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Fumonisin B₂ production by *Aspergillus niger* from Thai coffee beans

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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 B₂ (FB₂) and B₄ when grown on Czapek Yeast Agar with 5% NaCl. Small amounts (1-9.7 ng/g) of FB₂ were detected in 7 of 12 selected coffee samples after ion-exchange purification and LC-MS/MS detection. Two samples also contained FB₄. This is the first record of freshly isolated *Aspergillus niger* strains producing fumonisins and the first report on the natural occurrence of FB₂ and FB₄ in coffee.

Key words: Fumonisin B₂, Coffee beans, *Aspergillus niger*

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Introduction

Fumonisin 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). Fumonisin 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 B₂ 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 B₁, B₂ and B₃ on plant extract agars, but *A. niger* produces fumonisin B₂ 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 themselves.

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°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.*,

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3 1 2007) and kept in collection for further studies.
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8 3 *Determination of extrolite production by liquid chromatography-UV-mass spectrometry of*
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10 4 *fungi cultures*
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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 7 7 days at 25°C. Subsequently, 5 plugs of culture (1 cm²) were sampled, and moved to a 2-ml vial,
15 8 where it was extracted using ultra-sonication for 60 min with 0.75 ml 75% methanol, and
16 9 subsequently filtered through a 0.45 µm syringe filter (Frisvad *et al.* 2007).
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27 11 Solvents were HPLC grade and all other chemicals were analytical grade unless otherwise stated.
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29 12 Water was purified from a Milli-Q system (Millipore, Bedford, MA). LC-DAD-HRMS was
30 13 performed on an Agilent 1100 system equipped with a photo diode array detector (DAD) and a
31 14 50×2 mm i.d., 3 µm, Luna C₁₈ II column (Phenomenex, Torrance, CA). The LC system was
32 15 coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester,
33 16 UK), with a Z-spray ESI source (2).
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43 18 Samples were analyzed in ESI⁺ using a water-CH₃CN gradient system starting with 0.3 ml/min
44 19 flow of 30% CH₃CN which was increased linear to 60% in 5 min, then increased to 100% in 1
45 20 min while also increasing the flow to 0.5 ml/min, holding this for 2 min. The water was buffered
46 21 with 10 mM ammonium formate and 20 mM formic acid and the CH₃CN with 20 mM formic
47 22 acid (Nielsen and Smedsgaard, 2003; Nielsen *et al.*, 2005). One scan function (1 s) was used
48 23 with a potential difference of 50 V between the skimmers and using a scan range of *m/z* 100 to
49 24 900. Reference standards of fumonisin B₁, B₂ and B₃, AAL toxin TB₁ and TA₁, Malformins A, B
50 25 and C, ochratoxin A, and Asperazine were also co-analyzed in the sequences. Source of

reference standards: Certified standards of 50 µg/mL of FB₁ and fumonisin B₂ were obtained from Biopure, Tulln, Austria. FB₃ 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 B₂ 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 ($\pm m/z$ 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 B₁, B₂ and B₃ 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 min 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

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3 1 washed with 3 ml methanol-water (7:3 v/v) and 2 ml methanol, and the fumonisins eluted with
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5 2 2.4 ml methanol containing 1% acetic acid. Samples were then evaporated to dryness with
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7 3 nitrogen flow and redissolved in 250 µl acetonitrile-water (3:7 v/v) (modified from the EN
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9 4 13585:2001).
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14 6 Sub-samples of 5 µl were analyzed by LC-MS/MS on an Agilent HP 1100 liquid chromatograph
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16 7 system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer
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18 8 (Micromass, Manchester, UK) with ESI source. The separations was performed on a Gemini C6-
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20 9 phenyl column (Phenomenex, 50 × 2 mm, 3 µm) fitted with a security guard system and using a
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22 10 linear gradient starting from 20 % acetonitrile in water (both 20 mM formic acid) to 55%
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24 11 acetonitrile for 6 minutes at a flow rate of 300 µL/min, which was then increase to 100%
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26 12 acetonitrile in 30 sec and a flow of 0.5 ml/min keeping this fro 3.5 min before returning to the
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28 13 start conditions in 6 min. Tandem mass spectrometry was performed in ESI⁺ at a source flow at
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30 14 700 L/hr nitrogen at 350°C. Nitrogen was also used as collision gas, and the MS operated in
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32 15 MRM mode at the following transitions: FB₂ quantifier m/z 706 → 336 cone 50V, collision 40
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34 16 V, dwell time 50 ms, qualifier m/z 706 → 512 a, cone 50V, collision 25 V, dwell time 100 ms;
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36 17 FB₄ quantifier m/z 690 → 320 cone 50V, collision 35 V, dwell time 50 ms, qualifier m/z 690 →
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38 18 514 a, cone 50V, collision 30 V, dwell time 100 ms; and FB₁ and position analogues quantifier
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40 19 m/z 722 → 334 cone 50V, collision 40 V, dwell time 50 ms, qualifier m/z 722 → 528 a, cone
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42 20 50V, collision 25 V, dwell time 100 ms.
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46 22 Quantification was done from spiked samples, which were spiked with 30 to 100 µl acetonitrile
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48 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
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50 24 grinded coffee and stored for 1-4 days prior to extraction. Samples and spiked samples were
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52 25 extracted and analyzed 4 times on different days.
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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 B₂ as well as fumonisin B₄ (same retention time and tandem spectrum as from a *Fusarium* extract). FB₄ were relative to FB₂ levels in the

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4 1 range 0-40% with most being in the 10-20% range. Most of the tested *A. niger* isolated from
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6 2 Thai coffee beans (13 out of 17 isolates) produced fumonisin B₂ in the CYAS culture medium in
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8 3 amounts of 0.4 to 2 µg/cm² (Table 2). This indicates that these *A. niger* isolates may also
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10 4 produce fumonisin B₂ in coffee cherries or beans. All *A. niger* isolated from Northern Arabica
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12 5 coffee bean samples could produce fumonisin B₂, while in some isolates from Southern Robusta
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14 6 coffee samples no fumonisin B₂ was found. More molecular studies are needed in order to
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16 7 compare the differences in these isolates at the genotypic level. A high percentage of infection
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18 8 by *A. niger* as determined after surface disinfection of the green coffee beans indicated that *A.*
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20 9 *niger* actually grows actively in the coffee beans.
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27 11 LC-HRMS detection of fumonisin B₂ has been shown to have detection limit of ca. 25 ng/cm²
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29 12 culture, and a relative standard deviation better than 30%, and an apparent recovery better than
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31 13 80% (Frisvad, Nielsen *et al.* unpublished). A chromatogram example of fumonisin B₂ detection
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33 14 in *A. niger* 1F6 is shown in figure 2. Figure 3 shows the comparison of the tandem spectrum of
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35 15 FB₂ from *A. niger* and reference standard.
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41 17 In agreement with Samson et al. (2007) most of the isolates of *Aspergillus niger* from coffee
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43 18 beans produced funalenone, kotanin, orlandin, aurasperone B and other naphtho-γ-pyrones,
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45 19 tensidol B and pyranonigrin A (Table 3). Two isolates produced ochratoxin A (1F1 and 1F6) in
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47 20 addition to FB₂ and thus could produce two important mycotoxins.
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53 22 *Analysis of coffee beans*
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55 23 Screening for B type fumonisins with the RIDASCREEN® ELISA test kits also indicated that
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57 24 FB₂ was present in some of the coffee bean samples (results not shown) in levels up to 77 ng/g
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59 25 and it was thus decided to confirm this by LC-MS/MS which is much more specific. Since both
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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.

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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^2 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 FB₁ and FB₂ was 0.98 min.
12 R1 additionally contained FB₄ as shown in figure 4 where the un-smoothed chromatographic
13 profiles can be seen from the FB₂ and FB₄ identifications.

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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).

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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 B₂. 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

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 B₁ and B₂ 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 B₂ in the extract

Figure 3.

Tandem spectra (40 V collision), of fumonisin B₂ 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 FB₂ (A and B) as well as FB₄ (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
<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 FB₁ and FB₃, however retentions times do not match reference standards.

Table 2. Fumonisin B₂ production from *A. niger* isolates from Thai coffee beans.

Strain number	Fumonisin Production (µg/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 B₂ 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

Tandem spectra (40 V collision), of fumonisin B₂ 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).