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Analysis of Fumonisin B_1 in *Fusarium proliferatum*-Infected Asparagus Spears and Garlic Bulbs from Germany by Liquid Chromatography−Electrospray Ionization Mass Spectrometry§

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Fusarium proliferatum is one of a group of fungal species that produce fumonisins and is considered to be a pathogen of many economically important plants. The occurrence of fumonisin B_1 (FB_1) in *F. proliferatum*-infected asparagus spears from Germany was investigated using a liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-MS) method with isotopically labeled fumonisin FB_1-d_6 as internal standard. FB_1 was detected in 9 of the 10 samples in amounts ranging from 36.4 to 4513.7 ng/g (based on dry weight). Furthermore, the capability of producing FB_1 by the fungus in garlic bulbs was investigated. Therefore, garlic was cultured in *F. proliferatum*-contaminated soil, and the bulbs were screened for infection with *F. proliferatum* and for the occurrence of fumonisins by LC-MS. *F. proliferatum* was detectable in the garlic tissue, and all samples contained FB_1 (26.0−94.6 ng/g). This is the first report of the natural occurrence of FB_1 in German asparagus spears, and these findings suggest a potential for natural contamination of garlic bulbs with fumonisins.

KEYWORDS: Fumonisins; *Fusarium proliferatum*; toxins; asparagus; garlic; liquid chromatography−electrospray ionization mass spectrometry

INTRODUCTION

Fusarium proliferatum (Matushima) Nirenberg, a member of the *Liseola* Woll section, is a causal agent of diseases of various economically important plants. It is mostly found as a colonizer of maize (*1*) but, furthermore, of wheat, sorghum (*2*), date palm (*3*), rice (*4*), and asparagus (*Asparagus officinalis* L.) (*5*). The presence in useful plants is a potential risk to human health because many isolates of *F. proliferatum* can produce fumonisins (*6*). The fumonisins are a group of structurally related toxic metabolites mainly produced by *Fusarium verticillioides* (formerly *F. moniliforme*), which occurs worldwide and grows primarily on corn (*7*). Furthermore, fumonisins are produced by other *Fusarium* spp. such as *Fusarium proliferatum* (*8*). *F. moniliforme* and *F. proliferatum* are the two major species that produce fumonisins; however, both species are relatively different. Toxicological studies of the most prevalent isomer of the fumonisins, fumonisin B_1 (FB_1; **Figure 1**), proved clearly its causality for some animal diseases including equine leucoencephalomalacia (ELEM) in horses (*9*) and pulmonary edema in swine (*10*). Furthermore, it is hepatotoxic and carcinogenic in rats (*11*), and recently a National Toxicology Program (NTP) long-term feeding study provided clear evidence for the carcinogenic activities of FB_1 in the liver of female mice and in the kidney of male rats (*12*). FB_1 has also been implicated in high incidences of esophageal cancer in humans in South Africa and China (*13*, *14*).

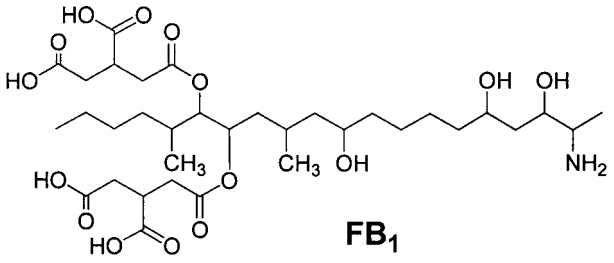

Figure 1. Structure of fumonisin B_1 (FB_1).

As far as we know, FB_1 produced by *F. proliferatum* has been verified as a natural contaminant in maize (*15*−*17*), rice (*18*), and asparagus (*5*). Further isolates of *F. proliferatum* taken from wheat, sorghum (*2*), and date palm (*3*) produced fumonisins when they were cultured on autoclaved medium. These findings suggest that human exposure to FB_1 is not restrained to consumption of corn and corn-based foods. To estimate and minimize the human risk, it is important to know which useful plants are susceptible to a fungal infection and if infection implicates a contamination with fumonisins. Therefore, a sensitive and reliable method for the determination of fumonisins is required. The great majority of the already existing methods are conceived to determine fumonisins from corn and corn-based

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matrices using the technique of precolumn fluorescent labeling with *o*-phthaldialdehyde (OPA). The major disadvantages of this method using OPA derivatization are the instability of the derivates and, even more, the fluorescent interferences caused by matrix components, making a definite and accurate quantification difficult.

One objective of this study was to develop a liquid chromatography−mass spectrometry (LC-MS) method allowing a reliable and quantitative determination of fumonisins, especially of FB_1 in vegetables. Because much lower levels of fumonisins are likely to be found in vegetables compared to corn and corn products, a more sensitive method is needed. As tentative plants we used asparagus and garlic. The demand for asparagus in Germany has greatly increased in recent years, and this leads to an expansion of the area under cultivation. Growers report more and more frequently on postcultivation problems and premature senescence. Therefore, it was in our interest to know how far *F. proliferatum* is involved in these problems and if asparagus spears are contaminated with fumonisins. The infection of asparagus plants with *F. proliferatum* alone or together with *F. oxysporum* f. sp. *asparagi* and other *Fusarium* species causes a destructive disease called *Fusarium* crown and root rot (*19*−*23*). Thus, we collected asparagus plant material of a selected German stand, which was damaged by rot and growth depression. Altogether three *Fusarium* species were isolated from the spears (*F. oxysporum*, *F. proliferatum*, and *F. sambucinum)* (*24*). To investigate the natural occurrence of fumonisins in German asparagus, samples that were infected by *F. proliferatum* were subsequently analyzed by LC-MS.

In addition, we focused our attention on garlic (*Allium sativum* L.), because we had previously demonstrated the occurrence of *F. proliferatum* in garlic bulbs from the German market (*25*). Therefore, it was of interest to know if *F. proliferatum* has the capability of producing fumonisins in garlic bulbs. In a model experiment we cultured garlic bulbs in *F. proliferatum*-contaminated soil, and after they had grown to ripeness, we investigated the occurrence of *F. proliferatum* (*25*) and fumonisins in the infected garlic bulbs.

MATERIALS AND METHODS

Analytical Standards. FB_1 was purchased from Alexis Biochemicals (Grünberg, Germany). Deuterium-labeled fumonisin B_1 (FB_1-d_6) was isolated from *F. moniliforme* culture material as described previously (*26*). 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 (TFA) were from Merck (Darmstadt, Germany). All other chemicals (of analytical purity) were obtained from Fluka (Deisenhofen, Germany) or Sigma-Aldrich (Steinheim, Germany). SAX Isolute solid-phase extraction cartridges (500 mg, 3 mL) were from ICT (Bad Homburg, Germany).

Asparagus Spears. Asparagus spears that were damaged by rot and growth depression were collected from fields of an asparagus-producing area of western Germany (Rheinland-Pfalz) in July 2000.

Garlic Bulbs. *F. proliferatum*, which was isolated before from garlic bulbs (see below), was incubated at 20 °C for 10 days on a wheat kernel medium. The infected wheat kernels were then mixed with a soil substrate, and garlic plants were cultured for 5 months in this mixture.

Fungal Isolation and Identification. Asparagus and garlic tissues were surface-disinfected and placed on plates containing a slight nutrient agar (SNA) medium selective for *Fusarium* (*27*). The identification of *Fusarium* spp. was performed using the method of Nirenberg (*27*). After incubation at 20 °C for 10 days, *F. proliferatum* could be identified in several asparagus and garlic samples. For fumonisin analysis asparagus and garlic samples were frozen at −20 °C.

Apparatus. Chromatographic separation was performed by an Applied Biosystems 140b LC 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.* The stalks of white asparagus were cut into small pieces (about 1 × 1 cm), ground in a laboratory blender, and dried by lyophilization. The sample was divided into two subsamples (0.2−1.5 g of dried sample, 3.1−24.4 g based on wet weight), and to each was added a known amount of FB_1-d_6 (100 ng/g) as isotopically labeled standard. The samples were then extracted by blending for 3 min with 15 mL of methanol/0.1 M hydrochloric acid (HCl) (3:1, pH ∼1.7) in an Ultra-Turrax disperser followed by centrifugation at 4000 rpm for 10 min. The supernatant was adjusted to pH 5.8−6.2 with 0.25 M sodium hydroxide solution (NaOH), and 4 mL was applied to a SAX cartridge preconditioned with 8 mL of methanol followed by 8 mL of methanol/water (3:1, adjusted to pH 5.8 with 0.1 M HCl). The cartridge was washed with 8 mL of methanol/water (3:1, adjusted to pH 5.8 with 0.1 M HCl), followed by 8 mL of methanol. Finally, fumonisins were eluted with 10 mL of 1% methanolic acetic acid. The eluate was evaporated to dryness under reduced pressure (40 °C, 330 mbar) and redissolved in 200 μL of methanol/water (60:40). All samples were at least analyzed in duplicate.

Entire garlic bulbs were ground in a laboratory blender, and to subsamples (4−5 g) was added a known amount of FB_1-d_6 (100 ng/g) as isotopically labeled standard. The samples were then extracted by blending for 3 min with 7 mL of methanol/0.1 M HCl (3:1, pH ∼1.7) in an Ultra-Turrax disperser followed by centrifugation at 4000 rpm for 10 min. The supernatant was adjusted to pH 5.8−6.2 with 0.25 M NaOH, and 4 mL was applied to a SAX cartridge. Preconditioning of the cartridge and isolation of fumonisins were performed as described above. The garlic samples were finally redissolved in 200 μL of acetonitrile/water (30:70). All samples were at least analyzed in duplicate.

Mass Spectrometric Analysis of FB_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) for asparagus samples and for garlic samples on a Waters Symmetry C18 column (150 × 2.1 mm i.d., 5 μm; Waters, Milford, MA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was 0.05% TFA in methanol (asparagus samples) or in acetonitrile (garlic samples). LC was programmed for asparagus samples as follows: isocratic step at 60% B for 1 min followed by a linear gradient to 99% B at 6 min. For garlic samples the LC program consisted of a linear gradient starting from 30% B to 45% B at 15 min followed by a linear gradient to 99% B at 2 min. The columns were both washed for 3 min with 100% solvent B 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 210 °C. Nitrogen served both as sheath (70 psi; 1 psi = 6894.76 Pa) and as auxiliary gas (10 units). The mass spectrometer was operated in the SIM mode, detecting the protonated molecular ions $[M + H]^+$ of FB_1 (*m*/*z* 722), FB_2 (*m*/*z* 706), and FB_3 (*m*/*z* 706) at a total scan duration of 1.0 s. For quantitative determination of FB_1 the protonated molecular ions $[M + H]^+$ of FB_1 (*m*/*z* 722) and FB_1-d_6 (*m*/*z* 728) were detected at a total scan duration of 1.0 s. Quantitative evaluations were based on the peak area ratios of FB_1 in comparison to that of FB_1-d_6, which served as isotopically labeled internal standard (for a detailed description see refs *26*, *30*, and *31*).

System Calibration and Recovery. The calibration was performed as described previously (*28*, *30*, *31*). The recovery was determined with standard solutions in matrix by adding 100 ng/g FB_1 to asparagus and garlic samples known to contain no FB_1. The samples ($n = 3$) were prepared as described above, and the recovery was specified to be 96−104% for garlic samples and 79−92% for asparagus samples. The good

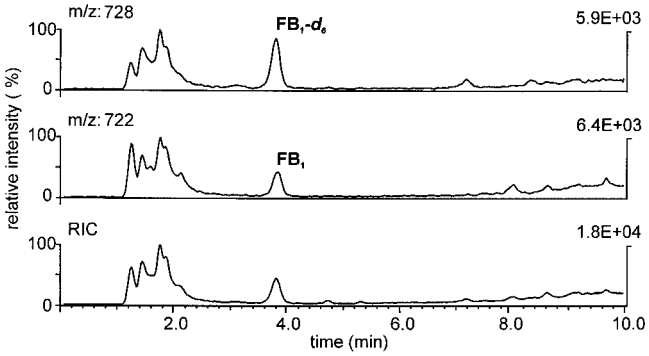

Figure 2. LC-ESI-MS analysis of an asparagus sample (**Table 1**, sample 3B). Monitored *m/z* ratios were 722 (FB_1) and 728 (FB_1-d_6). RIC = reconstructed ion chromatogram.

Table 1. Concentrations[a] of FB_1 in Various Asparagus Samples

	FB_1 (ng/g ± SD)	
sample	A	B
1	315.9 ± 2.2	4513.7 ± 5.3
2	99.7 ± 5.6	43.2 ± 4.1
3	249.1 ± 1.3	285.4 ± 7.8
4	nd[b]	nd[b]
5	72.3 ± 6.1	77.8 ± 21.8
6	267.2 ± 17.8	131.2 ± 0.7
7	74.9 ± 3.4	50.7 ± 7.3
8	36.4 ± 1.8	597.0 ± 0.9
9	123.4 ± 5.0	1863.6 ± 15.3
10	366.5 ± 14.0	3444.8 ± 28.6

[a] Based on dry weight; SD, standard deviation. [b] Not detected.

recovery resulted from the use of isotopically labeled FB_1-d_6 as internal standard.

RESULTS AND DISCUSSION

The combination of liquid chromatography with electrospray mass spectrometry is a proven tool for the analysis of fumonisins (*26*, *28*−*31*). On the basks of our experiences we developed a method for the qualitative and quantitative determination of fumonisins in vegetables, specifically in asparagus spears and garlic bulbs. Using a mixture of methanol/0.1 M HCl (3:1) for extraction and a strong anion exchanger (SAX) for sample cleanup, we applied a method that had been proven to be highly selective for fumonisin analysis (*26*). Because of the high water content of asparagus spears (90−95%), samples had to be lyophilized before cleanup to avoid a dilution of the extraction solution. The matrices of corn and corn-based food differ from those of vegetables. Thus, for the separation of the analytes we tested two different gradients that had been suitable for corn matrices: an acetonitrile/water and an methanol/water gradient (*30*, *31*) (see Materials and Methods). **Figures 2** and **3** show LC-ESI-MS chromatograms of an asparagus (**Table 1**, sample 3B) sample and of a garlic sample (**Table 2**, sample 1), demonstrating the separation of fumonisins B_1 from matrix components. Both gradients proved to be as suitable for the vegetable matrices as they were for the corn matrices. Quantitative analysis was carried out using labeled fumonisin FB_1-d_6 as internal standard. The high accuracy of this method has recently been proven and described in detail for the simultaneous determination of fumonisins (*30*, *31*).

To investigate the occurrence of fumonisins in white asparagus, samples which were damaged by rot and growth depression were collected from selected German cultivation areas. The asparagus samples showed colonization with *F. proliferatum* and other *Fusarium* spp. depending on the geographical origin

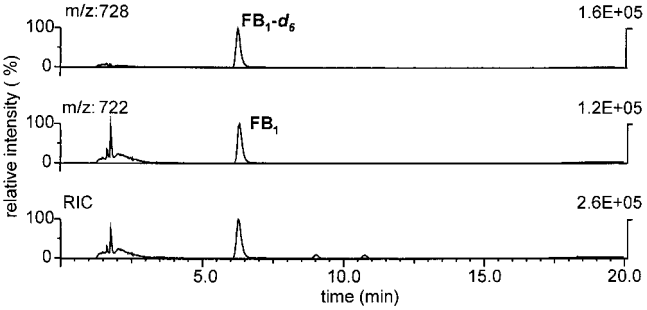

Figure 3. LC-ESI-MS analysis of a garlic sample (**Table 2**, sample 1). Monitored *m/z* ratios were 722 (FB_1) and 728 (FB_1-d_6). RIC = reconstructed ion chromatogram.

Table 2. Concentrations of FB_1 in Various Garlic Bulbs

sample	FB_1 (ng/g ± SD[a])
1	94.6 ± 2.5
2	26.0 ± 1.2
3	47.6 ± 1.1
4	27.7 ± 1.6

[a] SD, standard deviation.

(*24*). Fungal contamination was most evident at the lower ends of the stalks. Asparagus stalks that were infected with *F. proliferatum* were analyzed for FB_1 using the above-described method (for analysis, the upper part of the stalks was used). The results are summarized in **Table 1**. Fumonisin B_1 could be detected in 9 of the 10 analyzed asparagus samples in concentrations ranging from 36 to 4514 ng/g (based on dry weight). It is worth mentioning that in some cases the FB_1 concentrations in the asparagus subsamples (**Table 1**, A and B) differ extremely from each other. This can be explained by the fact that the fumonisin-producing fungi are not uniformly spread over the spears and subsamples were randomly taken from different sides of the upper part of the stalks (as mentioned above, fungal contamination was most evident at the lower part of the stalks; however, this part was not used for fumonisin analysis). Besides fumonisin B_1, FB_2 and FB_3 were found in six samples in lower concentrations compared to FB_1 (data not shown because an isotopically labeled standard is not available for FB_2 and FB_3 and the calibration was performed only for FB_1).

Furthermore, the capability of producing fumonisins by *F. proliferatum* in garlic bulbs was investigated. Therefore, garlic was cultured in *F. proliferatum*-contaminated soil for 5 months (see Materials and Methods). The garlic bulbs were then surface-disinfected and screened for *F. proliferatum*. Using the method of Nirenberg (*27*), *F. proliferatum* could be detected in the garlic tissue. The bulbs were then screened for fumonisins by LC-MS, FB_1 was found in all four garlic samples (**Table 2**) in concentrations ranging from 26 to 94 ng/g (all samples contained lower concentrations of FB_2 and FB_3; however, as mentioned above, accurate quantification was performed only for FB_1). The finding that *F. proliferatum* is able to infect garlic bulbs cultured in contaminated soil and the occurrence of fumonisins in infected bulbs suggest a potential for natural contamination of garlic with fumonisins. This is supported by the fact that the *F. proliferatum* strain used in this study was isolated from commercially available garlic bulbs (*25*).

This is the first report of the natural occurrence of fumonisins in asparagus spears from Germany, and, furthermore, our findings suggest a potential for the natural contamination of garlic bulbs with fumonisins.

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