Evaluation of *Aspergillus oryzae* species for the production of *candidate* mycotoxins listed by the Ministry of Agriculture, Forestry and Fisheries, Japan

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農林水産省のその他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

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) evaluates and regulates the mycotoxins found in foods and food products consumed by humans and animals (WHO Technical Report Series, 2002). Mycotoxins are known to be toxic secondary metabolites naturally produced by certain filamentous fungi. In Japan, risk assessment and regulatory guidelines on *high-risk* mycotoxins or mycotoxins considered to be most prioritized or important have been strictly implemented by the Ministry of Agriculture, Forestry and Fisheries (MAFF). Although there are other toxic secondary metabolites categorized as *candidate* mycotoxins by MAFF on the basis of insufficient substantial experimental studies on their effects to humans and animal health (no risk-assessment and no standard values have been set to date), these *candidate* mycotoxins are still worth of our attention particularly those may be possibly found in fermented foods and beverages such as miso, soy sauce, and sake.

The production of Japanese fermented food products commonly used the filamentous fungus, *Aspergillus oryzae* (Murakami et. al., 1976), which on the opposite is closely related to *Aspergillus flavus* (Machida et. al., 2005; Rokas et. al., 2007), a plant pathogen known for its mycotoxin production. It is therefore important to determine the safety of *A. oryzae* industrial strains based on their mycotoxin production. To evaluate the food safety, we investigated the production of prioritized 15 mycotoxins according to MAFF list for risk assessment among the *A. oryzae* species (Bahena-Garrido et. al., 2020). However, the production of sterigmatocystin could not be examined in the previous report because pure chemical standard was not available. In this study, we further investigated *candidate* mycotoxins in the MAFF list. Unlike aflatoxins which are notoriously produced by *A. flavus*, citrinin is produced by toxic strains of several *Penicillium*, *Aspergillus* and *Monascus* spp. (Bennett and Klich, 2003) forming lemon yellow crystals. Citrinin is considered the major causal agent of the yellow rice disease described for a long time in Japan (Kushiro, 2015). The major target organ of citrinin is kidney and its ingestion results to weight loss, because of renal degeneration (Gil-Serna et. al., 2019). In addition, ergot alkaloids are mycotoxins mainly produced by several

fungal species in the genus *Claviceps* (Tudzynski et. al., 2001; Gröcer and Floss, 1998). These ergot alkaloids contain a diverse category of secondary metabolites that have been classified into three groups as clavines, amides of lysergic acid, and ergopeptines. Ergot poisoning in humans and domestic animals is known as ergotism and this disease may cause strange hallucinations and feeling of itchy and burning skin (Money, 2016). Example of ergot alkaloids is the family of ergochrome including secalonic acids (secalonic acid A-G) (Zhang et. al., 2008) and in particular, secalonic acid F is produced by *Aspergillus japonicus* and *Aspergillus aculeatus* (Andersen et. al., 1977; Zeng et. al., 2004).

In this study, we efficiently screened and examined the production of 6 *candidate* mycotoxins, such as citrinin, ergot alkaloids (ergocornine, α-ergocryptine, ergocristine, and secalonic acid F) and sterigmatocystin among the 13 *A. oryzae* strains, by adopting a tandem approach of direct-infusion high-resolution mass spectrometry (DI-HRMS) and liquid chromatography quadruple time-of-flight mass spectrometry (LC-Q/TOF-MS) as previously reported (Bahena-Garrido et. al., 2020).

Materials and Methods

A. oryzae industrial strains and culture conditions

The following *A. oryzae*, RIB40, RIB128, RIB143, RIB163, RIB301, RIB306, RIB430, RIB915, RIB1108, RIB1172, RIB1178, RIB1187, RIBOIS01 strains were grown on 11 culture conditions as described in the previous report (Bahena-Garrido et. al., 2020).

The mycotoxin pure standards and the *m/z* values, metabolite extraction and analyses by DI-HRMS and LC-Q/TOF-MS of *A. oryzae* industrial strains

Pure standard of mycotoxins (≥ 98% purity) was commercially obtained from the following: citrinin (Cayman Chemical, USA), ergocornine and α-ergocryptine (Biopure^{TM}, Romer Labs Division Holding GmbH, Austria), ergocristine (TRC Toronto Research Chemicals, Inc.), secalonic acid F (AdipoGen, Switzerland) and sterigmatocystin (LKT Laboratories, Inc., USA). Powder of chemical standards was either dissolved by MS grade acetone, ethanol or methanol (99.5%, Wako Co., Japan) as described by company's manual.

Furthermore, metabolite fractions from all *A. oryzae* species grown under different conditions were extracted and examined following the method described in our previous report (Bahena-Garrido et. al., 2020). The *m/z* values were based on the theoretical monoisotopic mass of each metabolite calculated by ChemCalc (Patiny and Borel, 2013) (Table 1) at negative and positive ionization modes for both DI-HRMS and LC-Q/TOF-MS. Similar to the previous report, the detection of the *candidate* mycotoxin peaks in DI-HRMS screening method was conducted 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. The peak detection was carried out according to default setting. The validation of the screened *candidate* mycotoxin peaks was performed using the LC-Q/TOF-MS with selected ion monitoring (SIM) mode. The peak detection was performed based on default parameters and the peak sensitivity was set as 6-fold higher intensity against background noise.

Results and Discussion

To evaluate the safety of *A. oryzae* species based on the production of *candidate* mycotoxins (Table 1), we determined the presence or absence of these metabolites among 13 *A. oryzae* species adopting a tandem approach of DI-HRMS and LC-Q/TOF-MS analyses. Table 1 includes mycotoxins such as

citrinin, ergot alkaloids and sterigmatocystin which may be putatively produced by the *A. oryzae* strains. The production of sterigmatocystin was evaluated in this study because chemical standard was finally obtained. Furthermore, culture conditions and growth substrates were considered based on our previous 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 *candidate* mycotoxins were initially screened based on the theoretical monoisotopic mass at a maximum 10 ppm mass accuracy (as m/z ± 10 ppm) at both negative (N) and positive (P) ionization modes (Table 1). We observed high peak intensity (peak area) of *candidate* mycotoxins from *A. oryzae* strains while other mycotoxins were rarely detected in any strain grown in any culture condition (Table 1 and Figure 1). For example, citrinin peaks at N and P ionization modes were frequently and putatively detected in most strains cultivated in almost all culture conditions, while one *candidate* mycotoxin, ergocristine was only detected in RIB1172 cultivated in *soy sauce-koji* 6 days (Figure 1-D). In detail, highest peak values (>10,000) of putative citrinin metabolite were detected in almost all *A. oryzae* stains grown in zeolite (Figure 1-K) and in similar manner, this mycotoxin was also detected in RIB306 grown *soy sauce-koji* 2 days (Figure 1-C), RIB143, RIB163, RIB306, RIB915 and RIB1108 in oatmeal 6 days (Figure 1-F) and RIB1178 in corn (Figure 1-G). While relatively observed at lower peak intensity values (<5,000), ergot alkaloids such as ergocornine and α/β-ergocryptine were also detected in several *A. oryzae* strains grown in *rice-koji* 2 and 6 days, oatmeal 6 days and in zeolite (Figure 1-A, B, F, K) by DI-HRMS screening. Additionally, sterigmatocystin was detected at relatively lower peak intensity values (<5,000) in several *A. oryzae* strains cultivated in *soy sauce-koji* 2 days, oatmeal 6 days, corn, YES, CYA, WATM and zeolite (Figure 1-C, F, G, H, I, J, K).

Although, we observed peaks of a number of these mycotoxins using the DI-HRMS, we further confirmed the putative production of citrinin, ergot alkaloids (i.e. ergocornine, ergocristine secalonic acid F) and sterigmatocystin in *A. oryzae* by another method known as the targeted LC-Q/TOF-MS analysis in which these mycotoxin peaks observed were compared against the available pure standards (Table 1 and Figure 1) and the retention time (RT). In the case of α/β-ergocryptine, only α-ergocryptine could be confirmed by LC-Q/TOF-MS because it was the only available chemical standard. By

Table 1. The list of *candidate* mycotoxins as evaluated by JECFA* and MAFF*

Metabolite name	Chemical formula	Theoretical monoisotopic mass (m/z)**		DI-HRMS	LC-Q/TOF-MS
		Positive mode	Negative mode		
Citrinin	$C_{13}H_{14}O_5$	251.0919	249.0763	□ (13/7)	n.d.
Ergocornine	$C_{31}H_{39}N_5O_5$	562.3029	560.2873	□ (2/1)	n.d.
α/β-Ergocryptine	$C_{32}H_{41}N_5O_5$	576.3186	574.3029	□ (4/4)	n.d.
Ergocristine	$C_{35}H_{39}N_5O_5$	610.3029	608.2873	□ (1/1)	n.d.
Secalonic acid F	$C_{32}H_{30}O_{14}$	639.1714	637.1557	□ (3/1)	n.d.
Sterigmatocystin	$C_{18}H_{12}O_6$	325.0712	323.0555	□ (9/7)	n.d.

* Joint Food and Agriculture Organization and the World Health organization (FAO/WHO) Expert Committee on Food Additives (JECFA); Ministry of Agriculture, Forestry and Fisheries (MAFF)
** 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
(/): number of strains (*left side*) and conditions (*right side*) of/, in which the metabolite was detected per analysis.
n.d.: not detected

A.

	Rice-koji 2 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
3	α-Ergocryptine																										

B.

	Rice-koji 6 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
3	α-Ergocryptine																										

C.

	Soy sauce-koji 2 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
6	Sterigmatocystin																										

D.

	Soy sauce koji 6 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
4	Ergocristine																										

Peak intensity values

< 5,000 5,000 - 10,000 10,000 <

Figure 1. DI-HRMS screening of *candidate* mycotoxins and validation by LC-Q/TOF-MS among the *A. oryzae* species in all culture conditions

(# metabolites detected by DI-HRMS were further confirmed using the LC-Q/TOF-MS. Empty cells show metabolites were not detected).

E.

	Oatmeal 2 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										

F.

	Oatmeal 6 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
3	α-Ergocryptine																										
6	Sterigmatocystin																										

G.

	Corn 7 days	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
6	Sterigmatocystin																										

H.

	YES	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
6	Sterigmatocystin																										

Peak intensity values

< 5,000 5,000 - 10,000 10,000 <

Figure 1. (continued)

I.

	CYA	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
6	Sterigmatocystin																										

J.

	WATM	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
6	Sterigmatocystin																										

K.

	Zeolite	RIB40		RIB128		RIB143		RIB163		RIB301		RIB306		RIB430		RIB915		RIB1108		RIB1172		RIB1178		RIB1187		RIBOIS01	
No.	**DI-HRMS**	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	Citrinin																										
2	Ergocornine																										
3	α/β-Ergocryptine																										
4	Ergocristine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										
	LC-Q/TOF-MS#																										
1	Citrinin																										
2	Ergocornine																										
3	α-Ergocryptine																										
5	Secalonic acid F																										
6	Sterigmatocystin																										

Peak intensity values

< 5,000 5,000 - 10,000 10,000 <

Figure 1. (continued)

adopting DI-HRMS and LC-Q/TOF-MS tandem approach of analyses, we further substantiated the non-production or no detection of these *candidate* mycotoxins and sterigmatocystin among the *A. oryzae* strains (Table 1 and Figure 1) grown in various culture conditions. It may appear that various false positives of peaks were found by DI-HRMS analysis because metabolites may have same molecular formula but different chemical structures frequently observed as natural products in the extracted samples. The LC-Q/TOF-MS could further distinguish the difference of these chemical structures by the difference of the RT. Hence, the validation of these putative mycotoxin peaks (this time from the reduced number of samples) by using the LC-Q/TOF-MS in this tandem approach of analyses was considered essential and efficient.

Taken together, the result of our study here corroborated the safety of *A. oryzae* strains with that previously reported non-production of *high-risk*, notorious mycotoxins such as aflatoxins (Tominaga et. al., 2006; Kiyota et. al., 2011; Bahena-Garrido et. al., 2020) among the *A. oryzae* species grown in different

industrial conditions.

Conclusion

In this report, we investigated the production of 6 *candidate* mycotoxins in *A. oryzae* species based on the MAFF list of risk assessment. We evaluated the possible production of these mycotoxins using a tandem approach as reported in the previous paper. Based on the DI-HRMS data and further validation by LC-Q/TOF-MS, our results indicate no significant traces of these *candidate* mycotoxins. Thus, we conclude that these 6 mycotoxins were not detected in *A. oryzae* species grown in all 11 culture conditions.

Summary (in *Japanese*)

本報告では、農林水産省により今後リスク評価を実施するとされていたカビ毒のうち６つについて、昨年度報告したDI-HRMSのデータおよび、LC-Q/TOF-MSをもちいて生産性の検討を行った。その結果、麹菌群は米麹を含む11の培養条件で、これら６つのカビ毒は検出されなかった。

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References

Andersen R, Buechi G, Kobbe G and Demain A., Secalonic acids D and F are toxic metabolites of *Aspergillus aculeatus* *J. Org. Chem.* **42**, 352－353 (1977).

Bahena-Garrido SM, Saito R, Komatsu Y et. al., Report of the Research Institute of Brewing **192**, 46－57 (2020) https://www.nrib.go.jp/data/pdf/nrl/192-05.pdf

Bennett, J.W. and Klich, M., Mycotoxins. *Clin. Microbiol. Rev.* **16**, 497－516 (2003).

Gil-Serna J, Vázquez C and Patino B., Mycotoxins | Toxicology. *Reference Module in Food Science*. 10.1016/B978-0-08-100596-5.22630-9 (2019).

Gröcer D and Floss H., *Chapter 5 Biochemistry of Ergot Alkaloids—Achievements and Challenges*. The Alkaloids: Chemistry and Biology Ed. Cordell G, Academic Press, **50**, 171－218 (1998).

Kiyota T, Hamada R, Sakamoto K, Iwashita K, Yamada O and Mikami S., Aflatoxin non-productivity of *Aspergillus oryzae* caused by loss of function in the *aflJ* gene product. *J. Biosci. Bioeng*. **111**, 512－517 (2011).

Machida M, Asai K, Sano M et. al., Genome sequencing and analysis of *Aspergillus oryzae*, *Nature*, **438**, 1157－1161(2005).

Kushiro M., Historical review of researches on yellow rice and mycotoxigenic fungi adherent to rice in Japan. *Mycotoxins*. **65**, 19－23 (2015).

Money N., Chapter 12 - Fungi and Biotechnology, The Fungi (Third Edition), Editor(s): Watkinson S, Boddy L, Money N, Academic Press, 401－424 (2016).

Murakami H., The study of Koji (in *Japanese*). *J. Brew. Soc. Jpn.* **1**, 1－31 (1976).

Patiny L and Borel A., ChemCalc: A building for Tomorrow's Chemical Infrastructure. *Chem. Inf. Model*. **53**, 1223－1228 (2013).

Rokas A, Payne G, Fedorova ND et. al., What can comparative genomics tell us about species concepts in the genus *Aspergillus*? *Stud. Mycol.* **59**, 11－7 (2007).

Tominaga M, Lee YH, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, Gotoh, K and Akita O., Molecular Analysis of an Inactive Aflatoxin Biosynthesis Gene Cluster in *Aspergillus oryzae* RIB Strains. *Appl. Environ. Microb*. **72**, 484－490 (2006).

Tudzynski P, Correia T, and Keller U., Biotechnology and genetics of ergot alkaloids. *Appl Microbiol Biotechnol* **57**, 593－605 (2001).

WHO Technical Report Series 906 (Evaluation of Certain Mycotoxins in Food), Fifty-Six Report of the Joint FAO/WHO Expert Committee on Food Additives (2002): https://apps.who.int/iris/bitstream/handle/10665/42448/WHO_TRS_906.pdf

Zhang W, Krohn K, Zia-Ullah, Flörke U, Pescitelli G, et. al., New mono- and dimeric members of the secalonic acid family: Blennolides A-G isolated from the fungus *Blennoria* sp. *Chemistry*. **14**, 4913 – 23 (2008).

Zeng, R Shiming, L and Shi, Y., Allelopathic effects of secalonic acid F produced by *Aspergillus japonicus* on *Zea mays*. *The Journal of Applied Ecology* **15**, 145 – 8 (2004).