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Formation of Fumonisin B_1−Glucose Reaction Product, *in Vitro* Cytotoxicity, and Lipid Peroxidation on Kidney Cells

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Fumonisin B_1 (FB_1) content in corn products decreases during the heating process in foods containing reducing sugars, mainly because of the formation of *N*-(carboxymethyl)fumonisin B_1. In this study, a rapid method has been developed for the determination of both compounds in corn products using a high-speed blender, Ultra-Turrax, for solvent extraction and liquid chromatography−tandem mass spectrometry. The kinetics of FB_1 degradation and the formation of the Maillard adduct were studied in a model system constituted by corn bread spiked with FB_1 and heated at 160, 180, and 200 °C for 3, 6, 10, 15, and 20 min. FB_1 decreased from 0.96 to 0.3 mg/kg and *N*-(carboxymethyl)fumonisin B_1 increased to 0.1 mg/kg. Cytotoxicity and lipid peroxidation were studied in monkey kidney cells (Vero cells). After 24 h exposure, FB_1 revealed an IC_{50} (median inhibitory concentration) of 55 ± 7 μM with neutral red uptake, but no IC_{50} was obtained after *N*-(carboxymethyl)fumonisin B_1 exposure at the studied concentrations. Lipid peroxidation was assessed using the thiobarbituric acid reactive substance (TBARS) method for 90 min and 24 and 48 h. FB_1 significantly increased the production of malondialdehyde in Vero cells exposed to 1 μM FB_1 after 24 h, while malondialdehyde increased after 5 μM *N*-(carboxymethyl)fumonisin B_1 exposure. These findings showed that the transformation products exhibit lower cytotoxicity than fumonisin B_1 and lipid peroxidation may be involved in the cytotoxicity induced by both toxins.

KEYWORDS: Fumonisin B_1; *N*-(carboxymethyl)fumonisin B_1; thermally treated food; Maillard reaction; cytotoxicity; lipid peroxidation and reactive oxygen species

INTRODUCTION

Fumonisins are a group of mycotoxins produced by certain *Fusarium* species, which have elicited interest and concern due to their toxicity and ubiquitous occurrence in corn and corn products (*1*, *2*). Fumonisin B_1 (FB_1) is approximately 70% of total fumonisins found in naturally contaminated samples, being the most important one because of its toxicity and incidence (*3*).

FB_1 is a diester of tricarballylic acid whose backbone is a polyhydric alcohol that is very similar to the structure of sphinganine. This similarity led to the hypothesis that the FB_1 mechanism of action is the interruption of sphingolipid biosynthesis. Inhibition of ceramide synthase by FB_1 causes an increase of sphinganine levels and a decrease of ceramide and complex sphingolipid levels which mediate in several cellular functions including cell−cell communication, cell growth, differentiation, and transformation of cells (*4*, *5*). The interruption of sphingolipid synthesis causes apoptosis in liver and kidney of rodents and also in cultured human cell lines (*6*). Furthermore, fumonisins appear to increase the rate of oxidation, induce the free radical intermediate production, and accelerate the chain reactions associated with lipid peroxidation. Membrane damage caused by lipid peroxidation may also contribute to the oxidative damage of DNA, which has often been implicated in carcinogenesis (*7*).

FB_1 causes several animal diseases such as equine leukoencephalomalacia (ELEM), pulmonary edema and hepatic syndrome in swine, and hepatic alteration in cattle (*8*). This toxin fed to rats has caused chronic interstitial nephritis and hepatocellular carcinoma (*9*). In addition, contaminated corn with fumonisins intended for human consumption has been associated with a higher incidence of primary liver and esophageal carcinoma in South Africa and China (*10*, *11*). Therefore, based on toxicological evidence, the International Agency for Research on Cancer has established that FB_1 is potentially carcinogenic to humans (group 2B) (*12*).

Because FB_1 is a molecule with high toxicity, it is important to study its stability under food processing condition and the production of new degradation products. Most of the studies on mycotoxin stability and structural elucidation of thermal breakdown products are focused on model systems based on treatments with different temperatures. Some of these studies are degradation of ochratoxin A during coffee roasting (*13*) or nivalenol, T-2 toxin, and deoxynivalenol during the baking and cooking process (*14*−*16*). Some authors have reported that FB_1

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decreases during heat treatment and long cooking periods depending on temperature, exposure time, contamination level, and reducing sugar level (*17*). *N*-(Carboxymethyl)fumonisin B_1 is one of the principal reaction products of the Maillard reaction between FB_1 and glucose (through the aliphatic primary amine of FB_1) in foods containing reducing sugars after heat treatment (*18*, *19*). The Schiff base initially produced by the reaction of FB_1 and D-glucose undergoes through the Amadori rearrangement to the β-ketoamine which is oxidized to *N*-(carboxymethyl)fumonisin B_1. Other derivatives such as *N*-(1-deoxy-D-fructosyl-1-yl)fumonisin B_1 have been described as the first stable product of FB_1 with D-glucose (*20*).

The aim of the present study was, first, to study the kinetic of FB_1 degradation after bread processing conditions and to identify the thermal degradation product *N*-(carboxymethyl)fumonisin B_1 in a model heating system. For this purpose, a simple and fast analysis method was optimized to analyze FB_1 and *N*-(carboxymethyl)fumonisin B_1 based on a solvent extraction with Ultra-Turrax and determination with liquid chromatography/electrospray ionization (ESI) tandem mass spectrometry operating at positive ion mode. Additionally, the cytotoxicity of FB_1 and *N*-(carboxymethyl)fumonisin B_1 was evaluated in established monkey kidney cells (Vero cells) by the neutral red viability assay as well as the lipid peroxidation effect induced by both substances using the thiobarbituric acid reactive substance (TBARS) assay. TBARS determines the aldehydic end products, such as malondialdehyde, an end product formed from a variety of lipid peroxides and secondary lipid oxidation products.

Studies conducted to compare the toxicity of FB_1 and its derivatives from the Maillard reaction are very scarce, and to our knowledge *in vitro* toxicity effects of the FB_1 adduct with glucose in animal cells have not been reported before.

MATERIALS AND METHODS

General Remarks. FB_1 was produced from fungal culture. The strains of *Fusarium verticillioides* F5845 and F5839 obtained from the fungal culture collection of the Istituto Toxine e Micotossine de Parassiti Vegetali (ITEM), Bari, Italy, were cultivated in a potato dextrose broth (PDB) (Scharlau, Barcelona, Spain) at 25 °C. After 7 days, it was transferred to 500 g of autoclaved corn and incubated at 25 °C during 30 days. FB_1 was extracted from corn culture using an Ultra-Turrax T18 homogenizer (IKA Labortechnik, Staufen, Germany) with MeOH−H_2O (70:30), filtrated, evaporated to dryness using a Büchi Rotavapor R-200 (Labortechnik, Flawil, Switzerland) at 40 °C and 80 mbar, and reconstituted to a final volume of 10 mL with MeOH−H_2O (50:50 v/v). For isolation of FB_1, the extract obtained was passed through a semipreparative column packed with XAD-16 Amberlite (800 m^2/g, 100 Å) (Sigma, St. Louis, MO) and analyzed by liquid chromatography−tandem mass spectrometry (LC-MS/MS) to know the fraction window that contained the FB_1.

These obtained fractions were pooled, concentrated under vacuum, and applied to a semipreparative column (250 mm × 10 mm i.d., 5 μm, Phenomenex Gemini C_{18}) connected to a liquid chromatograph. Once more, fractioned extracts obtained at different times were injected into a LC-MS/MS to know which contained the pure FB_1 and then weighed after solvent evaporation with a nitrogen stream. FB_1 purity was compared with a FB_1 standard curve obtained from Sigma Chemical Co. (St. Louis, MO). Purified FB_1 from *Fusarium* strains was used for degradation studies, and FB_1 standards were selected for toxicity studies. Acetic acid, liquid chromatography (LC) grade methanol, and acetonitrile were supplied by Merck (Darmstadt, Germany). Formic acid was from Panreac (Barcelona, Spain). Deionized water (518 MΩ cm resistivity) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA).

***N*-(Carboxymethyl)fumonisin B_1 Production.** The production of *N*-(carboxymethyl)fumonisin B_1 was generated according to Howard et al. (*19*). Briefly, *N*-(carboxymethyl)fumonisin B_1 was produced at 78 °C in 20 mL containing 2 mM FB_1, 50 mM potassium phosphate, pH 7.5, and 150 mM glucose. The tubes were incubated at 74−78 °C in a block heater overnight (12 h). The solutions obtained were adjusted to approximately pH 4, then 3 mL was added to a C_{18} cartridge, and *N*-(carboxymethyl)-fumonisin B_1 was eluted with 2 mL of acetonitrile−25 mM HCOOH (1:1 v/v) and evaporated to dryness. The residue was reconstituted to a final volume of 1 mL with methanol−water (50:50 v/v). The *N*-(carboxymethyl)fumonisin B_1 structure was confirmed using the LC-MS/MS and contained 10% of FB_1.

Analysis of FB_1 and *N*-(Carboxymethyl)fumonisin B_1. For the kinetic study, 3 g of corn flour was mixed with 0.6 mL of water, kneading to make circular bread, spiked at 1 mg/kg of FB_1, and then left during 15 min at room temperature. No fermentation was done; thus the toast obtained was very thin. After that, the resulting toast was treated at 160, 180, and 200 °C for 3, 6, 10, 15, and 20 min. For the extraction of FB_1 and *N*-(carboxymethyl)fumonisin B_1, samples were homogenized for a few minutes with 20 mL of methanol−water (70:30 v/v) using an Ultra-Turrax T18. The homogenate was decanted into centrifuge tubes and spun at 2500*g* for 5 min, and the obtained extracts were transferred into a 50 mL round-bottomed flask and concentrated using a Büchi Rotavapor R-200 to about 5 mL. Then, the extract was transferred to a 15 mL conical tube and evaporated to dryness at 55 °C with a gentle stream of nitrogen using a multisample Turbovap LV evaporator (Zymark, Hopkinton, MA). The residue was reconstituted to a final volume of 1 mL with methanol−water (50:50 v/v) and filtered through a 25 mm/0.45 μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before their injection into the LC-MS/MS system.

LC-MS/MS. LC separation was carried out on a 150 mm × 4.6 mm i.d., 5 μm Luna C_{18} analytical column preceded by a 4 mm × 2 mm i.d., 5 μm guard column of the same material, both from Phenomenex (Madrid, Spain). FB's separation was performed using gradient elution with water and methanol, both containing 0.5% formic acid.

A TQ mass spectrometer Quattro LC from Micromass (Manchester, U.K.) equipped with an LC Alliance 2695 system (Waters, Milford, MA) was used. Triple quadrupole conditions used for FB_1 analysis were described by Silva et al. (*21*). The triple mass spectrometer was operated in selected monitoring mode (SRM), choosing as precursor ion for FB_1 the m/z 722 $[M + H]^+$ and m/z 352 and m/z 334 as product ions. For *N*-(carboxymethyl)fumonisin B_1 analysis, the precursor ion was m/z 780 $[M + H]^+$, and the product ion was m/z 410. Optimized cone voltage and collision energy were 50 V and 40 eV, respectively.

Experimental Solutions and Cell Culture. The reagent grade chemicals and cell culture components used, Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin−EDTA solution, HEPES, neutral red (NR) dye, thiobarbituric acid (TBA), deferoxamine mesylate salt (DFA), di-*tert*-butylmethylphenol (BHT), potassium phosphate, glucose, and dimethyl sulfoxide were Sigma products (Sigma Chemical Co., St. Louis, MO). Fetal calf serum (FCS) was from Cambrex Co. (Belgium).

Vero cells (ATCC CCL-81), kidney epithelial cells derived from the African green monkey *Cercopithecus aethiops*, were cultured in the monolayer in 9 cm^2 polystyrene tissue culture dishes with DMEM supplemented with 25 mM HEPES buffer (pH 7.4), 10% heat inactivated FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin. Incubation conditions were 37 °C, 5% CO_2, and 95% relative humidity atmosphere.

Cytotoxicity Tests. Lysosomal function was estimated according to the relative uptake of NR by measuring the incorporation of the water-soluble dye into lysosomes. Vero cells were cultured into 96-well tissue culture plates by adding 0.2 mL/well of a suspension of 2×10^4 cells/mL. After the cells reached 65% confluence, the medium was replaced with fresh medium containing toxins at different concentrations (1−100 μM) and incubated for 24 h at 37 °C. NR assays were performed as previously described (*22*). Briefly, culture medium was replaced by 200 μL of fresh culture medium at 50 μg/mL NR, and the microplate was returned to the incubator for another 3 h to allow the uptake of NR into the lysosomes of viable cells. After that, medium was removed, and cells were fixed for 1 min with a formaldehyde−$CaCl_2$ solution. The NR absorbed by the cells was extracted after adding 0.2 mL of acetic acid−ethanol solution to the wells, and absorbance was measured at 540 nm with an automatic ELISA reader (Multiscan Labsystems, Helsinki, Finland).

Lipid Peroxidation Assay. Lipid peroxidation was performed by measuring malondialdehyde as described by Ferrer et al. (*23*). Cells were grown to 65% confluence in six-well plates, then the culture medium was replaced, and cells were treated with each compound (1, 5, and 10 μM)

individually for 90 min and 24 and 48 h of incubation. After that, the medium was removed, and cells were washed twice with phosphate-buffered saline (PBS), homogenized in 0.5 mL of 20 mM Tris and 0.1% Triton, then transferred to glass centrifuge tubes, and boiled (100 °C water bath for 30 min) under acid conditions in the presence of 0.5 mL of 0.5% TBA, 5 μL of 1.5 mM DFA, and 5 μL of 3.75% BHT. Samples were cooled and centrifuged (1287*g*, 15 min), and the absorbance was measured at 532 nm. Results were expressed as nanograms of malondialdehyde per milligram of protein. Determinations were made in quadruplicate.

The total protein content (μg/mL) was determined by the Bradford method (*24*) using the Bio-Rad DC protein assay at 690 nm.

Statistical Analysis. Statistical analysis of data was carried out using the SPSS version 13 (SPSS, Chicago, IL) statistical software package. Data were expressed as mean ± SD of four independent experiments. The statistical analysis of the results was performed by Student's *t*-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HSD posthoc test for multiple comparisons. Different levels of signification ($P \geq 0.05$ to $P \geq 0.0001$) in the MDA assay were considered statistically significant.

RESULTS AND DISCUSSION

Analytical Quality of the Method. Many of the FB_1 analysis methods reported use fluorescence detectors (*21*). As fumonisins do not have any suitable chromophores, they must be derivatized for their fluorescence detection with precolumn derivatives such as *o*-phthalaldehyde (OPA) or naphthalene-2,3-dicarboxaldehyde (NDA). However, the Maillard reaction product formed with a primary amine of FB_1 and reducing sugars does not react with OPA or NDA, so they cannot be analyzed with fluorescence detectors (*19*). **Figure 1** illustrates the structures of FB_1 and *N*-(carboxymethyl)fumonisin B_1. The two-stage mass spectrometry process (MS/MS) provides sensitivity and selectivity in fumonisin quantification. Triple quadrupole (QqQ) MS was selected as it is the most widely employed analyzer because it provides a good precision and accuracy when working in SRM (*21*).

After heating the dough obtained with corn flour spiked with FB_1 and water, a nonenzymatic browning reaction product was obtained with signals at *m*/*z* 428, *m*/*z* 392, and *m*/*z* 410 in PI mode, which corresponds to the *N*-(carboxymethyl)fumonisin B_1 spectrum (*25*). This fact was confirmed after analysis of *N*-(carboxymethyl)fumonisin B_1 synthesized as previously described. For the analysis of FB_1 and its adduct, protonated molecules (*m*/*z* 722) for FB_1 and (*m*/*z* 780) for *N*-(carboxymethyl)fumonisin B_1 were mass-selected by the first quadrupole and fragmented after collision-induced dissociation (CID). These transition ions are generated by the loss of water and tricarballylic acid (TCA) side chains from the alkyl backbone. For acquisition in SRM mode, the ions selected were *m*/*z* 352 − $[M + H - 2TCA - H_2O]^+$ and *m*/*z* 334 − $[M + H - 2TCA - 2H_2O]^+$ for FB_1 and *m*/*z* 410 − $[M + H - 2TCA - H_2O]^+$ for *N*-(carboxymethyl)fumonisin B_1.

A calibration curve was constructed for FB_1 and *N*-(carboxymethyl)fumonisin B_1 in a concentration range between 5 and 5000 μg/L with correlation coefficients higher than 0.9997. Precision was assayed by analyzing five spiked samples on the same day and on five different days. Relative standard deviations (RSDs) of FB_1 and *N*-(carboxymethyl)fumonisin B_1 were 4 and 6 for run-to-run precision and 10 and 12 for day-to-day precision, respectively.

The simultaneous determination of FB_1 and *N*-(carboxymethyl)fumonisin B_1 on corn flour and corn products was studied by means of an analytical method using an Ultra-Turrax homogenizer and LC-MS/MS with a triple quadrupole (QqQ) analyzer. Mean recovery obtained from cornbread-spiked samples at four levels ranged 70−87% for FB_1 and 67−86% for *N*-(carboxymethyl)fumonisin B_1, and RSDs were below 10% and 13%, respectively. Under the experimental conditions, the limits of detection (LODs) were 4 μg/kg for FB_1 and 10 μg/kg for *N*-(carboxymethyl)fumonisin B_1, and the limits of quantification (LOQs) were 12 μg/kg for FB_1 and 30 μg/kg for *N*-(carboxymethyl)fumonisin B_1. LODs correspond to the lowest concentration that gives a signal-to-noise ratio (S/N) of 3, and LOQs were calculated based on a signal-to-noise ratio (S/N) of 10. For quantification purpose, standards prepared in matrix samples were used since matrix effects cause enhancement or suppression of fumonisin response in LC-MS/MS (*21*). A LC-MS/MS chromatogram of a cornbread sample spiked at 0.1 mg/kg of FB_1 and 0.1 mg/kg of *N*-(carboxymethyl)fumonisin B_1 is illustrated in **Figure 2**.

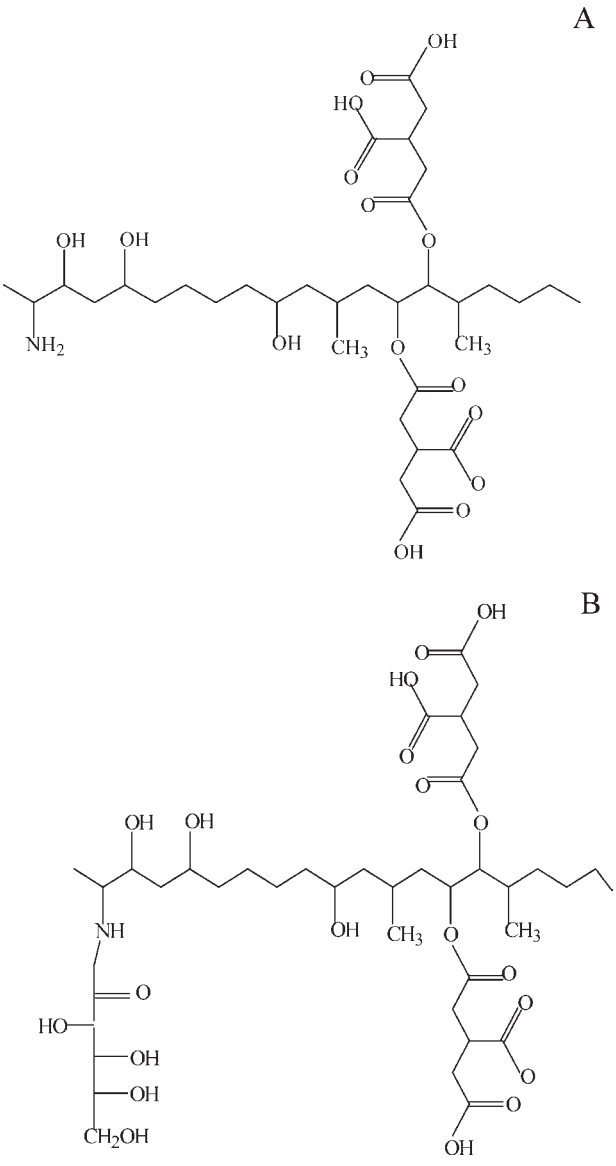

Figure 1. Structure of (**A**) FB_1 and (**B**) *N*-(carboxymethyl)fumonisin B_1.

Kinetic Studies. In an attempt to study the kinetics of degradation of FB_1 and the formation of the Maillard adduct *N*-(carboxymethyl)fumonisin B_1 under food processing conditions such as baking, different cornbreads spiked at 1 mg/kg of FB_1 were made at the laboratory. The analysis of the samples by LC-MS/MS revealed a degradation product among FB_1 and a reducing sugar. The experimental process was performed without adding glucose reproducing the cornmeal baking process. As no glucose was added, the product formed is considering that 0.5% of free glucose present in corn flour has reacted with the

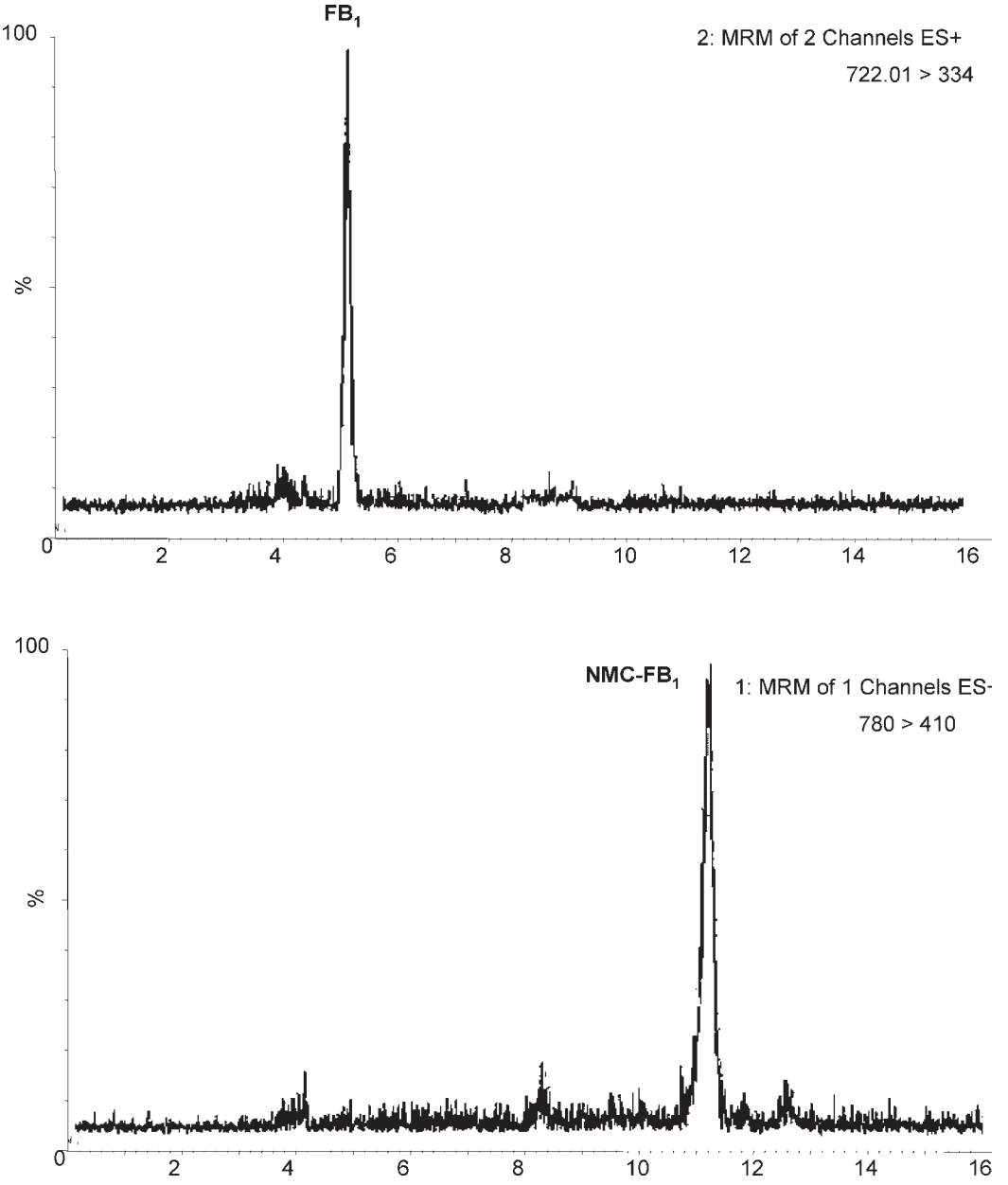

Figure 2. LC-MS/MS chromatogram of cornbread sample spiked at 0.1 mg/kg FB_1 and 0.1 mg/kg *N*-(carboxymethyl)fumonisin B_1.

fumonisin. The parameters varied were temperature (160, 180, and 200 °C) and time (3, 6, 10, 15, and 20 min). The reduction of FB_1 and formation of *N*-(carboxymethyl)fumonisin B_1 are proportional to the increment of temperature and time. Graphs with the results obtained are shown in **Figure 3**. The strongest degradation was found at 200 °C after 20 min with only 8% of the FB_1 left and the formation of 88 μg/kg of *N*-(carboxymethyl)fumonisin B_1. After 20 min at 160 °C, FB_1 decreased 53% and also was detected 52 μg/kg of *N*-(carboxymethyl)-fumonisin B_1. Previous reports that evaluated the production of *N*-(carboxymethyl)fumonisin B_1 in raw corn samples spiked with FB_1 treated under industrial conditions have shown that the addition of glucose and fructose increased the formation of the adduct whereas sucrose (nonreducing sugar) addition showed no reaction product (*19*, *25*). Castelo et al. (*26*) studied the effect of added sugars on FB_1 stability in extruded corn grits to produce breakfast cereals and snack food. The addition of glucose resulted in an important reduction of fumonisins (45−92%) in certain temperature, shear, and pressure conditions.

Howard et al. (*19*) indicated that the formation of *N*-(carboxymethyl)fumonisin B_1 is favored by alkaline conditions (pH > 7), requires molecular oxygen, and is catalyzed with several sugars whose ability to induce adduct formation is associated to the tendency to undergo nucleophilic attack on the carbonyl carbon. In a survey of corn products from German markets, six out of nine samples were contaminated with *N*-(carboxymethyl)-fumonisin B_1 (9.6−76 ng/g) (*25*).

Cytotoxicity Studies. In order to compare the cytotoxicity and peroxidation potential of FB_1 and *N*-(carboxymethyl)fumonisin B_1, neutral red and lipid peroxidation assays were performed simultaneously at the same conditions. **Figure 4** shows the concentration−response curves of Vero cells after 24 h exposure of FB_1 and *N*-(carboxymethyl)fumonisin B_1. The median concentration required for inhibition of cell growth by 50% (IC_{50}) was 55 ± 7 μM for FB_1, and no IC_{50} was detected for *N*-(carboxymethyl)fumonisin B_1 after 24 h exposure at the concentrations assayed. These results indicate that *N*-(carboxymethyl)fumonisin B_1 is less cytotoxic than FB_1. The presence in

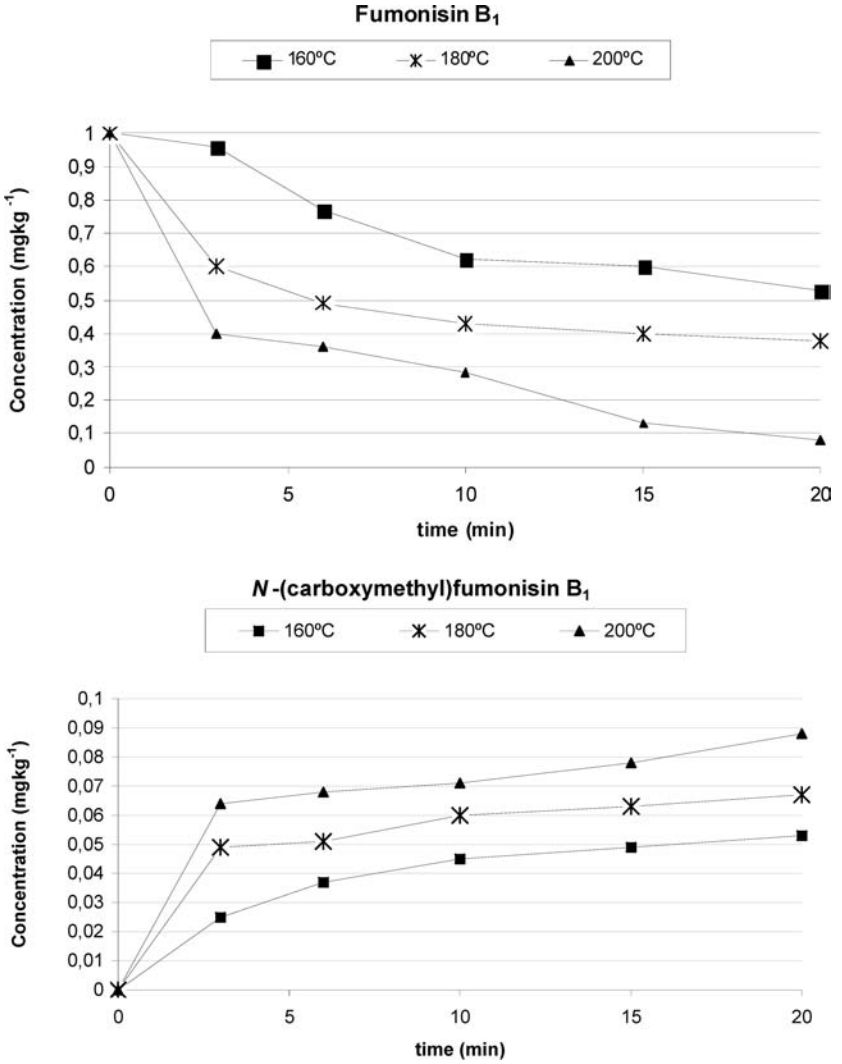

Figure 3. Effect of heat treatment on the degradation of FB_1 and production of *N*-(carboxymethyl)fumonisin B_1 in corn bread.

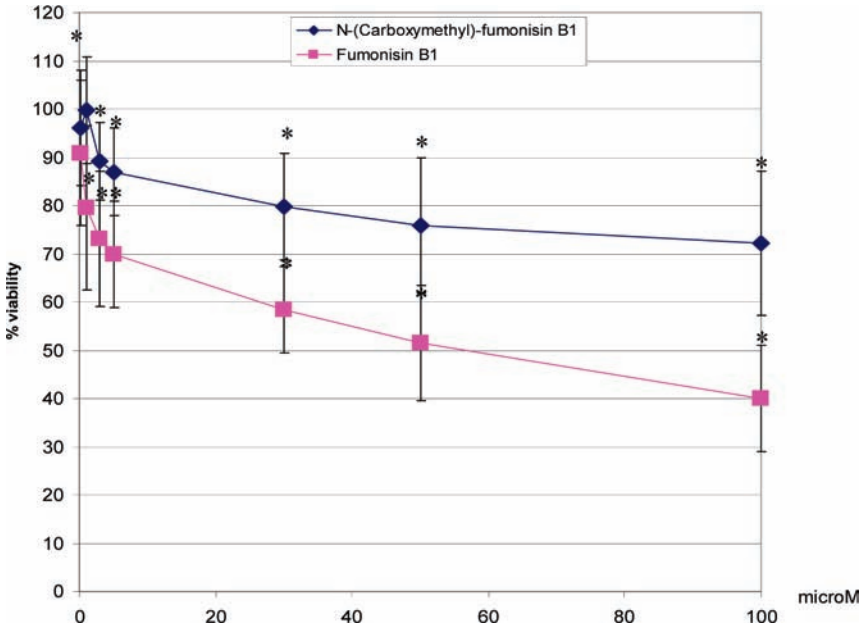

Figure 4. Inhibition of cell viability on Vero cells after 24 h exposure with FB_1 and *N*-(carboxymethyl)fumonisin B_1 by neutral red assay. Data expressed as percentage of the unexposed controls using six replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).

FB_1 of the amino group and the position of the hydroxyl group on C14/C15 have an important role in both the toxic and cancer initiating activities of FB_1. Moreover, the amino group facilitates the conjugation of FB_1 to protein carriers. The reaction of FB_1

with the reducing sugar D-glucose to form *N*-(carboxymethyl)-fumonisin B_1 can block the primary amine group of FB_1 and may detoxify this mycotoxin (*18*). In previous studies, FB_1 decreased cell viability of rabbit kidney cells (*27*), human esophageal epithelial cell (*28*), GT1−7 hypothalamic cells (*4*), and keratinocytes, but in other studies only incubations with high concentrations of FB_1 for 24 h induced a small decrease in the viability of C6 glioma cells and SHSY-5Y neuroblastoma (*4*). No cytotoxicity effects have been observed in the cerebral cell aggregate culture, in rat astrocytes, or in human fibroblast after FB_1 exposure (*4*).

FB_1 toxicity mechanism is based in the disruption of sphingolipid biosynthesis by the inhibition of ceramide synthase. Riley et al. (*5*) observed that the accumulation of sphinganine levels in the cell culture after FB_1 exposure at low levels was noncytotoxic due to their elimination, but prolonged exposure at high levels caused an increased cell death, being the disruption of sphingolipid metabolism a specific cytotoxic response.

In vivo studies of FB_1 acute toxicity are very scarce; generally, FB_1 is considered not acutely toxic through the oral route. McKean reported that male F344 rats by gavage with ≤46.4 mg/kg bw did not result in mortality; however, apparent toxic symptoms were noted (*29*). Howard et al. reported that FB_1 is a renal carcinogen in male F244/N rats and a hepatocarcinogen in female B6C3F1 mice when fed a concentration higher than 70 μmol/ kg; these effects were not observed after *N*-(carboxymethyl)fumonisin B_1 administration (*30*).

Several *in vivo* studies of acute and subacute effects of FB_1 and FB_1−glucose reaction products in swine and rats demonstrated that FB_1−glucose reaction products reduce FB_1 toxicity (*30*−*32*). In another study, a single screw extrusion of corn contaminated with FB_1 and glucose resulted in a significant reduction on FB_1 toxicity when fed to rats (*33*). There are no *in vitro* studies of *N*-(carboxymethyl)fumonisin B_1 cytotoxicity so it is difficult to compare our results with previous data, as only a short-term bioassay using brine shrimp (*Artemia salina*) showed a 100-fold reduced toxicity compared to FB_1 (*34*).

Lipid Peroxidation. Lipid peroxidation affects plasma membrane integrity as well as the nucleus and other cellular organelle membranes. FB_1 and *N*-(carboxymethyl)fumonisin B_1 lipid peroxidation was measured as malondialdehyde-production in Vero cells exposed to 1, 5, and 10 μM of each compound after 90 min and 24 and 48 h (**Figure 5**). Lipid peroxidation was increased in a concentration- and time-dependent manner after FB_1 exposure but slightly increased after *N*-(carboxymethyl)fumonisin B_1 exposure. After culture times of 90 min, increased malondialdehyde levels ranging from 47% to 153% of the control were observed in cells treated at dose levels of 5 and 10 μM FB_1, respectively ($P \leq 0.000$). However, increased malondialdehyde levels were observed at the three levels assayed, after 24 h from 24% to 197% ($P \leq 0.000$) and after 48 h from 106% to 329% of the control ($P \leq 0.000$). *N*-(Carboxymethyl)fumonisin B_1 induced lipid peroxidation at the three periods tested but only with two levels. At 5 μM, malondialdehyde levels increased from 20% ($P \leq 0.05$) to 38% ($P \leq 0.000$) of control and at 10 μM increased from 49% ($P \leq 0.000$) to 66% ($P \leq 0.000$) of control.

Tukey post hoc comparison indicates that the malondialdehyde production was statistically significant with FB_1 from concentrations of 1 μM up at 48 h exposure, whereas this effect was observed in *N*-(carboxymethyl)fumonisin B_1 only from 5 μM. The data clearly showed that the most effective in malondialdehyde production was FB_1 compared to *N*-(carboxymethyl)fumonisin B_1. In previous studies, increased malondialdehyde concentrations were obtained in SHSY-5Y cells after 24 h incubation with 100 μM FB_1 or after 72 h with 10 μM FB_1, as well as in GT1−7 and C6 cells exposed to 100 μM FB_1 for 144 h (*4*) and in Vero cells exposed to 0.14 μM FB_1 after 24 h (*7*).

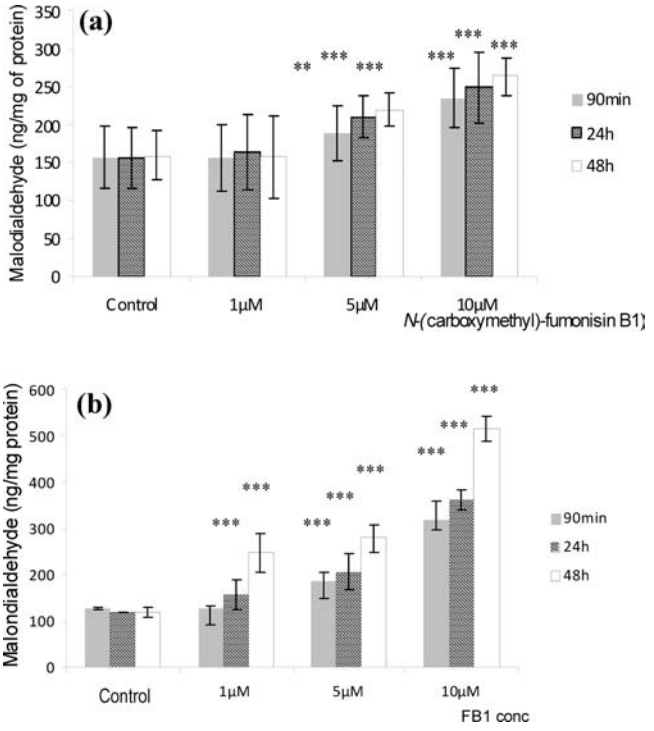

Figure 5. Malondialdehyde production by Vero cells exposed to 1, 5, and 10 μM (a) FB_1 and (b) *N*-(carboxymethyl)fumonisin B_1 during 90 min and 24 and 48 h. Results are expressed as the mean ± SD in ng of malondialdehyde/mg of protein measured by the Bradford method. Significantly different from the control: $P \leq 0.05$ (*), $P \leq 0.001$ (**), and $P \leq 0.000$ (***).

FB_1 induces lipid peroxidation in Vero cells to an extent that could alter the structure and function of the cellular membrane and block cellular metabolism leading to cytotoxicity. Lipid peroxidation may also be related to the disturbance of cell signaling processes, genotoxicity, mutagenicity, and tumor promotion caused by FB_1, since it has been shown that the diene conjugates formed during lipid peroxidation can interact with DNA (*7*). Mobio et al. (*35*) found that FB_1 could reduce C6 glioblastoma cell viability after 24 h, but cell death could be prevented by lipid-soluble antioxidant (vit E). These experiments confirmed the involvement of FB_1 lipid peroxidation on the cytotoxicity process that is also caused by sphingolipid accumulation. In other studies, *in vitro* findings in which FB_1 induced peroxidation in primary hepatocytes supported the increased level of TBARS in the liver of rats fed with different dietary levels of FB_1 (*36*). After simulating baking conditions in a spiked corn flour, the degradation of FB_1 and the formation of *N*-(carboxymethyl)fumonisin B_1 were demonstrated at different times and temperatures. The present results showed that FB_1 increased lipid peroxidation on Vero cells as well as decreased cell viability. However, *N*-(carboxymethyl)fumonisin B_1 is significantly less cytotoxic, and higher doses are required to induce lipid peroxidation compared to FB_1. These results corroborate previous studies concluding that the reaction of FB_1 and D-glucose produces a less toxic product *N*-(carboxymethyl)-fumonisin B_1, so heat treatment might be considered as a possible way for the detoxification of fumonisin-contaminated corn.

LITERATURE CITED

(1) Marasas, W. F. O. Discovery and occurrence of the fumonisins: A historical perspective. *Environ. Health Perspect.* **2001**, *109*, 230–243.

(2) D'Arco, G.; Fernández-Franzón, M.; Font, G.; Damiani, P.; Mañes, J. A survey of fumonisins B_1, B_2 and B_3 in conventional and organic retail corn products in Spain and Italy and estimated dietary exposure. *Food Addit. Contam.: Part B* **2009**, *2*, 146–153.
(3) Soriano, J. M.; Dragacci, S. Occurrence of fumonisins in foods. *Food Res. Int.* **2004**, *37*, 985–1000.
(4) Stockmann-Juvala, H.; Savolainen, K. A review of the toxic effects and mechanisms of action of fumonisin B_1. *Hum. Exp. Toxicol.* **2008**, *27*, 799–809.
(5) Riley, R. T.; Enongene, E.; Voss, K. A.; Norred, W. P.; Meredith, F. I.; Sharma, R. P.; Spitsbergen, J.; Williams, D. E.; Carlson, D. B.; Merrill, A. H., Jr. Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. *Environ. Health Perspect.* **2001**, *109*, 301–308.
(6) Dragan, Y. P.; Bidlack, W. R.; Cohen, S. M.; Goldsworthy, T. L.; Hard, G. C.; Howard, P. C.; Riley, R. T.; Voss, K. A. Implications of apoptosis for toxicity, carcinogenicity, and risk assessment: fumonisin B(1) as an example. *Toxicol. Sci.* **2001**, *61*, 6–17.
(7) Abado-Becognee, K.; Mobio, T. A.; Ennamany, R.; Fleurat-Lessard, F.; Shier, W. T.; Badria, F.; Creppy, E. E. Cytotoxicity of fumonisin B_1: implication of lipid peroxidation and inhibition of protein and DNA syntheses. *Arch. Toxicol.* **1998**, *72*, 233–236.
(8) Hussein, H. S.; Brasel, J. M. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* **2001**, *167*, 101–134.
(9) Domijan, A.; Zeljezic, D.; Peraica, M.; Kovacevic, G.; Gregorovic, G.; Krstanac, Z.; Horvatin, K.; Kalafatic, M. Early toxic effects of fumonisin B_1 in rat liver. *Hum. Exp. Toxicol.* **2008**, *27*, 895–900.
(10) Wagacha, J. M.; Muthomi, J. W. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *Int. J. Food Microbiol.* **2008**, *124*, 1–12.
(11) Sun, G.; Wang, S.; Hu, X.; Su, J.; Huang, T.; Yu, J.; Tang, L.; Gao, W.; Wang, J. S. Fumonisin B1 contamination of home-grown corn in high-risk areas for esophageal and liver cancer in China. *Food Addit. Contam.* **2007**, *24*, 181–185.
(12) IARC. Fumonisin B_1. In: *IARC monographs on the evaluation of carcinogenic risks to humans, some traditional herbal medicines, some mycotoxins, naphthalene and styrene*; IARC: Lyon, France, 2002; Vol. 82, pp 301−366.
(13) Cramer, B.; Königs, M.; Humpf, H. U. Identification and in vitro cytotoxicity of ochratoxin A degradation products formed during coffee roasting. *J. Agric. Food Chem.* **2008**, *56*, 5673–5681.
(14) Bretz, M.; Knecht, A.; Göckler, S.; Humpf, H. U. Structural elucidation and analysis of thermal degradation products of the *fusarium* mycotoxin nivalenol. *Mol. Nutr. Food Res.* **2005**, *49*, 309–316.
(15) Bretz, M.; Beyer, M.; Cramer, B.; Knecht, A.; Humpf, H.-U. Thermal degradation of the *fusarium* mycotoxin deoxynivalenol. *J. Agric. Food Chem.* **2006**, *54*, 6445–6451.
(16) Beyer, M.; Ferse, I.; Mulac, D.; Würthwein, E.-U.; Humpf, H.-U. Structural elucidation of T-2 toxin thermal degradation products and investigations toward their occurrence in retail food. *J. Agric. Food Chem.* **2009**, *57*, 1867–1875.
(17) Jackson, L. S.; Katta, S. K.; Fingerhut, D. D.; DeVries, J. W.; Bullerman, L. B. Effects of baking and frying on the fumonisin B_1 content of corn-based foods. *J. Agric. Food Chem.* **1997**, *45*, 4800–4805.
(18) Lu, Y.; Clifford, L.; Hauck, C. C.; Hendrich, S.; Osweiler, G.; Murphy, P. A. Characterization of fumonisin B(1)-glucose reaction kinetics and products. *J. Agric. Food Chem.* **2002**, *50*, 4726–4733.
(19) Howard, P. C.; Churchwell, M. I.; Couch, L. H.; Marques, M. M.; Doerge, D. R. Formation of *N*-(carboxymethyl)fumonisin B1, following the reaction of Fumonisin B_1 with reducing sugars. *J. Agric. Food Chem.* **1998**, *46*, 3546–3557.
(20) Poling, S. M.; Plattner, R. D.; Weisleder, D. *N*-(1-deoxy-D-fructos-1-yl)fumonisin B(1), the initial reaction product of fumonisin B(1) and D-glucose. *J. Agric. Food Chem.* **2002**, *50*, 1318–1324.
(21) Silva, L.; Fernández-Franzón, M.; Font, G.; Pena, A.; Silveira, I.; Lino, C.; Mañes, J. Analysis of fumonisins in corn-based food by liquid chromatography with fluorescence and mass spectrometry detectors. *Food Chem.* **2009**, *112*, 1031–1037.
(22) Fernández, M.; Ríos, J. C.; Jos, A.; Repetto, G. Comparative cytotoxicity of alachlor on RTG-2 trout and SH-SY5Y human cells. *Arch. Environ. Contam. Toxicol.* **2006**, *51*, 515–520.
(23) Ferrer, E.; Juan-García, A.; Font, G.; Ruiz, M. J. Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. *Toxicol. in Vitro* **2009**, *23*, 1504–1509.
(24) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(25) Seefelder, W.; Hartl, M.; Humpf, H. U. Determination of *N*-(carboxymethyl)fumonisin B(1) in corn products by liquid chromatography/electrospray ionization−mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 2146–2151.
(26) Castelo, M. M.; Jackson, L. S.; Hanna, M. A.; Reynolds, B. H.; Bullerman, L. B. Loss of fumonisin B1 in extruded and baked corn-based foods with sugars. *J. Food Sci.* **2001**, *66*, 416–421.
(27) Rumora, L.; Kovacić, S.; Rozgaj, R.; Cepelak, I.; Pepeljnjak, S.; Zanić Grubisić, T. Cytotoxic and genotoxic effects of fumonisin B_1 on rabbit kidney RK13 cell line. *Arch. Toxicol.* **2002**, *76*, 55–61.
(28) Bouhet, S.; Hourcade, E.; Loiseau, N.; Fikry, A.; Martinez, S.; Roselli, M.; Galtier, P.; Mengheri, E.; Oswald, I. P. The mycotoxin fumonisin B_1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. *Toxicol. Sci.* **2004**, *77*, 165–171.
(29) McKean, C.; Tang, L.; Tang, M.; Billam, M.; Wang, Z.; Theodorakis, C. W.; Kendall, R. J.; Wang, J.-S. Comparative acute and combinative toxicity of aflatoxin B1 and fumonisin B_1 in animals and human cells. *Food Chem. Toxicol.* **2006**, *44*, 868–876.
(30) Howard, P. C.; Couch, L. H.; Patton, R. E.; Eppley, R. M.; Doerge, D. R.; Churchwell, M. I.; Marques, M. M.; Okerberg, C. V. Comparison of the toxicity of several fumonisin derivatives in a 28-day feeding study with female B6C3F(1) mice. *Toxicol. Appl. Pharmacol.* **2002**, *185*, 153–165.
(31) Fernández-Surumay, G.; Osweiler, G. D.; Yaeger, M. J.; Hauck, C. C.; Hendrich, S.; Murphy, P. A. Glucose reaction with fumonisin B_1 partially reduces its toxicity in swine. *J. Agric. Food Chem.* **2004**, *52*, 7732–7739.
(32) Fernández-Surumay, G.; Osweiler, G. D.; Yaeger, M. J.; Rottinghaus, G. E.; Hendrich, S.; Buckley, L. K.; Murphy, P. A. Fumonisin B-glucose reaction products are less toxic when fed to swine. *J. Agric. Food Chem.* **2005**, *53*, 4264–4271.
(33) Voss, K. A.; Bullerman, L. B.; Bianchini, A.; Hanna, M. A.; Ryu, D. Reduced toxicity of fumonisin B_1 in corn grits by single-screw extrusion. *J. Food Prot.* **2008**, *71*, 2036–2041.
(34) Hartl, M.; Humpf, H.-U. Toxicity assessment of fumonisins using the brine shrimp (*Artemisa salina*) bioassay. *Food Chem. Toxicol.* **2000**, *38*, 1097–1102.
(35) Mobio, T. A.; Baudrimont, I.; Sanni, A.; Shier, T. W.; Saboureau, D.; Dano, S. D.; Ueno, Y.; Stey, P. S.; Creppy, E. E. Prevention by vitamin E and DNA fragmentation and apoptosis induced by fumonisin B1 in C6 glioma cells. *Arch. Toxicol.* **2000**, *74*, 112–119.
(36) Abel, S.; Gelderblom, W. C. Oxidative damage and fumonisin B_1-induced toxicity in primary rat hepatocytes and rat liver in vivo. *Toxicology* **1998**, *131*, 121–131.

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