12042 *J. Agric. Food Chem.* **2010,** *58,* 12042–12047
DOI:10.1021/jf103799q

JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

In Vitro Digestion Assay for Determination of Hidden Fumonisins in Maize

Chiara Dall'Asta, Claudia Falavigna, Gianni Galaverna,*
Arnaldo Dossena, and Rosangela Marchelli

Dipartimento di Chimica Organica e Industriale, Università degli Studi di Parma,
Parco Area Scienze 17/A, 43124 Parma, Italy

Hidden fumonisins have received great attention in the last years as they have been frequently found in maize products in addition to the free forms. Several papers have shown that interaction with macromolecular components such as protein and starch is at the base of the phenomenon: although the nature of the interaction (covalent or not) is still not clarified, the occurrence of hidden forms is generally revealed by the application of an alkaline hydrolysis procedure. In this study, an *in vitro* digestion model has been applied to raw maize to evaluate the possible release of hidden fumonisins under gastrointestinal conditions. Upon digestion of the food matrix, an increased amount of total detectable fumonisins was observed in comparison with the analysis on the nondigested matrix, an amount even higher than that calculated through the application of the hydrolysis procedure. Besides the analytical issues, our data have serious implications, since consumers may be exposed to a systematic higher risk than that estimated by conventional techniques.

KEYWORDS: Hidden fumonisins; *in vitro* digestion; bioaccessibility; *Zea mays*; *Fusarium*

INTRODUCTION

Fumonisins are secondary metabolites produced by a number of *Fusarium* species, mainly *Fusarium verticillioides*, *Fusarium nygamai*, and *Fusarium proliferatum* (*1*, *2*). Their toxicity is due to the structural similarity with sphingosine, which leads to the inhibition of ceramide synthase, causing the disruption of the biosynthesis of sphingolipids (*3*). Indeed, fumonisins are able to induce several diseases in animals, such as leukoencephalomalacia in horses (ELEM) and pulmonary edema in swine (PPE), and they are considered as an environmental risk factor for birth defects known as neural tube defects (NTDs) in humans (*4*, *5*). Since 2007, legal limits were established for fumonisins in raw commodities and food for human consumption in EU (EC No. 1126/2007) (*6*).

Fumonisins are relatively heat stable up to 100 °C, although it is known that processing induces significant decrease of the detectable mycotoxins: chemical degradation may take place via Maillard-type reactions at high temperature or hydrolysis via loss of the two tricarballylic moieties in the presence of alkali. Indeed, hydrolyzed fumonisins frequently occur in thermally treated plus alkali-treated products (tortillas and other nixtamalized products). Recent results have shown that their decrease might be due not only to chemical degradation but also to possible modifications of the mycotoxin structure by interaction with other food components, especially in thermally extruded products (corn flakes) (*7*). The presence of matrix-bound fumonisins, which escape routine analytical determinations, is a potential food safety concern as there is the possibility that, in addition to the fumonisins as such, hydrolyzed fumonisins or bound forms could be released upon gastrointestinal digestion, thus contributing to the overall toxicity. Indeed, in the past few years, several publications have demonstrated the presence of fumonisins potentially bound or strongly associated with proteins or other food components, which escape conventional analysis and can be determined only in an indirect way through the application of a hydrolysis step (*8*−*10*). Indeed, it has been observed that performing alkaline hydrolysis of contaminated corn products (especially, extruded products such as corn flakes) the amount of released hydrolyzed fumonisins was often higher than that stoichiometrically derived by the conversion of the fumonisins detectable by the routine analytical methods.

A possible explanation of these findings was proposed on the basis of *in vitro* experiments using methyl α-D-glucopyranoside and protected amino acids as model compounds for starch and proteins, respectively (*11*), which demonstrated the possibility of covalent bond formation between the tricarballylic moiety and hydroxyl groups of carbohydrates or amino groups of amino acids. Although many experiments revealed the presence of hidden forms via the indirect method of alkaline hydrolysis, direct experimental evidence of the occurrence of these covalently bound forms in foods was not obtained yet (*12*, *13*).

In this context, several authors have shown that, besides thermal effect that could give rise to covalent bond formation, there is also another masking phenomenon, based probably on a physical entrapment of the mycotoxins into the structure of macromolecular components (such as starch) (*8*, *9*, *14*), which may have strong influence on the accuracy of fumonisin measurement. Indeed, low recoveries and low accuracies were obtained using IAC cleanup, unless particular attention is not paid to an extractable,

*Corresponding author (tel, +390521906196; fax, +390521905472; e-mail, gianni.galaverna@unipr.it).

 © 2010 American Chemical Society

starch-bound fumonisin that behaved differently from fumonisins on the IAC (*15*).

Thus, other masking mechanisms such as complexation or physical entrapment should be taken into account for the evaluation of the occurrence of hidden fumonisins in nonthermally treated foods: indeed, several compounds such as lipids and flavors can be associated with protein or starch through noncovalent interactions (*16*). This kind of behavior may be also at the base of the difficulties in obtaining comparable and reproducible results using different analytical methods because such interactions may be differently broken during the extraction process, on account of different experimental parameters applied during extraction, thus leading to different recoveries of the analytes (*13*). Moreover, it has been demonstrated that instability of fumonisins in stored analytical samples, in particular, spiked samples used in collaborative method studies (*14*), may involve such an interaction.

Very recently, we reported for the first time the occurrence of hidden fumonisins in raw maize, suggesting that such noncovalent interactions were responsible for the phenomenon (*13*). In order to unravel this complicated matter, we used the following definitions: (1) bound fumonisins for those compounds which eventually involve a covalent linkage among the analyte and any matrix constituents; (2) hidden fumonisins only for noncovalently matrix-bound derivatives; (3) extractable fumonisins, noncovalently bound fumonisins which might actually be released applying common extraction procedures.

In order to verify the bioaccessibility of these hidden forms, Motta and Scott (*17*) applied an *in vitro* digestion model to corn flakes: low release of free FB1 (50%) was found in the chyme, and no contribution from bound forms was observed. More recently, applying the same protocol, a significant fraction of bound fumonisins was found in the chyme upon alkaline hydrolysis (*13*).

The aim of this study is to further investigate the occurrence of hidden fumonisins in raw maize by the systematic application of the *in vitro* digestion model (*18−21*) to mimic the gastrointestinal digestive process, giving thus an estimation of the native fumonisin bioaccessibility in the small intestine. We also compared the results obtained with the digestion approach with those obtained with the indirect alkaline hydrolysis method and with the extraction approach based on the use of a mixture of salts (QuEChERS) (*22*) in order to explore new analytical possibilities for the determination of hidden fumonisins.

MATERIALS AND METHODS

Chemicals. Fumonisin B_1, B_2, and B_3 mixed standard solution, 50 μg/mL each, in acetonitrile/water, 1:1 v/v, was purchased from Biopure (Tulln, Austria). All of the solvents used were of LC grade. Methanol was obtained from Carlo Erba (Milan, Italy), acetonitrile was from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Potassium hydroxide, potassium chloride, sodium chloride, ammonium chloride, 37% hydrochloric acid, potassium dihydrogen phosphate, sodium hydrogen carbonate, and dried calcium chloride were obtained from Carlo Erba (Milan, Italy), potassium thiocyanate and sodium sulfate were purchased from Riedel de Haën (Hannover, Germany), sodium dihydrogen phosphate monohydrate was from Fluka (Chemika-Biochemika, Basil, Switzerland), and magnesium chloride hexahydrate was obtained from Merck (Darmstadt, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (urea 98%, D-(+)-glucose 99.5%, D-glucuronic acid, D-(+)-glucosamine hydrochloride 99%, type III mucin from porcine stomach, uric acid, type VIII A α-amylase from barley malt, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas, and bovine and ovine bile) were purchased from Sigma (Stuttgart, Germany). The reference material containing fumonisins B_1 and B_2 at declared values of 2406 ± 630 and 630 ± 116 μg/kg, respectively, was a maize flour from Romer (Romer Laboratories Diagnostic GmbH, Tulln, Austria). Maize zein was from Fluka Chemika-Biochemika (Buchs, Switzerland), and maize starch was a commercial product from the market (Maizena, Unilever).

Preparation of Hydrolyzed Fumonisin Standard Solution. A standard solution of hydrolyzed fumonisins FB_1, FB_2, and FB_3 was prepared according to the hydrolysis procedure proposed by Dall'Asta et al. (*10*, *12*, *13*). Briefly, the procedure was as follows: 1 mL of the FB_1, FB_2, and FB_3 standard solution was evaporated to dryness. The residue was redissolved in 5 mL of 2 M KOH and allowed to react for 60 min at room temperature. After the hydrolysis, the mixture was extracted twice by liquid−liquid partition using twice 5 mL of acetonitrile. The organic-rich phases were pooled and evaporated under N_2 stream, and the residue was redissolved in 1 mL of acetonitrile/water, 1:1 v/v. The conversion yield was quantitative (*12*). Calibration curves were prepared by proper dilution of the standard solution.

Samples. Raw maize samples (n = 31) were collected in Italy over a 2 month period (September to October 2008) and are representative of several different maize hybrids grown under different agronomical conditions: they are indicated with the notations M1−M31. All maize samples were finely ground with an automatic miller (Braun GmbH, Italy). Maize flour, maize zeins, and maize starch were used as purchased.

Sample Preparation for the Analysis of Fumonisins. Extraction and analysis of fumonisins were performed according to Dall'Asta et al. (*10*, *12*, *13*). Briefly, 5 g of ground maize sample was blended in a high-speed blender (Ultraturrax T25; IKA, Stauffen, Germany) with 40 mL of water/methanol, 30:70 v/v, for 3 min at 6000 rpm and then filtered. After filtration on 0.45 μm nylon filters, 1 mL of extract was analyzed by LC-ESI-MS/MS.

Sample Preparation for the Analysis of Hydrolyzed Fumonisins. Aliquots (5 g) of the ground maize sample were blended in a high-speed blender (Ultraturrax T25; IKA, Stauffen, Germany) with 50 mL of 2 M KOH for 10 min at 6000 rpm and then stirred for 50 min. Then, 50 mL of acetonitrile was added, and after stirring for 10 min, two layers were formed which were separated by centrifugation at 3500 rpm for 15 min. A 2 mL portion of the acetonitrile-rich upper layer was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in water/methanol, 30:70 v/v, filtered through a 0.45 μm nylon filter, and analyzed by LC−MS/MS. Fumonisins obtained after sample hydrolysis were measured as the sum of hydrolyzed fumonisins B_1, B_2, and B_3. All of the results are expressed as the sum of FB_1, FB_2, and FB_3 equivalents, considering a correction factor due to the different molecular weight of native and hydrolyzed compounds and referred to as "total fumonisins after hydrolysis".

***In Vitro* Digestion Assay.** The preparation of artificial digestive juices (saliva, gastric juice, duodenal juice, and bile) was performed according to the original protocol of Versantvoort (*19*). Before each experiment, all digestive juices were heated at 37 ± 2 °C. The digestion started by adding 3 mL of saliva to 2 g of ground sample, followed by an incubation step of 5 min. Then, 6 mL of gastric juice was added, and the mixture was incubated for 2 h. Finally, 6 mL of duodenal juice, 3 mL of bile, and 1 mL of 1 M bicarbonate solution were added simultaneously to the mixture, and a final incubation step of 2 h was performed. During the *in vitro* digestion, the mixture was stirred by a magnetic stirrer to obtain a gentle but thorough mixing of the matrix with the digestive juices. The pH of the chyme varied in the range 6.5−7. At the end of the experiment the digestion tubes were centrifuged for 15 min at 3500 rpm, yielding the chyme as the supernatant and the digested matrix as the pellet. The concentration of fumonisins was determined in chyme after a desalting step through Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA). Briefly, after preconditioning with 2 mL of methanol followed by 2 mL of bidistilled water, 2 mL of chyme was loaded on the column, which was then washed again with 2 mL of bidistilled water. Fumonisins were eluted using 2 mL of water/acetonitrile, 1:1 v/v: preliminary experiments using 500 μg/mL standard fumonisin solution have shown recovery of 100%. The stability of fumonisins during digestion was checked comparing the amount of FB1 in the chyme after the digestion protocol with that of the control. Fumonisin B_1 was found to be completely stable under gastrointestinal digestion, obtaining a recovery after digestion of 100%. Then, a 1 mL portion of the solution containing fumonisins was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 1 mL of water/methanol, 30:70 v/v, prior to

analysis. For maize-based products (generally less contaminated than raw maize) 4 mL of chyme was applied to the cartridge. After elution with 4 mL of water/acetonitrile, 1:1 v/v, 3.5 mL of filtrate was evaporated, and the residue was redissolved in 1 mL of water/methanol, 30:70 v/v, for the analysis.

LC-MS/MS Analysis. LC-MS/MS analysis was performed by a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple quadrupole mass spectrometer with an electrospray source (Micromass; Waters, Manchester, U.K.) according to Dall'Asta et al. (*10*, *12*, *13*). Chromatographic conditions were the following: the column was a 250 mm × 2.1 mm i.d., 5 μm, XTerra C18; the flow rate was 0.2 mL/min; the column temperature was set at 30 °C; the injection volume was 10 μL; gradient elution was performed using bidistilled water (eluent A) and methanol (eluent B) both acidified with 0.2% formic acid: initial condition at 70% A, 0−2 min isocratic step, 2−5 min linear gradient to 45% B, 5−25 min linear gradient to 90% B, 25−35 min isocratic step at 90% B, 35−36 min linear gradient to 70% A, and reequilibration step at 70% A for 15 min (total analysis time: 50 min). MS parameters: ESI+ (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 50 V; extractor voltage, 2 V; source block temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow and desolvation gas flow (nitrogen), 50 L/h and 700 L/h, respectively. Detection was performed using a multiple reaction monitoring (MRM) mode by monitoring two transitions for each analyte, as follows: 722.4 → 334.4 (CE 40 eV), 722.4 → 352.3 (CE 35 eV) for FB1, 706.4 → 336.4 and 706.4 → 318.4 (CE 35 eV) for FB2 and FB3, 406.5 → 370.5 and 406.5 → 388.5 (CE 20 eV) for HFB1, 390.5 → 336.4 and 390.5 → 372.5 (CE 20 eV) for HFB2 and HFB3. The first transition reported was used for quantification, while the second transition was chosen as qualifier. For each sample, the entire procedure (preparation, cleanup, and digestion) was performed in duplicate (n = 2). Matrix-matched calibration curves (calibration range 10−1000 μg/kg) were used for extractable fumonisins, total fumonisins after digestion, and hydrolyzed fumonisin quantification.

QuEChERS-Based Extraction Method. The QuEChERS-like procedure enforced first of all in pesticide residues analysis (*22*) was employed for isolation of analytes. In this study, the protocol reported by Zachariasova et al. (*23*) has been used. Briefly, 4 g of homogeneous representative sample was weighted into the PTFE cuvette, and 12.5 mL of 0.1% (v/v) aqueous formic acid and 8 mL of acetonitrile were added. The suspension was shaken vigorously for 3 min. After addition of 1 g of NaCl and 4 g of $MgSO_4$, the mixture was shaken again. To separate aqueous and organic phase, the sample was centrifuged (5 min, 5000 rpm). The 2 mL aliquot of the upper organic phase was evaporated to dryness, and the residue was redissolved in water/methanol (3:7 v/v).

Statistical Analyses. Statistical analyses were performed using SPSS v.17.0 (SPSS Italia, Bologna, Italy) and OriginPro v.8.0 (OriginLab, Northampton, MA, USA). Data were statistically compared by using a one-way ANOVA test followed by a post hoc Tukey test (α = 0.05).

RESULTS AND DISCUSSION

Occurrence and Bioaccessibility of Hidden Fumonisins in Raw Maize Samples: *In Vitro* Digestion Assay. As hidden fumonisins were found to occur in raw maize samples (*10*, *13*) and *in vitro* digestion experiments were found to be able to release these forms, the aim of the present work was to widen the scope of the survey to many raw maize samples of different origin in order to verify the diffusion of the phenomenon.

The digestion assay used for the present study was based on Versantvoort et al. (*19*): in this model the chemical composition of digestive fluids, pH, and residence periods typical for each compartment (mouth, stomach, intestine) are reproduced to mimic in a simplified manner the physiological conditions in the human gastrointestinal tract during the digestion process. The model mimics all of the most important gastrointestinal digestion steps, apart from fermentation by intestinal microflora and permeation or transport across the intestinal barrier.

For this study, a large number of raw maize samples (n = 31) were collected in Italy over a 2 month period (September to October 2008). The selected samples are representative of several different maize hybrids grown under different agronomical conditions.

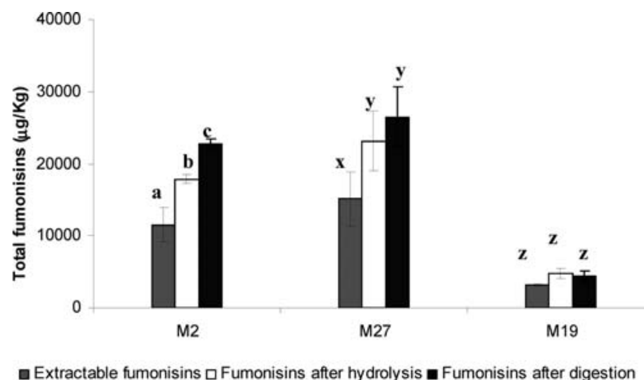

Figure 1. Comparison of the extractable fumonisins (sum of FB_1, FB_2, and FB_3), total fumonisins found after hydrolysis (measured as hydrolyzed fumonisins and expressed as sum of FB_1, FB_2, and FB_3 equivalents), and total fumonisins found in the samples after *in vitro* digestion (sum of FB_1, FB_2, and FB_3) obtained for several raw maize samples. Different letters designate statistically significant differences between data (α = 0.05).

As first, three of these maize samples were analyzed for the occurrence of free fumonisins with normal extraction procedures and of hidden fumonisins with the hydrolysis and the digestion approach. The data obtained are reported in **Figure 1** and expressed as the sum of FB_1, FB_2, and FB_3. All of the data were statistically compared by using a one-way ANOVA test followed by a post hoc Tukey test (α = 0.05). In all three samples fumonisins were found to occur, and upon hydrolysis, in two out of three samples a significant increase in total fumonisins was observed. Upon digestion, no hydrolyzed or partially hydrolyzed fumonisins were found in the chyme, but only native fumonisins were released from the matrix.

The analyzed samples gave three different results: in comparison with the amount of detectable free fumonisins, sample M2 and M27 showed a higher content of total fumonisins after digestion, whereas sample M19 seemed not to contain hidden fumonisins. For all of the samples, the contamination level after digestion was comparable or slightly higher than that found after hydrolysis.

These data confirmed that the gastrointestinal enzymes are able to destroy the matrix−fumonisin interactions, thus releasing the hidden forms. Moreover, as the release of the hidden forms using the hydrolysis approach gives similar results (slightly lower) to the digestion approach in this case, it could be argued that digestion is more efficient and that probably the two methods are able to destroy a similar masking phenomenon (covalent bonding or complexation).

Thus, we decided to apply the digestion protocol to all of the collected samples: for all of the considered samples, the occurrence and amount of fumonisins were measured by the normal extraction procedure (free fumonisins); then, maize samples were digested *in vitro*, and the amount of fumonisins was measured in the obtained chyme (total fumonisins after digestion). The results are summarized in **Table 1**.

As a general observation, total fumonisin levels measured after digestion were higher than those measured by the standard procedure (Tukey test, α = 0.05) in 20 out of 31 samples.

Thus, from this survey, it appears that the occurrence of hidden fumonisins in raw maize is a very general and common phenomenon: it is worth noting that applying the digestion protocol, fumonisins and not hydrolyzed fumonisins or other chemically modified derivatives were liberated in the medium. This fact is of

Table 1. Comparison of Extractable Fumonisins, Total Fumonisins after Digestion, and Hidden Fumonisins Found in Raw Maize Samples[a]

sample	extractable fumonisins[b] (μg/kg)	CV, %	total fumonisins[c] (μg/kg)	CV, %	hidden fumonisins[d] (μg/kg)	Tukey test[e] (p)
M1	1145	4.6	2501	1.8	1356	**
M2	11479	20.7	22755	2.8	11276	*
M3	6318	1.6	10116	10.7	3798	*
M4	999	0.9	2729	1.3	1730	**
M5	6407	3.8	18069	17.1	11662	*
M6	1515	1.8	2785	3.1	1270	**
M7	6369	10.8	6579	7.2	210	>0.05
M8	1997	2.8	1972	18.1	0	>0.05
M9	1180	12.5	2696	6.0	1516	**
M10	575	0.6	1578	0.6	1003	**
M11	6934	22.9	12755	1.7	5821	*
M12	2361	3.3	3829	3.7	1468	**
M13	1287	2.6	1135	18.8	0	>0.05
M14	4658	0.3	4677	2.4	19	>0.05
M15	2611	3.9	10734	2.5	8123	**
M16	5057	10.2	6766	13.3	1709	>0.05
M17	1702	2.3	2599	11.0	897	*
M18	4641	6.2	6074	5.8	1433	*
M19	3146	4.3	4427	15.4	1281	>0.05
M20	3523	7.6	5588	0.3	2065	**
M21	2871	11.4	2935	10.8	64	>0.05
M22	17014	5.4	40821	1.0	23807	**
M23	3258	9.7	4005	14.4	747	>0.05
M24	1981	1.7	2626	3.8	645	*
M25	576	3.5	2112	4.4	1536	**
M26	8518	0.4	9702	0.1	1184	**
M27	15067	25.2	26503	15.6	11436	>0.05
M28	5551	3.4	6212	9.1	661	>0.05
M29	9689	10.8	14406	28.8	4717	>0.05
M30	6887	2.8	12977	6.4	6090	**
M31	3749	2.7	6460	5.7	2711	**

[a] $n = 2$; *, $p < 0.05$; **, $p < 0.01$. [b] Extractable fumonisins: fumonisins obtained after routine analysis. [c] Total fumonisins: fumonisins obtained after digestion assay. [d] Hidden fumonisins: calculated difference among "total fumonisins" and "extractable fumonisins". [e] Tukey's test performed among total fumonisins and extractable fumonisins.

the utmost importance both for the analytical implications and also for the possible consequences on consumer's health. Indeed, it appears evident that the currently used analytical methods suffer for poor accuracy, as they are not able to reveal the occurrence of intact fumonisins hidden in the matrix. Moreover, consumers may actually be exposed to a higher level of contaminants in comparison with the exposure calculated on the base of the data obtained with the routine analytical procedure.

Analyzing the data, it is also very interesting that a strong correlation exists between amount of hidden forms and free extractable forms (Pearson's test: 0.914 at $\alpha = 0.01$): i.e., samples with high free extractable fumonisin levels showed, indeed, very high hidden fumonisin content after digestion. On the contrary, poorly contaminated samples showed lower or no increase in fumonisin levels after digestion.

The correlation between extractable fumonisins and total fumonisins is reported in **Figure 2**; according to the plot, when the free, extractable fumonisin amount is known, the total fumonisin concentration in the sample could be predicted with good agreement ($r^2 = 0.8703$). If this agreement will be confirmed by a larger amount of data, this could be a useful predictive tool for the estimation of the real level of fumonisins in maize.

***In Vitro* Digestion of a Certified Reference Material (Corn Flour).** In order to evaluate the potential impact of this problem on the accuracy of fumonisin analytical determination in food, we

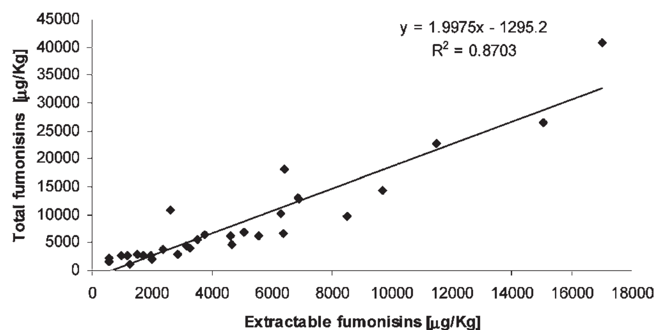

Figure 2. Correlation between extractable fumonisin concentration and total fumonisin concentration in maize.

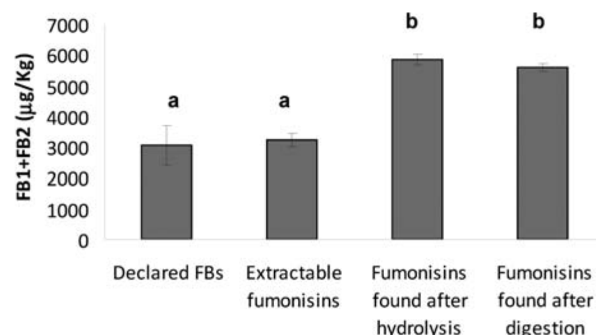

Figure 3. Comparison between extractable fumonisins (sum of FB_1 and FB_2) and total fumonisins (sum of FB_1 and FB_2) found after digestion for a certified reference material (FAPAS, maize sample). Different letters designate statistically significant differences between data ($\alpha = 0.05$).

analyzed a certified reference material, a maize flour with a declared contamination level of 2406 ± 630 μg/kg FB_1 and 630 ± 116 μg/kg FB_2, respectively. Certified reference materials are widely used to validate analytical methods and are currently highly considered, as they more correctly represent the real situation as far as the interactions between sample matrix and contaminant are concerned, in comparison with spiking experiments. Analysis of the sample with the normal extraction method gave good results in accordance with the declared contamination range (z score = 0.29).

Nevertheless, in order to check for the eventual occurrence of hidden forms in this sample, we applied the hydrolysis approach: the amount of total fumonisins calculated upon hydrolysis was higher (almost double) than the amount of free fumonisins detectable by the routine extraction procedure (**Figure 3**).

The increase, already observed in different maize products by several authors and also in raw maize samples by us, confirms that to ascertain the occurrence of these hidden forms represents a real analytical problem. From the point of view of food safety and of the potential risk for consumers, it has to be taken into consideration if these hidden forms could contribute to the overall toxicity or are characterized by a proper toxicity themselves. It is evident that if additional intact fumonisins, not detectable by the normal analysis, are released upon digestion, this would constitute one of the worst scenarios, as the consumer is really exposed to an higher level of toxicants.

In order to check if this is the case, we applied the *in vitro* digestion assay to the sample.

Applying the digestion protocol, the amount of detectable fumonisins in the chyme was found also almost double than that determined by the standard approach (**Figure 3**).

Data were statistically compared by using a one-way ANOVA test followed by a post hoc Tukey test ($\alpha = 0.05$). The amount of

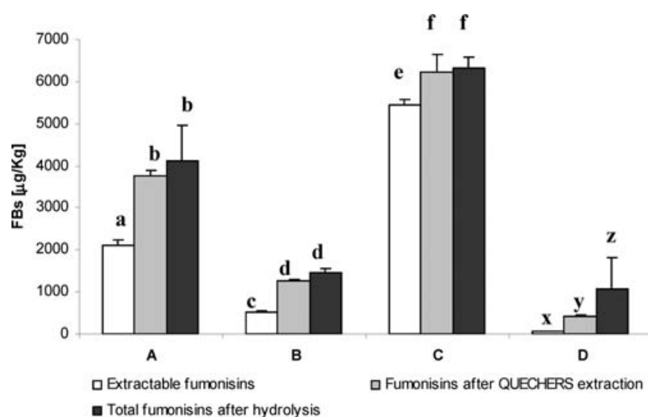

Figure 4. Comparison between the level of fumonisins found in raw maize (n = 4) after methanol/water extraction, after QuEChERS-like extraction, and after alkaline hydrolysis. Different letters designate statistically significant differences between data (α = 0.05).

total fumonisins after digestion and after hydrolysis was found to be statistically different from the free extractable ones (p = 0.011 and p = 0.019, respectively), whereas no significant difference was found between total fumonisins after hydrolysis and after digestion. These results showed that in the certified reference material hidden fumonisins occur which were not detected using standard approach but can be released upon digestion, thus potentially contributing to the overall toxicity of the contaminated product. Thus, although when setting an analytical method, recovery experiments by the spiking procedure show good performance of the method itself, a performance which can be further confirmed by using certified reference material; nevertheless, naturally contaminated maize samples invariably show the unreliability of this approach in determining the real contamination in the case of fumonisins.

As far as the nature of these masking interactions (covalent bonds, physical complexation, or entrapment into the food matrix), both the hydrolysis approach and the digestion protocol did not tell us much about this, although an entrapment into the food matrix (protein bodies, starch complexation, etc.) seems more feasible in this case since no thermal treatment was applied to raw maize samples or to maize flour, and as a result, the model proposed by Seefelder et al. (*11*) is not so feasible in this case.

On the basis of these considerations, we thought to try a different kind of extraction procedure, possibly capable of effecting a more powerful disaggregation of the analyte−matrix interactions.

Analysis of Raw Maize Samples by QuEChERS-like Approach. The QuEChERS approach was first developed for pesticide analysis and has been recently successfully proposed also for mycotoxin multiresidual determination (*23*). This approach is based on a partitioning of acetonitrile/water mixture induced by addition of inorganic salts. While the analytes are transferred into an organic phase, some more polar matrix impurities are left in an aqueous layer. Moreover, the addition of inorganic salts caused also protein denaturation through salting out effect.

In order to evaluate how the extraction procedure is able to affect the extractable fumonisins recovery, several raw maize samples (n = 4) were extracted using the QuEChERS-like approach, and the results were compared to those obtained by using the water/methanol-based procedure, as shown in **Figure 4**. Afterward, the same samples underwent alkaline hydrolysis for total fumonisin determination. According to the results, the amount of extractable fumonisins seemed to be strictly related to the extraction procedure. With the exception of sample D, the extractable fumonisins obtained by the QuEChERS-like method weres comparable to total fumonisins obtained after alkaline hydrolysis. Thus, the application of QuEChERS allowed for the recovery of an higher amount of fumonisins, suggesting that native fumonisins may be released from the matrix given the higher disaggregation capacity of this extraction method. This effect could be partially based upon the protein salting out effect caused by the inorganic salt addition, supporting the hypothesis of a noncovalent interaction between fumonisins and matrix constituents. Indeed, as a matter of fact, although total fumonisins found after alkaline hydrolysis could be ascribed either to the releasing from association complexes formed with the matrix macroconstituents or to the cleavage of covalently bound derivatives, the increased amount of extractable fumonisins found by applying the QuEChERS-like approach can be only due to the more efficient disaggregation of the matrix and to the destabilization of noncovalent interactions. Although further studies should be performed in order to clarify the nature of the occurring interactions, the results obtained applying the QuEChERS-like procedure support the hypothesis that a major part of hidden fumonisins in raw maize is strong physically entrapped into the food matrix and that this approach seems to be very promising in order to solve the analytical issue regarding hidden fumonisins in maize.

The digestion assay here applied allowed to demonstrate the release of native fumonisins from the food matrix during simulated gastrointestinal digestion and that total fumonisins after digestion are often in higher amount in comparison with total fumonisins detected by routine analytical methods. Total fumonisins found after digestion were generally comparable with fumonisin levels found after alkaline hydrolysis, the procedure usually applied to check for the presence of hidden forms. Thus, an analytical issue was introduced by our experiments, since currently used routine methods are unable to detect hidden fumonisins, opening a serious problem regarding risk assessment: consumers may be, as a matter of fact, concretely exposed to a higher risk than that evaluated by routine methods as these hidden forms may actually contribute to the overall toxicity being efficiently released during gastrointestinal digestion.

LITERATURE CITED

(1) Voss, K. A.; Riley, R. T.; Gelineau-van Waes, J. Trends in fumonisin research: recent studies on the developmental effects of fumonisins and *Fusarium verticillioides*. *Mycotoxins* **2005**, *55*, 91−100.

(2) Miller, J. D. Factors that affect the occurrence of fumonisin. *Environ. Health Perspect. Suppl.* **2001**, *109*, 321−324.

(3) Marasas, W. F.; Riley, R. T.; Hendricks, K. A.; Stevens, V. L.; Sadler, T. W.; Gelineau-van Waes, J.; Missmer, S. A.; Cabrera, J.; Torres, O.; Gelderblom, W. C.; Allegood, J.; Martínez, C.; Maddox, J.; Miller, J. D.; Starr, L.; Sullards, M. C.; Roman, A. V.; Voss, K. A.; Wang, E.; Merrill, A. H., Jr. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *J. Nutr.* **2007**, *134*, 711−716.

(4) Gelineau-Van Waes, J.; Voss, K.; Stevens, V.; Speer, M.; Riley, R. Maternal fumonisin exposure as a risk factor for neural tube defects. In: *Advances in food nutrition research*; Taylor, S., Ed.; Elsevier/Academic Press: Amsterdam, The Netherlands, 2009; pp 145−181.

(5) Missmer, S. A.; Suarez, L.; Felkner, M.; Wang, E.; Merrill, A. H., Jr.; Rothman, K. J.; Hendricks, K. A. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. *Environ. Health Perspect.* **2006**, *114*, 237−241.

(6) European Union. Commission Regulation (EC) No. 1126/2007. Maximum levels of certain contaminants in foodstuff. *Off. J. Eur. Union*, Sept 29, 2007, L255/14−L255/17.

(7) Humpf, H. U.; Voss, K. A. Effects of food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Mol. Nutr. Food Res.* **2004**, *48*, 255−269.

(8) Kim, E.-K.; Scott, P. M.; Lau, B.P.-Y. Hidden fumonisins in corn flakes. *Food Addit. Contam.* **2003**, *20*, 161−169.
(9) Park, J. W.; Scott, P. M.; Lau, B.P.-Y.; Lewis, D. A. Analysis of heat-processed corn foods for fumonisins and bound fumonisins. *Food Addit. Contam.* **2004**, *21*, 1168−1178.
(10) Dall'Asta, C.; Galaverna, G.; Aureli, G.; Dossena, A.; Marchelli, R. A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in corn and corn-based products. *World Mycotoxin J.* **2008**, *1*, 1−10.
(11) Seefelder, W.; Knecht, A.; Humpf, H.-U. Bound fumonisin B1: analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 5567−5573.
(12) Dall'Asta, C.; Galaverna, G.; Mangia, M.; Sforza, S.; Dossena, A.; Marchelli, R. Free and bound fumonisins in gluten-free food products. *Mol. Nutr. Food Res.* **2009**, *53*, 492−499.
(13) Dall'Asta, C.; Mangia, M.; Berthiller, F.; Molinelli, A.; Sulyok, M.; Schuhmacher, R.; Krska, R.; Galaverna, G.; Dossena, A.; Marchelli, R. Difficulties in fumonisin determination: the issue of hidden fumonisins. *Anal. Bioanal. Chem.* **2009**, *395*, 1335−1345.
(14) Kim, E. K.; Scott, P. M.; Lau, B. P.; Lewis, D. A. Extraction of fumonisins B_1 and B_2 from white rice flour and their stability in white rice flour, cornstarch, cornmeal, and glucose. *J. Agric. Food Chem.* **2002**, *50*, 3614−3620.
(15) Oh, K. S.; Scott, P. M.; Chung, S.-H. Incomplete recoveries of fumonisins present in naturally contaminated corn foods from an immunoaffinity column. *J. AOAC Int.* **2009**, *92*, 496−501.
(16) Momany, F. A.; Sessa, D. J.; Lawton, J. W.; Selling, G. W.; Hamaker, S. A.; Willett, J. L. Structural characterization of α-zein. *J. Agric. Food Chem.* **2006**, *54*, 543−547.
(17) Motta, E. L.; Scott, P. M. Bioaccessibility of total bound fumonisin from corn flakes. *Mycotoxin Res.* **2009**, *25*, 229−232.
(18) Oomen, A. G.; Rompelberg, C. J.; Bruil, M. A.; Dobbe, C. J.; Pereboom, D. P.; Sips, A. J. Development of an *in vitro* digestion model for estimating the bioaccessibility of soil contaminants. *Arch. Environ. Contam. Toxicol.* **2003**, *44*, 281−287.
(19) Versantvoort, C. H. M.; Oomen, A. G.; Van de Kamp, E.; Rompelberg, C. J. M.; Sips, A. J. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chem. Toxicol.* **2005**, *43*, 31−40.
(20) Brandon, E. F.; Oomen, A. G.; Rompelberg, C. J.; Versantvoort, C. H.; van Engelen, J. G.; Sips, A. J. Consumer product *in vitro* digestion model: bioaccessibility of contaminants and its application in risk assessment. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 161−171.
(21) Oomen, A. G.; Hack, A.; Minekus, M.; Zeijdner, E.; Cornelis, C.; Schoeters, G.; Verstraete, W.; Van de Wiele, T.; Wragg, J.; Rompelberg, C. J.; Sips, A. J.; Van Wijnen, J. H. Comparison of five *in vitro* digestion models to study the bioaccessibility of soil contaminants. *Environ. Sci. Technol.* **2002**, *36*, 3326−3334.
(22) Annastassiades M.; Mastovska K.; Lehotay S. J. Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. *J. Chromatogr. A* **2003**, *10*, 163.
(23) Zachariasova, M.; Lacina, O.; Malachova, A.; Kostelanska, M.; Poustka, J.; Godula, M.; Hajslova, J. Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Anal. Chim. Acta* **2001**, *662*, 51−61.

Received for review September 30, 2010. Revised manuscript received October 11, 2010. Accepted October 12, 2010. We kindly acknowledge the Network Laboratory SITEIA (Sicurezza, Tecnologie, Innovazione Agroalimentare), Emilia Romagna Region, for financial support.