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報　文

Liquid chromatographic detection of fumonisins in rice seed

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Abstract

Fumonisins are mycotoxins mainly produced by Fusarium *verticillioides*, which is a major pollutant to corn. On the other hand, rice adherent fungus *Gibberella fujikuroi* is taxonomically near to *F. verticillioides*, so that the potential risk of fumonisin contamination in rice is significant. Previously we developed an efficient extraction procedure (submergence extraction) and a sensitive detection method (liquid chromatography with tandem mass spectrometry (LC−MS /MS)) for the determination of fumonisins in brown rice. In this study, we attempted to detect fumonisins in rice seed in the husk by an extension of the method for fumonisin analysis in brown rice, according to the request to detect fumonisins in rice before threshing. As a result, extraction with submergence was found to be effective in rice seed as well as in brown rice, but more effective way was to use a different solid phase extraction (SPE) cartridge for the purification of fumonisins from rice seed extract. With submergence extraction and purification with the newly selected SPE cartridge, fine recovery was achieved for clean rice seed samples in spike and recovery tests. About five hundred grams of rice seed sample was divided to seven subsamples, and ten grams from each subsample was subjected to analysis. The conventional determination method, liquid chromatography using fluorescence detection (HPLC−FL) with precolumn derivatization, could not detect any fumonisins in any of the analytical samples, while fumonisins were detected in two of seven samples by use of LC−MS /MS. This is the first case of the natural occurrence of fumonisins in domestic Japanese rice. The importance of sampling in non−homogene−ous samples like rice seed was also suggested.

Key word: Fumonisin, rice, Gibberella fujikuroi, HPLC−FL, LC−MS /MS

Introduction

Mycotoxin contamination in cereals is a potential risk to human and animal health. Among several hundreds of mycotoxins, fumonisins are newly found *Fusarium* toxins which were first identified in 1988 and are among the most important mycotoxins regarding food and feed safety[1) 2)]. Currently, 28 structural fumonisin analogs are known, and the most abundant analogue in nature is fumonisin B1 (FB1), followed by fumonisin B2 (FB2) and fumonisin B3 (FB3)[3)]. Fumonisins are produced mainly by *Fusarium verticil−lioides*, a corn adherent fungi, and fumonisin contami−nation in corn has been observed in various areas of the world[4)]. FB1 has been implicated in disorders in

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animals such as leukoencephalomalacia in horses, pulmonary oedema syndrome in pigs, and showing nephrotoxicity, hepatotoxicity, and hepatocellular carcinogenicity in rats[5]. Furthermore, an association between high rates of human esophageal cancer and high concentration of fumonisin in corn has been reported in South Africa, the United States, and China [6)~9)]. In 2001, the Joint FAO /WHO Expert Committee on Food Additives and Contaminants (JECFA) established a Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisins (the sum of FB1, FB2, and FB3) of 2.0 μg/kg of body weight per day[10] and in 2002, the International Agency for Research on Cancer (IARC) evaluated FB1 derived from *F. verticillioides* as Group 2B, *i.e.* a possible human carcinogen[11]. Fumonisins have also been remarked as neurotoxins because they have been found to be a potential cause of neural tube defects in human babies since first reported in 1999 on the United States (Texas)-Mexico border, a high corn-consuming population[12].

Currently, corn and corn-based products are the only commodities which are known to contain significant amounts of fumonisins. Apart from corn-related products, fumonisins have occasionally been found in various cereals or food products including rice[13]. The fumonisin-polluted commodities reported other than corn, include rice, wheat, asparagus, cowpeas, maize, sorghum, millet, farro, black tea, and beer[13)~23)]. Rice is a cereal of tropical origin and generally tolerant to fungi compared to corn or wheat ; however, rice is susceptible to two kinds of fungi, *Pyricularia oryzae* and *Gibberella fujikuroi*. *P. oryzae* is the most destructive pathogen of rice worldwide, but it is not classified as a mycotoxin-producing fungus. On the other hand, an anamorph of *G. fujikuroi* is taxonomically close to *Fusarium verticillioides* (formerly *F. moniliforme*), a major fumonisins producer, and fumonisin production has been confirmed in *G. fujikuroi*[24)~26)], so that the potential risk of fumonisin contamination in rice is considered to be significant.

Although contamination of rice with fumonisin has been reported in the United States and it has been studied extensively in EU[13) 27)], little information has come from Asia. The present study describes the analysis for the contamination of major B type fumonisins (FB1, FB2 and FB3) in a *G. fujikuroi*-infected domestic rice grain with husks by application of our in-house validated method for the determination of fumonisins in rice[28]. So far, the most common and conventional detection method for fumonisins is HPLC separation with fluorescence (FL) detection for fluores- cent fumonisin derivatives using reagents like *o*-phthalaldehyde[29) 30)], while we have developed another detection method using liquid chromatography -tandem mass spectrometry (LC-MS /MS) coupled with an efficient extraction procedure for the determination of FB1, FB2, and FB3 in brown rice. In this study we compared the performance of LC-MS /MS detection to HPLC-FL detection for the detection of fumonisins in rice grain with husks, and also we examined the applicability of the extraction and purification procedures developed for brown rice to rice grain with husks.

Materials and methods

Samples

Rice seed, a part of which was considered to be infected with *G. fujikuroi*, was harvested in autumn 2007 in the Southern area of Japan.

Chemicals and standards

Standard FB1, FB2, and FB3 were purchased from Biopure Co. (Tulin, Austria). All other reagents were of analytical grade or HPLC grade. Stock solutions of all analytes (100 μg/ml each) were prepared in acetonitrile-H_2O solution (1 + 1, v/v) as recommended by AOAC Official Method 995. 15, a validated analytical method for fumonisins in corn[31], and were diluted to the desired concentration with acetonitrile-H_2O solution. Fumonisin stocks and standard working solutions were kept up to 6 months at 4℃.

Sample extraction and cleanup

A rice seed sample (ca. 500 g) was divided to seven aliquots and milled for 2 minutes by Waring Laboratory Blender (Model 7012S, Waring Commercial, CT, USA) to yield a fine material. An accurately weighed amount of each sample (10 g) was soaked with 12. 5 ml

of water in a 300 ml Erlenmeyer flask with a ground-in stopper for 0 or 30 minutes as described in *Extraction efficiency test and recovery test*. After submergence, 37. 5 ml of methanol was added into the flask, which was shaken for one hour by Laboratory shaker (TAITEC, Japan). The extract obtained by centrifugation was purified by solid-phase extraction (SPE) using two kinds of strong anion exchange (SAX) cartridges ; Sep-Pak Accell Plus QMA (Waters, CA, USA) or Bond Elut SAX (Varian, CA, USA). Five milliliters of filtrate was loaded onto the SPE-SAX cartridge followed by washing with 5 ml of methanol- water (3 + 1, v/v) and then by 5 ml of methanol. Fumonisins were eluted with 7 ml of 1 % methanolic acetic acid at a flow rate less than 1 ml /min. The eluate was collected in a 10 ml amber glass tube and evaporat- ed at 60℃ under a gentle flow of nitrogen. The tube was rinsed with methanol and evaporated to dryness to ensure that all acetic acid has evaporated. The residue was redissolved in 1.0 ml acetonitrile-water (1 + 1, v/v) and served as the sample solution for injection with precolumn derivatization (HPLC-FL) or for direct injection (LC-MS /MS).

Extraction efficiency test and recovery test

For the recovery test of spiked sample, 10 g of blank rice seed was weighed in a 300 ml flask with a ground-in stopper and submerged with 12.5 ml of water for 0 or 30 minutes. Then standard fumonisins solution (2. 0 ml of 2.5 μg/ml FB1, FB2, and FB3 mixture to make 0.5 mg /kg spiked sample) were added 5 minutes before the addition of methanol, followed by extraction and cleanup procedure as described above. Recovery was calculated by the equation as follows:

$$\text{Recovery}(\%) = (A - B) / \text{A X D X } 100$$

where A = signal of spiked sample, B = signal of blank sample, D = dilution factor.

Apparatus and determination conditions for HPLC-FL analysis

HPLC-FL analysis was performed using an LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a C_{18} L-column (250 x 4.6 mm i.d., 5 μm spherical particle size) (CERI, Tokyo, Japan), an auto-injector SIL-10A (Shimadzu) and a fluorescence detector RF-10AXL (Shimadzu) as described previously[28]. The mobile phase and injection were also same as used previously except for the reaction for precolumn derivatization. Injection with precolumn derivatization was conducted by programming, where 10 μl of fluorescent reagent mixture (0. 3 M *o*-phthalaldehyde in methanol- 0.1 M disodium tetraborate- 2-mercaptoethanol (1 + 5 + 0.05, v/v) and 10 μl of sample solution was mixed followed by injection of 5 μl. Fluorescence was detected with excitation at 335 nm and emission at 440 nm. Chromatographic data was analyzed using the Chromatopac system (Shimadzu).

Calibration curves were based on the analysis of standard working solutions in the range of 0.05-1.0 μg/ml (0.05, 0.1, 0.2, 0.5, and 1.0 μg/ml) for FB1, FB2, and FB3. The limit of detection (LOD) was defined as the concentration that was three times higher than the standard deviation of the blank signal. The LOD determined was 0. 08 mg /kg for FB1, FB2, and FB3. The LOQ was defined as twice the LOD.

Apparatus and determination conditions for LC-MS /MS analysis

An LC1100 HPLC system (Agilent Technologies, CA, USA) connected to a quadruple MS /MS system API 4000 Q-trap (Applied Biosystems, CA, USA) was used and data acquisition and mass spectrometric eval- uation were conducted by Analyst 1.4.1 software (Applied Biosystems) as described in our previous study[28]. HPLC gradient conditions were also same, except for the gradient ran from 10 % to 70 %. The injection volume was 5 μl. MS /MS conditions were also same as described previously with ionization by ESI positive mode. The detection was made by monitoring ion: 722.5/334.1 (FB1), 706.4/336.1 (FB2), and 706.4/336.2 (FB3), with the ion pairs representing the protonated precursor ion $[M+H]^+$ and the most abundant product ion $[M+H-2$ tricarboxylic acid (TCA)- $2H_2O]^+$ for FB1, and the protonated precursor ion $[M+H]^+$ and the most abundant product ion $[M+H-2TCA-H_2O]^+$ for FB2 and FB3.

Calibration curves were based on the analysis of standard working solutions in the range of 0.01-0.5 μg/

ml (0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 μg/ml) for FB1, FB2, and FB3. The LOD and LOQ were defined in the same way as those by HPLC-FL. The LOD determined was 0.02 mg /kg for FB1 and 0.01 mg /kg for FB2 and FB3. The LOQ was defined as twice the LOD.

Results and discussion

Applicability of extraction and purification procedures

The recoveries of each fumonisin spiked to rice seed samples are shown in Table 1. Spike and recovery tests were done in duplicate and the average value is shown. For the extraction of fumonisins from food samples, methanol-water (3 + 1, v/v) has often been used as the extraction solvent in the past, as adopted by the AOAC Official Method 995. 15[31) 32)]. However, in our previous study, this extraction solvent was not effective for rice, since the recovery ranged from 40 to 80 % for different rice lots[28)]. We therefore investigated another extraction technique using submergence in water prior to the addition of organic solvent that has been applied for the extraction of some pesticides[33)] and found this method effective in only 30 minutes submergence. In the present study, we found that the submergence for 30 minutes was also effective in rice seed as well as in brown rice in the spike and recovery test (Table 1, right column).

For the clean-up step for fumonisins, we used Sep-Pak Accell Plus QMA and Bond Elut SAX cartridges as the SPE-SAX cartridges, and found that the latter was more suitable for the purification of extracts of rice seed for better recovery (Table 1, upper row). In our previous study using brown rice[28)], there was no difference between Sep-Pak Accell Plus QMA and Bond Elut Sax cartridges in purification efficiency. Therefore, the difference may be derived from the matrix material not contained in brown rice, such as husks.

Occurrence of fumonisin in rice seed

G. fujikuroi is often found in rice, thus fumonisins potentially could occur in rice. The natural occurrence of fumonisins in rice was first reported by Abbas et al[13)]. in 1998 in Arkansas and Texas, USA. , and there are some other reports on fumonisins in rice[13) 23) 24)]. In the first report of rice contaminated with fumonisins in the USA[13)], it was shown by HPLC-FL and enzyme-linked immunosorbent assay (ELISA) that forty percent of rice with symptoms of Fusarium sheath rot disease were positive for FB1 at levels of 4.3 mg/kg. However, no recovery data for the analysis of fumonisins in rice matrix are published to date.

In our previous study[28)], we developed practical methods for the determination of fumonisins in rice by HPLC-FL or LC-MS /MS. HPLC-FL is useful for routine analysis due to its wider prevalence, while LC-MS/MS is useful for screening and quantitative analysis of samples with low contamination levels because the LOQ achieved by LC-MS/MS was five to ten times lower than that achieved by HPLC-FL. In the present study, we could detect fumonisins in rice seed, at the level ranging from 0.061 mg /kg to 0.101 mg /kg for FB1 and from 0.011 mg/kg to 0.027 mg /kg for FB2 by LC- MS /MS, which were not detected by HPLC-FL (Table 2, Fig. 1 b), Fig. 2 a)). According to the study of Abbas et al. , fumonisins were mainly contained in rice hulls (sometimes fed to livestock) and removed in white rice (most widely consumed in human diets)[13)]. However, this analysis was adopted for conventional methods using HPLC-FL or ELISA; therefore, low level

TABLE 1. Effect of submergence and SPE cartridges on the recovery of fumonisins from 0.5 mg /kg spiked blank rice seed

	Recovery (%)					
	Sep-Pak Accell Plus QMA			Bond Elut SAX		
Submergence period	FB1	FB2	FB3	FB1	FB2	FB3
0min	22	24	24	56	59	59
30 min	n.t.	n.t.	n.t.	68	70	71

n.t. = not tested

TABLE 2. Natural fumonisins in rice seed partially infected with *G. fujikuroi*

	Concentration by HPLC-FL (mg /kg)			Concentration by LC-MS /MS (mg /kg)		
Sample ID	FB1	FB2	FB3	FB1	FB2	FB3
# 1	n.d.	n.d.	n.d.	0.061	0.011	n.d.
# 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
# 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
# 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
# 5	n.d.	n.d.	n.d.	0.101	0.027	n.d.
# 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
# 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n. d. = less than the limit of detection

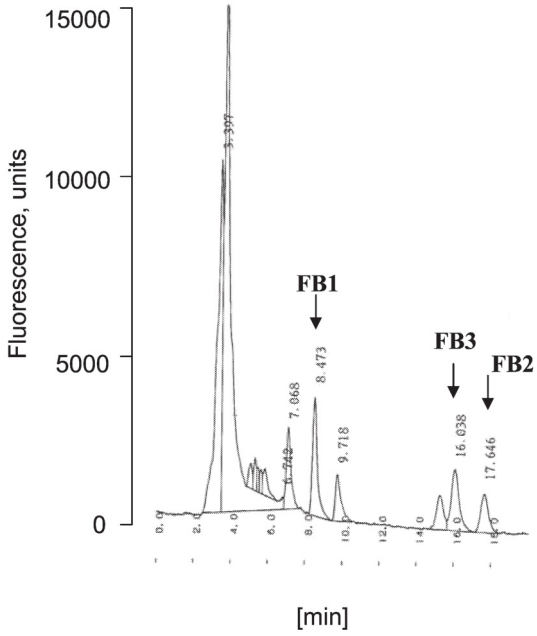

Fig 1. a) HPLC-FL chromatogram of 0.25 μg/ml each of FB1, FB2, and FB3 standards

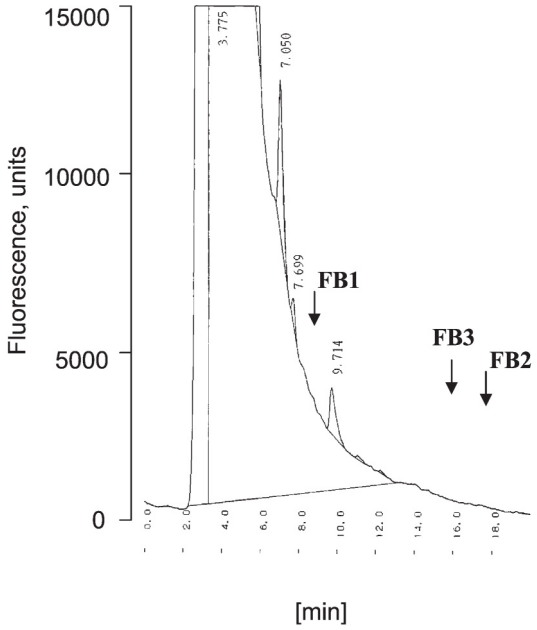

Fig 1. b) HPLC-FL chromatogram of rice seed extract # 1

contamination in white rice was likely overlooked. Moreover, they adopted for the same extraction procedure as corn, which might extract fumonisins from naturally polluted rice insufficiently. Thus, it will also be required to analyze the effects of processing and cooking on the concentration of fumonisins using a validated procedure and LC-MS /MS.

In summary, we successfully detected fumonisins from domestic rice seed for the first time by virtue of high sensitivity of LC-MS /MS. The observed variety in the amount of fumonisins extracted from each analytical sample implies the importance of sampling from non-homogeneous samples like rice seed (Fig. 2 a), b)). Since there is a genuine need for an analytical method for fumonisins in rice seed before threshing and blending with non-infected rice seed, it is essential to develop a validated analytical method for the determination of fumonisins in rice seed with an efficient extraction and purification method with low relative standard deviation. The amounts of fumonisins detected in this study were much lower than those generally found in corn, but the monitoring of fumonisins in rice should be carried out, since *G. fujikuroi* is one of the major rice-adherent fungi. Further study on the decomposition of fumonisins during processing as well as cooking is also required to assess and minimize the

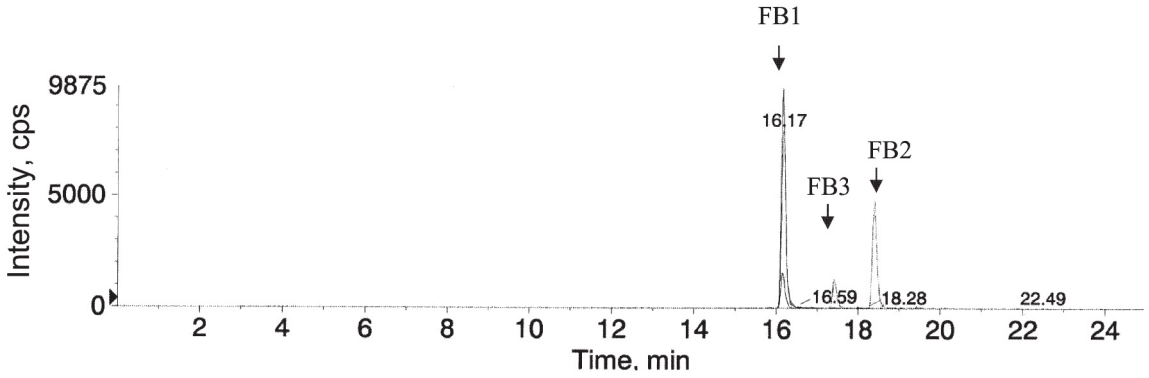

Fig 2. a) LC-MS/MS chromatogram of rice seed extract # 1

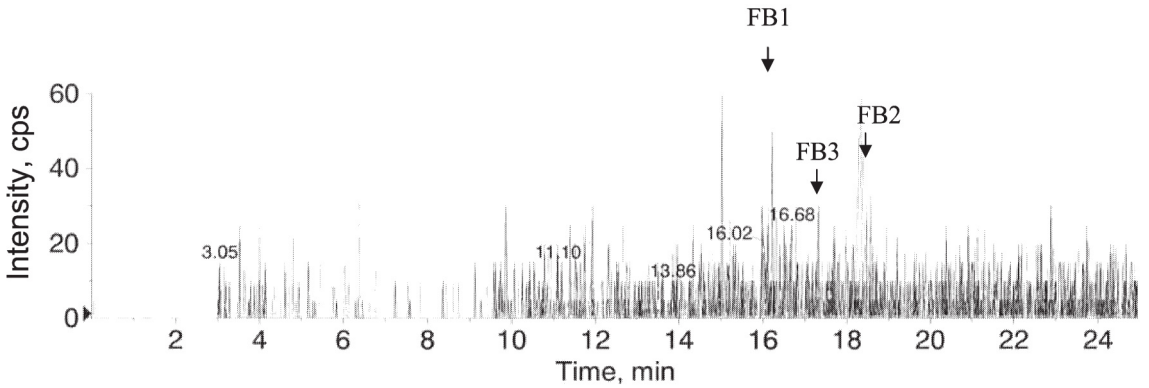

Fig 2. b) LC-MS/MS chromatogram of rice seed extract # 2

exposure to fumonisins in populations who live on rice as a staple diet.

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種籾中フモニシン類の液体クロマトグラフィーによる検出

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フモニシンは主にトウモロコシ赤カビ病菌 *Fusarium verticillioides* によって産生され，広くトウモロコシを汚染するカビ毒であるが，コメに付着するイネ馬鹿苗病菌 *Gibberella fujikuroi* は，*F. verticillioides* の近縁であるため，コメでフモニシン汚染が懸念されていた．われわれは以前，玄米中フモニシン定量のための効率的な抽出法（浸漬抽出）ならびに高感度な液体クロマトグラフィー–タンデム質量分析法（LC-MS/MS 法）を開発していた．今回，脱穀前段階でのフモニシン検出の要請があったため，玄米中フモニシン分析法のマトリクスエクステンションを試みた．その結果，抽出ステップでは，玄米と同様に浸漬抽出が有効であった．一方，精製ステップでは，玄米と異なる固相抽出カートリッジを用いるのが有効であり，ブランクの分析サンプルの添加回収試験において，良好な回収率が得られた．実際の種籾供試サンプル約500 g を 7 個のサブサンプルに分けて分析を行った結果，従来定量法であるプレカラム誘導体化機能付き液体クロマトグラフィー-蛍光検出法（HPLC-FL 法）では，各分析サンプル中のフモニシン量は検出限界未満であったが，LC-MS/MS 法では，7 点中 2 点が検出限界以上で測定可能であった．今回の結果より，国内産米で初めてフモニシンの検出に成功し，種籾のように不均一な試料におけるサンプリングの重要性も示された．