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Fumonisin monitoring in Thai red cargo rice by reversed-phase high-performance liquid chromatography with electrospray ionization ion trap mass spectrometry

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Abstract

The occurrence of fumonisins in red cargo rice from Thailand was studied by high-performance liquid chromatography with electrospray ionization ion trap mass spectrometry (LC-ESI-MS/MS). A quantification method for fumonisin B1 (FB1) was developed and the chromatogram of fumonisin B2 (FB2) was observed. The present method provides a sensitive detection limit at 1.0 ng g^{-1}. The limit of quantification was 5.0 ng g^{-1}. The recovery rate showed high yield of accuracy at 110.1±13.3, 89.3±11.1 and 91.9±4.6 % after fortification (n=5) at 50, 100 and 500 ng g^{-1}, respectively. Of the fifty eight samples from the retail markets, two samples were found to be naturally contaminated with FB1 at a trace level (lower than 5.0 ng g^{-1}). None of FB2 was found in any of the samples. This is the first report about the natural occurrence of FB in red cargo rice from Thai market.

Introduction

Foods and feeds contaminated with mycotoxins is a global serious problem that has high potential risk on human and animal health. Mycotoxins are toxics secondary metabolites produced by molds growing in foodstuffs and animal feeds. As known, the major mycotoxin-producing fungi are species of *Aspergillus*, *Fusarium*, and *Penicillium*. At present, the monitoring of mycotoxins contamination in staple food such as rice is highlighted. Currently, the occurrence of mycotoxins in rice, mostly known major toxin, has been reviewed (Tanaka *et al*., 2007).

Rice is one of the staple foods which is needed and consumed by human and animals all over the world. World rice production was recorded at 434.3 million tons (milled basis) in 2008/09 (Child, 2009). However, food safety is of interest to the consumer. Despite Thailand is the largest rice exporter in the world but data available on diversity of fungal flora and mycotoxin-producing fungi in Thai rice grains is scarce. At present, health organizations are focusing on natural substance contamination e.g. mycotoxins in grains. In Thailand, Pitt *et al*. (1994) suggested that Thai rice is susceptible to fungi following *Gibberella fujikuroi*, *Fusarium semitectum* and *Alternaria padwickii*. Fumonisins in rice were first detected in 1998 with Fusarium sheath rot disease (Abbas *et al*., 1998). Japanese scientists have developed a method to measure the level of fumonisins in rice, infected by *Gibberella fujikuroi*, by HPLC-Fluorescence (HPLC-FL) and LC-MS/MS and then detected the natural contaminant at 70.0-100.0 ng g^{-1} (in total) in 2 out of 6 samples (Kushiro *et al*., 2008). As known, the LC-MS/MS is a powerful instrument as a feasible analytical technique in the analysis of food contaminants including mycotoxins. The applicability of the LC-MS/MS method includes simple sample preparation, no derivatization is needed and a very small quantity of compounds can be detected. However, the disadvantage of LC-MS/MS is the matrix effect which obstructs the ionization of mycotoxins during analysis.

More recently, contamination of fumonisin in rice infected with *Fusarium* spp. was confirmed (Maheshwar *et al*., 2009). Commonly, the occurrence of mycotoxins in rice is at a minimal level and is not found as frequently as other cereals such as maize, wheat and barley (Tanaka *et al*., 2007). However, contaminations of certain mycotoxins in rice have been reported (Weidenboerner, 2000; Hussaini *et al*., 2007; Reddy *et al*., 2009). Red Cargo rice is a kind of unpolished rice similar to brown rice, only color of bran is red, purple or maroon. Unpolished rice is a healthy food which is richer in essential nutrients than milled rice, for instance vitamins (vitamin B1, B2, B3, B6 and E) and minerals (potassium, calcium, sodium, magnesium, iron, zinc, copper and manganese) (Anonymous1, 2001; Jiang *et al*., 2008). It has become a popular dish as a nourishing food

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in Asia, particularly in Thailand, and other parts of the world by cooking it either with or without normal polished rice. Red cargo rice retains its bran layer, and hence may be contaminated by mycotoxins such as fumonisins. Fumonisins are produced by *Fusarium* spp., known as pre-harvest or soil fungi. They reportedly have 28 structural analogs but FB1 is the major detected toxicant (Šegvić *et al*., 2001).

Fumonisins reportedly have a potential to affect sphingolipid biosynthesis (Wang *et al*., 1991). Moreover, they reportedly have hepatotoxicity effects and are classified in group 2B as a possibly carcinogenic substance to humans (IARC, 2002; Murphy, 2006). As well as in animals, fumonisins increasingly have become a problem in farm production. It causes softening of the white matter in the brain (leukoencephalomalacia) and is known as Moldy Corn poisoning in horses and Porcine Pulmonary Edema (PPE) in swine (Bucci and Howard, 1996; Haschek *et al*., 2001). To minimize the problem, the Scientific Committee on Food of the European Commission (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) allocated a provisional maximum tolerable daily intake of 2.0 ng g^{-1} of body weight to fumonisins B1, B2, and B3, alone or in combination(European Commission, 2003; WHO, 2002). Moreover, the maximum levels of total FB1 and FB2 range from0.2 to 2.0 μg g^{-1} in maize-based products and unprocessed maize and have been regulated by the European Union (European Commission, 2006). In addition, The Food and Drug Administration (FDA) has also released guidance levels of total fumonisins in corn and corn-based products at 2.0–4.0 μg g^{-1} for foods and 5.0–100 μg g^{-1} for animal feeds (FDA, 2001).

Despite unavoidable and long-term exposure even at low-doses, natural contamination of fumonisins is harmful for health but little information on the determination and detection of fumonisins in rice has been reported. Therefore, the aim of this study was to optimized detection method for fumonisins and limited survey of the occurrence of fumonisins, particularly the major toxicant (FB1), in Thai red cargo rice.

Materials and Methods

Chemical and reagents

A standard of Fumonisin B1 and B2 were purchased from Sigma Co. (St. Louis, MO., USA). Methanol, acetonitrile and acetic acid were purchased from Fisher Scientific (UK). For solid phase extraction, we used strong-anion-exchange cartridges (SAX, 500 mg, 3 ml, Varian, Inc. CA, USA) as a clean-up and collection step.

Red cargo rice sample and sample preparation

The occurrence of fumonisins contamination in Thai red cargo rice were investigated using 58 samples collected from retail markets in the central region of Thailand in 2009. Rice samples of 2 kg were collected from market and 10 sub-sampling (20 g each) were thoroughly mixed. Then, 10 g of mixed sampling was soaked under 5 mL of water for 30 min. Then, 15 mL of methanol was added and shaken at 150 rpm in an orbital shaker for 1 hr. The matrix was then centrifuged at 4500 rpm for 10 min and 5 mL of supernatants were collected. The extraction method was modified following Kushiro *et al*., (2008).To clean up the sample, a strong anion exchange (SAX) cartridges (500 mg sorbent, Varian, CA, USA) column was first prepared by washing with 10 mL of methanol and then precondition column was used with 10 mL of 75% methanol. The collected samples were loaded by passing into the column for 2 mL followed by 2 steps washing with 8 mL of 75% methanol and 4 mL of methanol, respectively. The elution was achieved by slowly passing 10 mL of 1% acetic acid in methanol. Then the eluate from SAX was transferred to evaporate under a gentle stream of nitrogen gas. The remainder was redissolved with 500 μL of methanol/water (50:50, v/v) and analysed with the LC-ESI-MS/MS.

Apparatus and method of quantification

Fumonisins were analysed by high-performance liquid chromatography coupled to electrospray ionization ion trap mass spectrometry. The HPLC system was carried out with an Agilent 1100 Series (Palo, Alto, CA, USA) equipped with a binary pump, a vacuum degasser and a autosampler. Gradient HPLC separation was performed on a Phenomenex Gemini C-18, 150 mm × 4.6 mm, 3 μm (Phenomenex, Macclesfield, UK) attached with a guard column, Phenomenex Gemini C-18, 3 mm, 3μm. The columns were controlled at 40°C. Solvent A was 0.05% acetic acid in water (v/v), and solvent B was 0.05% acetic acid in methanol. The gradient program was: 0-1 min, 30% B; 1-6 min linear gradient to 95% B; hold 95% B for 3 min; then equilibrate column with 30% B for 5 min between each injection at a flow rate of 0.2 mL min^{-1}. The column effluent was directly coupled to an Agilent 1100 MSD Trap SL mass spectrometer equipped with an atmospheric-pressure ESI source and an IT mass analyzer. Standard FB1 and FB2 were infused by syringe pump at a flow rate 20 μl min^{-1} for optimizing the mass spectrometer parameter. The MS^2 measurements of the protonated molecules were

made in full-scan mode. The spray needle voltage was +3.5 kV, the optimal capillary temperature was 350ºC, sheath gas pressure 80 psi, and auxiliary gas setting 40 units. To identify and confirm, at least three fragment specific ions were monitored.

No matrix effect of the rice sample appeared after using SAX clean up compared with chromatograms with fortified samples and standard samples. Thus, we obtained a standard calibration curve without a matrix. The method was linear (R^2>0.998) using triplicate standard solution at six different concentrations range from 5-1000 ng g^{-1} for the calibration curve. Accuracy, intra-day (n=5) and inter-day (n=3) precision were determined using quality control samples at concentration of 50, 100, and 500 ng g^{-1}. The recovery rate was reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Briefly, 10 g of blank red cargo rice, thus assumed not to contain fumonisins, were spiked with standard fumonisin B1 to obtain total levels of 50, 100, and 500 ng g^{-1}, followed by the extraction and clean-up described above. The limit of detection (LOD) was based on signal-to-noise ratio (S/N) that provided signals at three times above the background noise level. The limit of quantification (LOQ) was defined as the lowest concentration of standard calibration curve which was validated.

Table 1. Extraction recoveries of the method used for determination of FB1 in Thai red cargo rice

FB1 spiking level (ng g^{-1})	Recovery rate ± RSD[a]
50.0	110.1 ± 13.3
100.0	89.3 ± 11.1
500.0	91.9 ± 4.6
Mean of means	97.1 ± 11.3

[a]: relative standard deviation (n= 5 replicates)

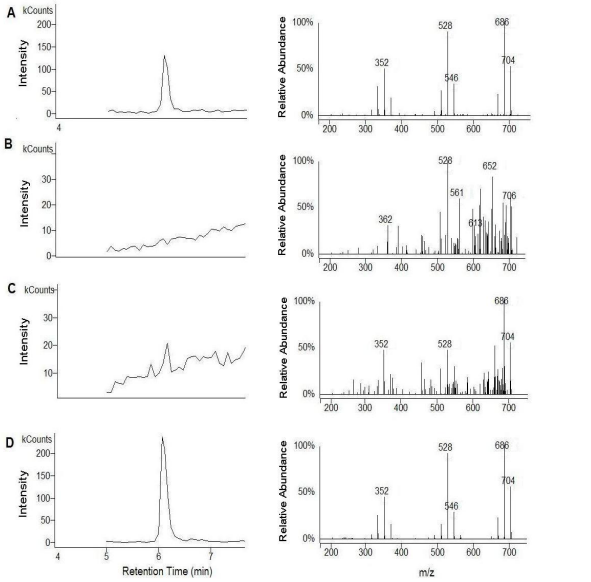

Figure 1. LC-ESI-MS/MS chromatograms of fumonisin B1 (m/z 722) and MS/MS spectrum; (A) standard of FB1 at 5.0ng g^{-1}, (B) Blank red cargo rice sample, (C) positive sample No.35, and (D) fortified sample at 50.0ng $g^{-1}$of FB1

Result and Discussion

Method of quantification

HPLC consisting of ESI/MS/MS permitted a highly sensitive and selective detection of fumonisin B1 in red cargo rice. FB1 and FB2 have highest intensive signal at m/z 722 and m/z 706 respectively. A typical chromatogram, showed in figure 1, was obtained from a rice blank sample (B) and FB1 spiked at 50 ng g^{-1} (D). Figure 1 (A) and 2 (A) showed the spectra of the product ions of FB1 and FB2 standards. The main fragment ions of FB1 were 686, 528 and 352. The fragmentation of FB2 showed the product ions at m/z 670, 512, and 336. These protonated ions were used to confirm FB1 and FB2 in red rice samples with retention time at 6.1 min for FB1 and 6.8 min for FB2. The mean recoveries of fortified samples were 89-110% (see table 1).

The biological-matrix effect in the LC-MS/MS analysis has been documented (Niessen, 2003; Spanjer *et al*., 2008). However, in the current method has no matrix effect. We observed neither signal suppression nor enhancement after the clean-up step by SAX-solid phase extraction column. The SAX cartridge has been extensively used in fumonisin detection as an excellent purification procedure (Shephard, 1998). However, the cartridge needs to control range of pH in the extracted sample to yield good recovery. Therefore, the current method adjusts acidic condition by adding 10 mL of 1% acetic acid into methanol for the elution process.

The smallest signal of FB1 that could be detected based on a signal-to noise ratio greater than three (LOD) was 1.0 ng g^{-1} and the limit of quantification was 5.0 ng g^{-1}. The mean recoveries of fortified samples were 89-110 %. The method provided good intra-day repeatability and inter-day reproducibility with acceptable relative standard deviation (RSD) range 4.6-13.3% and an excellent linearity (r^2) of the calibration curve at 0.9989. The parent and daughter fragment ion of FB2 in standard and samples were also investigated (see figure 2). The LOD of FB2 was 1.0 ng g^{-1}. Unfortunately, FB2 did not validate as there was insufficient data of spiked samples. Kushiro *et al*., (2009) who developed method for fumonisin detection in rice has limited the LOD level at 12 ng g^{-1}. The modified method reported here has better LOD (1 ng g^{-1}) might be due to using different solvent solution for preparing standard stock solution, mobile phase and even instrument sensitivity. Thus, the result

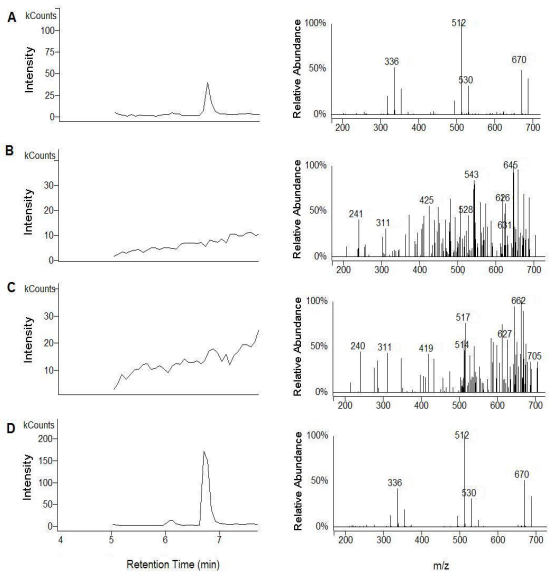

Figure 2. LC-ESI-MS/MS chromatograms of fumonisin B2 (m/z 706) and MS/MS spectrum; (A) standard of FB2 at 5.0ng g^{-1}, (B) Blank red cargo rice sample, (C) sample No.35, and (D) fortified sample at 50.0 ng g^{-1} of FB2

clearly showed that the fumonisin determination was well validated and appropriate to apply.

Occurrence of FB1 in Thai red cargo rice

It was estimated that mean of unpolished rice consumption by Thai was 25.60 g/person/day (Jankhaikhot, 2005). The current detection method was applied to quantify the presence of FB1 in red cargo rice by testing fifty eight samples collected from retail markets in the central region of Thailand. As results, rice has low risk of contamination with FB1 as it was found in 3.45% of the samples (2 of 58 samples) at a trace level (concentration between LOD and LOQ). In addition, none of the rice samples presented a signal of FB2 following product ions monitoring at m/z 670, 512 and 336 as shown in figure 2.

Although fumonisins contamination have been extensively studied in food commodities including maize, wheat, barley, cornflake, and wine (Castellá *et al*., 1999; Spanjer *et al*., 2008). As mentioned above, fumoinisins contamination in rice has also been documented (Abbas *et al*., 1998). In USA, high level of fumonisins found in rice (600-4,300 ng g^{-1}) has been recorded (Weidenboerner, 2000). In Japan, fumonisins has been found at the level ranging from 0.061-0.101 mg/kg for FB1 and from 0.011-0.027 mg/kg for FB2 in rice seed by LC–MS/MS detection (Kushiro *et al*., 2009). Nonetheless, data determination of fumonisins in rice, particularly in unpolished, is scarce.

Recently, the association of fumonisins producing a *F. verticillioides* isolate in paddy rice was confirmed (Maheshwar *et al*., 2009). However, in this present study *Fusarium* spp. was not isolated. More recently, focusing on fumonisins contaminated Thai rice, the FB1 has been detected (14 ng g^{-1}) only 1 sample from 100 rice sample in Thai black sweet rice (Bansal *et al*., 2011). In addition, as it's relatively low-level of fumonisin contamination, Thai rice has also been used as in-house reference material containing fumonisins (Awaludin *et al*., 2009). However, due to *Fusarium* spp. occurs worldwide on cereal grains, therefore, its mycotoxicology and its residue, particularly in rice, warrants further studied.

Regarding mycotoxogenic fungi in rice, rice is generally immune to fungal infection as compared to corn and wheat (Kushiro *et al*., 2009). However, a wide variety of fungi infected rice field and grain has been reported (Reddy *et al*., 2009). Thailand geographically has wide diversity. Thus, rice growing in field is commonly contaminated with various fungi and may also remain certain mycotoxins (Jankhaikhot, 2005). Although little data of fumonisins contamination in rice are available, other mycotoxins in rice such as aflatoxin, ochratoxin, deoxynivalenol, citrinin and zealarenone have been found (Weidenboerner, 2000; Tanaka, 2007). Reddy *et al*., (2009) found high incidence of aflatoxin contamination in rice in India. More recently, Fredlundet *et al*., (2009) reported a survey of mycotoxins in rice in the Swedish market identified the presence of aflatoxin and ochratoxin A. Moreover, co-occurrence of aflatoxin, citrinin and ochratoxin A in Vietnamese rice has been documented (Nguyen *et al*., 2007). Thus, a high specific quantification technique and monitoring program of mycotoxins in rice in order to protect human and animal health should be investigated.

Conclusion

The current method using LC-ESI-MS/MS is sufficient to detect fumonisin B in red cargo rice. As FB1 detected at very low level, fumonisins contamination in Thai red cargo rice may negligible. Although the maximum residue limit (MRLs) of mycotoxins in rice have not yet been established, to prevent risk of long-term and low-dose exposure of natural contamination, regular monitoring of mycotoxins in rice should be carried out. However, mould and mycotoxins contamination may variable occur in different condition. Data available on less-known mycotoxins produced by fungi-infected rice is scarce. Therefore, further study on the occurrence of mycotoxigenic fungi and related mycotoxins in rice should be investigated.

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