Simultaneous Determination of Fumonisin B_1 and Hydrolyzed Fumonisin B_1 in Corn Products by Liquid Chromatography/ Electrospray Ionization Mass Spectrometry

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A method for the simultaneous determination of fumonisin B_1 (FB_1) and its major hydrolysis product (HFB_1), which is known to be formed during alkaline treatment of fumonisin-containing corn meal, was devised to analyze the levels of these mycotoxins in corn products available on the German market. Liquid chromatography/electrospray mass spectrometry in combination with selected ion monitoring (SIM) was used for unambiguous detection of FB_1 and HFB_1 after extraction of samples with acetonitrile/methanol/water (25:25:50) and solid-phase C18 cleanup. Quantitation was carried out using labeled fumonisin FB_1-D_6 as an internal standard. The detection limits achieved with this method were 8 ng/g for HFB_1 (signal-noise ratio = 5:1) and 5 ng/g for FB_1 (s/n = 5:1) using the protonated molecule signals *m/z* 406 and 722 in the SIM mode. A screening of several corn-containing foodstuffs, among them extrusion products and alkali-processed corn food such as tortilla chips, showed HFB_1 and FB_1 contamination with levels of 8−80 and 5−450 ng/g, respectively.

Keywords: *Fumonisins; liquid chromatography/mass spectrometry; hydrolysis; corn products; mycotoxin*

INTRODUCTION

The fumonisins are a group of structurally related toxic metabolites of *Fusarium moniliforme*, one of the most common molds associated with corn worldwide (Marasas, 1996). Fumonisin B_1 (FB_1), the most prevalent isomer, has been proven to be responsible for the effects that occur in animals with the consumption of *F. moniliforme*-contaminated corn, for example, equine leukoencephalomalacia (ELEM) in horses (Kellerman et al., 1990) and pulmonary edema in swine (Harrison et al., 1990), and showed hepatotoxicity and carcinogenicity in rats (Gelderblom et al., 1991).

Extensive screenings of corn meal and corn-containing products for the presence of FB_1, FB_2, and FB_3 have revealed these toxins as important and widespread contaminants (Marasas, 1996). As fumonisins are as well asssociated with an increased rate of human esophageal cancer in South Africa (Rheeder et al., 1992) and China (Chu and Li, 1994), their presence in food is a potential risk to human health, particularly with respect to the fact that *F. moniliforme* toxins have recently been classified by the IARC in group 2B "possibly carcinogen" (IARC, 1993).

Fumonisin B_1 (Figure 1) is characterized by the presence of two tricarballylic acid (TCA) groups esterified to a 20-carbon backbone. It is relatively heat stable and persists through most of the conditions used in food manufacturing. However, the TCA side chains may be removed by alkaline treatment, yielding the hydrolyzed analogue HFB_1 (Murphy et al., 1996), the toxicity of which is not yet clear. Nixtamalization, a traditional treatment of corn with calcium hydroxide and heat, which is used for producing tortilla flour, has been shown to produce HFB_1 (Figure 1). When tested as a method for detoxifying fumonisin-contaminated corn, the nixtamalized culture material (containing HFB_1) showed toxicity similar to that of the untreated culture material (containing only FB_1) with respect to hepato- and nephrotoxicity (Hendrich et al., 1993; Voss et al., 1996). In a short-term liver cancer initiation/promotion model HFB_1 failed to initiate cancer but revealed greater cytotoxicity than FB_1 (Gelderblom et al., 1993). Regarding the mechanism of action on the molecular level, studies have shown the effective interruption of sphingolipid metabolism due to inhibition of the enzyme ceramide synthase by FB_1. HFB_1 is not as potent an inhibitor as FB_1 but was recently found to serve as a substrate for the ceramide synthase, forming an acylated hydrolyzed fumonisin, which was more toxic to a human colonic cell line than the parent compound HFB_1 and even FB_1 (Humpf et al., 1998).

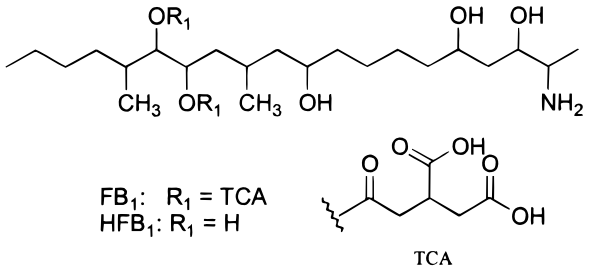

Figure 1. Structures of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1).

The presence of hydrolyzed fumonisin in nixtamalized corn meal (masa), tortilla chips, and canned yellow corn was recently established by Hopmans and Murphy (1993). With the overall toxicity still unclarified and the knowledge of this metabolic transformation, HFB_1 becomes a more important contributing factor in the risk

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assessment of fumonisins. Screening of corn-containing food seems to be advisable to record the exposure to HFB_1, especially in view of the growing popularity of snacks such as tortilla chips, nachos, and tacos. Several approaches for the analysis of HFB_1 in different matrixes have already been made. Earlier work is restricted to the detection of HFB_1 in corn-containing foods (Hopmans and Murphy, 1993) or HFB_1 and the partially hydrolyzed metabolites of FB_1 in feces (Shephard et al., 1994) as their fluorescent *o*-phthaldialdehyde (OPA) derivatives. For quantitative determination most of the so far known methods use as well precolumn fluorogenic labeling either with OPA (Scott and Lawrence, 1996; Thakur and Smith, 1996; Hopmans et al., 1997; Stack, 1998) or with naphthalene-2,3-dicarboxaldehyde (Maragos et al., 1997). Besides, the employment of liquid chromatography/fast atom bombardment mass spectrometry (Xie et al., 1997) or LC/ESI-MS (Newkirk et al., 1998) and an immunochemical approach utilizing a competitive direct ELISA (Maragos et al., 1996) are described. The sample workup follows in all cases a similar pattern, which is extraction with organic solvent, mainly acetonitrile/water 1:1 but also acetonitrile/methanol/water 25:25:50, methanol/phosphate buffer (Maragos et al., 1997) or acetonitrile/phosphate buffer (Stack, 1998), and then purification of the extract with solid-phase extraction cartridges. Depending on whether the analytical method should cover HFB_1 only or in addition FB_1 and FB_2, different cleanup procedures were employed. For the quantification of HFB_1 alone a C18 solid-phase extraction step is accepted as sufficient (Maragos et al., 1996), whereas for the analysis of FB_1, FB_2, and HFB_1 a combined workup using a SAX column followed by either an XAD or a C18 column was described (Thakur and Smith, 1996; Scott and Lawrence, 1996). A recent paper reports the use of a C18 cartridge for both FB_1 and its hydrolyzed analogue (Stack, 1998). Another possibility is the cleanup over immunoaffinity columns as performed by Newkirk et al. (1998) and Maragos et al. (1997). However, this requires the preparation of antibodies against HFB_1 as commercially available immunoaffinity columns are specified only for fumonisins B_1, B_2, and B_3.

In terms of sensitivity and accuracy the quantification of fumonisins as their fluorescent OPA derivatives, which is most widespread, provides a reasonable standard method with a limit of detection (LOD) of 50 ng/g or better (Shephard, 1998). Scott and Lawrence (1996) report detection limits in alkali-processed corn food of 10 and 20 ng/g for HFB_1 and FB_1, respectively. With the naphthalenedicarboxaldehyde (NDA) derivatives a lower detection limit of 10 ng/g could be achieved as stated by Maragos et al. (1997), whereas a competitive direct ELISA is even more sensitive, detecting HFB_1 at levels as low as 5 ng/g (Maragos et al., 1996). The most sensitive analytical determination of fumonisins B_1, B_2, and B_3 and hydrolyzed fumonisin yet reported is the method by Newkirk et al. (1998), which combines immunoaffinity column cleanup with LC/ESI-MS detection and attains an LOD of 2.5 ng/g. Recovery rates usually range between 70 and 126% and between 65 and 94% for HFB_1 and FB_1, respectively (Scott and Lawrence, 1996; Sydenham et al., 1995).

The major disadvantages of the methods based on OPA derivatization are the instability of the derivatives and even more the fluorescent interferences caused by matrix components, making a definite and accurate quantification difficult, especially with processed corn foods such as snacks and tortillas. Therefore, the objective of this study was to develop a sensitive method allowing a reliable and accurate quantitative determination of FB_1 and HFB_1 in processed corn-based food such as tortillas, tacos, nacho chips, and extrusion products for the assessment of the consumer's exposure to these mycotoxins.

EXPERIMENTAL PROCEDURES

Analytical Standards. Fumonisin B_1 was purchased from Alexis Biochemicals (Grünberg, Germany). Hydrolyzed fumonisin B_1 was produced from FB_1 according to the method of Hopmans et al. (1997) and further purified by means of preparative reversed-phase HPLC using an acetonitrile/water gradient. D_6-labeled fumonisin B_1 was isolated from *F. moniliforme* culture material as described previously (Lukacs et al., 1996). Stock solutions were prepared by dissolving 1 mg of the reference compound in 1 mL of acetonitrile/water (1:1) and further dilutions. Fumonisins are potential carcinogens and should be handled with care.

Reagents. Water, acetonitrile, and methanol, all of HPLC grade, and trifluoroacetic acid were from Merck (Darmstadt, Germany). Acetic acid (analytical purity) was obtained from Fluka (Deisenhofen, Germany). C18 solid-phase extraction cartridges (500 mg, 3 mL) were from ICT (Bad Homburg, Germany). Food samples were purchased in 1998 from local markets.

Apparatus. Chromatographic separation was performed by an Applied Biosystems 140b pump (Bai, Bensheim, Germany). For sample injection a SunChrom Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used. LC/ESI-MS analyses were conducted on a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and mass spectrometric evaluation were carried out on a personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT).

Analytical Procedures. *Sample Preparation*. Commercially available food samples (tortilla chips, nacho chips, taco shells, and extrusion products) were finely ground in a laboratory blender, and to 5 g subsamples was added a known amount of FB_1-D_6, serving as an internal standard for quantification. The samples were then extracted by blending for 3 min with 10 mL of methanol/acetonitrile/water (25:25:50) in an Ultra-Turrax TM disperser followed by centrifugation at 4000 rpm for 10 min. The supernatant was adjusted to pH 3.5 with 1 M hydrochloric acid, and 1 mL was applied to a C18 cleanup cartridge preconditioned with 2 mL of methanol and 1 mL of water. After washes with 2 mL of methanol/water (1:3) and 1 mL of methanol/water (1:1), each column was eluted with 1 mL of methanol containing 5% acetic acid (v/v). The eluate was evaporated under a gentle stream of nitrogen and redissolved in 200 μL of acetonitrile/water (1:1).

Mass Spectrometric Analysis of FB_1 and HFB_1. For LC/ESI-MS chromatographic separations were carried out on a Lichrospher 60-RP select B column (100 × 2.0 mm i.d., 5 μm; Knauer, Berlin, Germany) using a binary gradient. Solvent A was 0.05% TFA in methanol (v/v), and solvent B was 0.05% TFA in water (v/v). HPLC was programmed as follows: 0 min, 40% B; 1 min, 40% B; 7 min, 1% B. The column was washed for 3 min with 100% solvent A after each injection and equilibrated for 5 min at the starting conditions. The flow rate was set to 200 μL/min, and the injection volume was 10 μL. For pneumatically assisted electrospray ionization, the spray capillary voltage was set to 3.5 kV and the temperature of the heated capillary acting simultaneously as repeller electrode (20 V) was 250 °C. Nitrogen served both as sheath (60 psi; 1 psi = 6894.76 Pa) and as auxiliary gas (10 units). The mass spectrometer was operated in the selected ion monitoring (SIM) mode, detecting the positive ions $[M + H]^+$ of FB_1 (m/z 722), HFB_1 (m/z 406), and FB_1-D_6 (m/z 728) at a total scan

duration of 1 s. Quantitative evaluations were based on the peak area ratios of FB_1 or HFB_1 in comparison to that of FB_1-D_6, which served as an internal standard. Product ion spectra of HFB_1 were recorded using argon as collision gas at a pressure of 2.2 mTorr and a collision energy of −25 eV.

System Calibration, Detection Limit, and Recovery. The analytical system was calibrated with a standard curve for HFB_1, which was prepared as follows: a blank food sample, known to contain no HFB_1, was spiked with mixtures of HFB_1 and FB_1-D_6 in various weight ratios (1:3 up to 3:1), treated as described above, and analyzed. The resulting peak area ratios of the ions *m/z* 406 (HFB_1) to *m/z* 728 (FB_1-D_6) were plotted against the weight ratios and evaluated by means of a two-dimensional regression. The final concentrations of HFB_1 for each point of the curve were 50, 100, and 150 ng/g, which are equivalent to absolute injected amounts of 1, 2, and 3 ng, respectively.

The LOD was determined with standard solutions as well as in matrix. For the latter, a blank food sample was worked up according to the procedure described above and spiked with HFB_1 or FB_1 standard prior to the column eluate being redissolved with acetonitrile/water. The obtained extracts were injected, and thus the LOD was specified to be 8 ng/g for HFB_1 and 5 ng/g for FB_1 with a signal-noise ratio of 5:1. Recoveries were determined by adding 50, 70, 100, and 200 ng/g HFB_1 or 100 and 200 ng/g FB_1 to various food samples with known native contents of the analytes and submission to the analytical procedure. All analyses were performed in duplicate.

RESULTS AND DISCUSSION

Several studies (Doerge et al., 1994), along with our own results (Lukacs et al., 1996; Hartl et al., 1999), have proven the combination of liquid chromatography with electrospray mass spectrometry to be a useful tool for the analysis of fumonisins. As for the analytical determination of HFB_1, the great majority of the already existing methods uses the technique of precolumn fluorescent labeling with *o*-phthaldialdehyde for quantitation. Our experiences encouraged us to develop a method for the qualitative and quantitative determination of HFB_1, preferably along with FB_1, in corn-containing food, that is based upon LC/ESI-MS.

As known from our previous studies, the fumonisins are effectively ionized by the electrospray process, resulting mainly in the protonated molecule $[M + H]^+$. Figure 2A shows a typical electrospray mass spectrum of HFB_1 with the $[M + H]^+$ at *m/z* 406 as the main signal and its sodium and potassium adducts at *m/z* 428 and 444, demonstrating a behavior of this analyte similar to that of FB_1 and ensuring the applicability of the ESI process for HFB_1 analysis. The product ion spectrum of HFB_1, obtained by collision-induced dissociation of the protonated molecule using argon as collision gas, reveals signals at *m/z* 388, 370, 352, and 334, generated by the loss of one to four molecules of water (Figure 2B). In contrast to the product ion spectra of FB_1 and FB_2, which show characteristic fragments due to the loss of their TCA side chains, the HFB_1 product ion signals are too unspecific to be employed for the selected reaction monitoring (SRM) mode. Thus, we decided on the SIM mode, which is sufficiently specific and sensitive, as our results demonstrate.

Concerning the sample workup, several approaches have been followed. For sample extraction Scott and Lawrence (1996) described a mixture of acetonitrile/methanol/water (25:25:50) to be most effective for both HFB_1 and FB_1. For subsequent cleanup in most cases solid-phase extraction is used. We tested the combination of a C18 column (for HFB_1) following a SAX column

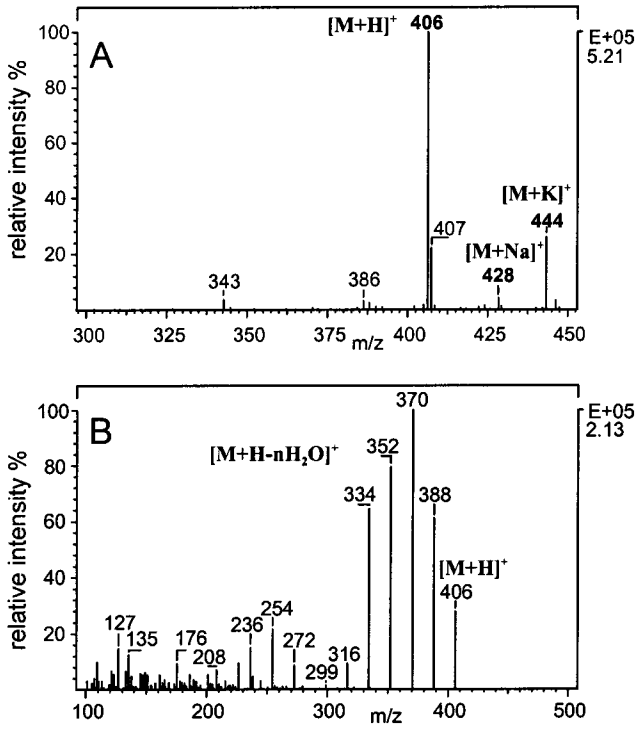

Figure 2. (A) Electrospray mass spectrum of hydrolyzed fumonisin B_1. (B) Product ion spectrum of HFB_1, obtained by collision-induced dissociation (CID) (−25 eV, 2.2 mTorr of Ar) of the precursor ion *m/z* 406 $[M + H]^+$.

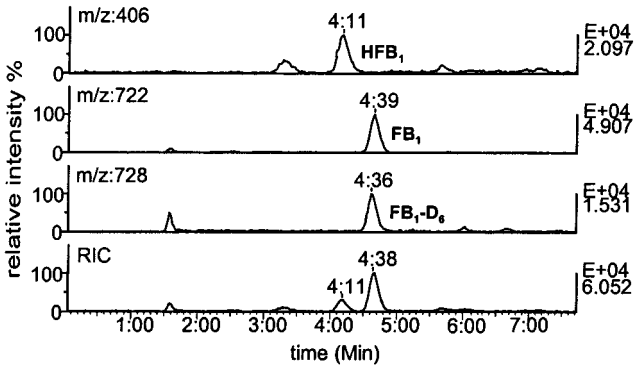

Figure 3. LC/ESI-MS analysis of a commercial taco shell sample containing 67.3 ng/g HFB_1 and 135.8 ng/g FB_1. *m/z* ratios 406 (HFB_1), 722 (FB_1), and 728 (FB_1-D_6) were monitored; RIC is reconstructed ion chromatogram.

(for FB_1) as well as the sole use of a C18 cartridge. Both methods gave comparable results; thus, for reasons of cost, time, and working effort, we decided on the latter one. With the C18 cartridge different solvent compositions for column washing and elution were tested, and the protocol described under Experimental Procedures was found to give the most satisfying results. Furthermore, the influence of pH was examined. When the sample extract was applied to the column with a pH of 6.5, parts of FB_1 and FB_1-D_6 (used as an internal standard) were found in the washings, whereas with a pH of 3.5 all analytes, FB_1, HFB_1, and FB_1-D_6, appeared exclusively in the cartridge eluate.

For chromatographic separations on a conventional reversed phase column we tested our standard methanol/water gradient, which was initially developed for the analysis of FB_1 (Lukacs et al., 1996). As the typical LC/MS chromatogram of a taco sample in Figure 3 demonstrates, the two analytes are successfully baseline

Table 1. Recovery of Fumonisins HFB_1 and FB_1 Added to Diverse Processed Corn-Containing Food[a]

HFB_1				FB_1			
sample	added, ng/g	found, ng/g	recovery, %	sample	added, ng/g	found, ng/g	recovery, %
tortilla chips 1	50	43.8	87.6				
taco shells 1	70	38.2	54.6				
extrusion product 1	100	57.2	57.2	tortilla chips 1	100	97.4	97.4
extrusion product 2	200	128.5	64.3	extrusion product 3	200	193.0	96.5

[a] To determine the recoveries, the food samples were first analyzed for their native concentrations of HFB_1 and FB_1 by the described method. FB_1 and HFB_1 standards were added, and duplicate samples of each spiking level were analyzed. Recoveries were calculated by subtracting the native levels from the values obtained after spiking.

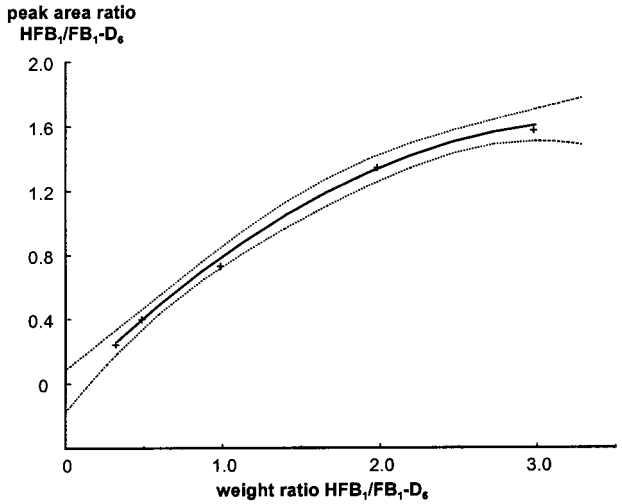

Figure 4. Calibration curve for HFB_1, showing the relationship between the weight ratios of HFB_1 to FB_1-D_6 and the resulting peak area ratios in the presence of genuine food matrix; the HFB_1 concentrations ranged from 50 to 150 ng/g.

separated, eluting after 4.11 min (HFB_1) and 4.39 min (FB_1 and its D_6-labeled isotopomer). However, because the peak areas for quantification are taken from the single-ion chromatogram of each individual analyte, baseline separation is not necessary.

The developed analytical procedure was further characterized by means of a calibration curve, the detection limit, and the recoveries. First, a calibration curve was made with pure mixtures of standard solutions of HFB_1 and FB_1-D_6 in different weight ratios (1:3, 1:2, etc., up to 5:1), each of it injected three times. When plotted against the resulting peak area ratios, a linear calibration curve resulted (correlation coefficient $r = 0.9978$). To seize the influence of the matrix encountered in genuine food samples, a second curve was obtained by spiking a representative blank sample as described under Experimental Procedures. The resulting curve is depicted in Figure 4. For the weight ratios of HFB_1/FB_1-D_6 1:3, 1:2, and 1:1 the curve is nearly linear with an average response factor of 1.25, which is still acceptable yet showing a trend that is even more pronounced with the weight ratios 2:1 and 3:1. There are two possible explanations for the finding that the resulting peak areas for HFB_1 are always smaller than expected: either the HFB_1 is more strongly discriminated during sample workup compared to the isotopically labeled FB_1 or its ionization in the electrospray process is hampered to a greater extend than that of FB_1-D_6 in the presence of matrix components. Subsequent investigations revealed that both factors do contribute. However, a ratio of HFB_1:FB_1-D_6 of close to 1:1 in the food samples ensures an accurate quantification. Samples that did not come up to this requirement due to very high amounts of HFB_1 were worked up a second time. A calibration curve for FB_1 was designated in a similar manner, resulting in a linear curve up to a weight ratio of FB_1:FB_1-D_6 of 5:1 (correlation coefficient $r = 0.9998$).

The LODs were also determined. With standard solutions amounts of 100 pg of FB_1 or HFB_1 could be detected (s/n = 5:1), whereas in the presence of a food matrix amounts of 5 ng/g FB_1 and 8 ng/g HFB_1 were required for detection with a signal-to-noise ratio of 5:1. The slightly lower sensitivity for HFB_1 in matrix is a consequence of the interfering components causing a higher increase of the background in the mass range around m/z 400 (for HFB_1) than in the mass range around m/z 700 (for FB_1).

The recoveries for FB_1 were determined in two different food samples (an extrusion product and tortilla chips), and for HFB_1 four samples were analyzed (extrusion products, tortilla chips, and taco shells) as described under Experimental Procedures. The results are listed in Table 1 and represent the average of four values (duplicate sample workup, each injected two times). In the case of hydrolyzed fumonisin, the recoveries obviously depended substantially on the food type with extrusion products giving the poorest results. For FB_1 the recovery rates in either food matrix were more than satisfying. The HFB_1 recoveries reported in the literature range from 70% (Scott and Lawrence, 1996) to 100% (Maragos et al., 1997). However, the analytical technique of quantification via peak areas of the fluorescent derivatives is basically different from our approach by referring to an internal standard, and the methods can scarcely be compared to each other. The major advantages of the LC/MS method are its sensitivity and selectivity. Concerning the sensitivity, the LOD for the *o*-phthaldialdehyde derivatives are in most cases ~10 ng/g (Scott and Lawrence, 1996; Stack, 1998) or higher. Only Maragos et al. (1996) reported an LOD of 5 ng/g achieved with ELISA, and Newkirk et al. (1998) could detect 2.5 ng/g with ESI-MS in rodent feed. The major disadvantage with ELISA is the tendency of overestimating fumonisin levels due to the presence of cross-reacting compounds. With fluorescence detection, identification and quantitation are sometimes impeded by interference of coeluting substances. In contrast, the mass spectrometric detection is more specific and allows unambiguous determination of the analyte. Especially the employment of the SIM technique provides a very selective method for analyzing trace compounds in complex matrixes, because the great majority of interfering substances are eliminated due to a different molecular weight. Therefore, the developed LC/MS technique combines sensitivity and selectivity and is not limited to a special kind of food matrix.

With the developed procedure various food samples from the local market were analyzed for their contents of HFB_1 and FB_1. The determined concentrations are

Table 2. Concentrations of HFB_1 and FB_1 in Various Processed Corn Products

sample	concentration, ng/g ± SD[a]	
	HFB_1	FB_1
nacho chips	83.4 ± 5.7	184.8 ± 21.4
corn chips	76.6 ± 6.8	67.2 ± 2.3
tortilla chips 1	49.3 ± 3.9	114.4 ± 6.7
tortilla chips 2	nd[b]	92.2 ± 2.6
tortilla chips 3	43.9 ± 0.7	119.0 ± 3.1
taco shells 1	77.4 ± 4.0	39.3 ± 4.9
taco shells 2	67.3 ± 5.5	135.8 ± 4.9
extrusion product 1	nd	nd
extrusion product 2	nd	448.9 ± 79.2
extrusion product 3	17.4 ± 4.1	342.0 ± 15.1
corn waffle bread	8.5 ± 0.5	<5

[a] The values represent the average of four measurements; SD, standard deviation; amounts reported were not corrected for recovery. [b] Not detected.

listed in Table 2, each value representing the average of four analyses (derived from duplicate sample workup, each injected two times) ± standard deviation (SD). Nearly all samples, apart from one extrusion product, contained either HFB_1 or FB_1. In two samples, tortilla chips and an extrusion product, only FB_1 could be detected. All other foodstuff were contaminated with both FB_1 and HFB_1, but the FB_1 amounts were usually higher than the HFB_1 contents. Regarding the various product groups the extrusion products remarkably contain only low levels (8−17 ng/g) or no HFB_1 at all. Presumably, the temperatures and the heating time are not sufficient for cleavage of the TCA side chains. During the extrusion process temperatures usually do not exceed 120 °C, and the heating time is very short. Investigations on the heat stability of fumonisins showed that substantial degradation of the mycotoxin to the partially and fully hydrolyzed species requires heating at 150 °C for at least 40 min (Jackson et al., 1996). In contrast to the extrusion products, all foodstuff based on nixtamalized corn meal had substantially higher levels of the hydrolyzed fumonisin, ranging from 40 to 80 ng/g. Nixtamalization is a treatment during which the corn is submitted to heat and alkaline conditions, leading to an effective cleavage of the ester bonds and resulting in the decomposition of FB_1 to its fully hydrolyzed analogue. This has already been proved in former experiments (Murphy et al., 1996), so our results are in agreement.

CONCLUSION

An LC/ESI-MS method was developed for the qualitative and quantitative simultaneous determination of FB_1 and HFB_1 in processed corn-containing food. For quantitation isotopically labeled fumonisin FB_1-D_6 was utilized as an internal standard. Various food samples from the local market were analyzed for the presence of FB_1 or HFB_1, and most of them were found to be contaminated with either mycotoxin or both. The method was demonstrated to be sensitive, reliable, and useful for unambiguous determination of fumonisins in complex matrixes such as encountered in processed food. The results clearly show the widespread presence of FB_1 and HFB_1 in these kinds of products, indicating a possible exposure of the average consumer to these contaminants. With respect to the toxicity and the increasing popularity of such snacks, these findings should be considered when the health risk arising from the consumption of these foods is assessed. This is the first report of a screening for HFB_1 of corn-containing products from the German market.

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