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Review

Presence of unreported carcinogens, Aflatoxins and their hydroxylated metabolites, in industrialized Oaxaca cheese from Mexico City

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ABSTRACT

Aflatoxins (AFs) are toxic secondary metabolites of the fungi *Aspergillus flavus, A. parasiticus* and *A. nomius*. The fungi produce these AFs in cereals, oilseeds and spices. AFs have damaging effects on all organisms, including humans, and their symptoms can be classified as acute (vomiting, hemorrhage and death) or chronic (immunodepression, Reye syndrome, Kwashiorkor, teratogenesis, hepatitis, cirrhosis, and various cancers). Basic AFs (AFB_1, AFB_2, AFG_1, and AFG_2) are metabolized in the liver or by microbes that produce hydroxylated metabolites (AFM_1, AFM_2, and AFP_1) and aflatoxicol (AFL), soluble in water and easy to dispose. Thus, AFs can be excreted in fluids, such as milk. AFs are not destroyed in the process of making cheese.

The purpose of this study was to identify and quantify the AFs present in 30 samples of industrialized Oaxaca-type cheese sold in Mexico City. The average concentrations of AFs detected in the 30 samples of industrialized cheese were as follows: AFB_1 (0.1 μg kg^{-1}) in 20% (6/30); a trace amount of AFB_2 (0.01 < LOD) in only 3% (1/30); AFG_1 (0.14 μg kg^{-1}) in 10% (3/30); AFG_2 (0.6 μg kg^{-1}) in 30% (9/30); AFM_1 (1.7 μg kg^{-1}) in 57% (17/30); AFP_1 (0.03% μg kg^{-1}) in 3% (1/30); and AFL (13.1 μg kg^{-1}) in 97% (29/30). AFB_1 and AFL were the most abundant aflatoxins in Oaxaca-type cheese. However, eight aflatoxins were present, contributing an average of 15.7 μg kg^{-1} AFs distributed among the 30 samples. The risk assessment analysis showed that there was no substantial risk for cancer due to AFs in industrialized Oaxaca cheese from Mexico City.

1. Introduction

Cheese is an economically important commodity worldwide. In Mexico, 76,696 tons of Oaxaca-type cheese were produced in 2005 (INEGI, 2006, 2008). Most of the information about aflatoxins (AFs) in cheese is related to industrial production and sale through formal commercialized channels. However, most of the Oaxaca-type cheese consumed in Mexico is handmade artisanally. The AFs and hydroxylated metabolites have been reported (Vargas-Ortiz et al., 2017) but not in industrialized cheese.

The cheese consumption by inhabitant in Mexico is 3.8 kg per year (CDIC, 2016) where the calculation includes non-consumers. In particular for Oaxaca cheese, the reported average consumption by inhabitant is 47.8 g per day and 17.448 kg per year (Hernández-Camarillo et al., 2016b) when only consumers are considered. Oaxaca cheese, also known as thread cheese, is a filament cheese paste type that is produced in artisanal or industrialized ways. In 2010, Oaxaca cheese was the second most ingested cheese in Mexico after fresh cheese (González-Córdova et al., 2016). Industrialized Oaxaca cheese is produced with pasteurized milk, which is acidified at 32–40 °C before the rennet is applied to obtain a stretched cheese curd. The fermented cheese curd (pH 5.2 to 5.3) (Torres and Chandan, 1981) is stretched, and the dough is then kneaded and submerged in hot water (80 °C) (Aguilar-Uscanga et al., 2006) until threads of 3–6 cm in width are formed. The filaments are then dried with salt and are molded as braided hair or balls followed by cooling and packaging into plastic bags (De Oca-Flores et al., 2009).

Approximately 35 years ago, thread cheeses originated from ranches where they were made with pure milk without any preservers and

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Abbreviations

%	Percentage
% CV	Variation coefficient percentage
< LOD	Below limit of detection
>LOD	Above limit of detection
° C	Centigrades
ACN	Acetonitrile
AFB_1	Aflatoxin B1
AFB_2	Aflatoxin B2
AFG_1	Aflatoxin G1
AFG_2	Aflatoxin G2
AFL	Aflatoxicol
AFM_1	Aflatoxin M1
AFM_2	Aflatoxin M2
AFP_1	Aflatoxin P1
AFs	Aflatoxins
b1	Slope value
b_1	Value of the slope
bo	Ordinate to origin
cm	Centimeters
Forms of AFL	A (Ro) and B
g (s)	Gram (s)
HPLC	High Performance Liquid chromatography
HPLC-FL	High Performance Liquid chromatography and Fluorescence
HBV	without Hepatitis B Virus
HBV +	with Hepatitis B Virus
IAC	Immunoaffinity Columns
IC(β)	Confidence interval for the slope to origin
LOD	Limits of detection
LOQ	Limits of quantification
LOQ	Limits of quantification
MeOH	Methanol
min	Minute
mL	Milliliter
MO	Missouri
Ng	Nanograms
Nm	Nanometers
NY	New York
OH−	Hydroxyl
PBS	Phosphate buffered saline
pH	Hydrogen potential
R^2	Determination coefficient
Rpm	Revolutions per minute
RT	Retention time
SD	Standard deviation
ST	Sterigmatocystin
$S_{y/x}$	Standard deviation of the regression
UK	United Kingdom or England
USD	United States dollars
UV	Ultraviolet
v/v	Volume to volume
WI	Wisconsin
$\mu g\ kg^{-1}$	Micrograms per kilogram
$\mu g\ L^{-1}$	Micrograms per liter
µL	Microliters

extenders. Currently, most of the cheeses produced in Mexico are standardized where the ingredients are not pure milk, and in some cases, liquid milk is not used to produce them (González-Córdova et al., 2016). In Mexico, industrial cheese production is concentrated into two industries that generate 50% of production and distribute their products with different trademarks. An additional 18% of production is generated by large industries in which the principal product is not cheese production, and the remaining production is distributed among medium and small industries dedicated to cheese production (Hervás, 2012). With regard to Oaxaca cheese produced by industry, 52 products have been reported with 47 presented as Oaxaca cheese and 5 presented as Oaxaca cheese imitations (Profeco, 2012).

The base for the elaboration of Oaxaca cheese is liquid cow milk. However, the industrialization of the Oaxaca cheese production uses additives and stabilizers to reduce costs and to acquire some sensorial attributes.

Aflatoxins (AFs) are toxic secondary metabolites, such as bis-dihydrofuran coumarins, produced mainly by the fungi *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, which can contaminate field crops, such as maize that is used as cattle fodder or as an ingredient of balanced feed. These fungi produce aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) (Creppy, 2002).

AFB_1 is a recognized mutagen and carcinogen in humans (IARC, 2002) that can produce other damaging effects, such as immunodepression, hepatitis, cirrhosis, abortions, and fetus malformations. These basic AFs are not soluble in water, and the livers of ruminants can metabolize them to reduce their toxicity by adding an OH− group to form hydroxylated metabolites, such as Aflatoxins M_1 (AFM_1), M_2 (AFM_2), P_1 (AFP_1) and aflatoxicol (AFL), which are water soluble and can be excreted from the body through milk (Hayes et al., 1977). Therefore, the milk used to produce dairy products, such as Oaxaca cheese, can contain the four basic aflatoxins and their mentioned hydroxylated metabolites, Fig. 1.

Moreover, maize flour can be added to the dough to thicken and curdle it, and this practice, although legal in Mexico (NOM, 2010), can add extra AFB_1 to cheese. Industrialized Oaxaca cheeses are produced in many Mexican States and are distributed throughout the country, resulting in the entire population being exposed to the AFs in these cheeses. The present research evaluated the presence of the four basic AFs and their four hydroxylated metabolites in 30 trademark industrialized Oaxaca cheeses of Mexico.

2. Materials and methods

2.1. Sampling

The study consisted of 30 samples from 30 trademarks of 750 g of Oaxaca-type industrialized cheese purchased in groceries and markets in Mexico City.

The cheeses were refrigerated immediately after sampling and were subjected to a drying process for a period of 2 days. Samples of Oaxaca-type cheeses were purchased in September 2016. Each cheese sample was manually unthreaded, and they were placed in a tray drier at 40 °C so the AFs were not affected. The dried samples were stored frozen until AF extraction and chemical analyses were performed.

2.2. Chemical extraction method for aflatoxins

The R-Biopharm (R-Biopharm Rhone Ltd., 2012) method, which is recommended for use with Total Aflatoxin Easi-Extract Immunoaffinity Columns (IAC) (R-Biopharm Rhone Ltd, Glasgow, Scotland, UK), was performed according to the following protocol.

Samples (15 g) of dry, ground Oaxaca-type cheese were blended (Waring ETL laboratory blender 7010S model WF 2211214, Torrington, CT, USA) for 2 min at high speed with a mixture of 100 mL of MeOH/water (80:20 v/v) and 2 g of NaCl to clarify the extract. The mixture was centrifuged at 4500 rpm for 15 min, and an equivalent of 1 g of supernatant of sample was dissolved in phosphate buffered saline (PBS)

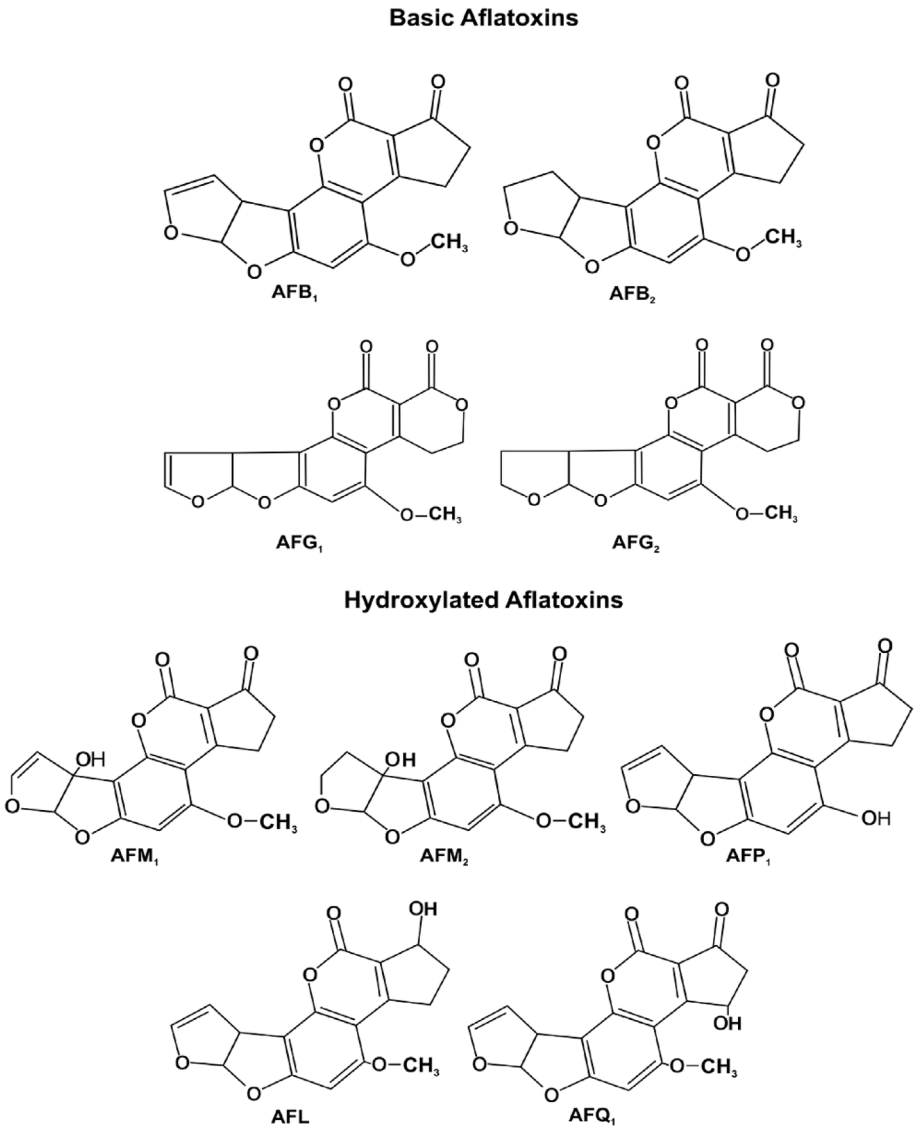

Fig. 1. Chemical structure of basic aflatoxins and their hydroxylated metabolites.

at pH 7.4 at a proportion of 1:4 (v/v) and homogenized for 1 min in a vortex. IAC was equilibrated with 20 mL of PBS at pH 7.4 applied at a flux of 5 mL/min. Each sample was then independently added to a new column. AFs were eluted using 1.5 mL of HPLC-grade MeOH followed by 1.5 mL of distilled water with reflux. The eluate was dried at 40 °C in an oven (F135A Novatech Model, Mexico City, Mexico) and then derivatized.

2.3. Derivatization

Derivatization is a process to increase the AF fluorescence of AF standards to generate calibration curves and to quantify AFs in cheese samples (Kok, 1994; Akiyama et al., 2001). The derivatization reaction with trifluoroacetic acid is the transformation of AFB_1 and AFG_1, which are less fluorescent, into their hemiacetals B_{2a} and AFG_{2a}, which are highly fluorescent. AFB_2 and AFG_2 are not affected by this reaction due to their saturated structure (Akiyama et al., 2001). Eight dry AF standards, namely AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFP_1 and AFL (Sigma-Aldrich; St. Louis, MO, USA), were used to determine the AFs' linearity and percentage of recovery validation. The AF standards were dissolved in 200 µL of HPLC-grade acetonitrile (ACN), and 800 µL of derivatization solution was them added. The derivation solution was prepared with 5 mL of trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA), 2.5 mL of glacial acetic acid (Merck, Naucalpan, Estado de Mexico, Mexico) and 17.5 mL of deionized distilled water, and the mixture was then vortexed (Vortex G-560, Bohemia, NY, USA) for 30 s. The vials containing the dry eluates were heated in a vapor bath at 65 °C for 10 min. The derivatized samples were cooled to room temperature, and triplicate 60 µL samples were analyzed by HPLC with fluorescence (HPLC-FL).

2.4. Validation of the extraction method

The validation of the analytical methods and the analyses of the 30 Oaxaca-type cheese samples were performed using known parameters (García et al., 2002).

2.5. Linearity of the system (calibration curves)

Solutions with different concentrations of AFs were prepared from a 1000 ng AFM stock. The 0.25 mg AFM standards were diluted with benzene: acetonitrile (98:2 v/v) according to a previously reported methodology (AOAC, 2006) to prevent decomposition of pure AFs.

a. The spectrophotometer (Genesys 10 UV Thermo Electron Corporation; Madison, WI, USA) was calibrated before the

experiments to measure the absorbance of the AFM standard solutions from 357 to 360 nm.

b. The following formula was applied to calculate 1000 ng stock solutions of each AF concentration (AOAC, 2006):

$$\text{AF } (\mu g\ mL^{-1}) = \frac{\text{Absorbance} \times \text{molecular weight} \times 1000 \times \text{Correction factor of the equipment}}{\text{Extinction coefficient}}$$

c. Twelve concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng) of the 8 different AFs were independently created from the 1000 ng stock solution. These standard dilutions were then used to plot the analytic signal (area below the curve of each chromatographic peak) against the AF concentrations. The curve equation and statistical parameters were obtained. The slope value (b1), ordinate to origin (bo), determination coefficient (R^2), confidence interval for the slope to origin (IC(β)), variation coefficient percentage (% CV), standard deviation (SD), and the limits of detection (LOD) and of quantification (LOQ) and were calculated using Excel 2003.

2.6. Limits of detection (LOD) and quantification (LOQ)

The LOD of the equipment was established in relation to the noise in the chromatogram. The LOD equals the AFM_1 concentration that gives a signal that is three times greater than the noise. The LOQ equals the AFM_1 concentration that is 10 times greater than the noise (Cruz-Rueda, 2016).

To calculate the LOD, the following equation was used:

$$\text{LOD} = \frac{3.3 \times S(y/x)}{b_1}$$

The LOQ was calculated using the following equation:

$$\text{LOQ} = \frac{10 \times S(y/x)}{b_1}$$

where $S_{y/x}$ is the standard deviation of the regression, and b_1 is the value of the slope (Cruz-Rueda, 2016).

2.7. Recovery percentages

The recovery percentage is a measure of the accuracy of the method and expresses the proximity between the theoretical and experimental values. The arithmetic average, standard deviation, percentage of variation coefficient and confidence interval were calculated. To obtain accurate measurements, the AFs of the samples of dried, ground Oaxaca-type cheese in 1 g aliquots diluted in PBS (1:4 v/v) were individually spiked with three different concentrations (5, 20 and 40 μg kg^{-1}) of the eight individual AF standards (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, and AFP_1) and AFL. One aliquot without spiked AF was used as the control, which gave the basal contamination level. The samples were individually processed using the R-Biopharm extraction method (R-Biopharm Rhone Ltd., 2012). AFs were purified and concentrated using an IAC, derivatized, and quantified by HPLC-FL, and the percentage of recovery for each AF was obtained. After the derivatization mixture was cooled to room temperature, triplicates of each sample (60 μL) were injected into the HPLC-FL.

2.8. HPLC quantitation

The chromatographic system was an Agilent Series 1200 HPLC (Agilent Technologies, Inc., USA) and consisted of an isocratic pump (Model G1310A), a fluorescence detector (Model G1310A Series DE62957044, Agilent Technologies, Inc., USA) set to an excitation wavelength from 357 to 360 nm and an emission maximum of 450 nm, and an autosampler (G1329A Series DE64761666). The chromatography column was a VDS Optilab VDSpher 100 C18–E 5 μm 250 × 4.6 mm maintained at room temperature (22 °C) with a mobile phase of water:ACN:methanol (65:15:20 v/v/v) that was degasified for 30 min by vacuum filtration and added at a flux of 1.0 mL/min.

2.9. Statistical analysis

Kruskal-Wallis analysis was performed to identify differences in the concentrations of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFP_1 and AFL among the 30 samples. We performed Pairwise Wilcoxon Rank Sum T test to identify where the differences occurred.

2.10. Risk assessment

Risk assessment include 4 stages:

1) Danger identification of the biological, chemical or physical agents that can damage human health and that are present in some food (WHO/FAO, 2006).
2) Characterization of the danger: the qualitative or quantitative evaluation of the damage to health caused by biological agents in food (WHO/FAO, 2006). Evaluations are realized with dose-response studies. Epidemiological studies gave enough evidence of the synergism between the hepatitis B virus (HBV) and AFB_1 to cause liver carcinogenesis. The calculations were on the potency of AFB_1 to cause liver cancer. WHO established the potency of AFB_1 to cause liver cancer was 0.013 in non Hepatitis B Virus (HBV^-) population and 0.328 in HBV + population in 100,000 inhabitants per year for each ng/kg exposition (WHO, 1998).
3) Exposition evaluation: is the qualitative or quantitative evaluation of the ingestion of biological, chemical or physical agents through food (WHO/FAO, 2006).
4) Risk characterization: Is the estimation of the occurrence probability and seriousness of the adverse effects on health in a certain population, in relation to the danger identification, characterization and exposure (WHO/FAO, 2006).

The exposure to a damaging agent to health through food ingestion, depends on the contamination levels and the quantity of food ingested. In our case, the exposure to AFB_1 through the ingestion of industrialized Oaxaca cheese, is calculated multiplying the sample contamination with the daily consumption, and the result is divided by the body weight. The equation is:

Exposure: (Contamination level) (ingested amount)/Body weight.

The unities of exposure are (ng kg^{-1} Body weight/per day) (Shephard, 2008).

In 2016 a report informed that the consumption per capita of cheese in Mexico was of 3.9 kg/year (CDIC, 2018), that would be 10.68 g daily. In Mexico, the National Chamber of Dress Industry (CANAIVE) reports an average weight of 74.8 kg for men and 68.7 kg for women in a sample of 5000 inhabitants (CANAIVE, 2012). An average weight from these data is 71.75 kg for adult population.

Population risk can be calculated multiplying exposure by average potency of HBV^+ and HBV^-; for populations of developing countries where a prevalence of HBV^+, is 25% (WHO, 1998). The risk can be calculated with the equation:

Population risk = Exposure x Average potency

For the risk calculations for Aflatoxicol (AFL) we used the same potency values than those applied for AFB_1, because AFL can transform itself into AFB_1 and viceversa. For AFM_1 the methodology of the mention potencies referred by WHO was used, being 0.03 for HBV^+ and 0.001 for HBV^- (JECFA, 2001).

3. Results and discussion

The validation parameters, including limit of detection (LOD), retention time (RT), coefficient of determination R^2 and recovery percentage, of aflatoxins and hydroxylated metabolites in Industrialized Oaxaca cheese are shown in Table 1.

AFB_1 was not a product of cow liver metabolism but was due to the addition of maize starch during the manufacturing of the cheese. This addition is legal (NOM, 2010) in Mexico and accepted for fresh cheeses, but it is not good practice from the point of view of AF contamination.

The average amount of AFB_1, the most carcinogenic AF, was high in artisanal Oaxaca cheese (11.2 ng) (Vargas et al., 2017) but low in industrialized Oaxaca cheese (0.1 μg kg^{-1}), maybe from the transformation of AFB_1 into AFL, which is the interconverting hydroxylated metabolite form (Karabulut et al., 2014), leaving only traces of AFB_1. The formation of AFL was high in both artisanal (19.1 μg kg^{-1}) and industrialized Oaxaca cheese (13.1 μg kg^{-1}). AFt of artisanal and industrialized cheese had a difference of 18.2 μg kg^{-1} (Vargas-Ortiz et al., 2017). The aflatoxins (μg kg^{-1}) and hydroxylated metabolites (μg kg^{-1}) in the industrialized Oaxaca cheese of Mexico City are shown in Table 2.

The different amount of AFs in both processes, the artisanal and industrialized Oaxaca cheeses are shown in Table 3.

Considering several aflatoxin biosynthetic pathways (Sweeney and Dobson, 1999; Trail et al., 1995; Minto and Towsend, 1997; Bennett et al., 1997), norsolorinic acid is the first stable precursor in the pathways. The polyketide undergoes approximately 12–17 enzymatic conversions through a series of pathway intermediates. Following the formation of versicolorin B, the pathway branches. One branch forms AFB_1 and AFG_1, which contain dihydrobisfuran rings and are produced from demethyl sterigmatocystin, and the other branch forms AFB_2 and AFG_2, which contain tetrabisfuran rings and are produced from dihydro-demethyl-sterigmatocystin. The step from vesiconal to versicolorin B and A as well as the transformation of versicolorin to AFG_1 and AFG_2 might be where the industrial cheese process changes from the artisanal process because the artisanal Oaxaca cheese (Vargas et al., 2017) had less AFG_1 (0.03 μg kg^{-1}) and AFG_2 (0.2 μg kg^{-1}) than the industrial cheese (AFG_1, 0.14 μg kg^{-1}; and AFG_2, 0.6 μg kg^{-1}), Table 3.

While AFs are produced only by certain strains of *A. parasiticus, A. flavus* and *A. nomius*, numerous ascomycetes and deuteromycetes, including *A. nidulans,* produce the sterigmatocystin (ST) mycotoxin, which is the penultimate intermediate in the AF biosynthetic pathway. The ST pathway is believed to include at least 15 enzymatic activities involving each enzyme activity from the AF pathway and the penultimate steps involving the conversion of ST to AF. AFs bind to proteins and, in the case of cheese, to the milk casein via a hydrophobic interaction (Brackett and Marth, 1982), resulting in the presence of AFM_1 in cheese. AFs resist high temperature without denaturalization, and AFM_1 can withstand temperatures up to 320 °C, indicating that it can withstand pasteurization (60 °C) and ultrapasteurization (160 °C) with acidification, which are used during the processing of cheeses (Deveci, 2007; Oruc et al., 2006).

The present study showed the presence of the eight different AFs in addition to AFM_1, the only reported AF in dairy products worldwide. Nonetheless, other AFs certainly contribute to the real ingested amount and to the risk associated with AFs. The established regulations for the maximum tolerable levels of AFs in milk and dairy products have been established (El Khoury et al., 2011). AFM_1, AFM_2, AFB_1 and AFL have been reported in Mexican milk (Carvajal et al., 2003a,b), so their presence in cheese was expected. The purpose of this research was to investigate different basic AFs (AFB_1, AFB_2, AFG_1, AFG_2) and their hydroxylated metabolites (AFM_1, AFM_2, AFP_1 and AFL), which have not been reported in industrialized cheese, and to identify their importance as carcinogens.

3.1. Quantification of all AFs

The averages of the AFs and hydroxylated AFs detected in the 30 samples of trademarks of industrialized cheese were as follows: AFB_1 (0.1 ng g^{-1}) in 20% (6/30); a trace amount of AFB_2 (0.01 < LOD) in only 3% (1/30); AFG_1 (0.14 ng g^{-1}) in 10% (3/30); AFG_2 (0.6 ng g^{-1}) in 30% (9/30); AFM_1 (1.7 ng g^{-1}) in 57% (17/30); AFP_1 (0.03% ng g^{-1}) in 3% (1/30) and AFL (13.1 ng g^{-1}) in 97% (29/30). AFB_1 and AFL were the most abundant aflatoxins in Oaxaca-type cheese. However, eight aflatoxins were present, contributing to an average of 15.7 μg kg^{-1} AFs distributed among the 30 samples (Fig. 2 and Table 2). All AFs were present in the industrialized cheeses (Fig. 3).

According to the Tukey's Test analysis ($P < 0.05$), AFB_1 and AFL were the most abundant AFs. Other AFs, such as AFB_2 (0.01 μg kg^{-1}) and AFP_1 (0.03 μg kg^{-1}) were present in trace amounts, and AFG_2 (0.6 μg kg^{-1}) appeared more frequently in 30% (9/30) of the samples. AFL (13.1 μg kg^{-1}), which was present in 97% of the samples, can be formed through the enzymatic or synthetic reduction of AFB_1, and it has high toxicity and carcinogenicity (Karabulut et al., 2014).

AFL was the most abundant hydroxylated metabolite in the artisanal and industrialized cheeses. AFL is produced by several fungi, including *Aspergillus flavus*, *A. parasiticus, A. niger*, *Eurotium herbariorum*, *Rhizopus* spp. and other non-aflatoxicogenic *A. flavus* (Nakazato et al., 1991), by the reduction of the 1-keto group of AFB_1 (Detroy and Hesseltine, 1970). AFL is equally carcinogenic as AFB_1, so its formation is not a significant detoxification mechanism (Schoenhard et al., 1981; Peraica et al., 1999). AFL has approximately 70% less mutagenicity than AFB_1 (Coulombe et al., 1982), and its structure has two forms, A (Ro) and B, both of which are produced from the biological reduction of AFB_1. AFL A is 18 times less toxic than AFB_1 in the duckling biliary hyperplasia assay, and the biological activity of AFL B is unknown (Schoenhard et al., 1981; Peraica et al., 1999). AFL is the major metabolite of AFB_1 in many plants and animals, and it has been detected in milk (Carvajal et al., 2003b; Detroy and Hesseltine, 1968; Cole and Cox, 1981; Hsieh, 1983), fermented dairy products (Megalla and Mohran, 1984), cereals and nuts (Saito et al., 1984), eggs (Trucksess et al., 1983), blood (Wong and Hsieh, 1978a,b; Kumagai et al., 1983), human brain (Oyelami et al., 1995), the sera and liver of humans with kwashiorkor and marasmic kwashiorkor in Ghana and Nigeria (Apeagyei et al., 1986; Hendrickse et al., 1989; De Vries et al., 1990; Oyelami et al., 1998), human urine (Lovelace et al., 1982), urine of heroin addicts (Hendrickse et al., 1989), a breast-fed infant with neonatal hepatitis (Coulter et al., 1986), the muscle of broiler chickens fed with contaminated diets (Fernández et al., 1994), and poultry fed chronic low doses of mycotoxins with the liver having the highest levels (Micco et al., 1988). AFB_1 and the AFM_1 and AFL hydroxylated metabolites accumulate in the tissues and urine of calves (Van der Linde et al.,

Table 1
Validation parameters, including limit of detection (LOD), retention time, coefficient of determination R^2 and recovery percentage, of aflatoxins and hydroxylates in industrialized Oaxaca cheese.

Aflatoxin	LOD (ng g^{-1})	Linearity (Calibration Curves)		Recovery percentage
		Retention time (minutes)	R^2	
AFB_1	0.01	7.085–8.849	0.9986	97%
AFB_2	0.02	17.452–20.228	0.9988	95%
AFG_1	0.05	5.722–5.876	0.9626	93%
AFG_2	0.05	11.215–14.513	0.9946	96%
AFM_1	0.01	8.514–9.549	0.9834	95%
AFM_2	0.05	20.208–22.447	0.9946	97%
AFP_1	0.05	15.563–19.318	0.9960	95%
AFL	0.01	3.032–5.569	0.9978	98%

LOD = Limit of detection; R^2 = Coefficient of determination.

Table 2
Averages of triplicate counts of reported and unreported aflatoxins in industrial Oaxaca-type cheeses of Mexico City.

Oaxaca cheese trademark samples	Basic Aflatoxins				Hydroxylated aflatoxins				AFt
	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	AFP_1	AFL	
1	0	0	2.6	2.3	5.0	0	0	0	9.9
2	0	0	0	0	0	0	0	7.2	7.2
3	0	0	0	0	0	0	0	6.3	6.3
4	0	0	0	0	0	0	0	6.2	6.2
5	0.04	0	0	0	0	0	0	8.8	8.8
6	0	0	0	0	2.8	0	0	11.2	14.0
7	0	0.3	0	2.5	3.7	0	0	13.9	20.4
8	0.3	0	0	0	3.5	0	0	11.8	15.6
9	0	0	0	3.1	3.8	0	0	19.1	26.0
10	0	0	0	0.9	4.4	0	0	21.5	26.8
11	0	0	0	0	3.8	0	0	25.5	29.3
12	0	0	0	4.1	1.3	0	0	18.9	24.3
13	0.5	0	0	0.8	1.4	0	0	16.2	18.9
14	0	0	0.03	0	0	0	0	8.6	8.6
15	0.3	0	0.1	0	1.4	0	0	11.2	13.0
16	0	0	0	0	0	0	0	5.7	5.7
17	0	0	0	0	0	0	0	3.2	3.2
18	0	0	0	0	3.9	0	0	22.0	25.9
19	0.1	0	0	0.9	1.2	0	0.8	16.2	19.2
20	0	0	0	2.6	0	0	0	21.2	23.8
21	0	0	0	0.9	3.8	0	0	7.1	11.8
22	0.1	0	0	0	3.6	0	0	19.3	23.0
23	0	0	0	0	0	0	0	22.3	22.3
24	0	0	0	0	3.8	0	0	24.9	28.7
25	0	0	0	0	1.2	0	0	23.2	24.4
26	0	0	0	0	3.6	0	0	5.1	8.7
27	0	0	0	0	0	0	0	6.5	6.5
28	0	0	0	0	0	0	0	8.8	8.8
29	0	0	0	0	0	0	0	9.5	9.5
30	0	0	0	0	0	0	0	11.0	11.0
Aflatoxin average	**0.1**	**0.01**	**0.14**	**0.6**	**1.7**	**0**	**0.03**	**13.1**	**15.6**

AFt = Total Aflatoxins.

1964; Sabino et al., 1995). AFL–DNA adducts produced *in vivo* are identical to those produced by AFB_1 and have similar molecular dosimetry responses and toxicity to the target organ (Bailey et al., 1998).

AFL is a more potent toxin than AFM_1, which can reconvert with AFM_1, becoming AFL M_1 (Bailey et al., 1994). AFL-induced hepatocellular carcinomas in rats and fish have a lower tumor incidence than those induced by AFB_1 (IARC, 1997). There is an interconversion of AFB_1 and AFL mediated by intracellular enzymes in rat blood (Chang et al., 1985), guinea pigs (Liu et al., 1993) and sharks, which reconvert 30% of AFL to AFB_1 (Troxel et al., 1997a,b; Bodine et al., 1989), as well as cultured human epidermal cells (Walsh et al., 1992). AFL converts into AFB_1, which is the most carcinogenic and toxic of all AFs (Nakazato et al., 1990). AFL is oxidized readily back to AFB_1, so it can serve as a 'reservoir' for AFB_1 *in vivo*, thereby prolonging the effective lifetime in the body (Wong and Hsieh, 1980a,b). If pH has a role in the AFB_1–AFL interconversion, it could act in the normal human digestion of milk where pepsin lowers the pH. The genotoxicity of AFM_1 has been demonstrated, and the carcinogenic potency of AFM_1 is 2–10% weaker than that of AFB_1 (FSCJ, 2013). AFM_1 contamination was the second highest with an average of $1.7\,\mu g\,kg^{-1}$ in 57% of Oaxaca-type industrialized cheese samples (Table 2), which was consistent with the results obtained for other types of cheese, such as cream cheese (Prandini et al., 2009), white pickled cheese (Oruc et al., 2006), sheep curd (Battacone et al., 2005), Grana Padano cheese (Manetta et al., 2009), parmesan (Pietri et al., 2016), Turkish kashar cheese (Tekinşen and Eken, 2008), and Serbian hard cheese (Škrbić et al., 2015). No AFM_2 contamination was found in the tested industrialized cheeses. There have been several studies (Chopra et al., 1999) on carryover from cows fed AFB_1-contaminated rations to AFM_1 in milk showing that the degree of toxicity and carcinogenicity of AFs is B1 > G1 > B2 > G2.

Table 3
Differences in Aflatoxins between the artisanal cheese from Veracruz and the industrialized Oaxaca cheeses from Mexico City.

Origin in Veracruz sample	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	AFP_1	AFL	AFt
Average of AFs in 30 artisanal Oaxaca cheeses in Veracruz[a]	11.2	0.02 < LOD	0.03	0.2	3.0	0.2	0.1	19.1	33.9
Average of AFs in 30 industrialized Oaxaca cheeses in Mexico City	0.1	0.01	0.1	0.6	1.7	**0**	0.03	13.1	15.7
Difference between artisanal and industrialized cheeses	**11.1**	**0.01 < LOD**	**- 0.07**	**- 0.4**	**1.3**	**0.2**	**0.07**	**6.0**	**18.2**

[b] AFt = Total Aflatoxins.
[a] Data from Vargas-Ortiz et al. (2017).

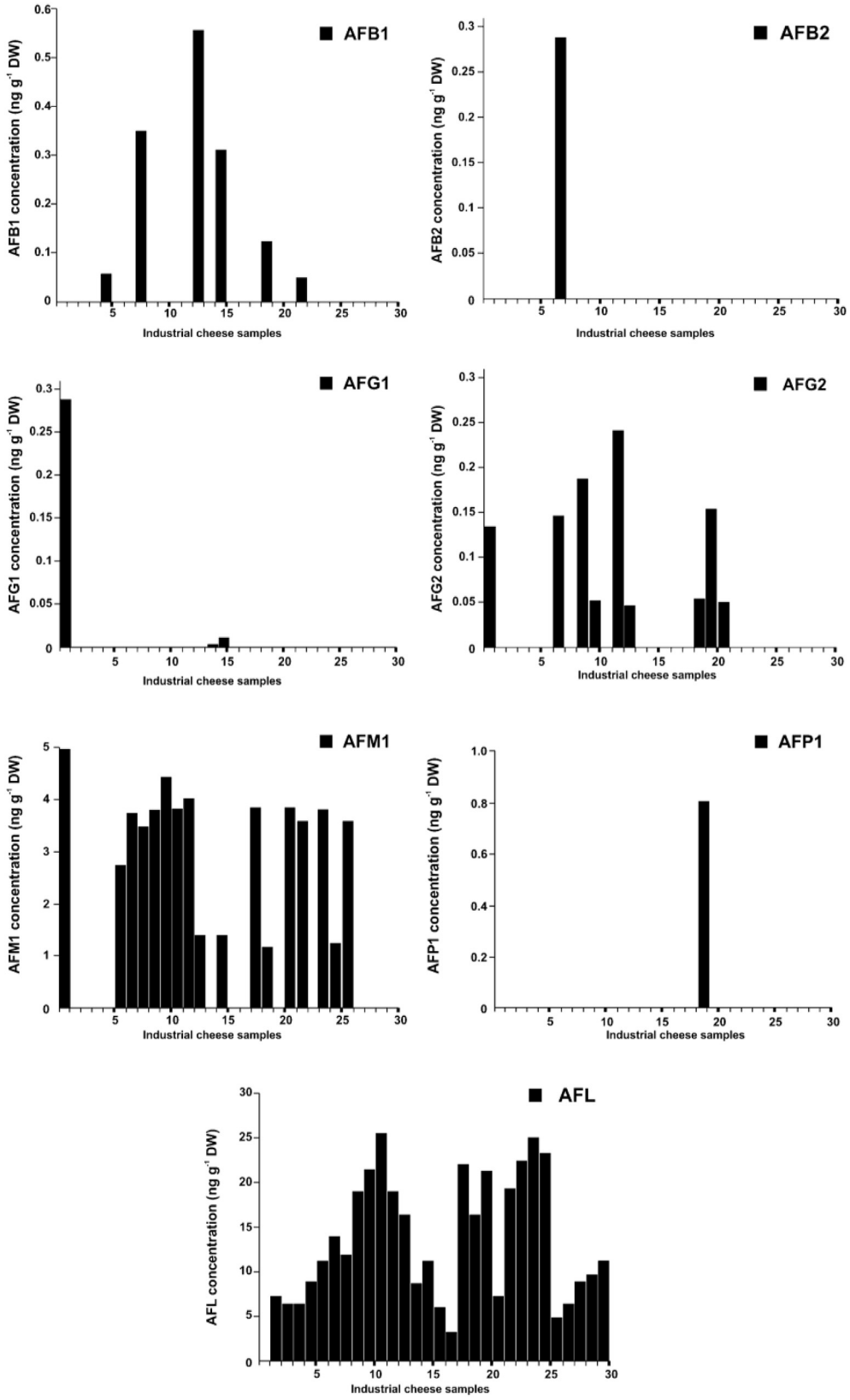

Fig. 2. Frequency and type of aflatoxins in the industrial Oaxaca cheese samples of Mexico City. There was no sample with AFM_2.

3.2. Statistical analysis

There were statistically significant differences in all the aflatoxins. For AFB_1, AFB_2, AFG_1, AFM_1, AFP_1, and AFL, the levels of aflatoxin greater than zero were all different from zero and all equal among themselves. These differences were not significant when using Bonferroni correction (Table 4).

For AFG_2, the results were similar. In the Covadonga trademark

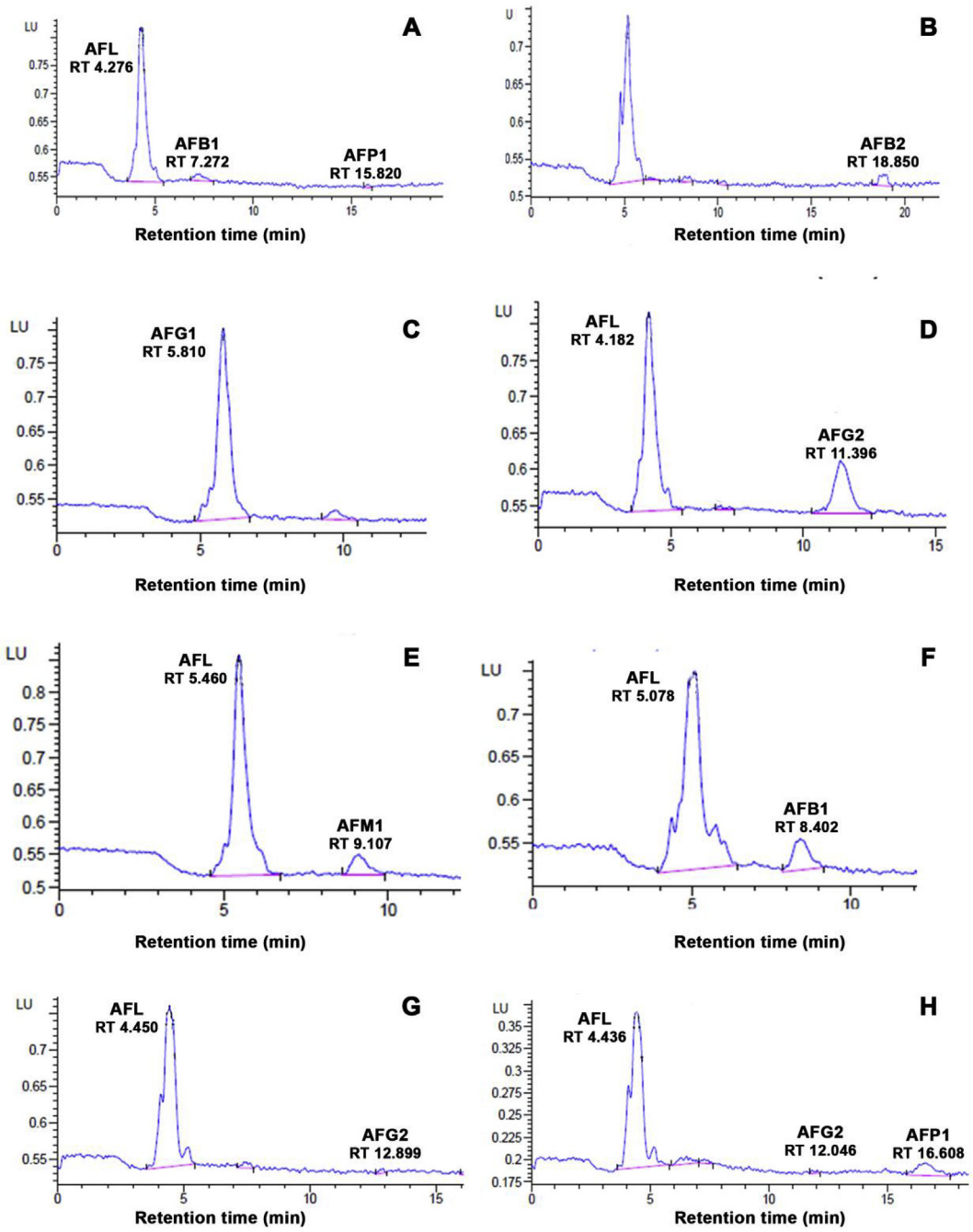

Fig. 3. Chromatograms of aflatoxins in industrial Oaxaca cheeses from Mexico City. A) Sample 13 with AFL, AFB_1 and AFP_1; B) Sample 7 with AFB_2; C) Sample 1 with AFG_1; D) Sample 12 with AFL and AFG_2; E) Sample 10 with AFL and AFM_1; F) Sample 11 with AFL and AFB_1; G) Sample 19 with AFL and AFG_2 Em 425 nm Exc 360 nm; H) Sample 19 with AFL, AFG_2 and AFP_1 Em 450 nm Exc 360 nm.

cheese, however, AFG_2 was not different from zero although different from all the samples that had any trace of this aflatoxin.

In the comparison of all the samples of artisanal cheese to the industrial cheese, there were no significant differences, except for AFB_1, where the levels in the artisanal cheese were greater. With regard to AFM_2, industrial cheese did not have any traces of this hydroxylated metabolized AF.

Therefore, the Food Safety Commission of Japan in 2013 (FSCJ, 2013) concluded that AFB_1 present in animal feed is extremely unlikely to affect the health of humans who have consumed contaminated milk or other livestock products. However, AF and the hydroxylated metabolites are also genotoxic carcinogens and are more likely to be found in livestock products, so AFB_1 contamination in feed and AFM_1 contamination in milk need to be reduced as much as possible. The intake of milk per 1 kg of body weight is higher in infants than in other age groups (FSCJ, 2013), suggesting that AFL in milk might still be a health hazard, particularly for infants whose staple diet is milk-based.

Risk assessment parameters for AFB_1, AFL and AFM_1 have been compared. The virtually safe dose for AFL is 1.7 times higher than that for AFB_1. The incidence of hepatocellular carcinoma in rats and fish dosed with AFL is lower than that in animals treated with AFB_1 at the same dosage (Kuiper-Goodman, 1990).

AFM_1 was the second most abundant AF after AFL. AFM_1 is classified as Group 2B as possibly carcinogenic to humans (IARC, 1997). AFM_1 has been found in Mexican milk (Carvajal et al., 2003a), so its presence in cheese was expected where most AFs were metabolized to

Table 4
Kruskal Wallis Statistical analysis, Chi square and p values of the eight aflatoxins, four basic aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) and four hydroxylated metabolites (AFM_1, AFM_2, AFP_1, AFL), found in the industrialized Oaxaca cheese samples.

Aflatoxins	Kruskal Wallis statistics Chi-square (29)	p-value
AFB_1	88.946	< 0.001
AFB_2	88.970	< 0.001
AFG_1	81.908	< 0.001
AFG_2	85.684	< 0.001
AFM_1	86.581	< 0.001
AFM_2	–	–
AFP_1	88.970	< 0.001
AFL	87.905	< 0.001
AFt	87.745	< 0.001

AFL. AFB_1 is present in milk at trace levels (0.05 μg L^{-1} to 0.42 μg L^{-1}) in 5.2% of 290 samples tested (Carvajal et al., 2003a) and is not considered a health risk, but cheese had more concentrated amounts (0.04–49.2 μg kg^{-1}) with an average of 11.2 μg kg^{-1} in the 30 samples of artisanal cheese analyzed before, and can be considered a health risk (Hernández-Camarillo, 2016a,c).

The AFP_1 hydroxylated metabolyte was present in trace amounts in one sample. Together with AFL, AFP_1 is not considered as toxicologically important in many countries. Polish and European Union legislations (Commission Regulation No. 152/98) agree that all food should be free from AF (Postupolski et al., 1999).

Oltipraz reduces AFB_1 adduct biomarkers (Li et al., 2000) and inhibits AFM_1 production by bovine hepatocytes (Kuilman et al., 2000), so it can be used to lower the risk related to cheese consumption. It is necessary to balance the availability of cheese in relation to the health risk, not only for cancer but also for other diseases, such as immune suppression, hepatitis and cirrhosis. Therefore, mycotoxin regulation is difficult and incomplete.

AFs are recurrent and occasionally unavoidable contaminants of milk and cheese, and their thermal stability invalidate both pasteurization and ultrapasteurization as effective control methods. The best control strategy is to keep raw materials and feed under obligatory mycotoxin regulation. In the case of cheese, it is recommended not to add maize flour during the manufacturing process.

Although the legislation regarding maximum tolerance levels has attempted to decrease the level of AFM_1 contamination in cheeses and there is no direct evidence of human toxicity resulting from the consumption of cheese contaminated with AFs, the problem of ingesting AFB_1 and AFL is still present mainly in artisanal, more than in the industrialized Oaxaca cheeses.

3.3. Risk assessment

The risk assessment taken from the equation presented in Methods shows:

$$3 \times 0.25 + 0.01 \times 0.75$$

Average potency = 0.0825 cancers per year per 100,000 inhabitants per ng AFB_1 kg^{-1} body weight day $^{-1}$. In Table 5 we have the Exposure and the liver cancer risk, that in the industrialized cheese did not reach the value of 1, showing that they represent no substantial risk for cancer, except for AFL that had an average of exposure of 3.77. In the case of the consumption of artisanal cheese, we found previously (Hernández-Camarillo, 2016a,c), that the risk assessment revealed that the population at higher risk to AFM_1 and AFM_2 was children, followed by the adolescents and adult women.

The AFB_1 contamination in industrialized Oaxaca cheese represented no substantial risk for liver cancer. On the other side aflatoxicol (AFL) is much higher in the samples, although AFB_1, AFM_1 and AFL in industrialized cheese still had values lower than 1. In the 93.3% of the samples of industrialized Oaxaca the contamination level due to AFB_1 and AFM_1 is of no concern, then the exposure is not important as well (FAO-WHO, 2004). Without exposure there is no substantial risk. Nevertheless, in the same samples AFL is present with low risk values. Therefore, the industrialized Oaxaca cheese consumption represents a no substantial risk to promote the hepatocarcinoma formation. The conclusion of the present research is that both artisanal and industrialized Oaxaca cheeses have aflatoxins, but the quantities have different risks, the ingestion of artisanal cheese is a risk for cancer, but the ingestion of industrialized Oaxaca cheese represents a no substantial risk.

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Table 5
Dietary exposure of aflatoxins (AFB_1, AFL and AFM_1) in industrialized Oaxaca cheese consumed by Mexican population. Risk assessment assumes an adult body weight of 71.75 kg.

Trademarks of industrialized Oaxaca cheese	Exposure: ng kg^{-1} body weight per day			Liver cancer risk/100,000 inhabitants[a]		
	B1	AFL	M1	B1	AFL	M1
1	0.00	0.00	0.74	0.00	0.00	0.01
2	0.00	1.07	0.00	0.00	0.09	0.00
3	0.00	0.94	0.00	0.00	0.08	0.00
4	0.00	0.92	0.00	0.00	0.08	0.00
5	0.01	1.31	0.00	0.00	0.11	0.00
6	0.00	1.67	0.37	0.00	0.14	0.00
7	0.00	2.06	0.56	0.00	0.17	0.00
8	0.05	1.76	0.37	0.00	0.15	0.00
9	0.00	2.90	0.57	0.00	0.24	0.00
10	0.00	3.19	0.66	0.00	0.26	0.01
11	0.07	3.79	0.47	0.01	0.31	0.00
12	0.00	2.73	0.19	0.00	0.22	0.00
13	0.07	2.41	0.20	0.01	0.20	0.00
14	0.00	1.29	0.00	0.00	0.11	0.00
15	0.05	1.62	0.18	0.00	0.13	0.00
16	0.00	0.85	0.00	0.00	0.07	0.00
17	0.00	0.48	0.00	0.00	0.04	0.00
18	0.00	3.28	0.57	0.00	0.27	0.00
19	0.02	2.48	0.19	0.00	0.20	0.00
20	0.00	3.15	0.00	0.00	0.26	0.00
21	0.00	1.05	0.57	0.00	0.09	0.00
22	0.01	2.88	0.38	0.00	0.24	0.00
23	0.00	3.31	0.00	0.00	0.27	0.00
24	0.00	3.71	0.57	0.00	0.31	0.00
25	0.00	3.45	0.19	0.00	0.28	0.00
26	0.00	0.71	0.37	0.00	0.06	0.00
27	0.00	1.01	0.00	0.00	0.08	0.00
28	0.00	1.29	0.00	0.00	0.11	0.00
29	0.00	1.42	0.00	0.00	0.12	0.00
30	0.00	1.65	0.00	0.00	0.14	0.00
Average	**0.02**	**3.77**	**0.46**	**0.001**	**0.31**	**0.001**

Arévalo for library information.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.046.

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