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Quantifying the Levels of the Mutagenic, Carcinogenic Hydroxylated Aflatoxins (AFM_1 and AFM_2) in Artisanal Oaxaca-Type Cheeses from the City of Veracruz, Mexico

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Abstract

Aflatoxins (AF) are fungal secondary toxic metabolites that have mutagenic and carcinogenic effects in humans. Aflatoxin B_1 (AFB_1), the most toxic Aflatoxin, contaminates cattle feed and can be metabolized and excreted as the hydroxylate Aflatoxin M_1 (AFM_1). Aflatoxin B_2 (AFB_2) is excreted as Aflatoxin M_2 (AFM_2) in milk, and dairy products such as cheese can concentrate these carcinogens. Artisanal Oaxaca-type cheeses were sampled in Veracruz City, Mexico in 2016, and three different extraction methods (AOAC 2006, R-Biopharm 2016 and Cavaliere et al. 2006) -which were representative of many other reported methods- were selected for testing and validation. The R-Biopharm method was chosen and used to analyze the 30 samples that were derivatized and quantified by HPLC. The validation methods gave limits of detection (LODs) of 0.01 ng g^{-1} for AFM_1 and 0.05 ng g^{-1} for AFM_2; the limits of quantification (LOQs) for each Aflatoxin were four times the respective LOD. The recovery percentages were 95% for AFM_1 and 93% for AFM_2. The retention times were in the range of 8.514 to11.849 min for AFM_1 and 20.208 to 22.447 min for AFM_2. The extraction method, derivatization, and quantification (which was achieved using an HPLC-fluorescence detector) showed that 16 of the 30 samples (53%) were contaminated with AFM_1, at concentrations ranging from 0.01 to 44 ng g^{-1}, whereas AFM_2 contamination was less frequent. AFM_2 contamination was found in only 6/30 samples (= 20%) of the 30 samples and the ranges were three samples with traces below the LOD, and another three above the LOD with concentrations ranging from 0.67 to 3.43 ng g^{-1}. These two ranges of AF contamination surpassed the tolerance limits stated by NAFTA (0.5 µg kg^{-1}) and by Codex Alimentarius and the European Union (0.05 µg kg^{-1}).

Keywords: Aflatoxins M_1 and M_2; Artisanal Oaxaca cheese; Carcinogens, Cheese contamination

Introduction

In 2014, Veracruz State was the sixth leading state in Mexico for milk production, processing 693,950 L of raw cow milk. The dairy industry is the third most important food industry in Veracruz State, and 53% of the milk produced here is sold as cheese. It is estimated that 11,130 tons of milk are produced in Veracruz State each year [1]. Handmade production of dairy products processes 51% of the milk for cheese (827,120 L), whereas industrial production processes 21% of the milk [2].

No quality control is in place in the artisanal non-industrialized milk industry, rendering handmade cheeses susceptible to Aflatoxin (AF) contamination a health risk for consumers; however, no published studies have measured AF levels in cheese.

AFs are relatively small molecules (MW < 700) and are fungal secondary metabolites [3]. Structurally, AFs are bis-dihydro furanocoumarins, and they are produced by *Aspergillus* fungi, such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius* [4]. These fungi produce spores that contain AFB_1, AFB_2, AFG_1 and AFG_2, which can contaminate field crops or storage warehouses; these toxins are a potential health risk for animals and humans [4]. AFB_1 can contaminate cereals such as maize, sorghum, rice, barley and wheat, i.e., the grains that are used to make nutritionally balanced feed for dairy cows. AFB_1 is a Group I (proven) carcinogen for humans [5]. When a cow eats AFB_1-contaminated feed, it excretes the toxin as AFM_1 hydroxylate and AFM_2 hydroxylate in its milk (Figure 1). The liver metabolizes AFB_1 by adding a hydroxyl group, which increases the water solubility of AFB_1 and facilitates its excretion in urine or milk. This reaction also lowers the toxicity of AFB_1 by converting it into AFM_1. AFB_2 in the feed is metabolized to AFM_2 and excreted in the milk. AFM_1 is metabolized by the cytochrome P450 enzymes that are located in the endoplasmic reticulum [6,7].

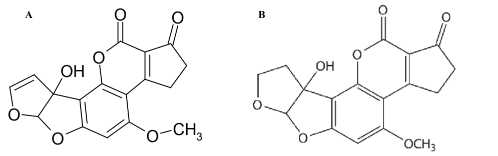

Figure 1: The structure of Aflatoxin M_1 (A) and Aflatoxin M_2 (B).

The warm tropical weather of the State of Veracruz favors fungal growth and AF production (Figure 2). Cheese made from AFM_1 contaminated milk has higher AFM_1 concentrations than are found in the original milk [8]. Milk and cheese are the vehicles for the introduction of AFM hydroxylates into the human diet [9]. There are

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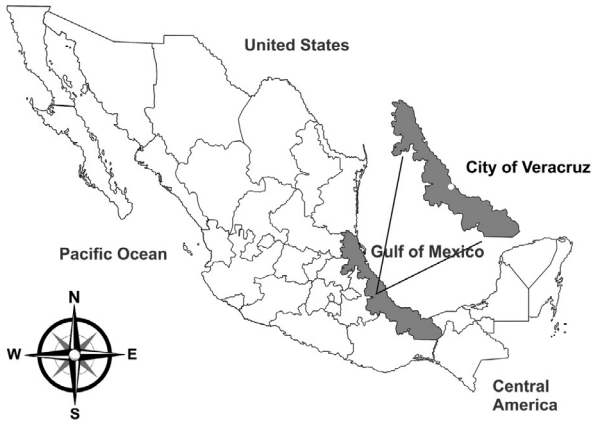

Figure 2: State and City of Veracruz in Mexico.

many cases of AFM_1 contamination of cheese in Brazil, Turkey, Mexico, Kuwait, Lebanon, Italy, and Serbia [8,10-14]. The stability of AFM_1 is not affected during the elaboration of cheese. AFM_1 seems to bind to casein, and so cheese curd has higher levels of AFM_1 than milk and is a risk factor when added during cheese elaboration [15]. AFM_1 is 2 to 10% less carcinogenic than AFB_1, but it is classified as a Group 2B (possible) human carcinogen because it is hepatotoxic, mutagenic and carcinogenic. Owing to its toxicity, many countries have established maximum tolerance levels [5]. The European Regulatory Commission 1881/2006 established a maximum limit of 0.05 μg kg^{-1} for AFM_1 in milk, whereas the USA has set a limit of 0.5 μg kg^{-1} [16,17]. The maximum tolerance limit of AFM_1 in cheese in some European countries, including Switzerland, France, Austria and Turkey, is 0.25 μg kg^{-1} [16]. Oaxaca-type cheese is produced by acidifying milk, adding curd and making dough that is submerged in hot water (65° to 70° C) until strands are formed [18]. Oaxaca is one of the more popular cheeses consumed in the City of Veracruz [19]. In Mexico, the presence of AFM_1 in milk has been reported, but the presence of AFM_1 in artisanal cheeses consumed in Veracruz has not been studied [20]. The purpose of this research was to quantify the levels of AFM_1 and AFM_2 in artisanal Oaxaca-type cheeses sampled in Veracruz.

Materials and Methods

Sampling

The study consisted of 30 samples of Oaxaca-type cheese, (each weighing 750 g), that were purchased in groceries and markets of the Port of Veracruz, Mexico (Figure 3). A Matlab algorithm was performed to randomly select the places from which samples were purchased. Samples of Oaxaca-type cheeses were purchased in Veracruz City, in March 2016, which is in the dry season, when the cows are fed with nutritionally balanced feed; the rest of the year, cows typically eat grass.

Methods tested for the extraction of AFM_1 from cheese

A bibliographic data base was consulted, and three different extraction methods for cheese were selected; these methods were representative of many other reported methods as they used the same solvents in different quantities. The three methods are summarized below.

1) The AOAC (2006) method is representative of other methods, all of which were performed using chloroform, diatomaceous earth and salt. In this study, the AOAC method was performed according to the following protocol [21,22].

Samples of dry, ground Oaxaca cheese (weighing 15 g each) were blended (Waring ETL laboratory blender 7010S Model WF 2211214 Torrington, Connecticut USA) with 5 g of diatomaceous earth, 100 mL of chloroform and 2 g of NaCl for 1 minute. This mixture was centrifuged (ALC 4235 Cool Working System, Milano, Italy) at 4500 rpm for 15 min, and 6.7 mL of the supernatant (equivalent 1 g of sample) was diluted in 24 mL of pH 7.4 phosphate-buffered saline (PBS). The diluted supernatant was applied to an immunoaffinity column (IAC), which was previously balanced with 20 mL of PBS. The sample was then washed with 20 mL of distilled water, and air was passed through the IAC to dry it. AFM_1 and AFM_2 were eluted from the agarose gel using HPLC-grade MeOH. The eluates were dried at 40°C in an oven (Novatech Model BTC 9100, Houston Texas, USA) and were derivatized.

2) The R-Biopharm (2012) method is almost the same as that used in a study by Iha et al., and uses methanol, water and salt [23,24]. This method has been recommended for use with Total aflatoxin Easi-Extract IACs (R-Biopharm Rhône Ltd., Glasgow, Scotland, UK). In this study, the R-Biopharm method was performed according to the following protocol.

Samples of dry, ground Oaxaca cheese (weighing 15 g each) were blended with a mixture of 100 mL of MeOH/water (80:20 v/v) and 2 g NaCl (to clarify the extract) for 2 minutes at high speed. The mixture was centrifuged at 4500 rpm for 15 min, and 6.7 mL of the supernatant (equivalent to 1 g of sample) was dissolved in 24 mL PBS. Before samples were added, each IAC that was used to detect total aflatoxin levels was balanced with 20 mL of PBS at pH 7.4, which was applied at a flux of 5 mL/min. The buffered sample was passed through the IAC, and AFM_1 and AFM_2 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 and then derivatized.

3) The Cavaliere et al. method uses acetone and silica gel with activated charcoal columns, methanol and water. In this study, the Cavaliere et al. method was performed according to the following protocol [25].

Samples of dry, ground Oaxaca cheese (weighing 15 g each) were

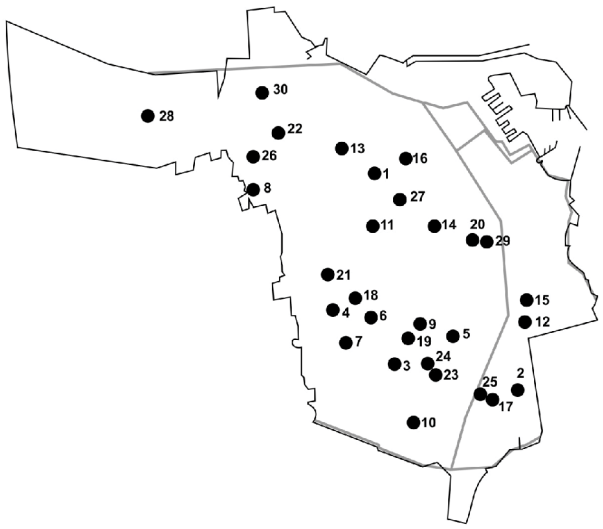

Figure 3: Sampling points (●) for Oaxaca type cheeses in the City of Veracruz, Mexico in 2016.

blended with acetone (150 mL), as recommended for fresh cheese. From this mixture, 6.7 mL of the supernatant (equivalent to 1 gram of sample) was analyzed. The sample extraction included a preliminary matrix solid-phase dispersion-extraction step before the solid-phase extraction clean-up step, which was achieved using a Carbograph-4 cartridge. The cheese sample was applied to the cartridge, which was washed with 5 mL of methanol containing 2% acetic acid. AFM_1 was eluted from the cartridge using 10 mL dichloromethane/methanol/acetic acid (88:10:2 v/v/v). The eluate was dried in an oven, derivatized, and 60 µL was used for HPLC.

Derivatization

We used established methods to derivatize AFs and thereby increase their fluorescence [26]. The dry AFM_1 and AFM_2 standards (Sigma-Aldrich, St. Louis MO, USA) were dissolved in 200 µL acetonitrile (ACN), and 800 µL derivatizing solution was added. The derivatizing solution was composed of 5 mL trifluoroacetic acid (Sigma-Aldrich, St. Louis MO, USA), 2.5 mL glacial acetic acid (Merck, Naucalpan, Estado de Mexico, Mexico) and 17.5 mL deionized distilled water, and was vortexed (Vortex G-560, Bohemia NY, USA) for 30 seconds. The vials containing the dry eluates were heated in a vapor bath at 65°C for 10 min. The samples were cooled to room temperature, and triplicates of 60 µL were analyzed by HPLC.

Validation of the extraction method

The validation of the analytical method was performed using known methods [27]. To decide which method was the best for further analysis of the 30 Oaxaca cheese samples, the following parameters were taken into account.

Linearity of the System (Calibration curves)

Solutions of different AFM_1 and AFM_2 concentrations were prepared from a stock concentration of 1000 ng AFM. For the 0.25 mg AFM standards, dry dusts produced by *Aspergillus flavus* were diluted with benzene:acetonitrile (98:2 v/v), following known methodology, so that the pure AFMs did not decompose [21].

a. The spectrophotometer that we used (Genesys 10 UV Thermo Electron Corporation. Madison Wisconsin, USA) was calibrated before the experiments to measure the absorbance of the AFM standard solutions at 357 nm.

b. The following formula was applied to calculate AFM concentrations:

Concentration of AFM (µg mL^{-1}) = Absorbance × molecular weight × 1000 × Correction factor of the equipment /Extinction coefficient.

c. We calculated the inverse of the AFM concentration (1/x), which represents the amount of AFM that has to be added to 1 mL of MeOH.

d. The amount of AFM was subtracted from 1000 ng to give the volume of MeOH needed for a 1000 ng stock solution.

e. Twelve concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng) of AFM were created from the 1000 ng stock solution. These samples were then used to plot the analytic signal (area below the curve of each chromatographic peak) against the AFM_1 and AFM_2 concentrations; the curve equation and statistical parameters were obtained. The slope value (b_1), ordinate to origin (b_o), determination coefficient (R^2), confidence interval for the slope to origin ($IC(\beta_1)$), variation coefficient percentage (% CV), standard deviation (SD), and the LOD and LOQ were calculated using Excel 2003.

LOD and 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 [27].

To calculate the LOD, the following equation was used:

$$\text{LODThe LOQ was calculated using the} = \frac{3.3\,x\,S_{(y/x)}}{b_1}$$

LODThe LOQ was calculated using the following equation:

$$\text{LOQ} = \frac{10\,xS_{(y/x)}}{b_1}$$

where $S_{y/x}$ is the standard deviation of the regression, and b_1 is the value of the slope.

Recovery percentage

The recovery percentage is a measure of the accuracy of the method and expresses the proximity between theoretical and experimental values. The recovery percentage of each spiked sample was calculated by dividing the recovered AFM quantities by the spiked amount. The arithmetic average, standard deviation, percentage of variation coefficient and confidence interval were calculated for the population media. To obtain accurate measurements, 15 g samples of dried, ground Oaxaca cheese and aliquots containing 1 g of each sample were spiked with three different concentrations (5, 20, and 40 µg kg^{-1}) of AFM_1; one aliquot without spiked AFM_1 was used as the control. The samples were individually processed with each one of the three extraction methods, as explained above. The control sample gave the basal level of contamination [21,23,25]. The AFM_1 of 1 g of sample — measured with and without spiking to account for the basal AFM_1 contamination — diluted in PBS (1:4 v/v) was purified and concentrated using an IAC and quantified by HPLC. The percentage of recovery (i.e., the accuracy) of each method was obtained.

Extraction, purification and concentration of AFM_1

After the validation of the three extraction methods, AFM_1 extraction was performed using the R-Biopharm Rhône methodology, and the obtained. AFM_1 or AFM_2 were then derivatized. When the derivatizing mixture cooled to room temperature, triplicates of 60 µL of each sample were analyzed by HPLC [23].

HPLC quantitation

The AOAC (2006) HPLC method was previously tested [21]. 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); which was set to an excitation wavelength of 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 x 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.

Statistical analysis

The statistical analysis was performed using Minitab version

16. Variance analyses with the Tukey test at 95% were performed in triplicate, considering each cheese as an experimental unit. The graphs showing the data from the Tukey test and the standard deviations were produced using Kaleidagraph version 3.5. A Kruskal-Wallis analysis was performed, to find differences on the concentrations of AFM_1 and AFM_2 among the 30 samples. Wilcoxon Rank Sums statistical analysis of both Aflatoxins AFM_1 and AFM_2

Results and Discussion

AFB_1 is a genotoxic carcinogen that is the most carcinogenic of all aflatoxins, and animal experiments have shown that AFB_1 causes liver cancer in most species. AFM_1 is a metabolite of AFB_1 that is found in the milk of animals that have ingested AFB_1. The liver is the target of the carcinogenic effects of AFM_1. The genotoxicity of AFM_1 has been demonstrated by *in vitro* and *in vivo* experiments. The carcinogenic potency of AFM_1 is 2 to 10% weaker than that of AFB_1 [28]. Hence, the Food Safety Commission of Japan (2013) has concluded that the AFB_1 present in animal feed is extremely unlikely to affect the health of humans that have consumed contaminated milk or other livestock products. However, AFM_1 and its 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. In particular, attention should be paid to the fact that the intake of milk per 1 kg of body weight is higher in infants than in other age groups [28].

Validation parameters

The LOD for AFM_1 was 0.01 ng g^{-1}, the range for the retention times was 8.514 to 8.769 min, the R^2 was 0.9834, and the recovery percentage was 95%. For AFM_2, the LOD was 0.05 ng g^{-1}, the range for the retention times was 20.208 to 22.447 min, the R^2 was 0.9946, and the recovery percentage was 93% (Table 1).

The recovery percentages of each extraction method are presented in Table 1; examination of these criteria led to the decision to use the R-Biopharm extraction method for the rest of the study. This method was safer than the AOAC method, which, dissolved the plastic of the blender cover and used more dangerous solvents (Figure 4) (Table 2).

Quantification of AFM in the Oaxaca-type cheese samples

The levels and SDs of AFM_1 and AFM_2 in the media are presented in Table 2 and Figure 4. AFM_1 contamination was found in more samples (16 out of 30, 53%) the AFM_1 levels ranged from 0.1 to 44 μg kg^{-1}. The AFM_2 contamination (6 out of 30, 20%); Table 2 and Figure 4 were three samples, in the range from 0.67 to 3.43 μg kg^{-1}, above the LOD, and another three samples had traces below LOD. In the Kruskal-Wallis analysis the differences among both aflatoxin groups were significant

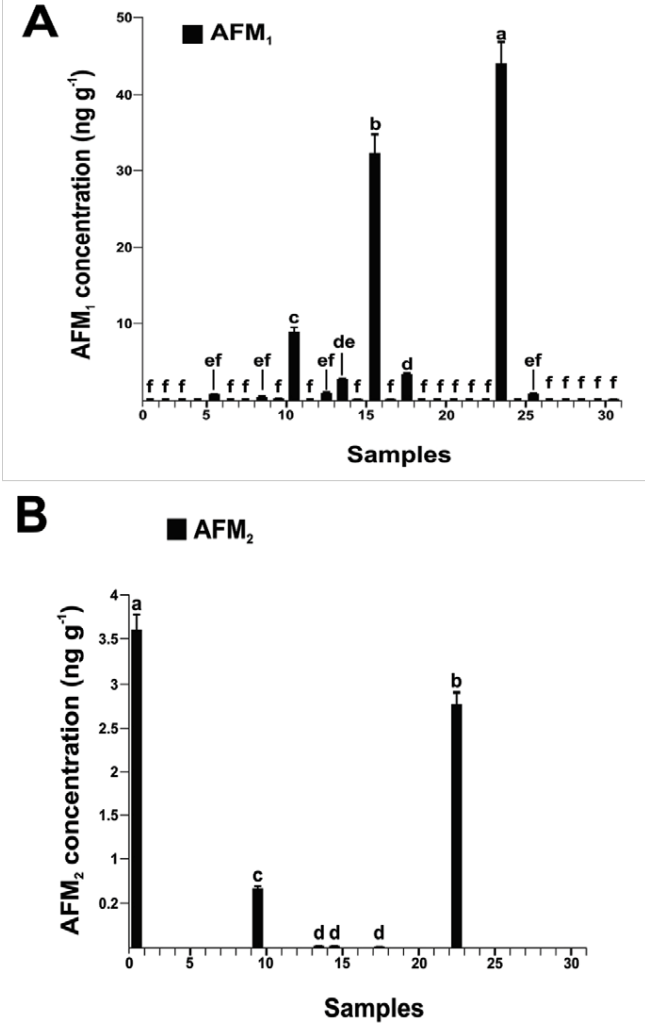

Figure 4: (A) Presence of AFM_1 and (B) AFM_2, in the artisanal Oaxaca cheese samples consumed in the City of Veracruz in 2016.

Method	Recovery percentages of the extraction methods for Oaxaca cheese				
	Basal AFM_1	AFM_1 (ng g^{-1}) spiked	Total AFM_1	Recovered AFM_1	Recovered percentage
AOAC (5 ng g^{-1})		5	11.82	4.86	41.1
AOAC (20 ng g^{-1})		20	26.82	26.37	98.3
AOAC (40 ng g^{-1})		40	46.82	42.70	91.2
Average of controls AOAC	6.82				**77 %**
Biopharm (5 ng g^{-1})		5	9.71	8.98	92.5
Biopharm (20 ng g^{-1})		20	24.71	24.51	99.2
Biopharm (40 ng g^{-1})		40	44.71	42.26	94.5
Average of controls Biopharm	4.71				**95 %**
Cavaliere et al. (5 ng g^{-1})		5	9.04	6.0	66.4
Cavaliere et al. (20 ng g^{-1})		20	24.04	16.86	70.1
Cavaliere et al. (40 ng g^{-1})		40	44.04	41.38	94.0
Average of controls Cavaliere	4.04				**85 %**

AFM_1 = Aflatoxin M_1; AFM_2 = AFlatoxin M_2; AFMt = Total M aflatoxins; SD = Standard deviation

Table 1: Recovery percentages of the three extraction methods for Oaxaca cheese.

Sampling point	Origin	AFM_1		AFM_2		AFMt
			Media		Media	
1	Tlalixcoyan	0.03 ± 0.01[b]	0	3.62 ± 0.27[a]	3.44	3.47
2	Jamapa	0	0	0	0	0
3	Jamapa	0	0	0	0	0
4	La Mixtequilla	0.01 ± 0[a]	0	0	0	0.01
5	Soledad de Doblado	0.76 ± 0.02[a]	0.72	0	0	0.76
6	Veracruz Port	0	0	0	0	0
7	Veracruz Port	0	0	0	0	0
8	Veracruz Port	0.44 ± 0.03[a]	0.41	0	0	0.43
9	Veracruz Port	0.04 ± 0.02[b]	0	0.68 ± 0.09[a]	0.65	0.70
10	La Antigua	8.95 ± 0.49[a]	8.50	0	0	8.95
11	Veracruz Port	0	0	0	0	0
12	Mixtequilla	0.97 ± 0.06[a]	0.92	0	0	0.97
13	Soledad de Doblado	2.66 ± 0.19[a]	2.53	< LOD	0	2.66
14	Tlalixcoyan	0.05 ± 0.01[a]	0	< LOD	0	0.05
15	Tlalixcoyan	32.19 ± 2.60[a]	30.58	0[b]	0	32.19
16	Veracruz Port	0.10 ± 0.01[a]	0.08	0	0	0.10
17	Malibran, Ver.	3.38 ± 0.17[a]	3.21	< LOD	0	3.37
18	Mixtequilla	0	0	0	0	0
19	Tierra Blanca	0	0	0	0	0
20	Boca del Río, Ver.	0	0	0	0	0
21	Veracruz Port	0	0	0	0	0
22	Soledad de Doblado	0	0	2.77 ± 0.76[a]	0.013	2.76
23	Veracruz Port	43.99 ± 2.90[a]	41.79	0[b]	0	43.99
24	Soledad de Doblado	0.01 ± 0[a]	0	0[b]	0	0.01
25	(Malibran), Ver.	0.72 ± 0.21[a]	0.680	0[b]	0	0.72
26	Veracruz Port	0	0	0	0	0
27	Veracruz Port	0	0	0	0	0
28	Veracruz Port	0	0	0	0	0
29	(La Joya) Acajete,	0	0	0	0	0
30	Veracruz Port	0.04 ± 0.01[a]	0	0[b]	0	0.04

AFM_1 = Aflatoxin M_1; AFM_2 = Aflatoxin M_2; AFMt = Total M aflatoxins; SD = Standard deviation. Different letters in the same row represent significant difference considering (ANOVAA with Tukey´s test, $P<0.05$).

Table 2: Concentrations of AFM_1 and AFM_2 (μg kg^{-1}) in artisanal Oaxaca cheese from Veracruz.

($P < 0.01$) for AFM_1 but less significant for AFM_2 (Table 3). We perform Wilcoxon Rank Sums test to find difference for every pair of samples. The letters in the last column denotes no statistical difference among the samples and in particular letter e denotes the samples that are not significant different than zero (Table 4). For AFM_2 just 6 samples have values different than zero the same statistics are given (Table 5). There are not significant differences among the samples and therefore we can conclude that all the samples are not significant different than zero (Figure 5 and 6). Some chromatograms of AFM_1 are in Figure 5 and from AFM_2 in Figure 6.

These two ranges surpass the tolerable limits stated by NAFTA (of 0.5 μg kg^{-1}) and by Codex Alimentarius and the European Union (0.05 μg kg^{-1}) [16]. A previous study has also reported high levels of AFM_1 in 27% of samples of fresh panela cheese from Mexico, but we found high AFM_1 levels in 53% of samples of Oaxaca-type cheese [11]. To date, no human cases of food poisoning have been associated with the consumption of contaminated cheese, even though high levels of carcinogens can be found in cheese.

Filamentous fungi are important for surface mold-ripened and core mold-ripened cheeses. The degradation of milk constituents, such as proteins and lipids, can lead to improvements in the texture, flavor, and nutritional quality of cheeses [15]. In *Aspergillus* spp., aflatoxins act as chemical signals for communication, competitive weapons to defend the fungal habitat, and inhibitors of the growth and reproduction of competitors within the same trophic niche [29]. In the cheese ecological niche, spoilage fungi and fungal cultures are in competition. The presence of competing microorganisms, including bacteria and yeast, is essential for mycotoxin

Aflatoxin	Chi-square (29df)	p-value
AFM_1	69.397	0.0001
AFM_2	40.157	0.0814

Table 3: Results of the Kruskal-Wallis analysis.

Sample	Mean	Median	Standard deviation	Standard error	
4	0.013333	0	0.023094	0.013333	a,e
24	0.013333	0	0.023094	0.013333	a,e
9	0.026667	0	0.046188	0.026667	a,e
1	0.033333	0	0.057735	0.033333	a,b,e
12	0.923333	0	1.59926	0.923333	a,b,c,e
10	8.49667	0	14.7167	8.49667	a,b,c,e
25	0.683333	0.1	1.0981	0.633991	a,b,c,e
14	0.053333	0.06	0.050332	0.029059	a,b,c,e
30	0.036667	0.02	0.028868	0.016667	a,b,c
16	0.076667	0.08	0.015275	0.008819	b,c
8	0.41	0.08	0.588982	0.340049	b,c
5	0.72	0.09	1.11727	0.645058	b,c
13	2.52667	2.7	0.33546	0.193678	c
17	3.20333	3.36	0.551936	0.31866	c
15	30.58	25.54	11.1867	6.45862	d
23	41.79	37.13	11.0066	6.35464	d

Table 4: Wilcoxon Rank Sums of the AFM_1 contamination of the samples of Oaxaca-type cheese from Veracruz.

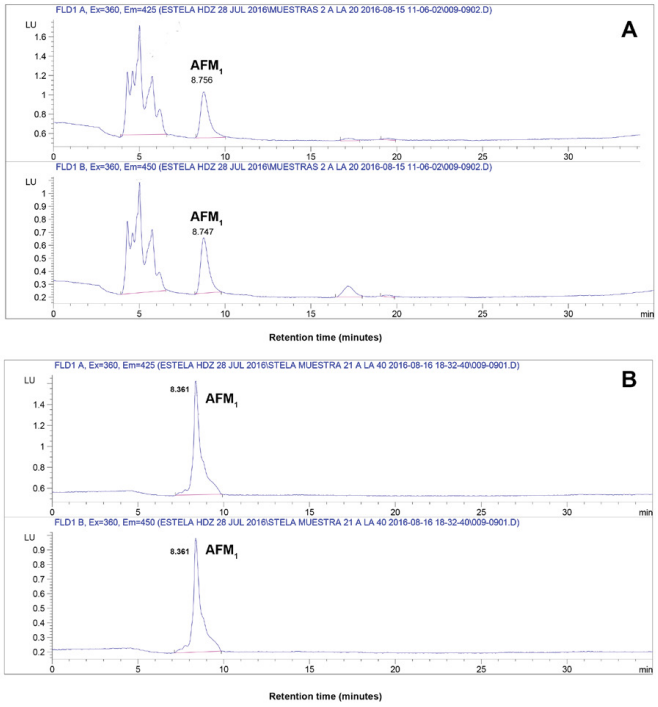

Figure 5: Chromatograms of AFM_1 from samples 10 (A) and 23 (B) of Oaxaca-type artisanal cheeses of Veracruz.

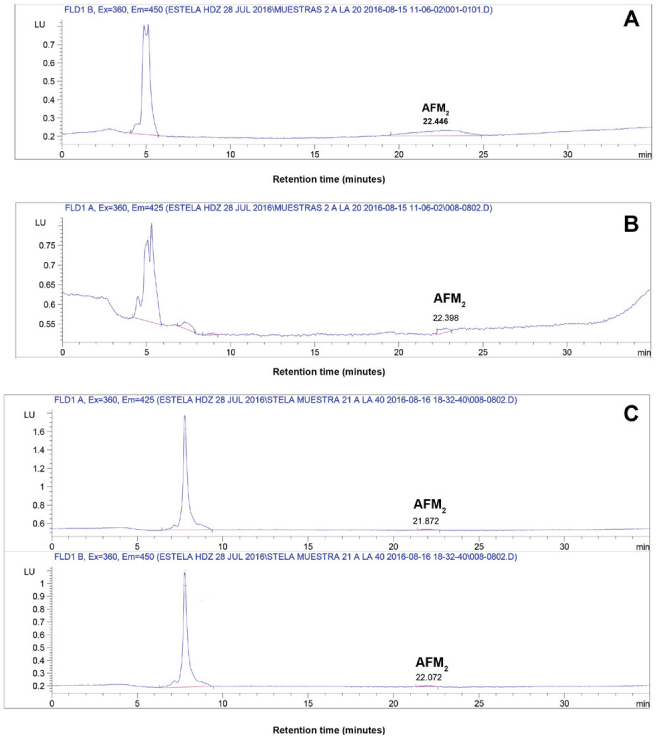

Figure 6: Chromatograms of AFM_2 traces from samples 1 (A), 9 (B) and 22 (C) of Oaxaca-type artisanal cheeses of Veracruz.

production as demonstrated in a study showing that serial transfers on culture media in laboratory conditions (i.e., without competitors and natural stress conditions) result in the loss of AF production in strains

Sample	Mean	Median	Standard deviation	Standard error
17	0.006667	0	0.011547	0.006667
13	0.016667	0	0.028868	0.016667
14	0.023333	0.02	0.025166	0.01453
9	0.643333	0	1.11429	0.643333
22	2.62	2.43	2.71998	1.57038
1	3.43333	0	5.94671	3.43333

Table 5: Wilcoxon Rank Sums statistical analysis of AFM_2

of *A. flavus* [15,30]. Nutrient depletion and competition with other fungi stimulate AF production, and early production of AF allows molds to rapidly colonize the environment. Lethal dose (LD_{50}) values range from 0.5 to 10 mg kg^{-1} depending on the type of AF. Different animal species have different susceptibilities to chronic and acute AF toxicity, which can ultimately lead to liver cancer [31].

Many countries monitor AFM_1 levels in milk and dairy products with different results. High AF levels of up to 850 ng kg^{-1} have been found in cheeses from certain regions of Turkey [9]. In the Bursa Province, Turkey, the AFM_1 levels in local cheese samples exceeded 250 $ngkg^{-1}$ [32]. In the South of Spain, AFM_1 was detected at concentrations between 20 and 200 µg g^{-1} in 16 out of 35 samples (45%) of local cheese [33]. In a study of cheeses in Campinas, Brazil, AFM_1 concentrations were low, and only 4 out of 204 samples were contaminated [10]. In Argentina, 2 out of 50 samples were positive for AFM_1, with levels of 0.33 and 0.20 µg kg^{-1} [34]. The consumption of AFB_1-contaminated or AFB_2-contaminated feed by dairy cows is known to result in the excretion of the monohydroxylated derivatives AFM_1 and AFM_2 in the cows' milk within a few hours [6]. If cows ingest 300 ng g^{-1} AFB_1, they will produce milk containing 1-3 ng g^{-1} AFM_1 24 h later. Other study has reported that the normal carry-over is approximately 0.4-0.6%, so an intake of ≥70 µg AFB_1 by cows results in the production of milk containing AFM_1 levels greater than the regulatory limit (0.05 µg kg^{-1}) [35]. The amount of AFM_1 formed depends on the individual cow, and its excretion in milk decreases one day after the consumption of AFB_1-contaminated feed ceases, although traces can be found over two or three more days. The conversion ratio of AFB_1 to AFM_1 varies from 1:100 to 1:300 [36].

The health risk of AFM_1 should not be underestimated. Ammonia treatment is the only method that can reduce AF levels; this method can destroy 95% to 98% of AFs and is used to decontaminate animal feed in various countries [37].

Cold temperatures (5 to 7°C) prevent fungal growth and mycotoxin production [15]. Other chemicals such as pimaricin (also known as natamycin) -which is produced by the actinobacterium *Streptomyces natalensis* — delay fungal growth and are used as preservatives to control mycotoxin production [15,38].

In conclusion, the artisanal Oaxaca-type cheeses of the city of Veracruz were contaminated with AFM_1 (53%) at concentrations ranging from 0.01 to 44 µg kg^{-1}, and less frequently with AFM_2 (20%) at concentrations ranging from 0.67 to 3.43 µg kg^{-1}. These results are relevant from the public health point of view. There are no protections from the authorities to devise guidelines to protect the general public from the risk of consumption of Oaxaca-type cheese with aflatoxin. In Veracruz cattle eat grass around 8 months of the year and this fact lower the risk of aflatoxin contamination. Balanced feed for cattle should be decontaminated from aflatoxins. Decontamination of feed includes a dry and cool storage place where the fungi cannot grow, or to control the fungal growth with the application of glucomannans or ammonia.

Although the legislation regarding maximum tolerance levels has attempted to decrease the level of AFM_1 contamination in cheeses, and although there is no direct evidence of human toxicity resulting from the consumption of cheese contaminated with AFM_1, the problem is still present in fresh cheeses such as the artisanal Oaxaca cheese, as we described in this manuscript.

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