Unreported Aflatoxins and Hydroxylate Metabolites in Artisanal Oaxaca Cheese from Veracruz, Mexico

Vargas-Ortiz M 1,2, Carvajal-Moreno M 1*, Hernández-Camarillo E 1,3, Ruiz-Velasco S 4 and Rojo-Callejas F 5

1Laboratorio C-119 De Micotoxinas, Departamento de Botánica, Instituto de Biología, Ciudad Universitaria, Universidad Nacional Autónoma de México (UNAM), CP 04510 CdMx, México.
2CONACYT-CIAD (Centro de Investigación en Alimentación y Desarrollo), Coordinación Cullacán, Carretera El Dorado Km 5.5, Col. Campo El Díez, Cullacán Sinaloa 80110, México.
3Instituto Tecnológico de Veracruz, Tecnológico Nacional de México, SEP, Calzada Miguel Angel de Quevedo 2779, Col. Formando Hogar, CP 91897 H. Veracruz, Ver., México.
4Departamento de Química Analítica, Facultad de Química, Ciudad Universitaria, Universidad Nacional Autónoma de México (UNAM), CP 04510 CdMx, México.
5Departamento de Química Analítica, Facultad de Química, Ciudad Universitaria, Universidad Nacional Autónoma de México (UNAM), CP 04510 CdMx, México.

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). The common AFs (AFB1, AFB2, AFG1, AFG2) are metabolized in the liver or by microbes that produce hydroxylates (AFM1, AFM2, AFP1, aflatoxicol (AFL)), which makes them soluble in water. This means that AFs can be excreted in fluids such as milk or urine, and AFs are not destroyed in the process of making cheese.

Other AFs can also be excreted in milk, but they have not been reported until now. The purpose of this study was to identify and quantify the AFs present in 30 samples of artisanal Oaxaca-type cheese sold in the City of Veracruz. The average concentrations of AFs detected in the 30 samples of artisanal cheese were AFB1 (11.2 ng g⁻¹) in 77% (23/30); AFL (19.1 ng g⁻¹) in 70% (21/30); AFG1 (0.2 ng g⁻¹) in 63% (19/30); AFM1 (3.0 ng g⁻¹) in 53% (16/30); AFB2 (0.1 ng g⁻¹) in 50% (15/30); AFM2 (0.2 ng g⁻¹) in 20% (6/30); AFG2 (0.03 ng g⁻¹) in 13% (4/30); and a trace amount of AFL. In only 3% (1/30) AFB1 and AFL, are the most abundant AFs in Oaxaca-type cheese, although eight AFs were present, contributing to an average of 33.9 AFs distributed among the 30 samples. Cheese can therefore be associated with a certain degree of risk for cancer development.

Keywords: Aflatoxins; Fresh cheese; Carcinogens; Food contamination

Introduction

Cheese is an economically important commodity worldwide. In Mexico, 76,696 tons of Oaxaca-type cheese were produced in 2005, with a value of 2,700 million Mexican pesos [1,2]. 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 artistically, and there have been no reports about AFs in cheese and the quantities sold in Mexico.

The State of Veracruz is the sixth largest producer of milk in Mexico [3], and 53% of the total milk produced in the state is used without pasteurization to produce artisanal cheeses [4], which are sold in large cities such as the Port of Veracruz. One of the main artisanal cheeses produced in this region is Oaxaca-type cheese, which is made in the same way throughout the country. The process begins with warming milk to temperatures between 18°C and 25°C; the milk is then heated to 38°C, and rennet is added (9-12 mL for 100 L of milk). The milk is then acidified with acetic acid at pH 5.5, and upon curdling the curds are cut into 2-cm squares. The curds are left to rest for 25 min and are later shredded by hand and left to acidify for 20 min. The whey is drained, and the curds without whey are melted when mixed with hot water (60°C). The product is stretched by hand to form threads of 3 cm to 6 cm wide and then cooled with water (18°C). The cheese is left to drain, and salt is added (11 g to 50 g salt per 1 kg of threads). Finally, ball hanks are formed with the cheese threads [5].

Aflatoxins (AFs) are toxic secondary metabolites that chemically correspond to bis-dihydro-furane coumarins and have well-known physicochemical properties [6]. AFs are mainly produced by the fungi Aspergillus flavus, A. parasiticus and A. nomius [7]. The common AFs found in cereals, which are present in balanced cow feed, are aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), which have blue fluorescence, and aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), which have green fluorescence [8].

The acute symptoms of AFs include vomiting, miscarriage, hemorrhage, diarrhea and death, and chronic symptoms include immunosuppression, fetal malformation, hepatitis B and C, cirrhosis, and carcinoma of the liver [9,10], cervix [11], colorectal system [12], breast [12] and pancreas [12]. AFs are considered potent carcinogens, and the International Organization for the Research on Cancer (IARC) classified them as Grade I in humans [13].

AFs can be present in balanced cattle feed [14] in countries with tropical weather, where high humidity and warm temperatures in storage warehouses facilitate fungal growth, as well as in agricultural
and unregulated local markets [15]. Cheese is an important source of nutrients for humans, and it is frequently contaminated with AFs [16]. AFs are present in the cereals and oilseeds that are used as ingredients in feed and silage for cattle [17]. They are common in countries where storage, harvest and climate conditions are suitable for fungal growth and in countries without food regulation legislation [17,18].

When dairy cattle consume fodder contaminated with AFB<sub>1</sub> or AFB<sub>2</sub>, these toxins are rapidly absorbed. The animal's liver and the microbial metabolism mitigates the damaging effects of the toxins by introducing a hydroxyl (OH-) into the AF molecule, forming hydroxylate metabolites that allow the toxins to be dissolved in water and expelled from the body via urine or milk. In this way, AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> from balanced feed of ruminants or from fodder are biotransformed into AFM<sub>1</sub>, AFM<sub>2</sub>, AFP<sub>1</sub> and aflatoxicol (AFL) [19], and these less toxic but still carcinogenic hydroxylate metabolites are secreted in cow's milk (Figure 1).

**Aflatoxins in cheese**

AFs bind to proteins such as milk casein via a hydrophobic interaction [20]. Therefore, AFM<sub>1</sub> is present in cheese when contaminated milk is used. AFM<sub>1</sub> distributes in a 40% to 60% ratio between curd and whey, depending on the cheese-making method [21]. AFM<sub>1</sub> can withstand temperatures up to 320°C before decomposing and is resistant to thermal treatments, such as pasteurization, ultrapasteurization and acidification, that are used during the processing of cheeses [22,23]. Although AFM<sub>1</sub> is less toxic than AFB<sub>1</sub>, it is still carcinogenic, and it is frequently reported in dairy products. Nonetheless, other AFs certainly contribute to the risk associated with AFs due to the high consumption of dairy products. Therefore, many countries have established regulations for the maximum tolerable levels of AFs in milk and dairy products [24]. AFM<sub>1</sub>, AFM<sub>2</sub>, AFB<sub>1</sub> and AFL have been reported in Mexican milk [25,26]. Oaxaca–type cheese has economic, cultural and alimentary importance; therefore, the detection of AFs in artisanal cheeses commercialized in Veracruz is necessary. The purpose of this research was to find other AFs (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) and the hydroxylate metabolites AFM<sub>1</sub> and AFM<sub>2</sub> for comparison as well as AFP<sub>1</sub> and AFL, which have not been reported, and to discuss their importance as carcinogens.

**Materials and Methods**

**Sampling**

The study consisted of 30 samples of 750 g of Oaxaca–type artisanal handmade cheese purchased in groceries and markets in the City of Veracruz. The cheese samples originated from 5 townships (Acajete, Medellin, Tlalixcoyan, Soledad and Veracruz) of the State of Veracruz, Mexico (Figure 2). These townships practice dual-purpose cattle raising for both meat production and small artisanal cheese making with no sanitary legislation. A Matlab algorithm was used to randomly select the places from which the samples were purchased in the City of Veracruz. The cheeses were refrigerated immediately after sampling and were subjected to a drying process for a period of less than 12 hours. Samples of Oaxaca–type cheeses were purchased in March 2016, which is in the dry season when the cows are fed nutritionally balanced feed; during the rest of the year, cows typically eat grass.

Each cheese sample was manually unthreaded, the cheese samples were placed in a tray drier, and the dry samples were stored frozen until AF extraction and chemical analysis were performed.

**Chemical extraction method for Aflatoxins**

The R-Biopharm [27] method has been recommended for use.
with Total aflatoxin Easi-Extract Immunoaffinity Columns (IAC) (R-Biopharm Rhône Ltd., Glasgow, Scotland, UK). This method was performed according to the following protocol.

First, 15 g samples of dry, ground Oaxaca-type cheese were blended (Waring ETL laboratory blender 7010S model WF 2211214, Torrington, CT, USA) for 2 minutes at high speed with a mixture of 100 mL of MeOH/water (80:20 v/v) and 2 g NaCl to clarify the extract. The mixture was centrifuged at 4000 rpm for 15 min, and an amount of supernatant equivalent to 1 g of sample was dissolved in phosphate-buffered saline (PBS) at pH 7.4 at a proportion of 1:4 (v/v) and homogenized for 1 minute in a vortex. Before adding the samples, each IAC was equilibrated with 20 mL of PBS at pH 7.4 applied at a flux of 5 mL/min. The buffered sample was passed through the IAC, and 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 and then derivatized.

**Derivatization**

Derivatization is a process to increase the AF fluorescence [28,29] of AF standards to make calibration curves and to quantify the AFs in cheese samples Figure 3. The derivatization reaction with trifluoroacetic acid is the transformation of AFB1 and AFG1 that are less fluorescent, in their hemiacetals B2a and AFG2, that are highly fluorescent. AFB2 and AFG2 are not affected by this reaction due to their saturated structure [28].

Eight dry AF standards (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, AFP, and AFL from Sigma-Aldrich; St. Louis, MO, USA) that were used to determine the AFs’ linearity and percentage of recovery validation were dissolved in 200 μL of HPLC-grade acetonitrile (ACN), and 800 μL of derivatization solution was added. The derivation solution was prepared with 5 mL of 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 then vortexed (Vortex USA), 2.5 mL glacial acetic acid (Merck, Naucalpan, Estado de Mexico, Mexico) and 17.5 mL deionized distilled water and then 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 derivatized samples were cooled to room temperature, and triplicate 60-μL samples were analyzed by HPLC with fluorescence (HPLC-FL).

**Validation of the extraction method**

The validation of the analytical method and the analysis of the 30 Oaxaca-type cheese samples were performed using known parameters [30].

**Figure 3:** The derivatization reaction with trifluoroacetic acid that transforms AFB1 and AFG1, less fluorescent, in their hemiacetals B2a and AFG2, that are highly fluorescent. AFB2 and AFG2 are not affected due to their saturated structure.

**Linearity of the system (Calibration curves)**

Solutions with different concentrations of AFs were prepared from a stock concentration of 1000 ng AFM. The 0.25 mg AFM standards were diluted with benzene:acetone (98:2 v/v), following a previously reported methodology [31], so that the pure AFs did not decompose.

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 at 357 nm.

b. Different formulas [31] were applied to calculate 1000 ng stock solutions of each AF concentration:

c. Twelve concentrations (0.01, 0.05, 0.1, 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 (b), ordinate to origin (b), determination coefficient (R2), confidence interval for the slope to origin (IC[b]), 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 is equal to the AF concentration that gives a signal three times greater than the noise. The LOQ equals the AF concentration that is 10 times greater than the noise [32].

**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 (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, AFP, and AFL) and expressed as the real AF concentration. For this, a sample of 1 g was spiked with a solution of each AF concentration:

- **AFL**
  - AFB1
  - AFB2
  - AFG1
  - AFG2
  - AFM1
  - AFM2
  - AFP

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 Kaleida Graph version 3.5. Kruskal-Wallis statistical analysis was used to find significance and differences of AFs. The Wilcoxon Rank Sums test was performed to find differences for each pair of samples and Bonferroni corrections of the samples.

Results and Discussion

Validation parameters

The Limit of Detection (LOD) in ng g⁻¹ and recovery percentage

| Aflatoxin | LOD (ng g⁻¹) | Linearity (curves) | Retention time (minutes) | R² | Recovery percentage |
|-----------|--------------|--------------------|--------------------------|-----|---------------------|
| AFB₁      | 0.01         | 7.085-8.849        | 0.986                    | 97% |
| AFB₂      | 0.02         | 17.452-20.228      | 0.998                    | 95% |
| AFG₁      | 0.05         | 5.722-5.876        | 0.962                    | 93% |
| AFG₂      | 0.05         | 11.215-14.513      | 0.994                    | 96% |
| AFM₁      | 0.01         | 8.514-8.769        | 0.983                    | 95% |
| AFM₂      | 0.05         | 20.208-22.447      | 0.994                    | 97% |
| AFP₁      | 0.05         | 15.563-19.318      | 0.990                    | 95% |
| AFL       | 0.01         | 3.032-3.569        | 0.978                    | 98% |

Table 1: Validation parameters of the study of aflatoxins and hydroxylates.

Aflatoxins ng g⁻¹

| Origin in Veracruz | Sample | AFB₁ | AFB₂ | AFG₁ | AFG₂ | AFM₁ | AFM₂ | AFP₁ | AFL | AFB² |
|--------------------|--------|------|------|------|------|------|------|------|-----|------|
| Tlalixcoyan        | 1      | 0.1  | 0    | 0    | 0    | 0.03 | 3.43 | 0    | 71.0| 74.5 |
| Jamapa             | 2      | 0    | 0    | 0.5  | 0    | 0    | 0.32 | 10.6| 12.6 |
| Jamapa             | 3      | 0.1  | 0    | 0    | 0.1  | 0    | 0    | <LOD| 25.3| 25.5 |
| La Mixtequilla     | 4      | 0.03 | 0    | 0    | 0    | 0.01 | 0    | 13.9| 13.9 |
| Soledad de Doblando| 5      | 0.1  | 0    | <LOD| 0.7  | 0    | <LOD| 2.9  | 3.8 |
| Unknown            | 6      | 0.1  | 0    | <LOD| 0.3  | 0    | 0    | 9.9  | 10.3 |
| Unknown            | 7      | 0    | 0    | 0.1  | 3.1  | 0    | 0    | 0.02 | 3.2 |
| Unknown            | 8      | 0    | 0    | 0    | 0    | 0.04 | 0    | 2.1  | 2.5 |
| Unknown            | 9      | 0.6  | 0    | 0    | 0.6  | 0.03 | 0.8  | <LOD| 24.7| 26.6 |
| La Antigua         | 10     | 9.4  | 0.02 | 0    | 0.1  | 8.5  | 0    | 1.25 | 104.2| 123.5 |
| Unknown            | 11     | 0.04 | 0    | 0    | 0.5  | 0    | 0    | 0    | 12.4| 13.0 |
| La Mixtequilla     | 12     | 0    | 0    | 0    | 0    | 0    | 0    | <LOD| 47.9| 48.9 |
| Soledad de Doblando| 13     | 0    | 0    | 0.2  | 2.5  | <LOD| <LOD| 45.2| 47.9 |
| Tlalixcoyan        | 14     | 2.8  | 0    | 0    | 0.1  | 0.1  | <LOD| 45.2| 48.1 |
| Tlalixcoyan        | 15     | 0.02 | 0    | 0    | 0.1  | 30.6 | 0    | 0    | 30.6 |
| Unknown            | 16     | 0    | 0    | 0    | 0    | 0    | 0.1  | <LOD| 51.0| 51.1 |
| Malibrán market    | 17     | 37.1 | 0    | 0    | 0    | 3.2  | <LOD| 0    | 0    | 40.4 |
| Unknown            | 18     | 37.9 | 0    | 0    | 0.1  | 0    | 0    | <LOD| 0    | 38.0 |
| Tierra Blanca      | 19     | 38.7 | 0    | 0    | 0.6  | 0    | <LOD| 0    | 39.2 |
| Boca el Río        | 20     | 36.7 | 0    | 0    | 0    | 0    | 0    | <LOD| 0    | 36.8 |
| Unknown            | 21     | 49.2 | 0    | 0    | 0    | 0    | 0    | 0    | 49.2 |
| Soledad de Doblando| 22     | 37.9 | 0    | 0    | 0    | 2.6  | <LOD| 1.8  | 42.4 |
| Unknown            | 23     | 0    | 0    | 0    | 0.2  | 41.8 | 0    | 0    | 42.0 |
| Soledad de Doblando| 24     | 48.0 | 0    | 0    | 0.3  | 0.01 | 0    | 0    | 48.3 |
| Malibrán market    | 25     | 0.2  | 0    | 0    | 0.1  | 0.7  | 0    | 26.9| 27.8 |
| Unknown            | 26     | 0.3  | 0    | 0.7  | 0.1  | 0    | 0    | 20.0| 21.1 |
| Unknown            | 27     | 0.4  | 0    | 0    | 0.1  | 0    | 0    | <LOD| 24.7| 25.2 |
| Unknown            | 28     | 0.3  | 0    | 0    | 0    | 0    | 0    | <LOD| 16.2| 16.5 |
| La Joya, Jalapa    | 29     | 3.0  | 0    | 0    | 0.2  | 0    | 0    | <LOD| 17.4| 20.6 |
| Unknown            | 30     | 32.6 | 0    | 0    | 0.2  | 0.04 | 0    | <LOD| 0    | 32.8 |
| Sum*               |        | 335.3| 0.02 | 0.8  | 7.3  | 89.6 | 6.7  | 3.5  | 573  | 1016 |
| Average            |        | 11.2 | <LOD | 0.03 | 0.2  | 3.0  | 0.2  | 0.1  | 19.1 | 33.9 |

*Decreasing order of abundance of AFs in Oaxaca-type cheese: 1) AFL, 2) AFB₁, 3) AFM₂, 4) AFG₁, 5) AFG₂, 6) AFM₁, 7) AFB₂, and 8) AFB₁.

Table 2: Averages of triplicate counts of reported and unreported aflatoxins in artisanal Oaxaca-type cheeses of Veracruz.
Figure 4: Chromatograms of non-reported Aflatoxins found in Oaxaca-type cheese in the City of Veracruz. LU=Concentration in Luminiscence Units. The fluorescence data of AFs: a) AFB₁, AFB₂, AFM₁, AFM₂ Exc=360, Em=425 nm, and b) AFG₁, AFG₂ and AFP, at, Exc=360 nm and Em=450 nm. A) AFB, in sample 24 rep 1; B) AFL in sample 14 rep 1; C) AFL and AFB₁ sample 6 rep 2 and D) AFG₁ and AFG₂ sample 6 rep 2; E) AFM₁ and AFB₂ sample 10 rep 2 and F) AFP, sample 10 rep 2; G) AFL, AFB₁, AFP, and AFB₂ sample 10 rep 2; and H) AFB₁, AFM₁ and AFM₂ (<LOD) sample 17 rep 1.

Figure 5: Unreported aflatoxins (A) AFB₁, (B) AFG₁, and (C) AFG₂. The same letters represent samples statistically the same with Tukey test (P<0.05). No AFB₂ was detected.

Figure 6: Reported AFM₁ (A) and AFM₂ (B), and non-reported aflatoxin hydroxylates AFP₁ (C) and Aflatoxicol (D) in artisanal cheese of Veracruz. The same letters represent samples statistically the same with Tukey test (P<0.05).

two toxic metabolites are more frequent in Oaxaca-type cheese than are AFM₁ and AFM₂. Other AFs, such as AFP₁ and AFG₂, were present in trace amounts [19], and AFG₂ appeared more frequently. AFL can be formed through the enzymatic or synthetic reduction of AFB₁, and
Aflatoxin Chi-square (29 df) p-value
AFB1 44.897 0.0302
AFB2 29.000 0.4651
AFG1 26.180 0.6159
AFG2 41.971 0.0565
AFP1 42.646 0.0491

df=Degrees of freedom, p=Probability.

Table 3: Kruskal-Wallis statistical analysis to find significance and differences of aflatoxins.

Sample Mean Median Standard deviation Standard error Equal medians Bonferroni correction
4 0.0267 0.0462 0.0267 a a
11 0.0400 0.0693 0.0400 a a
1 0.0633 0.1097 0.0633 a a
16 0.0633 0.1097 0.0633 a a
6 0.0867 0.1155 0.0667 a a
8 0.0867 0.1501 0.0867 a a
3 0.0967 0.1674 0.0967 a a
26 0.1133 0.1963 0.1133 a a
5 0.1267 0.1250 0.0722 a a
25 0.1700 0.1700 0.0982 a a
28 0.2700 0.2764 0.1365 a a
27 0.3000 0.3000 0.1732 a a
9 0.3467 0.3402 0.1964 a a
14 2.7933 4.8382 2.7933 a a
29 3.0067 5.1731 2.9867 a a
10 9.3800 12.1648 9.3201 a a
17 14.2433 24.6702 14.2433 a b a
15 15.4467 26.7111 15.4127 a b c a
30 22.9200 26.6212 13.0603 a b c a
24 24.1833 41.8866 24.1833 a b c a
22 32.1767 27.9373 16.1507 a b c a
20 36.7400 13.6776 7.8969 a b b
18 37.6667 31.1666 4.8681 a b b
19 38.6467 38.3 0.9488 0.5478 a b b
21 49.1550 45.89 7.4182 4.2829 a b b

Table 4: Wilcoxon Rank Sums test to find difference for every pair of samples.

Sample Mean Median Standard deviation Standard error Equal medians Bonferroni correction
30 0.0033 0.0058 30 a, c a
9 0.0067 0.0116 9 a, c a
22 0.0067 0.0116 22 a, c a
5 0.0100 0.0100 5 a, c a
13 0.0100 0.0173 13 a, c a
19 0.0100 0.0173 19 a, c a
27 0.0100 0.0173 27 a, c a
12 0.0133 0.0231 12 a, c a
18 0.0200 0.0346 18 a, c a
20 0.0367 0.0635 20 a, c a
29 0.0400 0.0693 29 a, c a
2 0.1600 0.2771 2 a, b c a
7 22.9433 38.7044 7 a, b c a
16 0.0250 0.0229 16 a, b b
10 1.2500 1.4688 10 b b

Table 5: Statistics for AFP1 in the 15 samples that have values different than zero.

Sample Mean Median Standard deviation Standard error
7 0.0212 0.007 0.0250 0.0144
22 1.8033 0 3.1235 1.8033

Table 6: Wilcoxon Rank Sums of Values of 20 samples with AFL concentrations different from zero.

it has high toxicity and carcinogenicity [34]. Although the toxicity of AFL is 18 times lower than that of AFB1, both molecular structures have similar potency to form an exo-epoxide analogue that can bind to DNA [34]. AFL interconverts with AFB1, has electrochemical properties like those of AFB1, and these compounds have been experimentally demonstrated to have high carcinogenicity and toxicity.

AFLM contamination was in third place in Oaxaca-type cheese samples (Table 2), consistent with the results obtained for other kinds of cheese, such as cream cheese [35], white pickled cheese [36], sheep curd [37], Grana Padano cheese [38], parmesan [39], Turkish kasar cheese [40], and Serbian hard cheese [41]. AFLM contamination has been less frequently reported. There have been several studies [42] on carryover from cows fed AFLM-contaminated rations to AFLM in milk. The degree of toxicity and carcinogenicity of AFs is in the following order: B1>G1>B2>G2.

We performed a Kruskal-Wallis analysis to find differences in the concentrations of AFB1, AFB2, AFG1, AFG2, AFP1, and AFL among the 30 samples, as shown in Table 3. There were statistically significant differences among the samples for AFB1 and AFP1. For AFG2, the differences were not statistically significant at 5%, but if a different significance value is considered (10%, for example), the differences among the samples may be significant. Table 4 presents the Wilcoxon Rank Sums test to find the differences for each pair of samples for AFB1.

For AFP1, we found that 13 of the 15 samples had concentrations that were not significantly different from zero. When the Bonferroni correction was applied, these samples differed from the remaining four samples, 18 to 21.

For AFP1, we found that 13 of the 15 samples had concentrations that were not significantly different from zero. When the Bonferroni correction was applied, these samples differed from the remaining two (samples 16 and 10). It is important to note that most samples had at least one replicate different from zero. Samples 2 and 5 had two replicates different from zero, and samples 10 and 16 had three replicates different from zero. Table 5 shows the statistics for AFP1 in the 15 samples that had values different from zero. Table 6 shows the Wilcoxon Rank Sums of Values of 20 samples with AFL concentrations different from zero. The Wilcoxon Rank Sums test for all the pairs indicated that there is some inconsistency in the results of the tests.
AFB1 produces AFL [44]. AFL is equally carcinogenic as AFB1, so its formation is not a significant detoxification mechanism [45,46]. AFL has approximately 70% the mutagenicity of AFB1 [47], and it has two forms, A(Ro) and B, both of which are produced from the biological reduction of AFB1, and mainly by Tetrahymena pyriformis, Dactylum dendroides and Rhizopus spp. AFL is 18 times less toxic than AFB1, in the duckling biliary hyperplasia assay, and the biological activity of AFL B is unknown [48,49]. AFL is the major metabolite of AFB1, in many plants and animals, and it has been detected in milk [26,50], fermented dairy products [51], cereals and nuts [52], eggs [53], blood [54,55], human brain [56], the sera and liver of humans with kwashiorkor and marasmic kwashiorkor in Ghana and Nigeria [57-60], human urine [61], urine of heroin addicts [58], a breast-fed infant with neonatal hepatitis [62], the muscle of broiler chickens fed with contaminated diets [63], and poultry fed chronic low doses of mycotoxins, with the liver having the highest levels [64]. AFB1, AFM1, and AFL accumulate in the tissues and urine of calves [65,66], AFL–DNA adducts that were produced in vivo were identical to those produced by AFB1, and had similar molecular dosimetry responses and toxicity to the target organ [67]. Regarding DNA adduction and hepatocarcinogenicity in rainbow trout, the tumorigenic potencies were AFB1=1.00, AFL=0.936, AFM 1=0.086. AFL is a more potent toxin than AFM1, which can reconvert with AFM1, becoming AFL M1 [68]. AFL-induced hepatocellular carcinomas in rats and fish have a lower tumor incidence than those induced by AFB1 [69]. There is an interconversion of AFB1 and AFL, mediated by intracellular enzymes in rat blood [70]; guinea pigs [71]; sharks, which reconvert 30% of AFB1 to AFL [72-74]; and cultured human epidermal cells [75]. AFL converts into AFB1, which is the most carcinogenic and toxic of all AFs [76]. AFL is oxidized readily back to AFB1, so it can serve as a ‘reservoir’ for AFB1, in vivo, thereby prolonging the effective lifetime in the body [77]. If pH has a role in the interconversion AFB1–AFL, it could act in the normal human digestion of milk, where pepsin lowers the pH. The isomerization of AFL to AFB1 was observed in culture media with a low culture pH [78].

The genotoxicity of AFM1 has been demonstrated by in vitro and in vivo experiments. The carcinogenic potency of AFM1 is 2% to 10% weaker than that of AFB1 [78]. Therefore, the Food Safety Commission of Japan in 2013 [78] concluded that the AFB1 that is 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 hydroxylate metabolites are also genotoxic carcinogens and are more likely to be found in livestock products, so AFB1 contamination in feed and AFM1, 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 [78].

Risk assessment parameters for AFB1, AFL and AFM1 have been compared [79]. The virtually safe dose for AFL was 1.7 times higher than that for AFB1.

The incidence of hepatocellular carcinoma in rats and fish dosed with AFL was lower than that in animals treated with AFB1, at the same dosage. AFL in milk might still be a health hazard, particularly for infants whose staple diet is milk-based. AFM1 was not the most abundant AF, and the risk increases when the AFL contamination in milk is added. AFM1 is possibly carcinogenic to humans and was classified as Group 2B by the IARC (1997) [69].

AFM1 has been found in Mexican milk [25], and its presence in cheese was not unexpected, where most AFs were metabolized to AFL. Autumn milk was significantly more contaminated with AFL (p<0.0002), AFB1 had no significant correlation with season, and it is not clear if the presence of vegetable oil helped to decrease the AFL contamination [26]. AFB1 was generally present in milk at trace levels (0.05 mg L⁻¹ to 0.42 mg L⁻¹) in 5.2% of the 290 samples [26] and is not considered a health risk, but cheese had more concentrated amounts (0.04 to 49.2 ng g⁻¹), with an average of 11.2 ng g⁻¹ in the 30 samples and can be considered a health risk.

The hydroxylates AFB1 and AFL are not accepted 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 [80]. Oltipraz was shown to reduce AFB1, adduct biomarkers [81] and inhibit AFM1, production by bovine hepatocytes [82], so it can be used to lower the risk related to cheese consumption. It is necessary to balance the availability of milk in relation to the health risk, not only for cancer but also for other diseases, such as immune suppression, hepatitis and cirrhosis. This fact makes mycotoxin regulation difficult and very incomplete.

AFs are recurrent and occasionally unavoidable contaminants of milk, cereals and oilseeds, and their thermal stability rules out 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.

Conclusion

Although the legislation regarding maximum tolerance levels has attempted to decrease the level of AFM1, contamination in cheeses and although there is no direct evidence of human toxicity resulting from the consumption of cheese contaminated with AFs, the problem of ingesting AFB1 and AFL is still present in fresh cheeses, such as the artisanal Oaxaca cheese.

Acknowledgments

The authors thank the Instituto Tecnológico de Veracruz for the cheese sampling and the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM) for the data analysis. The authors also thank IBUNAM’s personnel: Noemí Chávez from the Secretaría Técnica, and Joel Villavicencio, Jorge López, Alfredo Wong, Celina Bernat, Diana Martínez and Julio César Montero provided valuable assistance with imaging, computer analysis and design. Additionally, we thank Georgina Ortega Leite and Gerardo Areválo for library information.

References

1. INEGI. Instituto Nacional de Estadística, Geografía e Informática (2006) Encuesta industrial mensual. Cantidad y valor de ventas.
2. INEGI. Instituto Nacional de Estadística, Geografía e Informática (2008) Encuesta industrial mensual. Cantidad y valor de producción de los productos elaborados. Encuestas tradicionales en establecimientos.
3. SIAP (Servicio de Información Agroalimentaria y Pesquera), México (2015) Boletín de Leche enero-marzo de 2015, México.
4. FUNPROVER, Fundación produce Veracruz (2010) Reporte Técnico de Proyecto. Estudio y análisis del mercado de los productos del sistema bovino de doble propósito en el Estado de Veracruz. Colegio de Postgraduados Veracruz, Xalapa, México.
5. Villanueva-Carduaj A, Esteban-Chávez M, Espinoza-Ortega A, Arrriaga-Jordán CM, Domínguez-López A (2012) Oaxaca cheese: Flavour, texture and their interaction in a Mexican traditional pasta filata type cheese. J Food 10:63-70.
27. Oruc HH, Cibik R, Yilmaz E, Kalkanli O (2006) Distribution and stability of aflatoxin M1 and aflatoxicol in milk, yogurt, and storage of white pickled cheese. Food Control 18:1103-1107.

26. Kamkar A (2006) A study on the occurrence of aflatoxin M1 in Iranian Feta cheese. Food Control 17:768-775.

25. Milčević DR, Škrinjar M, Baltić T (2010) Real and perceived risks for mycotoxin contamination in foods and feeds.Challenges for food safety control. Toxins 2:572-592.

24. Shephards GS (2003) Aflatoxin and food safety:Recent African perspectives. J Toxicol Toxin Rev 2:572-592.

23. Brackett RE, Marth EH (1982) Association of aflatoxin M1 with casein. Z Naturforsch 37c:127-132.

22. Devechi O (2007) Change in the concentration of aflatoxin M1 during manufacturing and storage of white pickled cheese. Food Control 18:1103-1107.

21. Oruc HH, Cibik R, Yilmaz E, Kalkanli O (2006) Distribution and stability of aflatoxin M1 during processing and ripening of traditional white pickled cheese. Food Addit Contam 23:190-195.

20. Tekingen KK, Eken HS (2008) Aflatoxin M1 levels in UHT milk and cheese consumed in Turkey. Food Chem Toxicol 46:3287-3289.

19. Škrbíč B, Antíč I, Živančev J (2015) Presence of aflatoxin M1 in white and hard cheese samples from Serbia. Food Control 50:111-117.

18. Chopra RC, Chabra A, Prasad KSN, Dumas A, Murthy TN, et al. (1999) Carryover of aflatoxin M1 in milk of cows fed aflatoxin contaminated ration. Indian J Anim Nutr 16:78-85.

17. Nakazato M, Morozumi S, Saito K, Fujimura K, Nishima T, et al. (1991) Production of aflatoxins and aflatoxicols by Aspergillus flavus and Aspergillus parasiticus and metabolism of aflatoxin B1 by aflatoxin-non-producing Aspergillus flavus. Eisai Kagaku 37:107-116.

16. Detroy RW, Hesseline C W (1970) Structure of a new transformation product of aflatoxin B1. Can J Biochem Physiol 48:830-832.

15. Schoenhard GL, Hendricks JD, Nixon JE, Lee DJ, Wales JH, et al. (1981) Aflatoxin-induced hepatocellular carcinoma in rainbow trout (Salmo gairdneri) and the synergistic effects of cyclopropenoid fatty acids. Cancer Res 41:1011-1014.

14. Peraica M, Radic B, Lubic A, Pavlovic M (1999) Toxic effects of mycotoxins in humans. Bull WHO 77:754-766.

13. Coulombe RA, Shelton DW, Sinnhuber RO, Nixon JE (1982) Comparative mutagenicity of aflatoxins using a Salmonella/trout hepatic enzyme activation system. Carcinogenesis 3:1261-1264.

12. Detroy RW, Hesseline CW (1968) Isolation and biological activity of a microbial conversion product of aflatoxin B1. Nature 219:967.

11. Cole RF, Cox RH (1981) Handbook of toxic fungal metabolites. NY Acad Press, USA.

10. Hsieh DPH (1983) Metabolism and transmission of mycotoxins. Proc of the Intern Symp on Mycotoxins (Cairo, Egypt), pp. 151-165.

9. Megalla SE, Mohran MA (1984) Fate of aflatoxin B1 in fermented dairy products. Mycopathologia 88:27-29.

8. Saito K, Nishijima Y, Yasuda K, Kamihara H, Ibe A, et al. (1984) Analytical method for aflatoxins and aflatoxicols in cereals, nuts and their products. Studies on mycotoxins in foods. XVI. J Food Hyg Soc Japan 25:112-117.
53. Truckses MW, Stoloff L, Young K, Wyatt RD, Miller BL (1983) Aflatoxicol and aflatoxin B1 and M1 in eggs and tissues of laying hens consuming aflatoxin contaminated feed. Poult Sci 62:2176–2182.

54. Wong ZA, Hsieh DPH (1978) Aflatoxicol: Major aflatoxin B1 metabolite in rat plasma. Science 200:325–327

55. Kumagai S, Nakano N, Aibara K (1983) Interactions of aflatoxin B1 and blood components of various species in vitro: interconversion of aflatoxin B1 and aflatoxicol in the blood. Toxicol Appl Pharmacol 67:292-301.

56. Oyelami OA, Maxwell SM, Adelusola KA, Aalakomda TA, Oyelese AO (1995) Aflatoxins in the autopsy brain tissue of children in Nigeria. Mycopathologia 132:35–38.

57. Apeagyei F, Lamplugh SM, Hendrickse RG, Affram K, Lucas S (1986) Aflatoxins in the livers of chickens with kwashiorkor from Ghana. Trop Geogr Med 38:273–276.

58. Hendrickse RG, Maxwell SM, Young R (1989) Aflatoxins and heroin. Proceedings of the international symposium on agricultural and biological aspects of aflatoxin related health hazards. J Toxicol: Toxicol Rev 8:89–94.

59. De Vries HR, Maxwell SM, Hendrickse RG (1990) Aflatoxin excretion in children with kwashiorkor or marasmus kwashiorkor: A clinical investigation. Mycopathologia 110:1–9.

60. Oyelami OA, Maxwell SM, Adelusola KA, Aalakomda TA, Oyelese AO (1998) Aflatoxins in autopsy kidney specimens from children in Nigeria. J Toxicol Environ Health, Part A 56:317–323.

61. Lovelace CEA, Njapau H, Saffer LF, Bayley AC (1982) Screening method for the detection of aflatoxin and metabolites in human urine: aflatoxins B1, G1, M1, B2a, G2a, aflatoxicols I and II. J Chromatogr 227:256–261.

62. Coulter JBS, Hendrickse RG, Lamplugh SM, MacFarlane SB, Moody JB, et al. (1986) Kwashiorkor — clinical studies in Sudanese children. Trans R Soc Trop Med Hyg 35:360–365.

63. Fernández A, Verde MT, Gascon M, Ramos JJ, Gómez J (1994), Aflatoxin and its metabolites in tissues from laying hens and broiler chickens fed a contaminated diet. J Sci Food Agric 65:407–414.

64. Micco C, Miraglia M, Onori R, Brera C, Mantovani A, et al. (1988) Long term administration of low doses of mycotoxins to poultry. 1. Residues of aflatoxin B1 and its metabolites in broilers and laying hens. Food Addit Contam 5:303–308.

65. Van der Linde JA, Frens AM, De Jongh M, Vees RO (1964) Inspection of milk from cows fed aflatoxin containing groundnut meal. Tijdschr Diergeneesk 89:1082–1088.

66. Sabino P, Puchio A, Milanez TV (1995) Aflatoxins B1, M1 and aflatoxicol in tissues and urine of calves receiving aflatoxin. Food Addit Contam 12:467–472.

67. Bailey GS, Dashwood R, Lovelam PD, Pereira C, Hendricks JD (1998) Molecular dosimetry in fish: quantitative target organ DNA adduction and hepatocarcinogenicity for four aflatoxins by two exposure routes in rainbow trout. Mutat Res 399:233–244.

68. Bailey GS, Lovelam PD, Pereira C, Pierce D, Hendricks JD, et al. (1994) Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B1 and aflatoxicol, two aflatoxins that form the same DNA adduct. Mutat Res 313:25–38.

69. IARC (International Agency for Research on Cancer) (1997) Naturally occurring aflatoxins (Group 1), aflatoxin M1 (Group 2B) 08/21/1997. Monographs on the evaluation of carcinogenic risks to humans. Some Naturally Occurring Substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins 56:5.1–5.5, Lyon France.

70. Chang WM, Lin JK, Wu KC, Hsiung KP (1985) In vitro interconversion of aflatoxin B1 and aflatoxicol by rat erythrocytes. Biochem Pharmacol 34:2566–2569.

71. Liu L, Nakatsu K, Massey TE (1993) In vitro cytochrome P450 monoxygenase and prostaglandin H-synthase mediated aflatoxin B1 biotransformation in guinea pig tissues: effects of beta-naphthoflavone treatment. Arch Toxicol 67:379–385.

72. Troxel CM, Buhler DR, Hendricks JD, Bailey GS (1997a) CYP1A induction by beta-naphthoflavone, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-p-dioxin and its influence on aflatoxin B1 metabolism and DNA adduction in zebrafish (Danio rerio). Toxicol Appl Pharmacol 146:69–78.

73. Troxel CM, Reddy AP, O’Neal PE, Hendricks JD, Bailey GS (1997b) In vivo aflatoxin B1 metabolism and hepatic DNA adduction in zebrafish (Danio rerio). Toxicol Appl Pharmacol 143:213–220.

74. Bodine AB, Luer CA, Gangjee SA, Walsh CJ (1989) In vitro metabolism of the pro-carcinogenic aflatoxin B1 by liver preparations of the calf, nurse shark and clearance skate. Comp Biochem Physiol 94C:447–453.

75. Walsh AA, Hsieh DPH, Rice RH (1992) Aflatoxin toxicity in cultured human epidermal cells: stimulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Carcinogenesis 13:2029–2033.

76. Nakazato M, Morozumi S, Saito K, Fujimura K, Nishina T, et al. (1990) Interconversion of aflatoxin B1 and aflatoxicol by several fungi. Appl Environ Microbiol 56:1465–1470.

77. Wong ZA, Hsieh DPH (1986) The comparative metabolism and toxicokinetics of aflatoxin B1 in monkey, rat and mouse. Toxicol Appl Pharmacol 55:115–125.

78. FSCJ, Food Safety Commission of Japan (2013) Food Safety. Official Journal of Food Safety Commission, November 20th, 2013.

79. Kuiper-Goodman T (1990) Uncertainties in the risk assessment of three mycotoxins:aflatoxin, ochratoxin, and zearalenone. Can J Physiol Pharmacol 68:1017–1024.

80. Postupolski J, Rybinska K, Szczesna M, Karlowski K, Ledzion E (1999) The review of the European Union documents relating to contamination of aflatoxins in food. Roczniki Państwowego Zakładu Higieny 50:57–67.

81. Li Y, Su J, Qin L, Egner PA, Wang J, et al. (2000) Reduction of aflatoxin B1 adduct biomarkers by Oltipraz in the tree shrew (Tupaia belangeri chinensis). Cancer Lett 154:79–83.

82. Kullman MEM, Mass RFM, Woutersen-van Nijenatten FMA, Fink-Gremmels J (2000) Inhibition of aflatoxin B1 production by bovine hepatocytes after intervention with oltipraz. Vet Quarterly 22:30–35.