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Paramee Noonim, Warapa Mahakarnchanakulb, Kristian F Nielsen, Jens Frisvad, Robert A Samson

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Complete List of Authors:	Noonim, Paramee; Kasetsart University, Department of Food Science and Technology; Prince of Songkla University, Surat Thani Campus, Faculty of Technology and Management Mahakarnchanakulb, Warapa; Kasetsart University, Department of Food Science and Technology Nielsen, Kristian; Center for Microbial Biotechnology, Department of Systems Biology Frisvad, Jens; Center for Microbial Biotechnology, Department of Systems Biology Samson, Robert; CBS Fungal biodiversity centre
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Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans

Paramee Noonim[a,b,c], Warapa Mahakarnchanakul[b], Kristian F. Nielsen[d], Jens C. Frisvad[d] and Robert A. Samson[a]

[a] CBS Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

[b] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, 10900 Bangkok, Thailand

[c] Faculty of Technology and Management, Prince of Songkla University, Surat Thani Campus, 84100 Surat Thani, Thailand

[c] Center for Microbial Biotechnology, Department of Systems Biology, Building 221, Technical University of Denmark, DK-2800 Kgs Lyngby, Denmark

Corresponding author: RA Samson
CBS Fungal Biodiversity Centre,
P.O. Box 85167, 3508 AD Utrecht, The Netherlands
Telephone + 31 30 2122656
Telefax + 31 30 2512097
E-mail: samson@cbs.knaw.nl

Abstract

During 2006 and 2007, a total of 64 Thai dried coffee bean samples (*Coffea arabica*) from two growing sites of Chiangmai Province, and 32 Thai dried coffee bean samples (*Coffea canephora*) from two growing sites of Chumporn Province, Thailand, were collected and assessed for fumonisin contamination by black Aspergilli. No *Fusarium* species known to produce fumonisin were detected, but black Aspergilli had high incidences on both Arabica and Robusta Thai coffee beans. Liquid chromatography (LC) with high resolution mass spectrometric (HRMS) detection showed that 67% of *A. niger* isolates from coffee beans were capable of producing fumonisins B_2 (FB_2) and B_4 when grown on Czapek Yeast Agar with 5% NaCl. Small amounts (1-9.7 ng/g) of FB_2 were detected in 7 of 12 selected coffee samples after ion-exchange purification and LC-MS/MS detection. Two samples also contained FB_4. This is the first record of freshly isolated Aspergillus *niger* strains producing fumonisins and the first report on the natural occurrence of FB_2 and FB_4 in coffee.

Key words: Fumonisin B_2, Coffee beans, *Aspergillus niger*

Introduction

Fumonisins are carcinogenic mycotoxins produced by several *Fusarium* species (Gelderblom *et al*., 1988; Marin *et al*., 2004) and have been reported in many food commodities especially corn (Marin *et al*., 2004). Fumonisins has been reported to cause a fatal disease in horses (leukoencephalomalacia) (Marasas *et al*., 1988), pulmonary edema in pigs (Haschek *et al.*, 2001) and possibly esophageal cancer in humans (Yoshizawa *et al*., 1994). Even though fumonisins are less acutely toxic compared to aflatoxins, they could be found in high concentration of mg/kg in corn compared to μg/kg for aflatoxins.

Coffee is one of the most consumed beverages in the world, and have been reported to be contaminated with ochratoxin A. Aflatoxin contamination in coffee beans has also been reported (Soliman, 2002). However, there have been many publications discussing the ecology of ochratoxin-producing fungi, manipulation of environmental factors (Batista *et al*., 2003; Palacios-Cabrera *et al*., 2004; Esteban *et al*., 2006) and control strategies to prevent or reduce ochratoxin contamination (Suarez-Quiroz, 2005). Ochratoxigenic species in coffee beans are generally known from *Aspergillus* species of the section *Circumdati* and *Nigri* (Joosten *et al*., 2001; De Moraes *et al*., 2003; Martins, 2005; Leong *et al*., 2007; Ilic *et al*., 2006; Taniwaki *et al*., 2006) and various methods have been developed for the detection of ochratoxin-producing fungi and ochratoxin contamination in coffee beans (Patiño *et al*., 2005; Lobeau *et al*., 2005; Satori *et al*., 2006).

Recently, fumonisin B_2 was detected in agar cultures of four important isolates of *Aspergillus niger* (Frisvad *et al*., 2007) including the culture ex type and three full genome sequenced cultures (Baker, 2006). It was found that while *Fusarium verticillioides* produces fumonisin B_1, B_2 and B_3 on plant extract agars, but *A. niger* produces fumonisin B_2 only on agar media with

high amounts of carbohydrate or NaCl.

As the results of a survey on ochratoxigenic species in Thai coffee beans, we found that black Aspergilli including *A. niger* were the predominant contaminating fungi. In this study, we investigate the presence of fumonisin producing black Aspergilli on coffee beans, as well as fumonisin production in the beans them selves.

Material and Methods

Sampling

There are two coffee growing regions in Thailand, the Northern and Southern region, which are different in varieties of coffee grown, geographical condition and climate during harvesting. In this study, two types of Arabica coffee beans, parchment and green coffee beans, from the North were collected from two selected farms in two different growing sites. Two types of Robusta coffee bean, dried coffee cherries and green coffee beans, from the South were collected from two selected farms in two different growing sites. Four samples of 0.5-1 kg of each type per farm were collected. A total of 64 samples were collected during the two harvesting year 2006 and 2007.

Mycological analysis

A total of 50 beans per sample were plated directly (5 per plate) onto Dichloran 18% Glycerol Agar (DG18) plates and Malt Extract Agar (MEA) plates (Samson *et al*., 2004a) with and without surface sterilization. The plates were incubated for 5-7 days at 25^{o}C, and then inspected for fungal growth. Of the many species encounter on these plates (Noonim et al., in prep.) potentially fumonisin producing species of *Aspergillus* section *Nigri* were isolated and identified to species level using morphology, physiology and molecular characteristics (Samson *et al*.,

2007) and kept in collection for further studies.

Determination of extrolite production by liquid chromatography-UV-mass spectrometry of fungal cultures

Representative isolates of each *Aspergillus* species in section *Nigri* were inoculated in Czapek Yeast Agar with 5% salt (NaCl) (CYAS) medium (Frisvad and Samson, 2004) and incubated for 7 days at 25°C. Subsequently, 5 plugs of culture (1 cm^2) were sampled, and moved to a 2-ml vial, where it was extracted using ultra-sonication for 60 min with 0.75 ml 75% methanol, and subsequently filtered trough a 0.45 µm syringe filter (Frisvad *et al*. 2007).

Solvents were HPLC grade and all other chemicals were analytical grade unless otherwise stated. Water was purified from a Milli-Q system (Millipore, Bedford, MA). LC-DAD-HRMS was performed on an Agilent 1100 system equipped with a photo diode array detector (DAD) and a 50×2 mm i.d., 3 µm, Luna C_{18} II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK), with a Z-spray ESI source (*2*).

Samples were analyzed in ESI^+ using a water-CH_3CN gradient system starting with 0.3 ml/min flow of 30% CH_3CN which was increased linear to 60% in 5 min, then increased to 100% in 1 min while also increasing the flow to 0.5 ml/min, holding this for 2 min. The water was buffered with 10 mM ammonium formate and 20 mM formic acid and the CH_3CN with 20 mM formic acid (Nielsen and Smedsgaard, 2003; Nielsen et al., 2005). One scan function (1 s) was used with a potential difference of 50 V between the skimmers and using a scan range of *m/z* 100 to 900. Reference standards of fumonisin B_1, B_2 and B_3, AAL toxin TB_1 and TA_1, Malformins A, B and C, ochratoxin A, and Asperazine were also co-analyzed in the sequences. Source of

reference standards: Certified standards of 50 µg/mL of FB_1 and fumonisin B_2 were obtained from Biopure, Tulln, Austria. FB_3 was a gift from Dr. Michael Sulyok, Center for Analytical Chemistry (IFA-Tulln, Austria) and other reference standards were available from previous studies in our laboratory (Nielsen and Smedsgaard, 2003).

The presence of fumonisin B_2 was detected in ESI^+ from the reconstructed ion chromatograms of the $[M+H]^+$ ion at *m/z* 706.39-706.41 (calc. mass 706.4014). Other metabolites were detected as the predominant ion in extracted ion chromatograms (± *m/z* 0.01).

A few samples were also analyzed by LC-tandem MS as described for the coffee samples below, except that the MS was operated in ESI^+ daughter ion scan mode using fragmentation potentials from 20 to 50 V.

ELISA Screening of fumonisins in coffee beans

Coffee bean samples were assessed for fumonisin contamination by using RIDASCREEN® Fumonisin ELISA test kits (r-biopharm) using the protocol for corn. All sample preparation and test procedures were according to manufacturer's instructions. Specificity for fumonisin B_1, B_2 and B_3 are 100, 40 and 100, respectively. The lower detection limit of the test kit was specified as 25 µg/kg. Assay was not validated nor tested on spiked samples.

LC-MS/MS *of fumonisins in coffee beans*

The coffee bean samples were frozen by liquid nitrogen and grinded for 2 minus in a domestic electrical coffee grinder. Subsamples of 1.0 g were then shaken with 10.0 ml methanol-water (7:3 v/v) in a falcon tube for 15 min and centrifuged at 9000 g for 3 min. Then a 5 ml subsample transferred to a 100 mg Strata SAX column (Phenomenex) which had previously been sequentially conditioned with 1 ml methanol and 1 ml methanol-water (7:3 v/v). Columns were

washed with 3 ml methanol-water (7:3 v/v) and 2 ml methanol, and the fumonisins eluted with 2.4 ml methanol containing 1% acetic acid. Samples were then evaporated to dryness with nitrogen flow and redissolved in 250 µl acetonitrile-water (3:7 v/v) (modified from the EN 13585:2001).

Sub-samples of 5 µl were analyzed by LC-MS/MS on an Agilent HP 1100 liquid chromatograph system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with ESI source. The separations was performed on a Gemini C6-phenyl column (Phenomenex, 50 × 2 mm, 3 µm) fitted with a security guard system and using a linear gradient starting from 20 % acetonitrile in water (both 20 mM formic acid) to 55% acetonitrile for 6 minutes at a flow rate of 300 µL/min, which was then increase to 100% acetonitrile in 30 sec and a flow of 0.5 ml/min keeping this fro 3.5 min before returning to the start conditions in 6 min. Tandem mass spectrometry was performed in ESI^+ at a source flow at 700 L/hr nitrogen at 350°C. Nitrogen was also used as collision gas, and the MS operated in MRM mode at the following transitions: FB_2 quantifier m/z 706 → 336 cone 50V, collision 40 V, dwell time 50 ms, qualifier m/z 706 → 512 a, cone 50V, collision 25 V, dwell time 100 ms; FB_4 quantifier m/z 690 → 320 cone 50V, collision 35 V, dwell time 50 ms, qualifier m/z 690 → 514 a, cone 50V, collision 30 V, dwell time 100 ms; and FB_1 and position analogues quantifier m/z 722 → 334 cone 50V, collision 40 V, dwell time 50 ms, qualifier m/z 722 → 528 a, cone 50V, collision 25 V, dwell time 100 ms.

Quantification was done from spiked samples, which were spiked with 30 to 100 µl acetonitrile solutions to final concentrations of 50, 25, 12.5, 5.0, 3.75, 2.50, 1.25, 0.500, 0.375, 0.00 ng/g grinded coffee and stored for 1-4 days prior to extraction. Samples and spiked samples were extracted and analyzed 4 times on different days.

Results and Discussion

Mycological analysis and identification of fungal isolates

Of all coffee bean samples analyzed, none of typical fumonisin-producing species (*Fusarium* spp.) were detected. Besides Penicillia and other saprophytes, *Aspergillus* spp. of section *Circumdati* and *Nigri* were the predominant species in the Arabica coffee samples with 77 and 75% infestation, respectively. In the Robusta coffee samples, *Aspergillus* spp. section *Nigri* was the predominant one with approximately 100% infection.

A diversity of black Aspergilli were observed (Fig. 1) in Thai coffee beans, including, *A. niger*, *A. carbonarius*, *A. tubingensis*, *A. foetidus*, *A. aculeatinus* and *A. sclerotiicarbonarius*. The latter two species were found to unrelated to the known taxa and proposed as new taxa (Noonim et al., 2008).

Considering each type of coffee beans, there are differences in the mycobiota observed. Arabica coffee had a higher incidence of *A. niger* and related taxa while in Robusta coffee, both *A. carbonarius* and *A. niger* were the dominant species. *A. carbonarius* and *A. sclerotiicarbonarius* were found only in Robusta coffee from Southern Thailand while *A. foetidus* was found only in Arabica coffee from the Northern region (Table 1.). These differences could be due to differences in the geography, climate and methods used for coffee processing in the two regions.

Mycotoxigenic potential of the Aspergillus species

Using LC-HRMS (Frisvad *et al*., 2007) a total of 82 isolates from 6 species were analyzed. Only *A. niger* isolates were found to produce fumonisin B_2 as well as fumonisin B_4 (same retention time and tandem spectrum as from a *Fusarium* extract). FB_4 were relative to FB_2 levels in the

range 0-40% with most being in the 10-20% range. Most of the tested *A. niger* isolated from Thai coffee beans (13 out of 17 isolates) produced fumonisin B_2 in the CYAS culture medium in amounts of 0.4 to 2 µg/cm^2 (Table 2). This indicates that these *A. niger* isolates may also produce fumonisin B_2 in coffee cherries or beans. All *A. niger* isolated from Northern Arabica coffee bean samples could produce fumonisin B_2, while in some isolates from Southern Robusta coffee samples no fumonisin B_2 was found. More molecular studies are needed in order to compare the differences in these isolates at the genotypic level. A high percentage of infection by *A. niger* as determined after surface disinfection of the green coffee beans indicated that *A. niger* actually grows actively in the coffee beans.

LC-HRMS detection of fumonisin B_2 has been shown to have detection limit of ca. 25 ng/cm^2 culture, and a relative standard deviation better than 30%, and an apparent recovery better than 80% (Frisvad, Nielsen *et al*. unpublished). A chromatogram example of fumonisin B_2 detection in *A. niger* 1F6 is shown in figure 2. Figure 3 shows the comparison of the tandem spectrum of FB_2 from *A. niger* and reference standard.

In agreement with Samson et al. (2007) most of the isolates of *Aspergillus niger* from coffee beans produced funalenone, kotanin, orlandin, aurasperone B and other naphtho-γ-pyrones, tensidol B and pyranonigrin A (Table 3). Two isolates produced ochratoxin A (1F1 and 1F6) in addition to FB_2 and thus could produce two important mycotoxins.

Analysis of coffee bens

Screening for B type fumonisins with the RIDASCREEN® ELISA test kits also indicated that FB_2 was present in some of the coffee bean samples (results not shown) in levels up to 77 ng/g and it was thus decided to confirm this by LC-MS/MS which is much more specific. Since both

a quantifier and a qualifier ion were used the method earns 4 identification-points accordingly to Council Directive 96/23/EC, which is required for forbidden compounds.

The detection limit of the LC-MS/MS method was approx 0.5 ng/g in spiked sample and the limit of quantification (LOQ) 1.25 ng/g (RSD < 30% for 4 replicates at this level). R^2 from the calibration curves were in the 4 experiments >0.985 (7 detected levels).

LC-MS/MS showed that the ELISA results were false positives, and of the 12 samples analyzed (4 times each), the most contaminated one (Robusta, R1) contained 9.7 ng/g, and the 3 other (SO.1C, Ch.3P, R8) between 1.9 and 1.3 ng/g (RSD ca. 40%, 95% level), while 2 were positive but below LOQ (R7 and R9). Chromatographic separation between FB_1 and FB_2 was 0.98 min. R1 additionally contained FB4 as shown in figure 4 where the un-smoothed chromatographic profiles can be seen from the FB_2 and FB_4 identifications.

More experiments are needed for the detection of the *Aspergillus* fumonisin in food commodities. An extensive survey of fumonisin producing black Aspergilli from other sources is in progress (Frisvad, Nielsen and Samson, personal communication).

One of the great concerns with fumonisin contamination of maize is that very large amounts of fumonisin may be produced by *Fusarium* species, but in accordance with the results of Frisvad *et al*. (2007), *Aspergillus niger* needs a relatively large amount of carbohydrate in the substrate to produce high amounts of fumonisin B_2. In contrast *Fusarium verticillioides* needs less carbohydrate to produce substantial amounts of fumonisins. Green coffee beans contain small amounts of carbohydrates, while coffee cherries contain some carbohydrate. Thus, widespread infection of *A. niger* in coffee beans does not necessarily represent a high risk for fumonisin

contamination, which is also indicated from the concentrations detected in very limited number of samples analyzed.

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Figure 1. Diversity of fungal population in coffee beans from direct plating of two types of coffee bean samples from 2 different regions of Thailand.

A. Arabica green coffee, MEA. B. Arabica green coffee, DG18. C. Robusta green coffee, MEA. D. Robusta green coffee, DG18. E. Robusta cherries, DG18. F. Robusta cherries, MEA. G. Arabica parchment coffee DG18. H. Arabica parchment coffee, MEA;

Figure 2. A. showing the total ion chromatogram (TIC) of a fumonisin B_1 and B_2 standard mix. B. extracted ion chromatogram m/z 706.39-706.41, from plug extract of *Aspergillus niger* 1F6 grown on CYAS for 7days. C. TIC of same extract, and D. mass spectrum of fumonisin B_2 in the extract

Figure 3.

Tandem spectra (40 V collision), of fumonisin B_2 peak from *A. niger* (A) extract and a reference standard (B).

Figure 4.

LC-MS/MS chromatograms of R1 sample, showing the MRM transitions from FB_2 (A and B) as well as FB_4 (C and D).

Table 1. Distribution and fumonisin producing abilities of *Aspergillus* spp. in section *Nigri* isolated from Thai coffee beans as determined by LC-MS

Arabica (Northern Thailand)	Robusta (Southern Thailand)	Fumonisin Production*	
		Positive	Fumonisin B_2
A. niger (44)	*A. niger* (28)	13/17	+++
A. tubingensis (19)	*A. tubingensis* (17)	0/13	-
A. foetidus (28)	-	0/15	-
A. aculeatinus (9)	*A. aculeatinus* (15)	0/14	-
-	*A. carbonarius* (35)	0/18	-
-	*A. sclerotiicarbonarius* (5)	0/5	-

Note: In brackets: percent of black Aspergilli isolates identified from each type of Thai coffee beans as determined on non-surface disinfected coffee beans.

* Other analogues are with accurate masses matching FB_1 and FB_3, however retentions times do not match reference standards.

Table 2. Fumonisin B_2 production from *A. niger* isolates from Thai coffee beans.

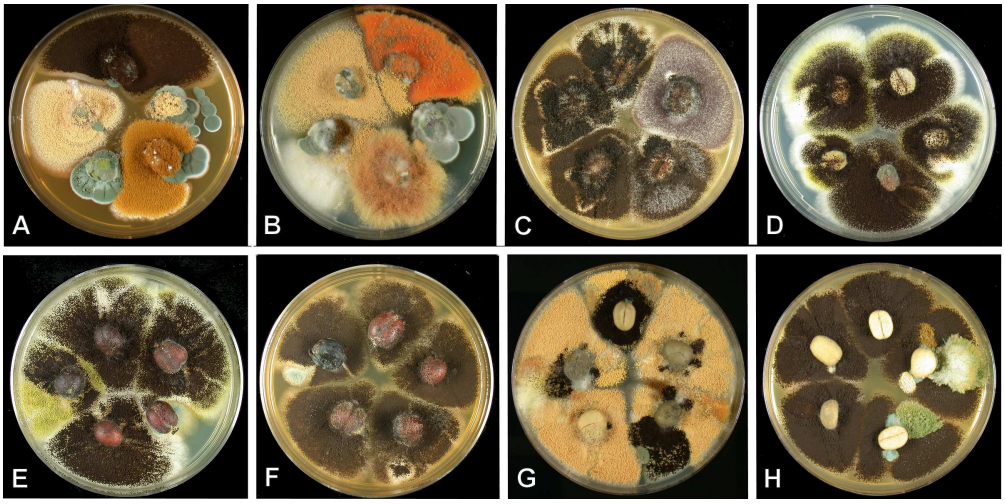
Strain number	Fumonisin Production ($\mu g/cm^2$)*	Coffee type, source
1B6	0.4	Arabica, Northern Thailand
1B7	0.7	Arabica, Northern Thailand
1B8	0.9	Arabica, Northern Thailand
1F1	0.9	Arabica, Northern Thailand
1F6	0.7	Arabica, Northern Thailand
1F7	0.5	Arabica, Northern Thailand
3A2	1.3	Arabica, Northern Thailand
3E2	0.8	Arabica, Northern Thailand
3G2	ND**	Robusta, Southern Thailand
3G7	ND	Robusta, Southern Thailand
3H2	ND	Robusta, Southern Thailand
3H3	1.3	Arabica, Northern Thailand
3H4	1.2	Robusta, Southern Thailand
3H7	2	Robusta, Southern Thailand
4C4	0.3	Robusta, Southern Thailand
4D4	ND	Robusta, Southern Thailand
6E3	2	Arabica, Northern Thailand

***Relative standard deviation 30%. **Not detected.**

Table 3. Extrolite production other than Fumonisin B_2 by isolates of *Aspergillus niger* from Thai green coffee beans:

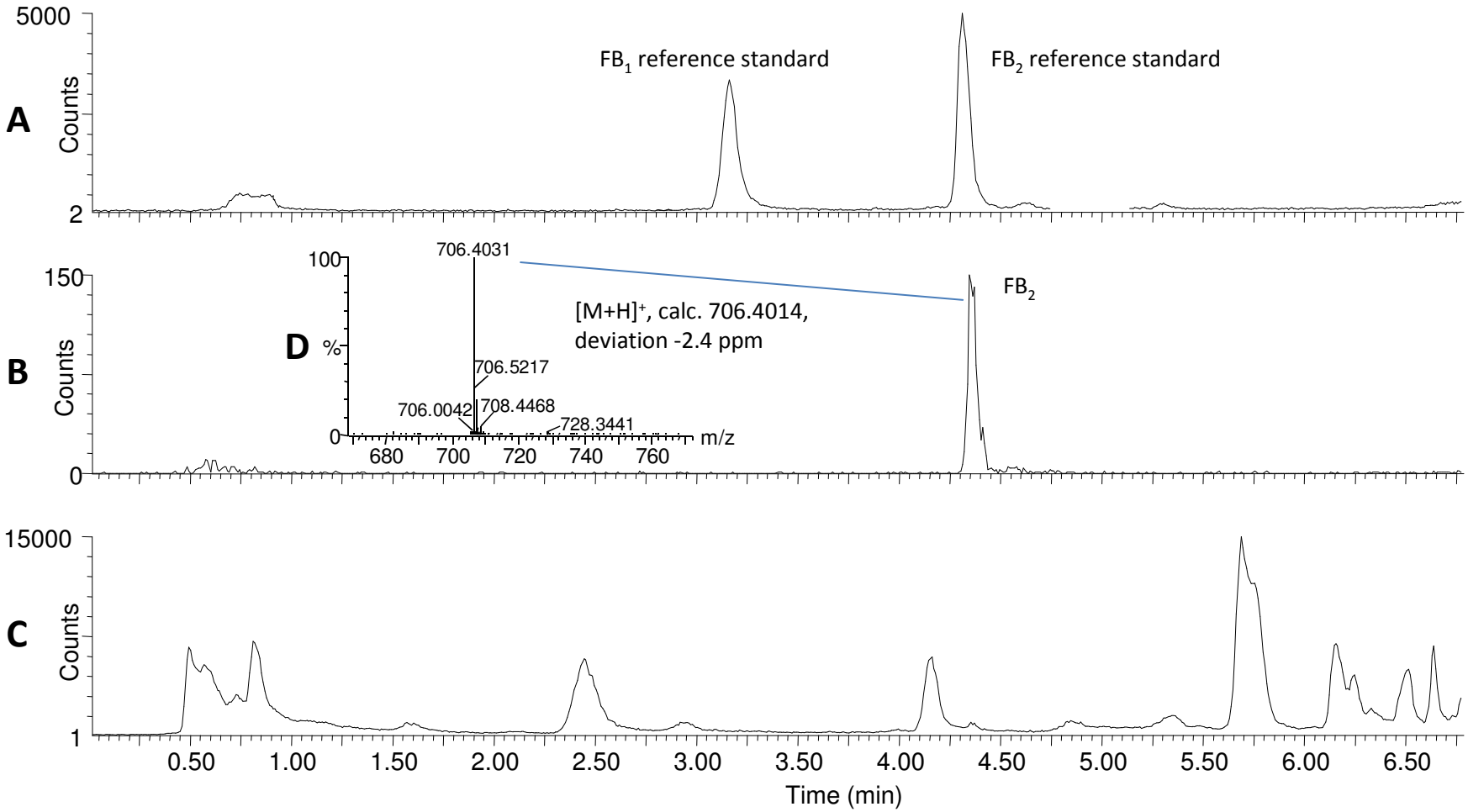
Strain	Extrolites							
1B6	AU-NA	FU	—	—	PY	TE	—	—
1B7	AU-NA	FU	KO	OR	PY	TE	—	—
1B8	AU-NA	FU	KO	OR	PY	TE	—	—
1F1	AU-NA	FU	KO	OR	PY	TE	OT A	OT B
1F6	AU-NA	FU	KO	OR	PY	TE	OT A	OT B
1F7	AU-NA	FU	KO	OR	PY	TE	—	—
3E2	AU-NA	FU	KO	OR	PY	TE	—	—
3G2	AU-NA	FU	KO	OR	PY	TE	—	—
3G7	AU-NA	FU	KO	OR	PY	TE	—	—
3H2	AU-NA	FU	KO	OR	PY	TE	—	—
3H3	AU-NA	FU	KO	OR	PY	TE	—	—
3H4	AU-NA	FU	KO	OR	PY	TE	—	—
3H7	AU-NA	FU	KO	OR	PY	TE	—	—
4C4	AU-NA	FU	KO	OR	PY	TE	—	—
4D4	AU-NA	FU	KO	OR	PY	TE	—	—

AU-NA = aurasperone B and other naphtho-γ-pyrones, FU = funalenone, KO = kotanin, OR = orlandin, PY = pyranonigrin A, TE = tensidol A, OT = ochratoxin.

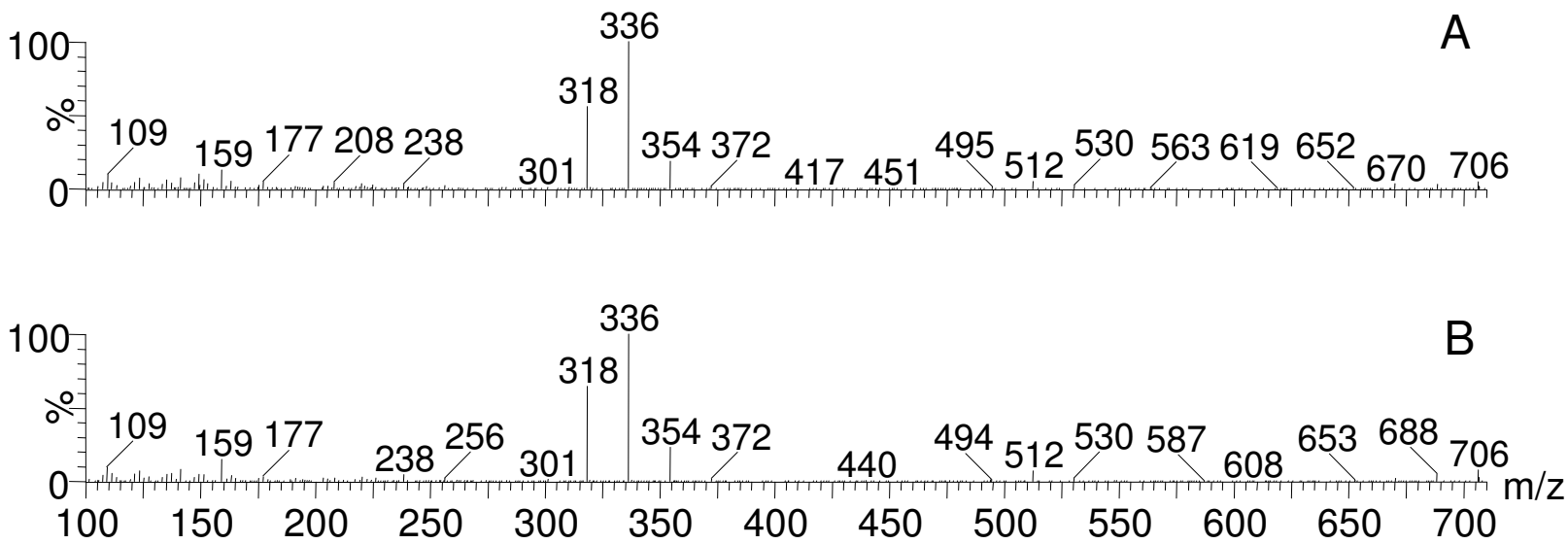


203x100mm (300 x 300 DPI)

Noonim et al. Figure 2

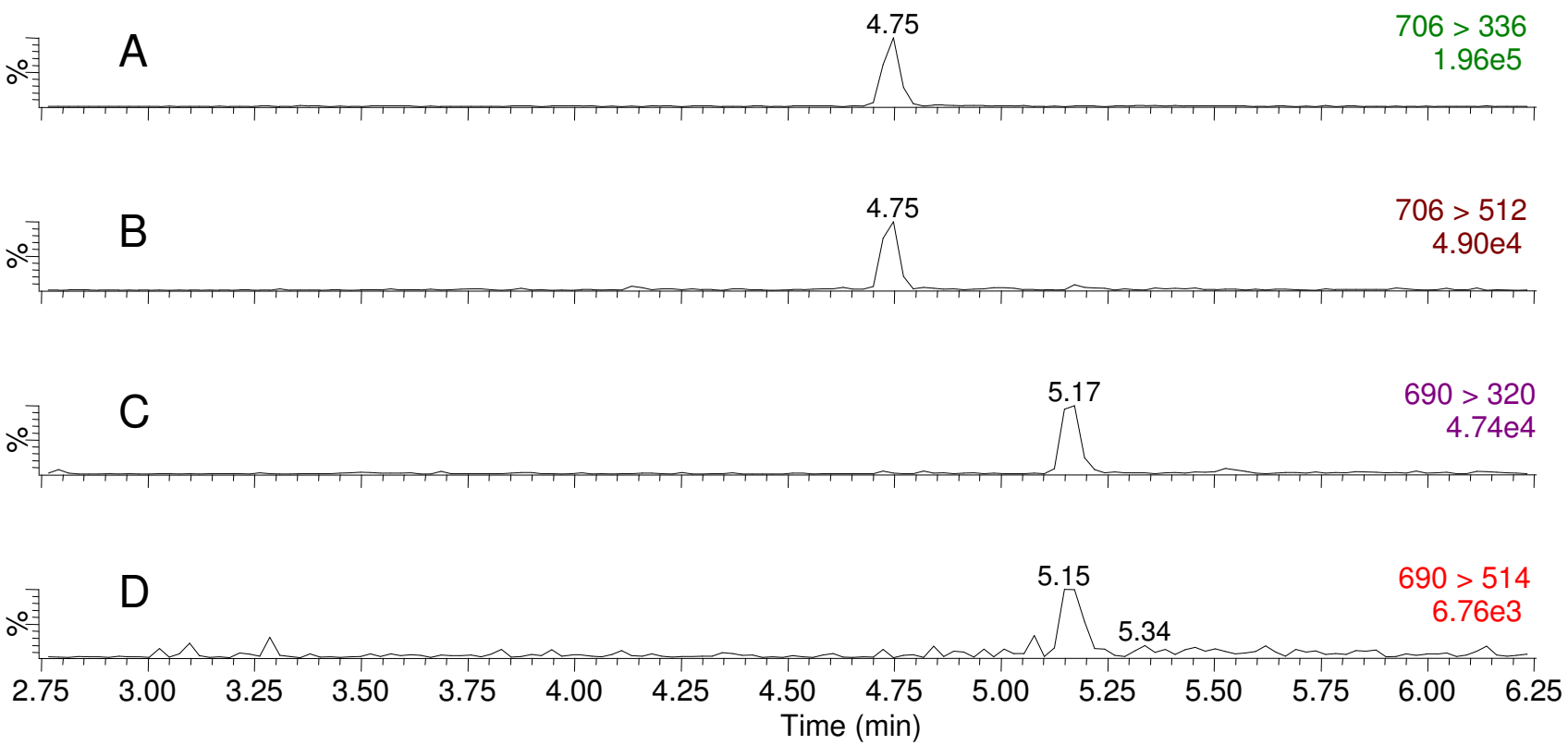


Noonim et al. Figure 3



Tandem spectra (40 V collision), of fumonisin B_2 peak from *A. niger* (A) extract and a reference standard (B).

Noonim et al. Figure 4



LC-MS/MS chromatograms of R1 sample, showing the MRM transitions from FB_2 (A and B) as well as FB_4 (C and D).