Food safety evaluation of *Aspergillus oryzae* species based on the regulation by Ministry of Agriculture, Forestry and Fisheries, Japan

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農林水産省が定める優先的にリスク管理を行うべきカビ毒のリストに基づいた *Aspergillus oryzae*種の安全性評価

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Introduction

Mycotoxins are toxic secondary metabolites naturally produced by certain filamentous fungal species. Consumption of mycotoxin-contaminated foods or livestock feeds, such as cereals and corn, can cause mycotoxicosis in domestic animals and humans. Therefore, it has public health significance. These fungal mycotoxins including aflatoxins, HT-2 toxin, deoxynivalenol and the like have been evaluated and regarded as unsafe compounds by the international scientific committee known as the Joint Food and Agriculture Organization and the World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) (WHO Technical Report Series, 2002). In Japan, risk management and regulatory guidelines on these mycotoxins have been strictly implemented by the Ministry of Agriculture, Forestry and Fisheries (MAFF).

Meanwhile, based on comparative genome analysis, *Aspergillus flavus* a plant pathogen notorious for aflatoxin production is interestingly closely related (at 99.5% genetic similarity) to *Aspergillus oryzae* (considered as a domesticated species of *A. flavus*) (Machida et al., 2005; Rokas et al., 2007). *A. oryzae* is the workhorse behind the production of most of the traditional Japanese fermented food products, such as miso, soy sauce, and sake (Murakami et al., 1976). While *A. oryzae* has been used for food fermentation in Japan for more than 1,000 years, some researchers already confirmed the non-aflatoxin production of *A. oryzae* strains due to their defective biosynthetic gene cluster (Tominaga et al., 2006; Kiyota et al., 2011). However, the production of other mycotoxins by *A. oryzae* has not been thoroughly evaluated based on the MAFF guideline. Therefore, to verify the safety of *A. oryzae* species for the production of fermented foods and beverages, we determined the presence or absence of these mycotoxins in *A. oryzae*. In detail, there were 13 *A. oryzae* strains selected based on the phylogenetic tree of 55 *A. oryzae* strains by genome array analysis (https://nribf21.nrib.go.jp/CAoGD//) (Iwashita, 2012) which later gave rise to 13 putative clades (Iwashita, 2012). It is further thought that these 13 *A. oryzae* selected from each distinct clade could represent all 55 *A. oryzae* strains in the said phylogenetic tree.

Furthermore, these *A. oryzae* strains were

grown under various conditions, including industrial condition of *rice-koji* and *soy sauce-koji* and were analyzed using a tandem approach of direct-infusion high-resolution mass spectrometry (DI-HRMS) for rapid analysis and screening of secondary metabolites and liquid chromatography quadruple time-of-flight mass spectrometry (LC-QTOF-MS) for further metabolite confirmation. In this report, we detected the secondary metabolite production of 13 *A. oryzae* strains particularly grown in industrial conditions.

Materials and Methods

A. oryzae industrial strains

The following *A. oryzae*, RIB40, RIB128, RIB143, RIB163, RIB301, RIB306, RIB430, RIB915, RIB1108, RIB1172, RIB1178, RIB1187, RIBOIS01 strains were used in the study. *A. flavus* NRRL 3357 was used as positive control.

Culture media preparation

Synthetic media such as YES (Yeast Extract Sucrose), CYA (Czapek Yeast Autolysate), WATM (Wickerham's Antibiotic Test Medium) were prepared as culture plates and zeolite in liquid culture. YES was composed of 20 g/L yeast extract, 150 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.005 g/L $CuSO_4 \cdot 5H_2O$, 0.01 g/L $ZuSO_4 \cdot 7H_2O$, 2% agar, pH 6.0. CYA contained 5 g/L yeast extract, 170 g/L sucrose, 3 g/L $NaNO_3$, 1.3 g/L $KH_2PO_4 \cdot 3H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.005 g/L $CuSO_4 \cdot 5H_2O$, 0.01 g/L $ZuSO_4 \cdot 7H_2O$, 2 % agar, pH 6.3. On the other hand, WATM consisted of 2 g/L yeast extract, 30 g/L sucrose, 3 g/L peptone, 2 g/L dextrose, 5 g/L corn steep solids, 2 g/L $NaNO_3$, 1 g/L $KH_2PO_4 \cdot 3H_2O$, 0.2 g/L KCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 2% agar, pH 7.0. Synthetic culture media were prepared as previously described by Rank et al, (2012). Furthermore, zeolite medium was composed of 3% glycerol, 1% of glucose, 0.5% of peptone, 0.2% NaCl and 1% of molecular sieve (0.5 nm).

Growth of *A. oryzae* industrial strains on each culture condition

1. *Rice-koji solid-state fermentation*

The growth of strains under *rice-koji* condition (plate-scale) was performed with some modifications from previously reported (Okazaki, 1979). Conidia suspension of each *A. oryzae* strain was added to 15 g rice (1×10^6 conidia/g rice) to be 30% water content in alpha-rice and immediately mixed well. *Rice-koji* was cultivated at 30℃ for 2 days and 6 days under saturated water vapor pressure.

2. *Soy sauce-koji solid-state fermentation*

The cultivation of strains under *soy sauce-koji* condition (also plate-scale) was conducted following the method of Yamasa soy sauce (personal communication, Watanabe J.). Briefly, 6 g of defatted soy in a glass of petri dish then added with 8.4 ml of deionized water and allowed to absorb water for 30 min. Later, 6 g of wheat was added and mixed. Glass plates were wrapped with aluminum foil and autoclaved at 120℃ for 40 min and cooled down. Conidia powder (1×10^6 conidia/g) of each *A. oryzae* strain was inoculated and grown at 30℃ for 2 days and 6 days under saturated water vapor pressure.

3. *Growth on corn and oatmeal*

Strains were cultivated on media with plant materials as primary compositions namely, corn and oatmeal substrates. All *A. oryzae* on plant substrates were grown at 30℃ under saturated water vapor pressure for 7 days (corn) and 2, 6 days (oatmeal).

4. *Growth on synthetic agar plate*

Strains were also grown on synthetic media (YES, CYA, WATM). All *A. oryzae* strains on culture plates were grown at 30℃ under saturated water vapor pressure for 6 days.

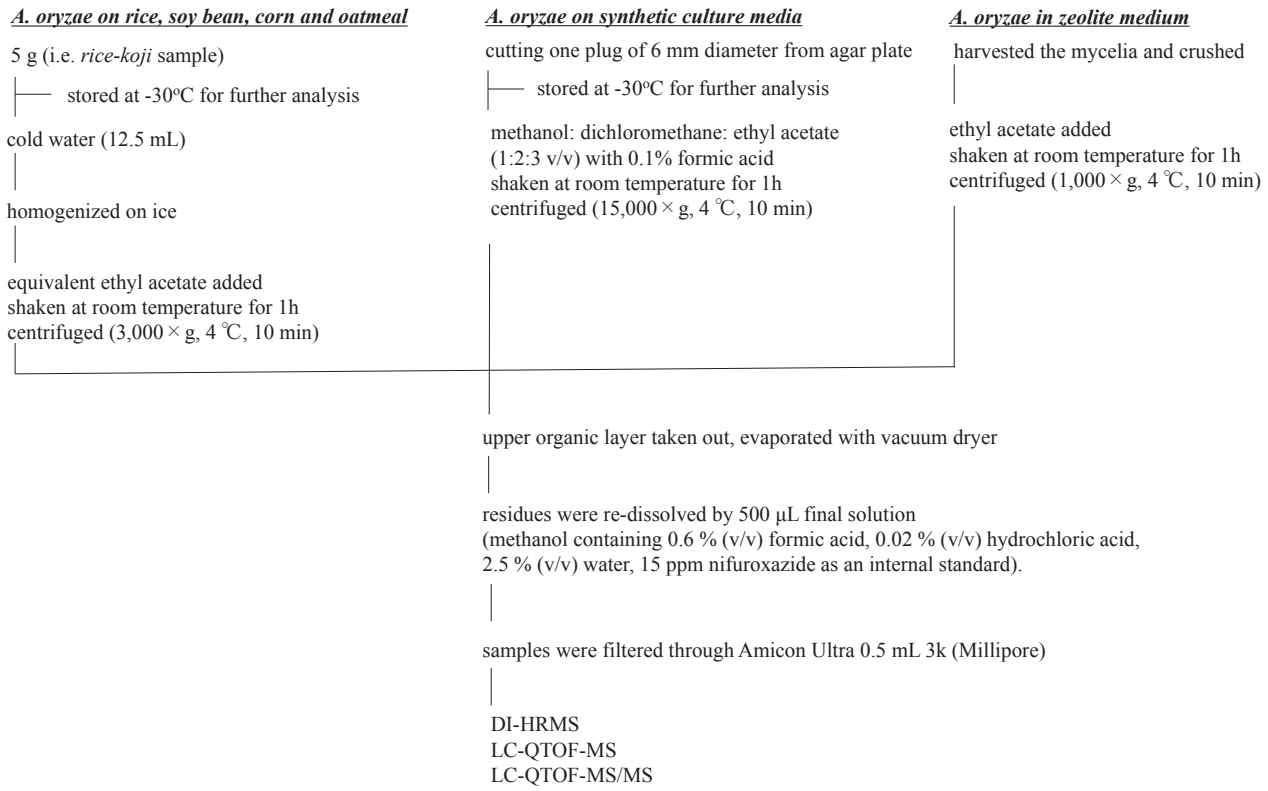

Figure 1. Extraction of mycotoxin metabolites from *A. oryzae* strains

Table 1. The list of mycotoxins evaluated by JECFA* as unsafe to human and animal health

Metabolite name	Chemical formula	Theoretical monoisotopic mass (m/z) **		DI-HRMS	LC-QTOF-MS	LC-QTOF-MS/MS
		Positive mode	Negative mode			
Patulin	$C_7H_6O_4$	155.0344	153.0187	n.d. (0/0)	−	−
Deoxynivalenol	$C_{15}H_{20}O_6$	297.1338	295.1181	□ (13/6)	n.d.	−
Nivalenol	$C_{15}H_{20}O_7$	314.1360	311.1130	□ (1/2)	n.d.	−
Aflatoxin B1	$C_{17}H_{12}O_6$	313.0712	311.0555	□ (4/2)	n.d.	−
Aflatoxin B2	$C_{17}H_{14}O_6$	315.0868	313.0712	□ (13/3)	□ (13/3)	n.d.
Aflatoxin G1	$C_{17}H_{12}O_7$	329.0661	327.0504	n.d. (0/0)	−	−
Aflatoxin G2	$C_{17}H_{14}O_7$	331.0817	329.0661	□ (13/2)	n.d.	−
Aflatoxin M1	$C_{17}H_{12}O_7$	329.0661	327.0504	□ (1/1)	n.d.	−
Fusarenon-X	$C_{17}H_{22}O_8$	355.1393	353.1236	n.d. (0/0)	−	−
Sterigmatocystin	$C_{18}H_{12}O_6$	325.0712	323.0555	nSTD	nSTD	−
Zearalenone	$C_{18}H_{22}O_5$	319.1545	317.1388	□ (9/2)	n.d.	−
Diacetoxyscirpenol	$C_{19}H_{26}O_7$	367.1756	365.1600	□ (11/6)	n.d.	−
Ochratoxin A	$C_{20}H_{18}ClNO_6$	404.0900	402.0744	n.d. (0/0)	−	−
HT-2 toxin	$C_{22}H_{32}O_8$	425.2175	423.2019	□ (13/8)	n.d.	−
T-2 toxin	$C_{24}H_{34}O_9$	468.2359	465.2124	□ (3/2)	X	−
Fumonisin B1	$C_{34}H_{59}NO_{15}$	722.3962	720.3800	n.d. (0/0)	−	−

* Joint Food and Agriculture Organization and the World Health organization (FAO/WHO) Expert Committee on Food Additives (JECFA)

** as calculated by ChemCalc (Patiny and Borel, 2013)

□: candidates were putatively detected at m/z ± 10 ppm in any strain grown in any culture condition

nSTD: no standard available

X: the pure standard was not detected

n.d.: not detected

− : no further analysis was conducted

(/) : number of strains (*left side*) and conditions (*right side*) of /, in which the metabolite was detected per analysis

Machine: LTQ Orbitrap XL (Thermo Fisher Scientific, USA)
ESI sources and parameters:

	Positive ion mode (P)	*Negative ion mode (N)*	*both modes*
spray voltage			5.0 kV
capillary temperature			330 ℃
capillary voltage	30 V	-30 V	
tube lens voltage	100 V	-60 V	
sheath gas flow rate			40 L/min
auxiliary gas flow rate			10 L/min

Mass range: 100-1000 mass to charge ratio (*m/z*)
Resolution: 60, 000
Sample: 5 μL each by direct-infusion for 1 min
Data collection: Xcalibur 2.2 software (Thermo Fisher Scientific, USA)

Figure 2. Specifications for DI-HRMS analysis

5. *Liquid culture*

A. oryzae strains were further cultivated in zeolite liquid culture and were shaken at 120 rpm at 30℃ for 4 days.

Mycotoxin metabolite extraction and analyses by DI-HRMS, LC-QTOF-MS and MS/MS of *A. oryzae* industrial strains

Extraction of mycotoxins

Mycotoxin metabolites from all *A. oryzae* grown under different conditions were extracted following the method previously described with some modifications (Nielsen et al., 2011) (Figure 1).

Acquisition of pure standards of mycotoxin metabolites and their m/z values

Pure standard of mycotoxins (99% purity) was commercially obtained from WAKO Co., Japan. Powder of metabolite standards was either dissolved by acetonitrile or methanol (Sigma-Aldrich) as described by company's manual. On the other hand, the m/z values were based on the theoretical monoisotopic mass of each metabolite compound calculated by ChemCalc (Patiny and Borel, 2013) (Table 1) at positive and negative ionization modes for both DI-HRMS and LC-QTOF-MS.

Direct-infusion high-resolution mass spectrometry (DI-HRMS)

For initial screening of the presence of putative mycotoxins, the extracted metabolites were analyzed by DI-HRMS with the specifications described in Figure 2. The peak intensity (as peak area) of each metabolite was extracted based on the monoisotopic mass (m/z) at a maximum of 10 parts per millions (ppm) mass accuracy (as m/z ± 10 ppm) at both ionization modes. These peak intensity values of metabolites were further categorized as the following: values < 5000, values within 5000-10000 range and values > 10000. We screened three replicates of each *A. oryzae* strain under per culture condition.

Liquid chromatography quadruple time-of-flight mass spectrometry (LC-QTOF-MS and MS/MS)

The samples together with the pure standard of the mycotoxin metabolites were further analyzed by LC-QTOF-MS. Detailed analysis was conducted as previously described with some modifications (Nielsen et al., 2011) and the specifications as shown in Figure 3. Peak identification was conducted using selected ion monitoring (SIM) mode and this time, peaks and retention time of samples were confirmed against the pure standards. LC-QTOF-MS/MS was further

Machine: ACQUITY UPLC Xevo Qtof® (Waters)
Column: Luna C18(2) column (length, 50 mm; inside diameter, 2 mm; particle size, 3 μm) (Phenomenex, USA)
LC parameters
column temperature: 40 ℃
flow rate: 0.3 mL/min
injection volume: 3 μL
solvent A: 0.1 % (v/v) formic acid in water;
solvent B: 0.1 % (v/v) formic acid in acetonitrile
gradient condition:

0-20 min	B 15-100 %
20-25 min	B 100 %
25-27 min	B 100-15 %
27-32 min	B 15 %

Source type: Electronspray Ionization (ESI)
Ion polarity: Positive and Negative
Nebulizer gas: Nitrogen
MS and MS/MS parameters
scan: 50-1000 m/z
scan rate: 0.2 s
capillary voltage: 2.4 kV
cone voltage: 15.0 V
source temperature: 140 ℃
desolvation temperature: 450 ℃
cone gas flow: 50 l/Hr
desolvation gas flow: 800 l/Hr.
collision-induced dissociation (CID) gas: Argon
collision energy: 25 V (aflatoxin B2)

Figure 3. Specifications for LC-QTOF-MS and MS/MS analyses

conducted to validate the correctness of intriguing peaks obtained by using only LC-QTOF-MS/SIM mode.

Results and Discussion

To evaluate *A. oryzae* on food safety, we determined the production of mycotoxin metabolites in 13 strains using a tandem approach of DI-HRMS and LC-QTOF-MS analyses. Culture conditions and growth substrates were considered in this study to mimic the industrial production process of fermented food products. These strains were also incubated on cereal substrates such as corn and oatmeal and synthetic media of YES, CYA, WATM and zeolite because these conditions were generally used for the study of secondary metabolite production.

By DI-HRMS analysis, the presence or absence of the mycotoxin metabolites were initially screened based on the theoretical monoisotopic mass at a maximum 10 ppm mass accuracy (as m/z ± 10 ppm) at both ionization modes. Using DI-HRMS screening, we observed high peak intensity (peak area) of some mycotoxins from *A. oryzae* strains while other mycotoxins were not detected (Table 1 and Figure 4) in any strain grown in any culture condition. For example, HT-2 toxin peak was detected in almost all strains under all culture conditions except in oatmeal 2 days, CYA and WATM. Also, the peak of zearalenone was putatively detected in several *A. oryzae* strains grown in *soy sauce-koji* (6 days). Furthermore, deoxynivalenol metabolite was detected in *soy sauce -koji* (6 days), oatmeal (2 days and 6 days) and in corn (Figure 4). We further confirmed the *A. oryzae* production of HT-2 toxin, zearalenone, deoxynivalenol and other mycotoxins by another method, the targeted analysis using LC-QTOF-MS and these metabolite peaks were compared against the pure standards as shown in Table 1. By adopting this tandem approach, we substantiated the non-production or no detection of these mycotoxin metabolites in *A. oryzae* strains (Table 1). In this approach, however, various false positives in the samples were found by DI-HRMS analysis because they may have same molecular formula but different chemical structures as frequently observed natural products. The LC-QTOF-MS could distinguish further the difference of these chemical structures by the difference of the retention time (RT).

On the other hand, one metabolite peak with RT and m/z from *A. oryzae* samples in *soy sauce-koji* agreed with that of aflatoxin B2 (AFLB2). Based on DI-HRMS data (Figure 5), AFLB2 was detected in almost all *A. oryzae* strains grown particularly in *soy sauce-koji* (2 and 6 days). To illustrate some examples, we observed AFLB2 peak intensity values at positive ionization mode in RIB128 (～16000), RIBOIS01 (～59000) and in *A. flavus* (～42000) under *soy sauce-koji* (6 days). These strains exceeded the peak intensity values of 10000 and were further categorized as 10000 < (indicated in black color) (Figure 5). On the contrary, we

A

	Rice-koji (2 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
7	Deoxynivalenol																										
8	Aflatoxin B1																										
9	Aflatoxin B2																										
10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

B

	Rice-koji (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
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5	Nivalenol																										
6	HT-2 toxin																										
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8	Aflatoxin B1																										
9	Aflatoxin B2																										
10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

C

	Soy sauce-koji (2 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
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9	Aflatoxin B2																										
10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

Peak intensity value

< 5000 5000 – 10000 10000 <

Figure 4. DI-HRMS screening of mycotoxins among *A. oryzae* strains in all culture conditions

D

	Soy sauce-koji (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
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5	Nivalenol																										
6	HT-2 toxin																										
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11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

E

	Oatmeal (2 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
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5	Nivalenol																										
6	HT-2 toxin																										
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10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

F

	Oatmeal (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
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10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

Peak intensity value

< 5000 5000 – 10000 10000 <

Figure 4. (continued)

G

	Corn (7 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
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11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

H

	YES (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
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11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

I

	CYA (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
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11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

Peak intensity value

< 5000　　5000 – 10000　　10000 <

Figure 4. (continued)

J

	WATM (6 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
7	Deoxynivalenol																										
8	Aflatoxin B1																										
9	Aflatoxin B2																										
10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

K

	Zeolite (4 days)	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	Metabolite name	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
1	Patulin																										
2	Zearalenone																										
3	T-2 toxin																										
4	Diacetoxyscirpenol																										
5	Nivalenol																										
6	HT-2 toxin																										
7	Deoxynivalenol																										
8	Aflatoxin B1																										
9	Aflatoxin B2																										
10	Aflatoxin G1																										
11	Aflatoxin G2																										
12	Aflatoxin M1																										
13	Ochratoxin A																										
14	Fumonisin B1																										
15	Fusarenon-X																										
16	Sterigmatocystin																										

Peak intensity value

< 5000　　5000 – 10000　　10000 <

Figure 4. (continued)

could not detect the production of AFLB2 shown as blanks (Figure 5) and other aflatoxins (aflatoxin B1, G2, M1) by DI-HRMS (Figure 4) among all the *A. oryzae* strains on corn substrates as well as grown in *rice-koji* (2 and 6 days) indicating their safety in *rice-koji* fermentation.

For the purpose of food safety, it was also extremely important then to determine if *A. oryzae* actually produced AFLB2 in fermentation condition such as in *soy sauce-koji*. Based on LC-QTOF-MS/SIM mode analysis, we observed that the peak of AFLB2 pure standard (STD) appeared at 4.38 min RT while the samples in *soy sauce-koji* showed conflicting peak RT ranging from 4.03 - 4.13 min against the STD (Figure 6). In spite of this, we remained skeptical that the peaks observed among *A. oryzae* strains could be the correct peak of AFLB2. We then exploited LC-QTOF-MS/MS method to validate these peaks as AFLB2 in detail including *A. flavus* on corn substrate (*in vitro*) as a positive control since *A. flavus* is known as a notorious aflatoxin producer during infection of corn, peanuts and other crops (Amaike and Keller, 2011).

Figure 6 shows the chromatogram and mass spectrum profile of all the samples under scrutiny and we consequently validated two things: 1) peaks found in RIB40, RIBOIS01 and

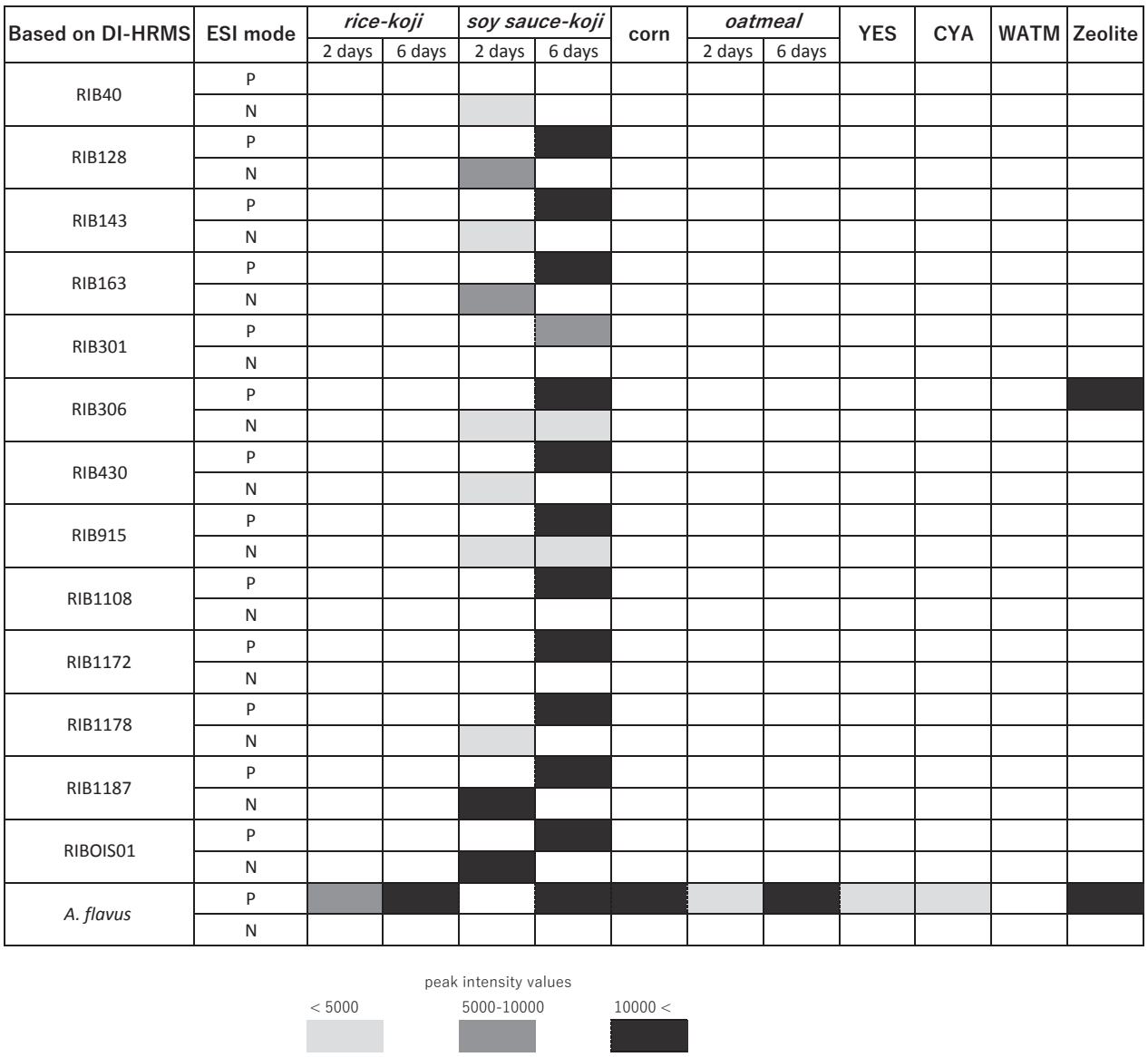

Figure 5. DI-HRMS profile of Aflatoxin B2 (AFLB2) only among *A. oryzae* strains in *rice-koji*, *soy sauce-koji* (both at 2 and 6 days), in corn and other culture conditions

RIB915 (*soy sauce-koji* 2 days) with 4.02- 4.13 min RT showed dissimilar MS/MS profile when compared against the AFLB2 STD (at 4.38 min RT) indicating that these strains did not certainly produce AFLB2 under *soy sauce-koji* condition, 2) In contrast, *A. flavus* on corn substrate (*in vitro*) sample showed the same peak RT (at 4.38 min) as indicated by arrows and highly similar MS/MS profile when compared against the AFLB2 STD indicating its AFLB2 production on corn substrate while it did not produce in *soy sauce-koji* condition. Taken together, our results were in agreement with the previous study of non-aflatoxin production of *A. oryzae* strains due to their defective biosynthetic gene cluster (Tominaga et al., 2006; Kiyota et al., 2011).

Conclusion

Based on the tandem approach of DI-HRMS and LC-QTOF-MS/SIM (and MS/MS), we could finally conclude that no significant traces of harmful secondary metabolites or mycotoxins found in all of the 13 *A. oryzae* industrial strains cultivated under different culture conditions. We further confirmed the safety of these *A.*

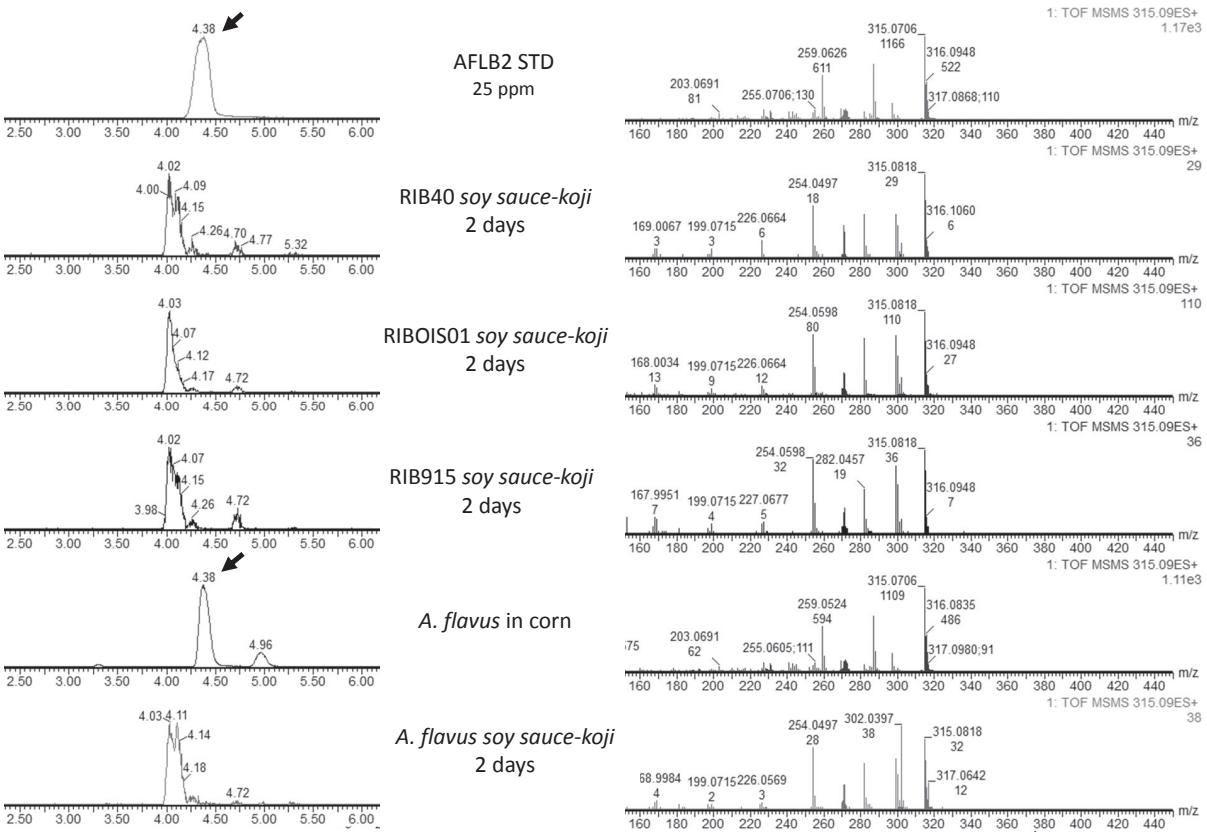

Figure 6. Chromatogram and the MS/MS spectra

oryzae strains for industrial production of fermented foods and beverages.

Summary (in *Japanese*)

麹菌は安全な菌株であると考えられており、アフラトキシンの非生産性については、多様な菌株で詳細に研究がなされ、遺伝子のレベルで非生産性が確認されている。しかし、農水省が優先的にリスク管理を進めるカビ毒について、麹菌群全体での生産性について解析した例は少ない。麹菌群はゲノムアレイに基づいた系統解析により13の系統に分かれる。そこで麹菌群全体の生産性について検討するために、我々は各系統から1株ずつ選抜し、米麹や醤油麹を含む11の培養条件により、農水省が優先的にリスク管理を進める15のカビ毒について生産の有無を確認した。本解析では、DI-HRMSを利用することにより、非生産株・条件を効率的に排除した。さらに、カビ毒と精密質量が一致したものについては、LC-QTOFMS/SIM解析を行い、溶出時間と精密質量による検出を行ない、両者が一致したものについては、さらにMS/MS解析を行った。この結果、全てのカビ毒について、全ての菌株、培養条件で検出されなかった。

Acknowledgment

We would like to thank Ms. Tomoko Amimoto from Hiroshima University for running our samples using the DI-HRMS analysis.

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