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4 1 **Fumonisin B<sub>2</sub> production by *Aspergillus niger* from Thai coffee beans**  
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11 4 **Paramee Noonim<sup>a,b,c</sup>, Warapa Mahakarnchanakul<sup>b</sup>, Kristian F. Nielsen<sup>d</sup>, Jens C. Frisvad<sup>d</sup>**  
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3 **Abstract**  
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8 During 2006 and 2007, a total of 64 Thai dried coffee bean samples (*Coffea arabica*) from two  
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10 growing sites of Chiangmai Province, and 32 Thai dried coffee bean samples (*Coffea canephora*)  
11  
12 from two growing sites of Chumporn Province, Thailand, were collected and assessed for  
13  
14 fumonisin contamination by black Aspergilli. No *Fusarium* species known to produce fumonisin  
15  
16 were detected, but black Aspergilli had high incidences on both Arabica and Robusta Thai  
17  
18 coffee beans. Liquid chromatography (LC) with high resolution mass spectrometric (HRMS)  
19  
20 detection showed that 67% of *A. niger* isolates from coffee beans were capable of producing  
21  
22 fumonisins B<sub>2</sub> (FB<sub>2</sub>) and B<sub>4</sub> when grown on Czapek Yeast Agar with 5% NaCl. Small amounts  
23  
24 (1-9.7 ng/g) of FB<sub>2</sub> were detected in 7 of 12 selected coffee samples after ion-exchange  
25  
26 purification and LC-MS/MS detection. Two samples also contained FB<sub>4</sub>. This is the first record  
27  
28 of freshly isolated *Aspergillus niger* strains producing fumonisins and the first report on the  
29  
30 natural occurrence of FB<sub>2</sub> and FB<sub>4</sub> in coffee.  
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38 **Key words:** Fumonisin B<sub>2</sub>, Coffee beans, *Aspergillus niger*  
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## 1 Introduction

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

9  
10 Coffee is one of the most consumed beverages in the world, and have been reported to be  
11 contaminated with ochratoxin A. Aflatoxin contamination in coffee beans has also been reported  
12 (Soliman, 2002). However, there have been many publications discussing the ecology of  
13 ochratoxin-producing fungi, manipulation of environmental factors (Batista *et al.*, 2003;  
14 Palacios-Cabrera *et al.*, 2004; Esteban *et al.*, 2006) and control strategies to prevent or reduce  
15 ochratoxin contamination (Suarez-Quiroz, 2005). Ochratoxigenic species in coffee beans are  
16 generally known from *Aspergillus* species of the section *Circumdati* and *Nigri* (Joosten *et al.*,  
17 2001; De Moraes *et al.*, 2003; Martins, 2005; Leong *et al.*, 2007; Ilic *et al.*, 2006; Taniwaki *et al.*,  
18 2006) and various methods have been developed for the detection of ochratoxin-producing fungi  
19 and ochratoxin contamination in coffee beans (Patiño *et al.*, 2005; Lobeau *et al.*, 2005; Satori *et*  
20 *al.*, 2006).

21  
22 Recently, fumonisin B<sub>2</sub> was detected in agar cultures of four important isolates of *Aspergillus*  
23 *niger* (Frisvad *et al.*, 2007) including the culture ex type and three full genome sequenced  
24 cultures (Baker, 2006). It was found that while *Fusarium verticillioides* produces fumonisin B<sub>1</sub>,  
25 B<sub>2</sub> and B<sub>3</sub> on plant extract agars, but *A. niger* produces fumonisin B<sub>2</sub> only on agar media with

1 high amounts of carbohydrate or NaCl.

2  
3 As the results of a survey on ochratoxigenic species in Thai coffee beans, we found that black  
4 Aspergilli including *A. niger* were the predominant contaminating fungi. In this study, we  
5 investigate the presence of fumonisin producing black Aspergilli on coffee beans, as well as  
6 fumonisin production in the beans themselves.

## 7 8 **Material and Methods**

### 9 *Sampling*

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

### 18 19 *Mycological analysis*

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

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2  
3 1 2007) and kept in collection for further studies.  
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6 2  
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8 3 *Determination of extrolite production by liquid chromatography-UV-mass spectrometry of*  
9  
10 4 *fungus cultures*

11  
12 5 Representative isolates of each *Aspergillus* species in section *Nigri* were inoculated in Czapek  
13 6 Yeast Agar with 5% salt (NaCl) (CYAS) medium (Frisvad and Samson, 2004) and incubated for  
14  
15 7 7 days at 25°C. Subsequently, 5 plugs of culture (1 cm<sup>2</sup>) were sampled, and moved to a 2-ml vial,  
16  
17 8 where it was extracted using ultra-sonication for 60 min with 0.75 ml 75% methanol, and  
18  
19 9 subsequently filtered through a 0.45 µm syringe filter (Frisvad *et al.* 2007).  
20  
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26  
27 10  
28 11 Solvents were HPLC grade and all other chemicals were analytical grade unless otherwise stated.  
29  
30 12 Water was purified from a Milli-Q system (Millipore, Bedford, MA). LC-DAD-HRMS was  
31  
32 13 performed on an Agilent 1100 system equipped with a photo diode array detector (DAD) and a  
33  
34 14 50×2 mm i.d., 3 µm, Luna C<sub>18</sub> II column (Phenomenex, Torrance, CA). The LC system was  
35  
36 15 coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester,  
37  
38 16 UK), with a Z-spray ESI source (2).  
39  
40  
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44 17  
45 18 Samples were analyzed in ESI<sup>+</sup> using a water-CH<sub>3</sub>CN gradient system starting with 0.3 ml/min  
46  
47 19 flow of 30% CH<sub>3</sub>CN which was increased linear to 60% in 5 min, then increased to 100% in 1  
48  
49 20 min while also increasing the flow to 0.5 ml/min, holding this for 2 min. The water was buffered  
50  
51 21 with 10 mM ammonium formate and 20 mM formic acid and the CH<sub>3</sub>CN with 20 mM formic  
52  
53 22 acid (Nielsen and Smedsgaard, 2003; Nielsen *et al.*, 2005). One scan function (1 s) was used  
54  
55 23 with a potential difference of 50 V between the skimmers and using a scan range of *m/z* 100 to  
56  
57 24 900. Reference standards of fumonisin B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>, AAL toxin TB<sub>1</sub> and TA<sub>1</sub>, Malformins A, B  
58  
59 25 and C, ochratoxin A, and Asperazine were also co-analyzed in the sequences. Source of  
60

1 reference standards: Certified standards of 50 µg/mL of FB<sub>1</sub> and fumonisin B<sub>2</sub> were obtained  
2 from Biopure, Tulln, Austria. FB<sub>3</sub> was a gift from Dr. Michael Sulyok, Center for Analytical  
3 Chemistry (IFA-Tulln, Austria) and other reference standards were available from previous  
4 studies in our laboratory (Nielsen and Smedsgaard, 2003).

5  
6 The presence of fumonisin B<sub>2</sub> was detected in ESI<sup>+</sup> from the reconstructed ion chromatograms of  
7 the [M+H]<sup>+</sup> ion at  $m/z$  706.39-706.41 (calc. mass 706.4014). Other metabolites were detected as  
8 the predominant ion in extracted ion chromatograms ( $\pm m/z$  0.01).

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

### 12 13 *ELISA Screening of fumonisins in coffee beans*

14 Coffee bean samples were assessed for fumonisin contamination by using RIDASCREEN<sup>®</sup>  
15 Fumonisin ELISA test kits (r-biopharm) using the protocol for corn. All sample preparation and  
16 test procedures were according to manufacturer's instructions. Specificity for fumonisin B<sub>1</sub>, B<sub>2</sub>  
17 and B<sub>3</sub> are 100, 40 and 100, respectively. The lower detection limit of the test kit was specified  
18 as 25 µg/kg. Assay was not validated nor tested on spiked samples.

### 19 20 *LC-MS/MS of fumonisins in coffee beans*

21 The coffee bean samples were frozen by liquid nitrogen and grinded for 2 min in a domestic  
22 electrical coffee grinder. Subsamples of 1.0 g were then shaken with 10.0 ml methanol-water  
23 (7:3 v/v) in a falcon tube for 15 min and centrifuged at 9000 g for 3 min. Then a 5 ml subsample  
24 transferred to a 100 mg Strata SAX column (Phenomenex) which had previously been  
25 sequentially conditioned with 1 ml methanol and 1 ml methanol-water (7:3 v/v). Columns were



1 washed with 3 ml methanol-water (7:3 v/v) and 2 ml methanol, and the fumonisins eluted with  
2 2.4 ml methanol containing 1% acetic acid. Samples were then evaporated to dryness with  
3 nitrogen flow and redissolved in 250  $\mu$ l acetonitrile-water (3:7 v/v) (modified from the EN  
4 13585:2001).

5  
6 Sub-samples of 5  $\mu$ l were analyzed by LC-MS/MS on an Agilent HP 1100 liquid chromatograph  
7 system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer  
8 (Micromass, Manchester, UK) with ESI source. The separations was performed on a Gemini C6-  
9 phenyl column (Phenomenex, 50  $\times$  2 mm, 3  $\mu$ m) fitted with a security guard system and using a  
10 linear gradient starting from 20 % acetonitrile in water (both 20 mM formic acid) to 55%  
11 acetonitrile for 6 minutes at a flow rate of 300  $\mu$ L/min, which was then increase to 100%  
12 acetonitrile in 30 sec and a flow of 0.5 ml/min keeping this fro 3.5 min before returning to the  
13 start conditions in 6 min. Tandem mass spectrometry was performed in ESI<sup>+</sup> at a source flow at  
14 700 L/hr nitrogen at 350°C. Nitrogen was also used as collision gas, and the MS operated in  
15 MRM mode at the following transitions: FB<sub>2</sub> quantifier m/z 706  $\rightarrow$  336 cone 50V, collision 40  
16 V, dwell time 50 ms, qualifier m/z 706  $\rightarrow$  512 a, cone 50V, collision 25 V, dwell time 100 ms;  
17 FB<sub>4</sub> quantifier m/z 690  $\rightarrow$  320 cone 50V, collision 35 V, dwell time 50 ms, qualifier m/z 690  $\rightarrow$   
18 514 a, cone 50V, collision 30 V, dwell time 100 ms; and FB<sub>1</sub> and position analogues quantifier  
19 m/z 722  $\rightarrow$  334 cone 50V, collision 40 V, dwell time 50 ms, qualifier m/z 722  $\rightarrow$  528 a, cone  
20 50V, collision 25 V, dwell time 100 ms.

21  
22 Quantification was done from spiked samples, which were spiked with 30 to 100  $\mu$ l acetonitrile  
23 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  
24 grinded coffee and stored for 1-4 days prior to extraction. Samples and spiked samples were  
25 extracted and analyzed 4 times on different days.

1

## 2 Results and Discussion

### 3 *Mycological analysis and identification of fungal isolates*

4 Of all coffee bean samples analyzed, none of typical fumonisin-producing species (*Fusarium*  
5 spp.) were detected. Besides *Penicillia* and other saprophytes, *Aspergillus* spp. of section  
6 *Circumdati* and *Nigri* were the predominant species in the Arabica coffee samples with 77 and  
7 75% infestation, respectively. In the Robusta coffee samples, *Aspergillus* spp. section *Nigri* was  
8 the predominant one with approximately 100% infection.

9

10 A diversity of black Aspergilli were observed (Fig. 1) in Thai coffee beans, including, *A. niger*,  
11 *A. carbonarius*, *A. tubingensis*, *A. foetidus*, *A. aculeatinus* and *A. sclerotii carbonarius*. The latter  
12 two species were found to unrelated to the known taxa and proposed as new taxa (Noonim et al.,  
13 2008).

14

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

21

### 22 *Mycotoxigenic potential of the Aspergillus species*

23 Using LC-HRMS (Frisvad *et al.*, 2007) a total of 82 isolates from 6 species were analyzed. Only  
24 *A. niger* isolates were found to produce fumonisin B<sub>2</sub> as well as fumonisin B<sub>4</sub> (same retention  
25 time and tandem spectrum as from a *Fusarium* extract). FB<sub>4</sub> were relative to FB<sub>2</sub> levels in the

8

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

10  
11 LC-HRMS detection of fumonisin B<sub>2</sub> has been shown to have detection limit of ca. 25 ng/cm<sup>2</sup>  
12 culture, and a relative standard deviation better than 30%, and an apparent recovery better than  
13 80% (Frisvad, Nielsen *et al.* unpublished). A chromatogram example of fumonisin B<sub>2</sub> detection  
14 in *A. niger* 1F6 is shown in figure 2. Figure 3 shows the comparison of the tandem spectrum of  
15 FB<sub>2</sub> from *A. niger* and reference standard.

16  
17 In agreement with Samson *et al.* (2007) most of the isolates of *Aspergillus niger* from coffee  
18 beans produced funalenone, kotanin, orlandin, aurasperone B and other naphtho-γ-pyrones,  
19 tensidol B and pyranonigrin A (Table 3). Two isolates produced ochratoxin A (1F1 and 1F6) in  
20 addition to FB<sub>2</sub> and thus could produce two important mycotoxins.

## 21 22 *Analysis of coffee beans*

23 Screening for B type fumonisins with the RIDASCREEN<sup>®</sup> ELISA test kits also indicated that  
24 FB<sub>2</sub> was present in some of the coffee bean samples (results not shown) in levels up to 77 ng/g  
25 and it was thus decided to confirm this by LC-MS/MS which is much more specific. Since both

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

3  
4 The detection limit of the LC-MS/MS method was approx 0.5 ng/g in spiked sample and the  
5 limit of quantification (LOQ) 1.25 ng/g (RSD < 30% for 4 replicates at this level). R<sup>2</sup> from the  
6 calibration curves were in the 4 experiments >0.985 (7 detected levels).

7  
8 LC-MS/MS showed that the ELISA results were false positives, and of the 12 samples analyzed  
9 (4 times each), the most contaminated one (Robusta, R1) contained 9.7 ng/g, and the 3 other  
10 (SO.1C, Ch.3P, R8) between 1.9 and 1.3 ng/g (RSD ca. 40%, 95% level), while 2 were positive  
11 but below LOQ (R7 and R9). Chromatographic separation between FB<sub>1</sub> and FB<sub>2</sub> was 0.98 min.  
12 R1 additionally contained FB<sub>4</sub> as shown in figure 4 where the un-smoothed chromatographic  
13 profiles can be seen from the FB<sub>2</sub> and FB<sub>4</sub> identifications.

14  
15 More experiments are needed for the detection of the *Aspergillus fumonisin* in food commodities.  
16 An extensive survey of fumonisin producing black *Aspergilli* from other sources is in progress  
17 (Frisvad, Nielsen and Samson, personal communication).

18  
19 One of the great concerns with fumonisin contamination of maize is that very large amounts of  
20 fumonisin may be produced by *Fusarium* species, but in accordance with the results of Frisvad  
21 *et al.* (2007), *Aspergillus niger* needs a relatively large amount of carbohydrate in the substrate  
22 to produce high amounts of fumonisin B<sub>2</sub>. In contrast *Fusarium verticillioides* needs less  
23 carbohydrate to produce substantial amounts of fumonisins. Green coffee beans contain small  
24 amounts of carbohydrates, while coffee cherries contain some carbohydrate. Thus, widespread  
25 infection of *A. niger* in coffee beans does not necessarily represent a high risk for fumonisin

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3 1 contamination, which is also indicated from the concentrations detected in very limited number  
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6 2 of samples analyzed.  
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8 3

9  
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4 1 **Figure 1.** Diversity of fungal population in coffee beans from direct plating of two types of  
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6 2 coffee bean samples from 2 different regions of Thailand.

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8 3 A. Arabica green coffee, MEA. B. Arabica green coffee, DG18. C. Robusta green coffee, MEA.  
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10 4 D. Robusta green coffee, DG18. E. Robusta cherries, DG18. F. Robusta cherries, MEA. G.  
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12 5 Arabica parchment coffee DG18. H. Arabica parchment coffee, MEA;  
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17 7 **Figure 2.** A. showing the total ion chromatogram (TIC) of a fumonisin B<sub>1</sub> and B<sub>2</sub> standard mix.  
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19 8 B. extracted ion chromatogram m/z 706.39-706.41, from plug extract of *Aspergillus niger* 1F6  
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21 9 grown on CYAS for 7days. C. TIC of same extract, and D. mass spectrum of fumonisin B<sub>2</sub> in the  
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23 10 extract  
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29 12 **Figure 3.**  
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31 13 Tandem spectra (40 V collision), of fumonisin B<sub>2</sub> peak from *A. niger* (A) extract and a reference  
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33 14 standard (B).  
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38 16 **Figure 4.**  
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40 17 LC-MS/MS chromatograms of R1 sample, showing the MRM transitions from FB<sub>2</sub> (A and B) as  
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42 18 well as FB<sub>4</sub> (C and D).  
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**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 <sub>2</sub>
<i>A. niger</i> (44)	<i>A. niger</i> (28)	13/17	+++
<i>A. tubingensis</i> (19)	<i>A. tubingensis</i> (17)	0/13	-
<i>A. foetidus</i> (28)	-	0/15	-
<i>A. aculeatinus</i> (9)	<i>A. aculeatinus</i> (15)	0/14	-
-	<i>A. carbonarius</i> (35)	0/18	-
-	<i>A. sclerotii carbonarius</i> (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<sub>1</sub> and FB<sub>3</sub>, however retentions times do not match reference standards.

**Table 2.** Fumonisin B<sub>2</sub> production from *A. niger* isolates from Thai coffee beans.

Strain number	Fumonisin Production (µg/cm <sup>2</sup> )*	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

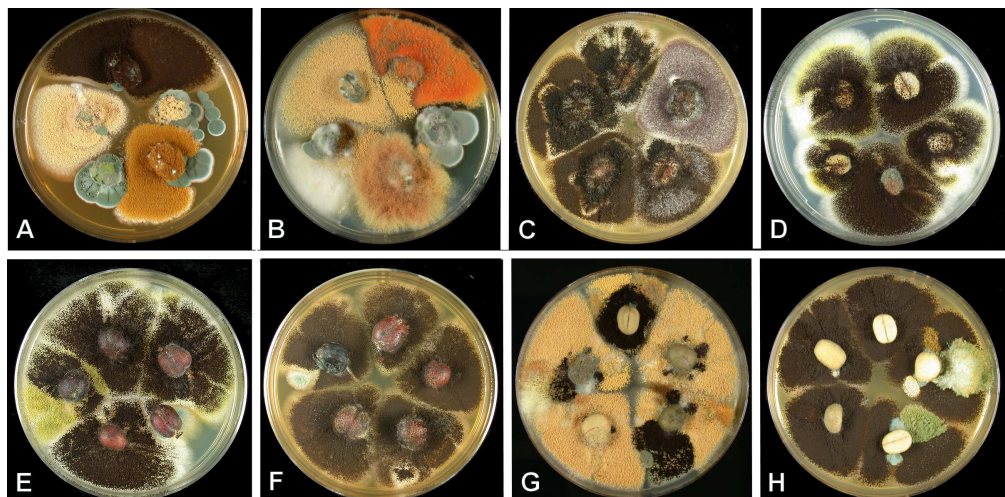
\*Relative standard deviation 30%. \*\*Not detected.

Table 3. Extrolite production other than Fumonisin B<sub>2</sub> by isolates of *Aspergillus niger* from Thai green coffee beans:

Strain	Extrolites							
	AU-NA	FU	—	—	PY	TE	—	—
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- $\gamma$ -pyrones, FU = funalenone, KO = kotanin, OR = orlandin, PY = pyranonigrin A, TE = tensidol A, OT = ochratoxin.

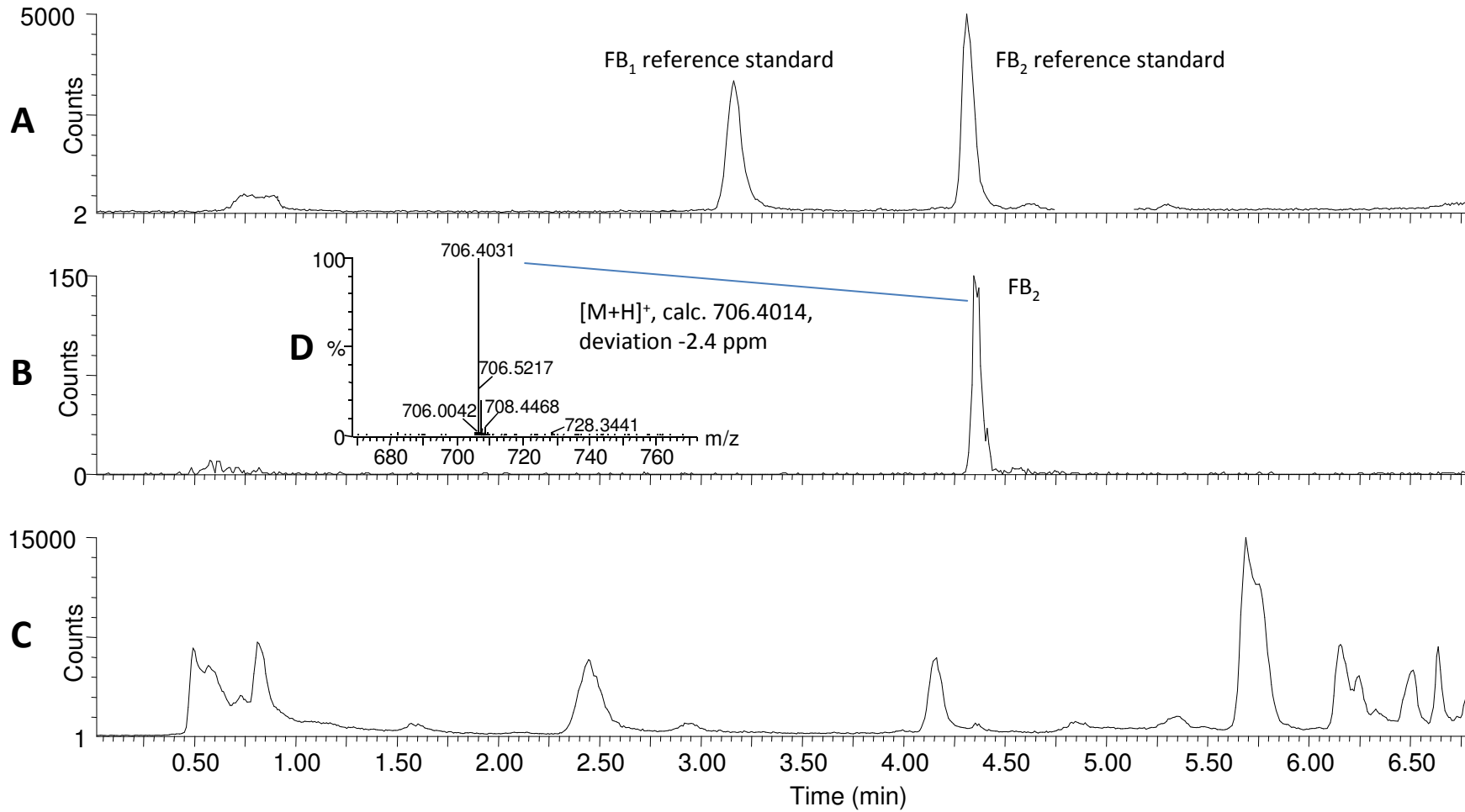
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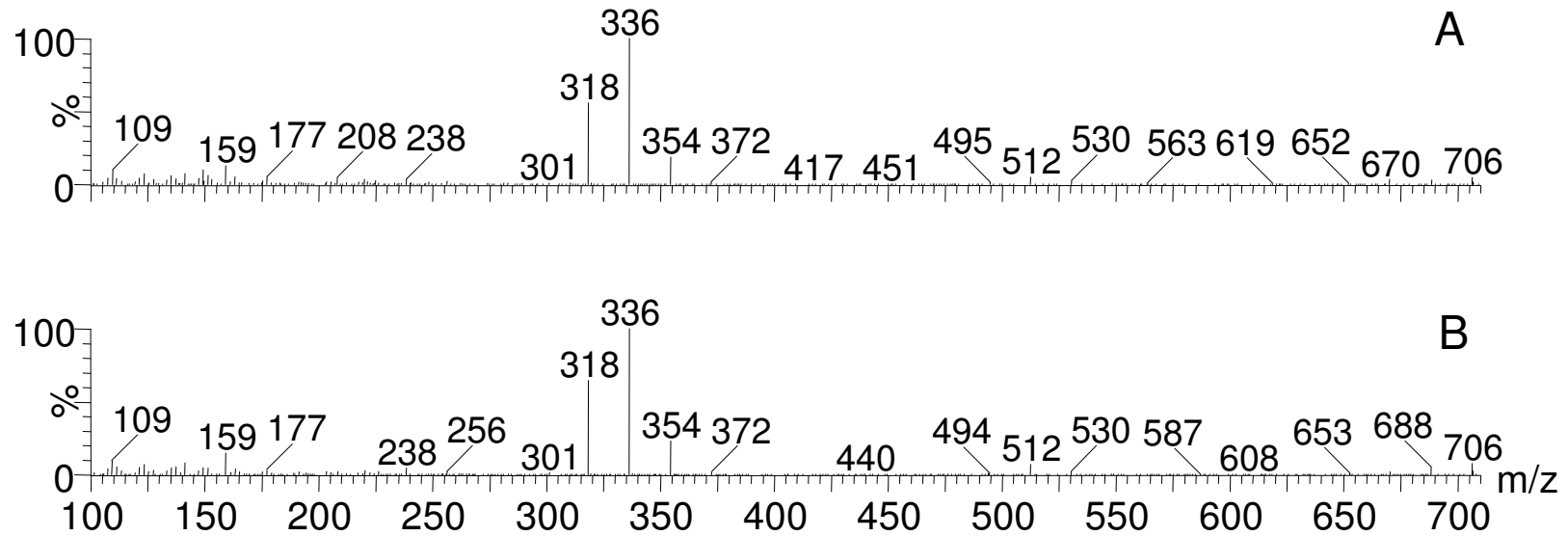


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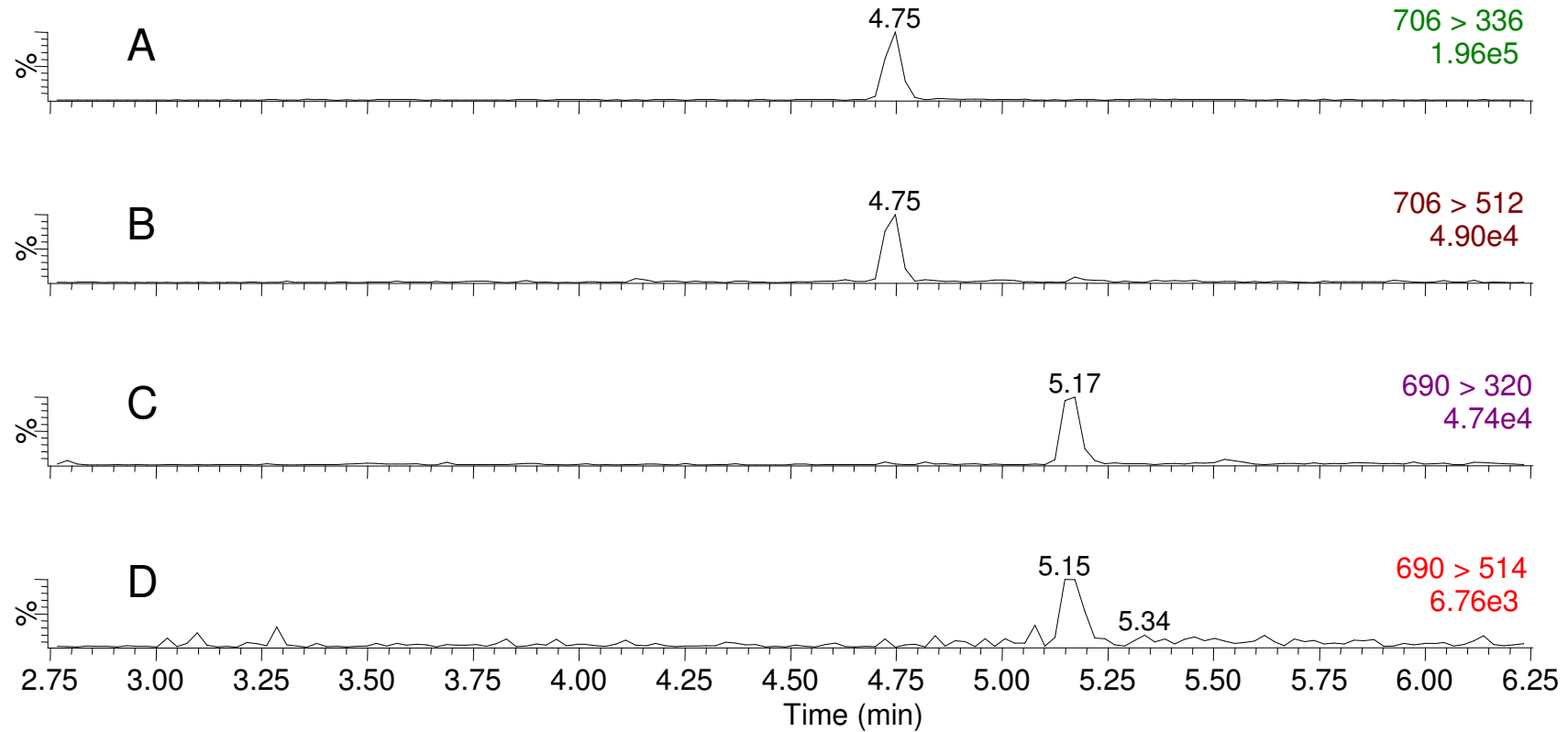
Noonim et al. Figure 2





Tandem spectra (40 V collision), of fumonisin B<sub>2</sub> 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).