEMS/Food cluster diets and contaminants database, expressed in ng/kg bw/day.

| Scenario | EDI (ng/kg bw/day) | | | |
|---|---|---|---|---|
| | Mean | | P95 | |
| | LB | UB | LB | UB |
| Present study[(a)] | 0.7 | 1.7 | 3.3 | 8.3 |
| Castillo-Urueta et al. [21][(a)] | 3.4 | 8.5 | 4.6 | 11.7 |
| Maize, cluster G05 [3] [(b),(c)] | 1.1 | 1.5 | 2.2 | 2.9 |
| Cluster G05 [3] | 2.5 | 5.0 | 5.0 | 9.9 |
| International [3] | 0.2 | 12.0 | 0.3 | 23.1 |

LB: Lower Bound; UB: Upper bound.
[(a)] EDI obtained from AFB1 concentrations in the samples (LB, UB) for both datasets (Table 3) combined with mean (193.8 g/day) and P95 (488.5 g/day) consumption data.
[(b)] P95 considered twice the value of the mean EDI for the LB and UB.
[(c)] The high percentile corresponds to P90 instead of P95 [3].

### 3.2. Exposure assessment

Table 4 presents the estimated daily intakes (EDI) obtained for a person of 70 kg bw when the mean and P95 consumption of nixtamalized maize, 193.8 and 488.5 g/day, respectively, are combined with the average concentrations of AFB1 in the samples (LB, UB) (Table 3). Table 4 also presents the estimates for AFB1 intake by the GEMS/Food cluster diets considering the contribution of maize alone in the cluster G05, the total AFB1 intake from all source for cluster G05, and the international dietary exposure range to AFB1 [3].

### 3.3. Risk characterization

#### 3.3.1. Margin of exposure (MOE)

The MOE values obtained using the EDI values presented in Table 3 and the $BMDL_{10}$ derived from the rat or the human data are shown in Table 5. Considering that the $BMDL_{10}$ derived from human data (870 ng/kg bw/day) is higher than the one derived from rat data (170 ng/kg bw/day) [36], the MOE values calculated from the human $BMDL_{10}$ are 5 fold higher than the values obtained using the rat $BMDL_{10}$ value (Table 5). All MOE values based on the rat $BMDL_{10}$ are substantially lower than the cut off value of 10,000 proposed for evaluation of the exposure when using a $BMDL_{10}$ from rodent studies [36].

#### 3.3.2. Estimated liver cancer risks

The liver cancer incidence estimates obtained by the quantitative cancer risk approach expressed in extra cases per 100,000 individuals per year are displayed in Table 6, while the extra cases expressed per million individuals upon lifetime exposure of 75 years are shown in Table 7. The values thus obtained reveal that for all exposure scenarios the estimated cancer risk exceeds the virtual safe value of one in a million upon lifetime exposure (Table 7) equivalent to 0.00133 cases

**Table 3**
Overview of the AFB1 occurrence data used in this Risk Assessment.

| Sample | N | LOD ng/g | LOQ ng/g | Samples > LOD (%) | Concentration (ng/g) | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | | LB[(a)] | UB[(b)] | |
| nixtamalized maize dough and flour | 88 | 1.0 | 3.0 | 4 (5%) | 0.2 ± 1 | 1.2 ± 1 | Present study |
| nixtamalized maize tortilla | 396 | 0.5 | 1.4 | 44 (11%)[(c)] | 1.2 ± 9 | 1.7 ± 9 | Castillo-Urueta et al. [21] |

LB: Lower Bound; UB: Upper bound.
[(a)] Samples below LOD set to zero in LB.
[(b)] Samples below LOD set to the numerical LOD value.
[(c)] The report from Castillo-Urueta et al. [21] indicates 39 positive samples with AFB1 values above LOQ, and 5 samples between the LOD and LOQ.

**Table 5**
Overview of the Margin of exposure (MOE) obtained using the EDI values from Table 4 and the rat $BMDL_{10}$ or human $BMDL_{10}$ values reported by EFSA [36].

| Scenario | MOE for animal $BMDL_{10}$[a] | | | | MOE for human $BMDL_{10}$[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | | P95 | | Mean | | P95 | |
| | LB | UB | LB | UB | LB | UB | LB | UB |
| Present study | 257 | 102 | 51 | 20 | 1317 | 522 | 263 | 104 |
| Castillo-Urueta et al. [21] | 50 | 20 | 37 | 15 | 257 | 102 | 187 | 74 |
| Maize, cluster G05 [3] | 153 | 117 | 76 | 58 | 781 | 597 | 390 | 299 |
| Cluster G05 [3] | 68 | 34 | 34 | 17 | 350 | 175 | 175 | 88 |
| International [3] | 992 | 14 | 496 | 7 | 5075 | 73 | 2538 | 38 |

LB: Lower Bound; UB: Upper bound.
[a] Rodent $BMDL_{10}$ of 170 ng/kg bw/day divided by mean and P95 estimated daily intakes (EDI) from Table 4.
[b] Human $BMDL_{10}$ of 870 ng/kg bw/ day divided by mean and P95 estimated daily intakes (EDI) from Table 4.

per 100,000 per year (Table 6).

## 4. Discussion

Mycotoxins are known for not being evenly distributed in food posing difficulties in determining the true concentration in a sample [41]. In this study the variability was reflected in the standard deviation of the triplicates from the samples above the LOQ; variability that diminished by including a fourth replicate analysed by an independent laboratory. Even so, recent data on AFB1 occurrence for nixtamalized products in Mexico are scarce, while a related risk assessment has, to the best of our knowledge, not yet been performed. In this study, we present up-to-date occurrence data on AFB1 from 88 nixtamalized maize samples quantified using LC–MS/MS. The data thus obtained could be compared to those from only one other study found in literature reporting on AFB1 levels in nixtamalized maize products from Mexico determined by using a fluorescence detector [21]. In spite of the different methods used, the results obtained are comparable with only a limited number of detected samples and a large number of non-detects. By a substitution method, lower and upper bound EDI values were calculated for both the mean and P95 nixtamalized maize consumption in the Mexican population. The EDI values obtained in this study were also compared to the estimates for AFB1 exposure from the GEMS/food cluster diets. The estimates obtained in the present study are within the range of the estimates based on the GEMS/Food cluster diets considering the contribution of maize alone in the cluster G05 (mean: 2.5–5.0 ng/kg bw/day, P95: 5.0–9.9 ng/kg bw/day) and the international dietary exposure for AFB1 estimated by JECFA [3] (mean: 0.2–12.0 ng/kg bw/day, P95: 0.3–23.1 ng/kg bw/day).

Using the EDI values thus obtained, the present study also performed a risk assessment using both the MOE approach and an approach based on the calculated liver cancer risk [36,3]. The results obtained revealed that MOE values based both on the rat and human $BMDL_{10}$ were all below 10,000 pointing at a priority for risk management. This still holds when it is considered that when using a human $BMDL_{10}$ value the cut off value of 10,000 may theoretically be lowered to a cut-off value of 1,000 because the value of 10,000 includes a factor 10 for interspecies differences, which may be no longer relevant when using a human $BMDL_{10}$ [31,36]. However, even when comparing the MOE values obtained with the human $BMDL_{10}$ to a cut-off value of 1,000, almost all MOE values are still below this lower cut-off value and thus raise a concern. The second approach used to evaluate the risk of exposure to AFB1, revealed that the AFB1 induced extra cancer incidences were above an extra risk of one in a million upon life time exposure, also pointing out a health concern. Thus, both approaches indicated a concern for human health at the levels of AFB1 exposure resulting from nixtamalized maize products consumed in Mexico, indicating a priority for risk management.

It is of interest to note that this concern also holds for the LB EDI values, calculated by setting the AFB1 level in the high percentage of non-detects found in the studies at zero. Thus, the small percentage of positives already results in estimated intakes that raise a concern in a subsequent risk assessment. Yet, the level of AFB1 in the samples of the present study and in the majority of the samples in the study of Castillo-Urueta et al. [21] were below the regulatory limit of 12 ng/g established in Mexico for total aflatoxins in nixtamalized maize products. This indicates that the Mexican regulatory limit may still result in levels of intake that raise a concern due to the high consumption of nixtamalized maize products. This also hold for the regulatory limit of the United States for total aflatoxins of 20 ng/g for maize [24], and even for the stricter levels adopted in the European Union of 2 ng/g for AFB1 and of 4 ng/g for total aflatoxins in ready to eat maize [25]. Consuming maize at the levels reported for the Mexican population of 193.8 g at the mean and 488.5 g at the P95 with an AFB1 level of 2 ng/g, would result

**Table 6**
Overview of the yearly estimated liver cancer risk for a population of 100,000 individuals resulting from AFB1 exposure in Mexico.

| Scenario | Population cancer risk (HCC/100,000 individuals/ year) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | | | | P95 | | | |
| | Central[a] | | Upper[b] | | Central[a] | | Upper[b] | |
| | LB | UB | LB | UB | LB | UB | LB | UB |
| Present study | 0.012 | 0.029 | 0.033 | 0.083 | 0.058 | 0.146 | 0.165 | 0.417 |
| Castillo-Urueta et al. [21] | 0.059 | 0.149 | 0.169 | 0.426 | 0.081 | 0.205 | 0.232 | 0.585 |
| Maize, cluster G05 [3] | 0.020 | 0.039 | 0.056 | 0.111 | 0.026 | 0.051 | 0.073 | 0.146 |
| Cluster G05 [3] | 0.044 | 0.087 | 0.124 | 0.249 | 0.087 | 0.174 | 0.249 | 0.497 |
| International [3] | 0.003 | 0.210 | 0.009 | 0.600 | 0.006 | 0.405 | 0.017 | 1.158 |

LB: Lower Bound; UB: Upper bound.
[a] Cancer incidences from central based cancer potency estimates.
[b] Cancer incidences from upper based cancer potency estimates.

**Table 7**
Overview of the estimated liver cancer risk expressed per million individuals per lifetime of 75 years resulting from AFB1 exposure in Mexico.

| Scenario | Population cancer risk (HCC/1 × $10^6$ individuals/ 75 years) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | | | | P95 | | | |
| | Central[a] | | Upper[b] | | Central[a] | | Upper[b] | |
| | LB | UB | LB | UB | LB | UB | LB | UB |
| Present study | 9 | 22 | 25 | 62 | 43 | 109 | 124 | 312 |
| Castillo-Urueta et al. [21] | 44 | 112 | 127 | 320 | 61 | 154 | 174 | 439 |
| Maize, cluster G05 [3] | 15 | 29 | 42 | 84 | 19 | 38 | 55 | 109 |
| Cluster G05 [3] | 33 | 65 | 93 | 187 | 65 | 131 | 187 | 373 |
| International [3] | 2 | 158 | 8 | 450 | 5 | 304 | 13 | 868 |

LB: Lower Bound; UB: Upper bound.
[a] Cancer incidences from central based cancer potency estimates.
[b] Cancer incidences from upper based cancer potency estimates.

for a 70 kg adult in an EDI of 6 ng/kg bw/day at the mean and 14 ng/kg bw/day at the P95. These EDI values would result in MOE values of 31 and 12 and in an extra HCC incidence of 73 and 524 extra HCC cases per million in a lifetime. This indicates that current regulatory limits may still not be low enough to eliminate concerns. It also supports, given that AFB1 is an unavoidable food contaminant, the conclusion that for AFB1 one should apply the ALARA principle, keeping levels and thus exposure as low as reasonably achievable. This suggestion is in line with the conclusion provided in the EFSA opinion on AFB1 for almonds, hazelnuts and pistachios which presented EDI values for AFB1 exposure of the European population amounting to 0.35 to 1.93 ng/kg/bw [36]. They also concluded that to apply the ALARA principle it would be essential to reduce the number of highly contaminated foods reaching the market and the exposure from food sources other than almonds, hazelnuts and pistachios [36].

Our findings give a particular insight to current AFB1 levels in nixtamalized maize in Mexico, suggesting a low prevalence of contamination, and confirming a pattern previously reported by Castillo-Urueta et al. [21]. The low prevalence may well be due to the nixtamalization process, considered a control strategy to reduce the aflatoxin levels, and to the establishment of regulatory limits in the early nineties [8]. Nonetheless, nixtamalization reduction depends on the initial levels of contamination and the process conditions, which can vary among producers between 29.5–90% [42,43]. For instance, unprocessed maize for human consumption analysed in Mexico in 1991 and 1998, had a 68.3% prevalence of AFB1 with concentrations ranging from 5.03 to 465.31 ng/g, and a 33.1% prevalence of total aflatoxins ranging from 1 to 18 ng/g, respectively [44,45]. The occurrence of AFB1 in non-nixtamalized maize seem especially relevant for the consumption of non-nixtamalized maize products (e.g. boiled or roasted maize). Even though the mean and P95 consumption per day per person is substantially lower (2.8–11.2 g/day/person or 0.04-0.16 g/day for a 70 kg bw person) than the consumption of nixtamalized maize products, the Mexican regulatory limit for total aflatoxins in non-nixtamaized products is higher (20 ng/g) [17,23]. Considering a worst-case exposure scenario in which all non-nixtamalized maize consumed by a 70 kg person would contain AFB1 at the regulatory limit of 20 ng/g, would imply an additional dietary exposure of AFB1 from maize of 0.8–3.2 ng/kw bw/day. Comparison of these values to the EDI values obtained in our study for the intake of AFB1 from nixtamalized maize amounting to 0.7–8.5 ng/kg bw/day, based on a mean maize consumption and 3.3-11.7 ng/kg bw/day for the P95 maize consumption, indicates that AFB1 exposure via exposure to nixtamalized maize adds substantially to the AFB1 exposure and accompanying health risks.

It is also of interest to observe, that the results of this study reveal that the risks from AFB1 occurrence in nixtamalized maize mainly originate from the high consumption of nixtamalized maize products rather than from high levels of the mycotoxin in the nixtamalized maize or the level of HBsAg + prevalence within the Mexican population. It should be noted, that we assumed that the maize consumption estimates from the population of Veracruz City were similar to the population of Mexico City because Mexico has an historic consumption of nixtamalized maize, particularly in the centre and south of the country [18]. In addition, both places are urban areas with a similar age and gender distribution [46].

It can thus be suggested that the cancer estimates presented in Table 7, represent a percentage of the estimated HCC incidence in 2018 in Mexico for both sexes and all ages predicted from The Global Cancer Observatory (GLOBOCAN). The HCC estimates from GLOBOCAN amount to 7265 liver cancer cases per year for a population of 130,759,070 individuals [27], corresponding to 4167 liver cancer cases/million individuals/75 years (5.6 HCC/100,000 individuals per year). By using the cancer risk estimates here presented it is suggested that the AFB1 exposure via nixtamalized maize products might account for 0.2%–10.5% of the HCC cases in Mexico for the low-end to high-end estimates, respectively. These liver cancer risk estimates differ not much from those obtained by Liu and Wu [26] for maize consumption in Mexico of 166–1007 cases/109 million individuals/year, equivalent to 114–693 liver cases/million individuals/75 years and also to 0.15-0.92 HCC/100,000 individuals per year (Supplementary Table S6), which corresponds to 2.7–16.6% of the estimated HCC incidence of 2018. The deviation in the estimates comes mainly from the higher AFB1 occurrence range taken by Liu and Wu [26] of 2.7–17 ng/g.

Some additional aspects are worth noting. Prevailing seasons in Mexico City are wet and dry [47] and in the present study, samples were all collected in the dry season. It has been reported that contamination levels in storage and during transport of the grain from different harvest times in Mexico is variable, and changes from year to year depending on the weather conditions [48,49]. Moreover, no differences were reported in the contamination proportion in processed maize products such as tortillas collected in a dry (April) and a wet (November) period [21]. Therefore, the time of sample collection may not have influenced the data to a substantial extent. Furthermore, the effects of the thermal treatment such as cooking, baking or frying were not considered for the samples in this study. Although maize was estimated to represent the largest commodity contributing to the total AFB1 exposure in Mexico because of its high consumption, mainly as nixtamalized maize [50], other food sources can contribute to AFB1 exposure in the Mexican diet such as rice, peanuts and chili [51,3,52].

Lastly, we focused this assessment solely on AFB1 because of its high toxicity, and because it is the mycotoxin most frequently found in contaminated food with aflatoxins, yet the co-exposure to other mycotoxins, such as Fumonisins, may add to the risks for liver damage as well [3]. For instance, levels of total fumonisins in nixtamalized maize dough from Mexico have been reported to amount to a total mean of 885 ng/g [53]. Considering the mean and P95 maize consumption used

in our study (193.8 and 488.5 g/person/day) this would result in an estimated daily intake of 2.5–6.2 μg/kw bw/day, exceeding the available Provisional Maximum Tolerable Daily Intake (PMTDI) of fumonisins of 2 μg/kg bw/day set by JECFA [3].

Future risk assessments should consider AFB1 occurrence of other food sources consumed by the Mexican population as well as co-exposure to other mycotoxins. Altogether, the assessment reveals the need for continued risk management of AFB1 in Mexico.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

## Acknowledgements

This work is financed by the Mexican National Council for Science and Technology (CONACYT) via the scholarship granted to Ixchel Gilbert Sandoval (No. 570901). Special thanks to Theo de Rijk and Ed Boers from RIKILT for their suggestions with the extraction procedure, and to Hilda Sandoval Cabrera and Pedro Ramírez Godínez for their help with the collection of samples.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2019.10.008.

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