Reduction of aflatoxin B_1 during *tortilla* production and identification of degradation by-products by direct-injection electrospray mass spectrometry

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Abstract

Objective. To determine the effect of pH, and exposure time over the inactivation of aflatoxin B_1 (AFB_1) during the *tortilla* making process as well as the degradative molecules generated. **Materials and methods.** Inactivation of AFB_1 in maize-dough with alkaline pH and in alkaline methanolic solutions was determined by HPLC. Kinetics of time exposure of AFB_1 in methanolic solution and the degradative products were analyzed by direct injection electrospray mass spectometry (DIESI-MS). **Results.** The alkaline pH of the maize-dough after *nixtamalización* between 10.2, and 30-40 minutes of resting at room temperature allows the 100% reduction of AFB_1. DIESI-MS analysis of the extracts indicated the presence of two degradation molecules from AFB_1. **Conclusion.** The alkaline pH of maize-dough and resting time are the principal factors involved in diminishing AFB_1 levels in *tortillas*. A procedure to the *tortilla* making process is proposed, which allows the reduction of remnant AFB_1, avoiding the accumulative effect over consumers.

Key words: aflatoxin B_1; degradation products; alkalinity; *Aspergillus*; Mexico

Moreno-Pedraza A, Valdés-Santiago L, Hernández-Valadez LJ, Rodríguez-Sixtos Higuera A, Winkler R, Guzmán-de Peña DL. Reducción de la aflatoxina B_1 durante la producción de tortilla y la identificación de los productos de degradación por espectrometría de masas con ionización por electrospray de inyección-directa (DIESI-MS). Salud Publica Mex 2015;57:50-57.

Resumen

Objetivo. Determinar el efecto del pH alcalino de la masa de maíz y el tiempo de exposición sobre la aflatoxina B_1 (AFB_1) durante la producción de tortillas e identificar los posibles productos de degradación mediante DIESI-MS. **Material y métodos.** La inactivación de la AFB_1 a pH alcalino y diferentes tiempos de exposición en masa nixtamalizada y en soluciones metanólicas fueron determinadas por HPLC. La cinética de degradación de AFB_1 y los productos de degradación en soluciones metanólicas se determinaron por DIESI-MS. **Resultados.** El pH alcalino de la masa y 30 a 40 minutos de reposo redujeron en 100% la AFB_1 adicionada. Se identificaron dos moléculas de degradación. **Conclusión**. Los principales factores involucrados en la disminución de la AFB_1 durante la producción de tortillas son la hidrólisis alcalina y el tiempo de reposo. Se propone un procedimiento para la producción de tortilla que reducirá la AFB_1 residual evitando el efecto acumulativo en los consumidores.

Palabras clave: aflatoxina B_1; productos de degradación; alkalinidad; *Aspergillus*; México

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Aflatoxin B_1 (AFB_1) is a fungal secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus*.[1] This mycotoxin is considered a potent genotoxic and carcinogenic substance, classified in the category 1 by the International Agency for Research on cancer in 1993.[2] Exposure to AFB_1 is related with acute non-viral hepatitis, aflatoxin-related immune suppression, liver cancer, nutrition-related problems and even death.[3,4]

Due to this important global issue, maximum levels of AFB_1 have been established at *Codex alimentarius* in animal and human foods in at least 99 countries.[5] The level of AFB_1 contamination varies widely: in Africa, 355μg of AFB_1/kg were reported in 2005 whereas much higher levels (955μg AFB_1/kg) were detected in Mexico in 1989.[3,6] Some Latin America countries have the regulatory limit of 5-20μg/kg, depending of the kind of food.[7] In Mexico maize is mainly used for human consumption. Around 12 millions tons are consumed per year, 6.3 millions tons of which in form of *tortilla* (in rural areas 217.9g/day and in urban areas 155g/day per capita).[8] According to the Mexican Norm,[9] the permitted maximum level of AFB_1 in Mexico is 20μg/kg in maize, and 12μg/kg in *harina nixtamalizada* (maize flour to make *tortillas*).

Nixtamalización is as old as Aztec civilization. It is estimated that *nixtamalización* began 1 200 years B.C.[10] The traditional *nixtamalización* has been reported highly effective in removing AFB_1 from spiked maize-dough. Using radioactive aflatoxin solution, it was observed that most of the radioactivity was discarded into the cooking liquid and the first wash, confirming the elimination of AFB_1 by-products.[11] The effectiveness of *nixtamalización* process was also reported by Mendez-Albores and colleagues.[12,13] They reported a reduction of AFB_1 of 92 and 93.2% respectively. *Nixtamalización* process can be divided into four steps: boiling maize with water and limestone, soaking the mixture; washing the cooking maize and milling *nixtamal* to obtain the maize-dough (*masa*).[14] Many reports have demonstrated that *nixtamalización* process inactivated AFB_1 by 85-95%.[6,15] However, for high aflatoxin contamination (520μg/kg), the percentage of AFB_1 inactivation was reduced to 93%,[6] therefore 7% of AFB_1 remains in the *tortilla*, representing a health hazard.[16] Mexican people would daily ingest 0.95μg of AFB_1 for each tortilla and 4.75μg AFB_1/5 *tortillas*.[6] There are some evidences that the pH has an essential role in the inactivation of AFB_1 in different substrates.[17] Hence, in the present work we analyzed the role of alkaline pH of maize-dough during the *tortilla* making process in the AFB_1 contamination. Additionally, we analyzed the AFB_1 degradation by-products generated during alkaline treatment.

Materials and methods

The AFB_1 standard was obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents, including acetone, acetonitrile, benzene, chloroform and methanol, were HPLC grade and obtained from J.T. Baker (Mallinckrodt Baker, Inc. Mexico). Limestone was obtained from a local market in Irapuato, Guanajuato, Mexico.

Preparation of maize-dough

White maize (1 kg) was boiled for 45 min at 90°C in 3L of water with 10g of lime (minimum content of $Ca(OH)_2$) = 90%) and left to soak overnight (18h at 24°C). Afterwards, the cooked maize was rinsed once with tap water (to remove pericarps) yielding 2kg of alkaline (*nixtamal*) maize ready for grinding. When this maize was ground into maize-dough, it had a pH of 10.2. Ten samples of maize-dough (50g each) were analyzed to determine the natural aflatoxin contamination.

Treatments of the maize-dough

Fifty grams samples of maize-dough (pH 10.2) were spiked with known amounts of AFB_1 standards solution 6.25μg/ 50g (125μg/kg of AFB_1). The AFB_1 was spiked in the middle of samples, after 5 min the corn dough was mixed with a spatula. Ten samples were maintained at room temperature (24°C), the other ten samples were flattened and cooked at 150°C during 5 min each side (*tortillas*). To evaluate the effect of the treatment over the spiked mycotoxin, AFB_1 determination and quantitation was done after 18h by HPLC-UV, HPLC(High-Pressure Liquid Chromatography with UV detector)-fluorescence and DIESI-MS. The negative control was maize-dough without addition of AFB_1.

Kinetic of time exposure of AFB_1 in alkaline maize dough pH 10.2

To determine the effect of alkaline pH of maize-dough over time, thirty samples (50g each) spiked with 115μg/kg of AFB_1, concentrations were measured in the spiked corn-dough at 0, 2, 6 and 18h after resting at 24°C. Six replicates of each treatment were used. Determination and quantitation was done by HPLC-UV and HPLC-fluorescence.

Effect of pH on AFB_1 standard solution

The standard solution of AFB_1 in methanol was used to analyze the effect of varying pH on aflatoxin stability. Three ml of AFB_1 standards were placed in glass tubes and 3ml of methanol adjusted to different pH values with 0.1 M NaOH (pH 8.0, 11.9 and 12.5) was added. A standard AFB_1 solution in non-buffered methanol was used as the reference. Three replicates for each pH value were prepared and they were placed in darkness at 14°C for 24 h. Afterwards, the liquid was removed by evaporation in water bath at 80°C and the contents re-dissolved in 1ml methanol. After filtration through an Extract CleanTM SPE C18 column (50mg per 1.5ml) (Grace Davison Discovery Science, Deerfield, Il 60015) the extracts were quantified by HPLC-UV and HPLC-fluorescence as described below.

Kinetic of time exposure of AFB_1 in alkaline methanolic solution

Methanol solution of standard AFB_1 (66.5µg/ml) at pH 10.5 was prepared and analyzed by DIESI-MS after different incubation times 10, 20, 30 and 40 min.

Extraction and quantification of aflatoxin by HPLC

Extraction of AFB_1 from samples of maize-dough was done according to the modification of the method 1 Association of Official Analytical Chemists (AOAC).[18] Quantitative determination of AFB_1 in the extracts were made by HPLC using a Supelco C18 column 4.6 x 250nm in a HPLC Agilent Technologies 1200 (ABC Instrumentación Análitica SA de CV), Software Agilent chemstation. The isocratic mobile phase was a mixture of water:acetonitrile:methanol (60:20:20 by volume). Elution of aflatoxin was recorded at 364nm. The detection level of this system was: 21ng AFB_1 in 20μl of extract.

Prederivatization with trifluoroacetic acid

In order to confirm the negative values of the extract treatments, a derivatization with trifluoroacetic acid was performed according to AOAC,[19] and a fluorescence detector was used at 360nm of excitation and 440nm of emission at 40°C in the Agilent 1200 HPLC equipment. The detection limit of this technique is 0.26ng of AFB_1 in 10μl of extract.

DIESI-MS identification of possible derivative AFB_1 products

Samples were filtered through Extract Clean TM SPE C18 column. Next, the samples were subjected to mass spectrometric analysis. The maize-dough and methanol extracts were analyzed by DIESI-MS,[20,21] with the following conditions: flow rate: 10μL/ min, spray voltage: 5kV, capillary temperature: 350°C, capillary voltage: 15V tube lens: 60V mode. The second technique was full positive scan. Open MS, ToppView version 1.10.0 and mMass 3.0 were used to data processing.

Data analysis

Each experiment was repeated three times, and aflatoxin levels were logarithmically transformed before statistical analysis, which was performed using the R version 2.15.2 (The R foundation for Statistical Computing).[22] Differences between values were evaluated by Tukey´s test (p=0.05) using SAS (version 6.12; SAS Institute, Cary, NC, USA).

Results

Effect of the *tortilla* making process over AFB_1

Ten non-spiked samples (containing low levels of AFB_1, identified as natural contamination level 0.034µg/50g) and ten more samples spiked with 6.25μg AFB_1/50g of corn, a value equivalent to 125μg/kg were used to make *tortillas*. Samples of maize-dough containing low levels of AFB_1 did not present any measurable AFB_1, suggesting 100% destruction. Meanwhile, 2.9μg/kg (0.145μg/50g) remained in those samples spiked with high levels of AFB_1 (125μg/kg). These data suggested that under the conditions used in this study, the *tortilla* making process destroys about 97.7% of aflatoxins (data not shown). When a more sensitive method (fluorescence determination) was used, a detection limit of 0.26μg/10μl was reached, the same samples presented 12.52μg AFB_1/kg corresponding to 90% of aflatoxin reduction. By DIESI-MS it was not found any ionized AFB_1 indicating 100% reduction.

On the other hand, the effect of temperature over AFB_1 content during flattening and cooking of the thin maize-cake was addressed. Samples of 50g of maize-

Table I
EFFECT OF CORN-DOUGH, pH 10.2 AT 24°C, OVER SPIKED AFB_1 STANDARD SOLUTION AT DIFFERENT TIMES. IRAPUATO, GUANAJUATO, MÉXICO, 2010

Spiked AFB_1 in maize-dough	*Exposure time*	*AFB_1 recovered µg/kg*-HPLC-UV*	*Reduction %*	*AFB_1 recovered µg/kg*-HPLC-Fluor*	*Reduction %*
0.0	0	0.0	0.0	0.0	0.0
115.0	0.5	0.0	100.0	0.36±0.22	99.81
115.0	2.0	0.0	100.0	0.34±0.14	99.70
115.0	6.0	1.28±0.58	98.88	0.46±0.19	99.60
115.0	18.0	0.60±0.36	99.48	0.14±0.10	99.80

* Aflatoxin data was logarithmically transformed: Log (1+X) before statistical analysis. Values of mean of 6 replicates

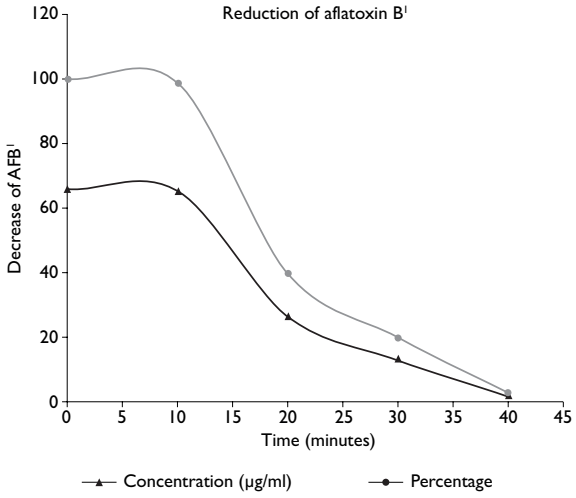


FIGURE 1. GRAPHIC SHOWING THE RESULT OF THE ANALYSIS BY DIESI-MS OF REDUCTION OF AFB1 OVER TIME IN ALKALIZED METHANOL pH 10.5

dough pH 10.2 with 30 min of resting at room temperature spiked with AFB_1 125µg/kg (6.25µg AFB_1/50 g) was used (data not shown).

Effect of alkaline conditions over AFB_1 reduction

The time-course of aflatoxin degradation in maize-dough at pH 10.2 suggested that most (~100%) of the AFB_1 was eliminated with 30 min exposure. Prederivatization of samples and fluorometric detection of AFB_1 yielded similar data (table I). Nevertheless, the kinetics of AFB_1 reduction analyzed with DIESI-MS in methanol solution indicated that the optimal time was 40 min (figure 1).

Table II
FATE OF AFB_1 STANDARD IN METHANOL SOLUTIONS UNDER DIFFERENT pH CONDITIONS. IRAPUATO, GUANAJUATO, MEXICO, 2011 (N=3)

AFB_1/methanol µg/mL	*Solution pH*	*AFB_1 recovered*		*Destruction of AFB_1 %*
		µg/mL	*%*	
0	7.7*	0.0±0.0[&]	0.0	0.0
7.02	8.0	5.70±0.9[§]	80.90	19.10
15.66	8.0	12.33±2.0[‡]	78.60	21.40
7.02	12.5	0.00±0.0[&]	0.00	100.00
15.66	11.8	1.5±1.3[#]	9.76	89.58

* Normal methanol pH. Alkaline pH was adjusted with 0.1 MNaOH. Values are mean of three replicates± standard deviation
‡,§,#,& Values with the same symbol are not significantly different (Tukey 0.05)

Tests with two concentration of AFB_1 in buffered solutions showed that pH values around neutral were much less effective in destroying aflatoxins than more alkaline conditions. At pH 8.0 only 19.10% of AFB_1 was reduced and 80% remained in the solution. However, at pH 11.8 and 12.5, the aflatoxin was reduced 90 and 100% respectively (table II).

Degradative products of AFB_1 under alkaline conditions

The alkaline treatment of standard AFB_1 produced at least two degradation molecules that were observed in the DIESI-MS spectra, these two increased peaks in the treatment were not presented in the control (where the AFB_1 was not subjected to alkaline conditions) (figure 2A). The molecular formula of modified-AFB_1 was established; the smallest mass peak was identified being m/z 301.25 corresponding to $C_{17}H_{16}O_5$

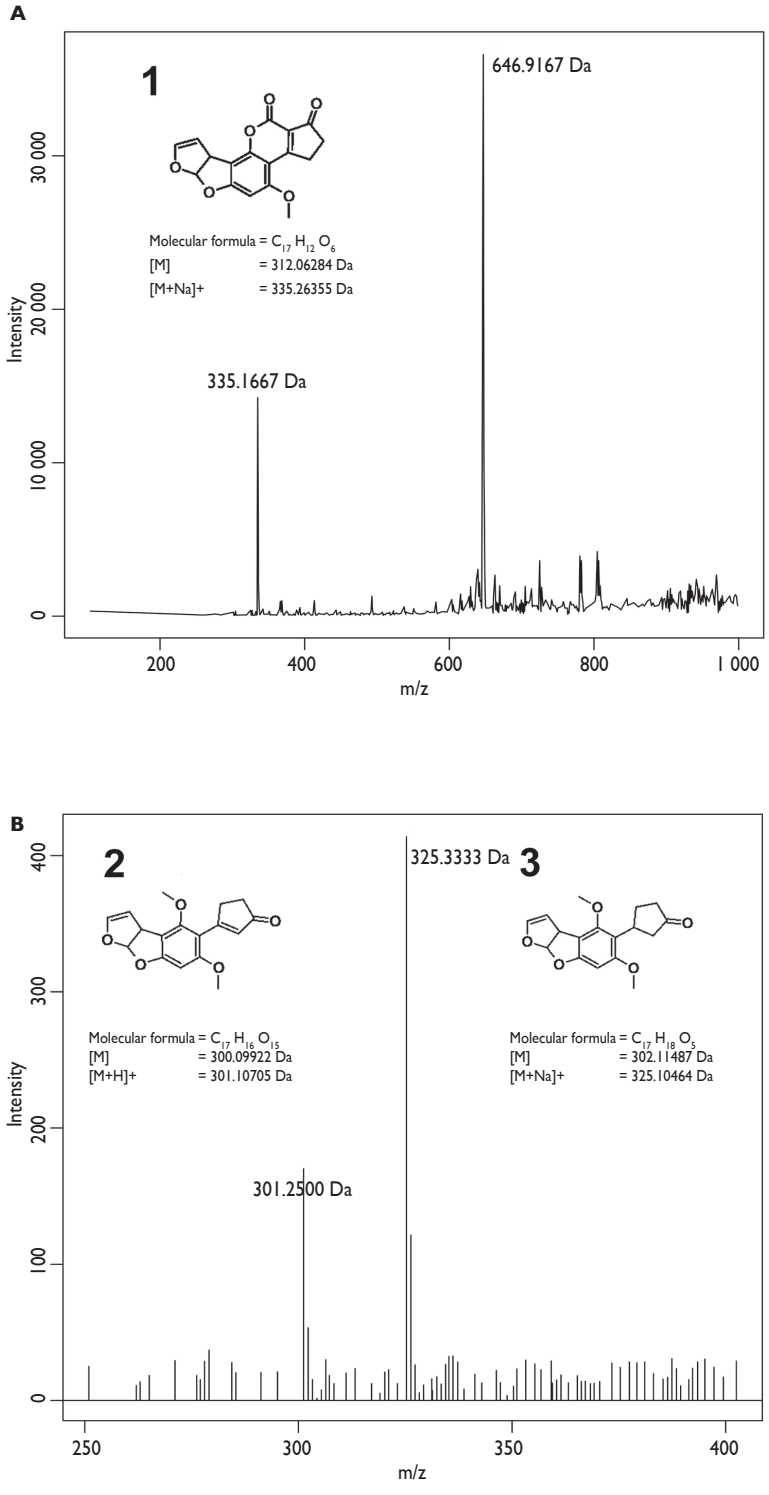


Figure 2. A. Reference spectrum of Aflatoxin B1: m/z 335.1667 shows a sodium adduct signal as well as a cluster with sodium was detected with a m/z of 646.9167 B. The spectrum indicates two picks resulted of the alkaline treatment. Molecule 2 corresponds to the m/z 301.2500 and molecule 3 m/z 325.333, molecules were calculated with the help of a draw molecular software showing two possible degradation by products

with a mass calculated of 300.30 (figure 2B-2). The largest mass peak was observed at m/z 325.33, the mass calculated for $C_{17}H_{18}O_5$ was 302.32, indicating a derivative of aflatoxin B_1 (figure 2B-3).

Discussion

Aflatoxin has been named, quite rightly, an invisible food hazard. Aflatoxin remnant contamination has been reported in *tortillas*,[11] and even when very small amounts of aflatoxin are consumed there is a risk, due to its accumulative effect. Chronic exposure to low levels of AFB_1 in patients with hepatitis B virus could contribute to the development of hepatocellular carcinoma.[23] In humans 1.4-2.3% of the AFB_1 ingested binds covalently to serum albumin, and ingestion of 2-6mg/day for a month can cause acute hepatitis and death.[24] Therefore, a procedure to obtain *tortilla* free of aflatoxin is very important, since its consumption has increased in the last years as a consequence of the migration of Latinamerican population, and the adoption of this food in those countries.

During the *tortilla* making process, two main degradation mechanisms of AFB_1 could be identified: one resulting from the alkaline pH of the maize-dough, the other due to the applied high temperature for cooking the flattened dough. Resting the dough at least 40 min (figure 1), at room temperature (24°C) at elevated pH (10.2) (tables I and II) eliminated nearby 100%, which indicates that alkalinity is the most effective component of the *tortilla* making process. The results strongly suggested that the effect of alkaline pH is the primary factor responsible for the AFB_1 destruction. The loss of fluorescence indicates a structure change of AFB_1 due to the alkaline conditions. It is well documented that alkalinity enhances the opening of the lactone ring of AFB_1 to form a substituted o-coumaric acid B.[17] However, also more drastic changes have been observed, for example, decarboxylation of the carboxylic acid and further decomposition.[17,25]

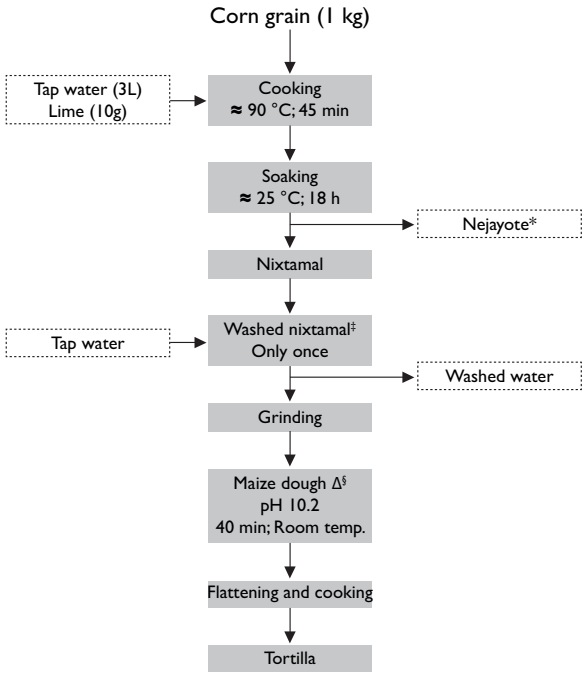
We observed similar effects: Highly alkaline conditions (pH 12.5) rapidly destroyed 100% of AFB_1 (table II). Those results confirmed the effectiveness of *nixtamalización* process in the reduction of AFB_1 as it has been reported by different authors.[11-13] However, several authors suggested that the non-fluorescent molecule formed during *nixtamalización* is able to reforming to AFB_1 when the *tortilla* reaches the stomach pH.[13,26] In opposition to this hypothesis, Anguiano-Ruvalcaba and colleagues[6] found that under gastrointestinal tract similar conditions (pH 1.5, 37 °C, and 30 min the time that food remains in the stomach) there is not a recovering of AFB_1. This conclusion is supported by Yates and colleagues.[27] They demonstrated by C-NMR that at low pH, the lactone rings closed but not via a simple reversal or the ring opening, rather, prolonged incubation times are necessary. Additionally, Anguiano-Ruvalcaba and colleagues[6] showed that young chicks fed with maize dough from *nixtamalization* did not present any symptoms compared with young chicks fed with contaminated dough. It is important to mention that only less of 10% remains during the *nixtamalización* since according with radiolabel AFB_1 assays, 90% is eliminated in the cooking liquid and the first wash.[11] In this way, only the remnant AFB_1 could have an accumulative effect in the consumers.

Temperature presented a stark contrast compared with alkalinity. It was observed that flattening and cooking of the thin maize-cake at 150°C during 5 min each side did not have any additional AFB_1 reduction. Higher decomposition levels can be obtained by cooking at 230 to 300°C.[28] Pérez-Flores *et al*.[29] used 270°C for 15 seconds to cook flattened maize-dough containing 69.62 ng AFB1/g, only 58% reduction in AFB_1 content was achieved (the pH of the maize-dough was 8.2). It is important to mention that temperatures above 150°C reduce the sensorial quality of the *tortillas*.

Three rinses produce a less alkaline maize-dough, Perez-Flores and colleagues[29] had suggested that higher amounts of lime should be used to obtain more alkaline maize-dough. However, addition of 1% lime and subsequent rinsing once with tap water produces a maize-dough with a pH about 10.2.

In order to find out the chemical modification that AFB_1 suffers during the process, samples containing AFB_1 standard under alkaline conditions were analized by mass spectrometry. The ionized AFB_1 has a monoisotopic weight of 335.27 m/z, corresponding to the sodium adduct (figure 2A). Two new signals at 301.25 m/z and 325.33 m/z (figures 2B-2 and 2B-3) indicated the generation of degradation products. Fragmentation of the ions was not possible due to their low abundance. However, both signals exhibit isotopic patterns which are consistent with compounds related to AFB_1. Revising possible chemical structures, we suggest the formation of compounds 2 and 3 (figure 2B), where compound 2 is known as aflatoxin D_1 a non-toxic one.[30]

In summary, the alkalinity of the maize-dough is the primary factor to aflatoxin removal. For this reason, toxin-free *tortillas* can be produced by alkaline conditions and at least 40 min resting time of the maize-dough. Changes in the procedure used to make *tortillas* (figure 3) and rigorous implementation of this regime is a simple but efficient



* *Nejayote* (cooking liquor) should be eliminated since it contains pericarp, where most of mycotoxins are eliminated (see discussion)

‡ *Nixtamal* has to be washed only once in order to preserve alkaline conditions (≈ pH 10.2)

§ Remnant AFB_1 is reduced during resting time of at least 15 min at room temperature

Figure 3. Proposed *tortilla* making process to remove 100% of AFB1 depicted

way of reducing or eliminating AFB_1 from maize-based foods. Our findings also might be transferred to reduce of AFB_1 of other maize food products.

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Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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