Free and Hidden Fumonisins in Hordei Fructus Germinatus and Their Transfer to the Decoction

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Abstract

Objective: This study aimed to determine the free and hidden fumonisins in Hordei Fructus Germinatus samples and to investigate the transfer rates of these mycotoxins from Hordei Fructus Germinatus to its decoction. Materials and Methods: The contamination levels of free and hidden fumonisins in a total of 60 Hordei Fructus Germinatus samples were analyzed using high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The decoction procedure was simulated with a highly contaminated Hordei Fructus Germinatus sample, and fumonisins in the resulting decoction preparation were determined by LC-MS/MS. Results: Among all the samples, 8.3% were contaminated with free fumonisins (FB, and/or FB2) and 13.3% were contaminated with total fumonisins (free + hidden, measured as hydrolyzed fumonisins, i.e., HFB, and/or HFB2). The concentrations of FB, and HFB, reached up to 83 and 95 µg/kg, respectively, whereas FB, and HFB, were detected only in traces. The transfer rates of free and total fumonisins from Hordei Fructus Germinatus to the decoction were 71.8% and 83.3% for FB, and FB2, respectively. In comparison, much lower transfer rates were found for total fumonisins, i.e., 38.2% and 24.7% for HFB, and HFB2, respectively. Conclusion: The incidence and contamination levels of free and hidden fumonisins in Hordei Fructus Germinatus samples were generally low. Regarding decoction preparation, the transfer rates of free fumonisins into the decoction were high, whereas a large part of hidden fumonisins were retained in Hordei Fructus Germinatus rather than migrating into water.

Keywords: Decoction, fumonisins, hidden fumonisins, Hordei Fructus Germinatus, liquid chromatography coupled to tandem mass spectrometry, transfer rate

Introduction

 Hordei Fructus Germinatus is the dried germinant grain of the plant Hordeum vulgare L. (commonly known as barley), belonging to the family Gramineae. Hordei Fructus Germinatus is produced by steeping barley grains in water and then keeping them under appropriate moisture and temperature for germination; the resulting barley malts are fried, without oil, to dryness. Hordei Fructus Germinatus is a widely used traditional Chinese medicine; it is usually used to facilitate digestion, as well as to help breastfeeding women terminate lactation.[1] Hordei Fructus Germinatus is listed among the traditional Chinese medicines that can be used as both medicine and food according to the National Health Commission of China.

The raw materials of Hordei Fructus Germinatus, barley grains, are susceptible to Fusarium head blight, a disease caused by fungi belonging to the genus Fusarium, which results in yield losses, quality reduction, and accumulation of mycotoxins.[2,3] Although infection of the grains by Fusarium fungi usually takes place in the field, fungi growth may continue after harvest with inappropriate storage practices. Specifically, the moisture conditions for barley germination could be especially favorable for fungal growth and mycotoxin production, leading to even higher contamination levels of mycotoxins.[4,5]

Among the various mycotoxins produced by Fusarium fungi, fumonisins have been reported to occur in barley grains

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and barley malt.[6-8] Fumonisins are a group of structurally related mycotoxins mainly produced by the species *Fusarium verticillioides* (formerly *Fusarium moniliforme*) and *Fusarium proliferatum*.[9] All fumonisins are highly polar compounds with a long aminopolyhydroxyalkyl chain that is diesterified with tricarballylic acid groups.[10] Based on differences in chemical structures, they are classified into four series, being fumonisin B$_1$ and B$_2$ (FB$_1$ and FB$_2$) of series B, the most abundant in food and feed.[11] FB$_1$ has been classified as a Group 2B carcinogen (possibly carcinogenic) to humans by the International Agency for Research on Cancer. Fumonisins are suspected to be related to the high incidences of human esophageal cancer in the Transkei region of South Africa and Linxian County of China.[12,13] They also exhibit different toxic effects in animals, including leukoencephalomalacia in horses, pulmonary edema in pigs,[14] and hepatocarcinogenicity in rats.[15]

In addition to free fumonisins, various forms of hidden fumonisins have been reported in food, where they could be chemically modified or bound to matrix macroconstituents through covalent bonds;[11] the latter is expected to be converted into their original form during digestion and thus exert toxicity to humans.[16]

As a herbal medicine, Hordei Fructus Germinatus is usually prepared by first boiling in water, and then the decoction (i.e., the resulting liquid) is taken, whereas the residue is discarded. In light of this, we consider it important to determine the transfer rates of free and hidden fumonisins from the original Hordei Fructus Germinatus into the final decoction.

The aims of this study were (a) to determine the contamination levels of free and hidden fumonisins in Hordei Fructus Germinatus samples and (b) to investigate the transfer rates of these mycotoxins from Hordei Fructus Germinatus to decoction.

**Materials and Methods**

**Chemicals and reagents**

Standards of FB$_1$ and FB$_2$ (50 µg/mL in acetonitrile) were purchased from Fermentek (Jerusalem, Israel); [13C$_{34}$]-FB$_1$ (25 µg/mL in acetonitrile) and [13C$_{34}$]-FB$_2$ (10 µg/mL in acetonitrile) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Various dilutions of the above standards were prepared in acetonitrile and stored at −20°C. The standards of hydrolyzed fumonisins (HFB$_1$ and HFB$_2$), as well as [13C$_{34}$]-HFB$_1$ and [13C$_{22}$]-HFB$_2$, were synthesized in the laboratory by alkaline hydrolysis followed by ethyl acetate extraction according to the method described by Dall’Asta et al.[17]

For high-performance liquid chromatography (HPLC) assays, acetonitrile, methanol, and ethyl acetate (all of HPLC grade) were purchased from Tedia (Fairfield, OH, USA); formic acid (HPLC grade) was obtained from Aladdin (Shanghai, China); and sodium hydroxide (analytical grade) was obtained from Sinopharm Chemical Reagent (Shanghai, China). Ultrapure water was produced by a Purelab Pulse system (Elga, Germany).

**Samples**

A total of 60 Hordei Fructus Germinatus samples were purchased from local traditional Chinese pharmacies and markets in the Zhejiang province, China, as well as from online stores in China. Each sample package weighed 500 g or 1 kg. All the samples were ground into a fine powder and screened through a sieve (<250 µm) before further treatment.

**Sample preparation of Hordei Fructus Germinatus**

For the determination of free fumonisins (FB$_1$ and FB$_2$), 1 g of each sample was extracted with 10 mL of acetonitrile/methanol/water (25:25:50, v/v/v) by shaking vigorously for 30 min and then centrifuged at 5000 rpm for 5 min. The supernatant was filtered through a 0.22 µm membrane filter, and an aliquot of the filtrate (usually 1.0 mL) was mixed with specific amounts of each labeled internal standard (usually 15 ng of [13C$_{34}$]-FB$_1$ and 10 ng of [13C$_{34}$]-FB$_2$) before HPLC injection.

Hidden fumonisins were determined by indirectly measuring total (free + hidden) fumonisins. The Hordei Fructus Germinatus samples were treated with an alkaline solution to transform both free and hidden fumonisins into hydrolyzed fumonisins (HFB$_1$ and HFB$_2$), and the latter were measured. The levels of HFB$_1$ and HFB$_2$ were expressed as equivalent of FB$_1$ and FB$_2$, respectively. Specifically, 1 g of each sample was weighed, to which [13C$_{34}$]-FB$_1$ and [13C$_{34}$]-FB$_2$ were added (usually 20 ng each). The mixture was hydrolyzed with 20 mL of 2 M NaOH, then extracted with 20 mL of ethyl acetate, and centrifuged at 5000 rpm for 5 min. After centrifugation, the glutinous middle layer was collected with the ethyl acetate layer and centrifuged again at 10,000 rpm for 10 min. The resulting ethyl acetate layer was collected, dried, redissolved in 2 mL of acetonitrile–water (1:1, v/v), and then filtered through a 0.22 µm membrane filter before HPLC injection. For a complete extraction of hydrolyzed fumonisins from the alkaline layer, a second extraction with ethyl acetate would be necessary. However, because the labeled standards of free fumonisins were added to the samples before the alkaline treatment, any loss of hydrolyzed fumonisins due to incomplete extraction would be compensated for by a proportional loss of labeled hydrolyzed fumonisins; therefore, a second extraction was omitted to save laborious work.

**Sample preparation of Hordei Fructus Germinatus decoction**

A highly contaminated Hordei Fructus Germinatus sample was used to simulate the decoction preparation procedure. The sample was obtained by inoculating a regular sample with a strain of *F. verticillioides* isolated from a moldy maize sample. After 1 month of fungi growth, the sample was dried in an oven under 100°C, then ground into a fine powder, and homogenized. The free and hydrolyzed fumonisins in the sample were determined as described above.
Decoction was prepared in triplicate; for each preparation, 30 g of the sample was added to 600 mL of water and boiled for 45 min. The resultant product (decoction + residue) was weighed, centrifuged, and filtered to separate the decoction from the residue. The weight of each decoction was 295 g, 355 g, and 392 g, respectively, which meant that for every gram of Hordei Fructus Germinatus, approximately 10–13 g of decoction was obtained. To maintain the decoction sample preparation similar to that of the Hordei Fructus Germinatus, 10 g of the decoction was mixed with 5 mL of acetonitrile and 5 mL of methanol; subsequent procedures were the same as those described for Hordei Fructus Germinatus samples for the determination of free fumonisins. For the determination of total fumonisins, 1.6 g of sodium hydroxide was dissolved in 10 mL of water and mixed with 10 g of decoction to obtain a 2 M NaOH mixture. Labeled internal standards were added to this mixture, and the subsequent procedures were similar to those described for Hordei Fructus Germinatus samples, with the exception of the second centrifugation step at 10,000 rpm, which was not necessary.

**Liquid chromatography coupled to tandem mass spectrometry conditions**

Liquid chromatographic separation was carried out on a Shimadzu LC-30AD system (Shimadzu, Kyoto, Japan) using an Agilent Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 µm i.d., Agilent, Santa Clara, CA, USA). The column oven was set at 40°C, injection volume was 5 µL, and flow rate was 0.8 mL/min. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (methanol). The free and hydrolyzed fumonisins were analyzed in separate runs, and in both cases, gradient elution was carried out with the following program: 0–3.0 min, 40% B; 3.0–8.0 min, 40%–90% B; 8.0–10.0 min, 90% B; 10.0–10.2 min, 90%–100% B; 10.2–12.0 min, 100% B; 12.0–13.0 min, 100%–40% B; and 13.0–15.0 min, 40% B. The LC system was interfaced to a Qtrap 5500 mass spectrometer (AB Sciex Inc., Foster City, CA, USA). The ion source parameters were set as described previously (Hu et al., 2019).[18] Detection of the analytes was performed in multiple reaction mode using the ion pairs presented in Table 1. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) chromatograms of a naturally contaminated Hordei Fructus Germinatus sample are shown in Figure 1.

**Method validation**

For each analyte, a seven-point calibration curve was generated by measuring a series of standard solutions, for which the molar ratios of the analyte and its isotope-labeled internal standard were 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, and 10:1, respectively. After LC-MS/MS measurement, response curves were obtained from molar ratios ($n_{analyte}/n_{standard}$) versus peak area ratios ($A_{analyte}/A_{standard}$), and response functions were calculated using linear regression. Recoveries were determined by spiking a blank Hordei Fructus Germinatus sample with the analytes at three concentrations (25, 50, and 500 µg/kg), each in triplicate. Limits of detection (LODs) and limits of quantitation (LOQs) were obtained by spiking a blank sample with different concentrations of analytes, and the LODs and LOQs were calculated based on signal-to-noise ratios of 3 and 10, respectively.

**RESULTS AND DISCUSSION**

**Method validation**

The calibration curves presented good linearity within the chosen molar ratios of analytes and internal standards (i.e., 0.1–10). The coefficients of correlation ($R^2$) of the response functions for $FB_1$, $FB_2$, $HFB_1$, and $HFB_2$, were 0.9998, 0.9989, 0.9993, and 0.9995, respectively. The mean recoveries at three spiking levels (25, 50, and 500 µg/kg) ranged from 91.2% to 104.1%, and the precision, expressed as relative standard deviation, ranged from 2.3% to 9.6%. These results were well in accordance with the performance criteria for $FB_1$ and $FB_2$, set by the European Commission Regulation No. 401/2006.[19] The LODs for $FB_1$, $FB_2$, $HFB_1$, and $HFB_2$, were 7, 5, 7, and 4 µg/kg, respectively, and their LOQs were 20, 17, 20, and 10 µg/kg, respectively.

**Free and hidden fumonisins in Hordei Fructus Germinatus samples**

A total of 60 Hordei Fructus Germinatus samples were analyzed for the presence of free ($FB_1 + FB_2$) and total fumonisins ($HFB_1 + HFB_2$); their contamination levels are summarized in Table 2. Regarding free fumonisins, 8.3% of the samples were contaminated with at least one of $FB_1$ and $FB_2$. As for total fumonisins, a higher incidence (13.3%) was found. The concentrations of $FB_1$ and $HFB_1$ in positive samples reached up to 83 and 95 µg/kg, respectively. In three samples, $HFB_1$ was detected in levels below LOQ, whereas $FB_1$ was not detected. For the other positive samples, $HFB_1$ levels were higher than those of $FB_1$, and the $HFB_1/ FB_1$ ratios varied between 1.14 and 3.57, results comparable with those previously reported in raw maize.[20,21] On the other hand, $FB_1$ and $HFB_1$ were detected only in traces, with values mostly below LOQs, suggesting that there were no

| Analyte     | Precursor ion (m/z) | Product ion (m/z) |
|-------------|---------------------|-------------------|
| $FB_1$      | 374.3               | 334.3             |
| $[^{13}C_3]FB_1$ | 374.4               | 334.5             |
| $FB_2$      | 318.3               | 336.2             |
| $[^{13}C_3]FB_2$ | 318.5               | 336.5             |
| $HFB_1$     | 370.5               | 390.5             |
| $[^{13}C_3]HFB_1$ | 390.5               | 391.5             |
| $HFB_2$     | 318.5               | 340.5             |
| $[^{13}C_3]HFB_2$ | 340.5               | 394.5             |

FB: Fumonisin, HFB: Hydrolyzed FB
quantifiable levels of hidden FB$_2$ in these Hordei Fructus Germinatus samples.

Previously, occurrences of free fumonisins have been reported in barley malt, but not in Hordei Fructus Germinatus. In a survey of brewer’s grain (by-product of barley malt after beer brewing) from Brazil, FB$_1$ was found in 72.5% (58 out of 80) of samples with a mean value of 226.5 µg/kg and a maximum level of 908 µg/kg.\cite{7} Gonzalez Pereyra \textit{et al.}\cite{22} investigated 51 samples of barley malt and brewer’s grain from Argentina, all of which were contaminated with 104–145 µg/kg of FB$_1$. In a more recent survey of barley malt from Brazil, lower incidence and contamination levels of fumonisins were reported, where out of 50 samples, only 5 were contaminated with no more than 13 µg/kg of FB$_1$ and only 1 sample was contaminated with 90 µg/kg of FB$_2$.\cite{8}

As for hidden fumonisins, to the best of our knowledge, this is the first report of their occurrence in Hordei Fructus Germinatus, as well as in barley malt. Overall, the contamination levels of fumonisins in Hordei Fructus Germinatus samples were much lower compared to those found in other matrices such as maize, where levels above 1000 µg/kg were frequently reported.\cite{18,21,23}

\textbf{Figure 1:} Liquid chromatography coupled to tandem mass spectrometry chromatograms of a naturally contaminated Hordei Fructus Germinatus sample: (a) fumonisins and their labeled internal standards; (b) hydrolyzed fumonisins and their labeled internal standards.
Currently, there are no regulations for the limits of fumonisins in Hordei Fructus Germinatus or in barley malt in either China or European Union (EU). In maize intended for direct human consumption, a maximum level of 1000 µg/kg for the sum of FB1 and FB2, is set by EU.[24] All of the Hordei Fructus Germinatus samples examined in this study were well below the limit, even if taking into account the hidden fumonisins.

**Transfer of free and hidden fumonisins from Hordei Fructus Germinatus to the decoction**

The levels of free and total fumonisins in the inoculated Hordei Fructus Germinatus and the decoction are given in Table 3. Here, the fumonisin concentrations in the decoction were recalculated to transform the units from “µg of fumonisin per 10 g of decoction” into “µg fumonisin per kg of the original Hordei Fructus Germinatus.”

For free fumonisins, the transfer rates from Hordei Fructus Germinatus to decoction were 71.8% and 83.3% for FB1 and FB2, respectively. The high transfer rates are not surprising, given that both FB1 and FB2 are highly soluble in water. A similarly high transfer rate of 83.4% was reported in a previous study on the transfer of ochratoxin A from the medicinal herb Astragalus propinquus root to decoction,[25] although ochratoxin A is less soluble in water than FB1 and FB2. In comparison, for total fumonisins, the transfer rates were 38.2% and 24.7% for HFB1 and HFB2, respectively. The considerably lower transfer rates of total fumonisins suggest that a large part of the hidden fumonisins were retained in the Hordei Fructus Germinatus residue rather than migrate into the water during boiling. In a previous report by Nian et al., the transfer rates of aflatoxins from Hordei Fructus Germinatus to decoction were studied, and the rates were similarly low for aflatoxins B1 and G1 (29.8 and 33.5%, respectively), and even lower with aflatoxin B2, which was only 6.3%.[26]

**Conclusion**

An LC-MS/MS method for the analysis of free and hidden fumonisins in Hordei Fructus Germinatus was established and validated; good recovery, precision, and LOQs were achieved. The method was applied to 60 Hordei Fructus Germinatus samples collected in China. The incidence and contamination levels of free and hidden fumonisins in the examined samples were generally low. In the decoction experiment, the transfer rates of free fumonisins from Hordei Fructus Germinatus to decoction were found to be high. In contrast, much lower transfer rates were found for total fumonisins, suggesting that a large part of hidden fumonisins were retained in Hordei Fructus Germinatus rather than migrate into water. Therefore, as a herbal medicine, hidden fumonisins existing in Hordei Fructus Germinatus would pose lower health risks to humans than free fumonisins.

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**Conflicts of interest**

There are no conflicts of interest.

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