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**Toxigenic potentiality of *Aspergillus flavus* and *Aspergillus parasiticus* strains isolated from black pepper assessed by an LC-MS/MS based multi-mycotoxin method**

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**Abstract**

A liquid chromatography triple quadrupole tandem mass spectrometry method was developed and validated to determine mycotoxins, produced by fungal isolates grown on malt extract agar (MEA). All twenty metabolites produced by different fungal species were extracted using acetonitrile/1% formic acid. The developed method was applied to assess the toxigenic potentiality of *Aspergillus flavus* (n=11) *and A. parasiticus* (n=6) strains isolated from black peppers (*Piper nigrum* L.) following their growth at 22, 30 and 37°C. Highest mean radial colony growth rates were observed at 30°C for *A. flavus* (5.21±0.68 mm/day) and *A. parasiticus* (4.97±0.33 mm/day). All of the *A. flavus* isolates produced aflatoxin B1 and O-methyl sterigmatocystin (OMST) while 91% produced aflatoxin B2 (AFB2) and 82% of them produced sterigmatocystin (STERIG) at 30°C. Except one, all the *A. parasiticus* isolates produced all the four aflatoxins, STERIG and OMST at 30°C. Remarkably high AFB1 was produced by some *A. flavus* isolates at 22°C (max 16-40 mg/kg). Production of mycotoxins followed a different trend than that of growth rate of both species. Notable correlations were found between different secondary metabolites of both species; $R^2$ 0.87 between AFB1 and AFB2 production. Occurrence of OMST could be used as a predictor for AFB1 production.

***Keywords:*** *Aspergillus flavus, Aspergillus parasiticus, aflatoxins, O-methyl sterigmatocystin, LC-MS/MS, malt extract agar*

## 1. Introduction

Aflatoxins are toxic secondary metabolites produced predominantly by the two species of *Aspergillus* section *Flavi*, *Aspergillus flavus* and *Aspergillus parasiticus*. Toxigenic *A. flavus* mainly produces aflatoxin B1 (AFB1) and B2 (AFB2), while *A. parasiticus* produces all the four aflatoxins (B1, B2, G1 (AFG1) and G2 (AFG2)). Aflatoxins are extremely carcinogenic, hepatotoxic, immunosuppressive and anti-nutritional contaminants (Williams et al., 2004). Among them, AFB1 has been identified as the potent natural hepatocarcinogen known and it has been designated as group 1 human carcinogen (IARC, 1993). Generally, *A. parasiticus* produces high concentration of aflatoxins and most of the strains isolated (>90%) are able to synthesize aflatoxins. On the other hand, only 40-50% of the *A. flavus* isolated from natural habitats were capable of producing aflatoxins (Schmidt-Heydt et al., 2010). Other aflatoxin producers in *Aspergillus* section *Flavi*, include the phylogenetically closely related *A. nomius*, *A. parvisclerotigenus*, *A. bombycis*, *A. pseudotamari*, *A. minisclerotigenes*, *A. arachidicola*, and *A. toxicarius* which have been encountered less frequently (Frisvad et al., 2005; Bennet and Klich, 2003; Varga et al., 2009).

Both *A. flavus* and *A. parasticus* are usually confined in tropical and subtropical regions. According to Pitt and Hocking (1999), *A. parasiticus* was only occasionally found in South East Asia, while widely distributed in soils and foodstuffs in the United States, Latin America, South Africa, India and Australia. On the other hand, *A. flavus* is a more aggressive and widely distributed species. Thus, ecological distribution of these species could be due to the fluctuation and regional trends in climate changes, which could also reflect on the regulation of mycotoxins biosynthesis (Schmidt-Heydt et al., 2010). A very good example for this could be the recent warning for maize contamination in Europe (Northern Italy) issued in 2012-2013 as a consequence of drought conditions favourable for *A. flavus* infection (Perrone et al., 2014). Moreover, mould growth and mycotoxin contamination could be influenced by several other factors like temperature, type of substrate, water activity, inoculum concentration, microbial interaction, physiological state of the mould etc. However, the influence of these factors on growth could be different from that of mycotoxin production (Garcia et al., 2009).

Aflatoxin production is favored particularly by warm climates. Exposure of the mature crop to favourable temperature and moisture conditions, either in the field or during transportation and storage, could be associated with increased toxin production. The optimum temperatures for aflatoxin production (24-30°C) can differ among *A. flavus* and/or *A. parasiticus* isolates while optimum temperature for growth ranges from 30-35°C (Gqaleni et al., 1997; Mousa et al., 2013). Moreover, the regulation of sterigmatocystin (STERIG) and especially aflatoxins production in *Aspergillus* generally require simple sugars, low pH, reduced nitrogen source and mild oxidative stress (Georgianna and Payne, 2009). Yeast extract and sucrose enriched yeast extract (YES) agar were found to enrich aflatoxins production by *A. parasiticus.* Wickerhams Antibiotic Test Medium (WATM) has also been found to induce high STERIG and aflatoxins production at 25°C incubation (Georgianna and Payne, 2009). Hence, the growth medium and temperature could play a significant role in the expression of particular secondary metabolites.

Interest in the variation in aflatoxin production by *Aspergillus* section *Flavi* has increased recently because atoxigenic strains could be used as bio-control agents to reduce the aflatoxins risk (Donner et al., 2010; Abbas et al., 2011; Yu, 2012; Alaniz Zanon et al., 2013; Tran-Dinh et al., 2014; Perrone et al., 2014). Since, it has been well documented that not all fungal strains are able to produce mycotoxins hence, this encouraged the use of modern detection and screening techniques for assessing the secondary metabolite/mycotoxin production potential of the *A. flavus* and *A. parasiticus* isolates of black pepper (*Piper nigrum* L.). Hence, a multi-mycotoxin analytical method using LC-MS/MS was developed with the prime objective to determine the secondary metabolite production by pure fungal cultures grown in malt extract agar (MEA), a growth medium widely used in mycology. The method was applied to assess the secondary metabolite production potential (AFG2, AFG1, AFB2, AFB1, STERIG and O-methyl sterigmatocystin (OMST)) of some *A. flavus* and *A. parasiticus* strains isolated from black pepper at different temperatures. Moreover, the possible correlations between different secondary metabolites production were assessed. This is the first study to apply a simple and straightforward confirmatory method on secondary metabolite analysis of pure *Aspergillus* cultures grown on malt extract agar and comprehensively evaluating the toxigenic potentiality of the two potent aflatoxin producing fungal species.

## 2. Materials and methods

### 2.1. Chemicals and reagents

LC-MS grade absolute methanol (MeOH) and analytical grade acetonitrile (MeCN) were purchased from VWR International (Zaventem, Belgium). Formic acid ULC-MS grade (99%) was supplied by Bio Solve B.V. Ammonium formate (±99%) was obtained from Sigma-Aldrich, Steinheim. Formic acid analytical grade (98-100%) was from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) tablets were supplied by Oxoid (Hampshire, England). Tween 80 (polyoxyethylenesorbitan monooleate) was obtained from Merck, Germany. Ultrafree®-MC centrifugal filter devices (0.22 µm) were obtained from Millipore (Bredford, MA, USA). Water was purified (18 MΩ) on a Milli-Q Plus apparatus (Millipore; Brussels, Belgium). All other chemicals and reagents used were of analytical grade.

### 2.2. Mycotoxin standards

Mycotoxin reference standards namely, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), HT-2 toxin (HT-2), alternariol methyl ether (AME), zearalenone (ZEN), sterigmatocystin (STERIG) and zearalanone (ZAN) were purchased from Sigma-Aldrich (Bornem, Belgium). NEO was obtained as solution (100 µg/mL) in MeCN. T-2 toxin (T-2) was purchased from Biopure (Tulln, Austria). Fumonisin B3 (FB3) was supplied by Promec Unit (Tygerberg, South Africa). Roquefortine C (ROQ C) was purchased from Enzo Life Science (Lorrach, Germany). O-Methyl Sterigmatocystin (OMST) was purchased from Chromodex (California, USA). FB2 and FB3 standards at a concentration of 1 mg/mL were prepared in MeCN/water (50/50, v/v). Stock solutions of DON, 3-ADON, 15-ADON, AFB1, AFB2, AFG1, AFG2, OMST, OTA, FB1, HT-2, T-2, ZEN, STERIG, ZAN and ROQ C and were prepared in MeOH at a concentration of 1 mg/mL. Stock solution of AME (1 mg $mL^{-1}$) was prepared in MeOH/dimethylformamide (60/40, v/v). All the stock solutions were stored for maximum one year at (-20)$^{o}$C except FB2 and FB3 which were stored at 4$^{o}$C.

From the individual stock standard solutions, working solutions were prepared by diluting them in MeOH. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations: AFB1, AFB2, AFG1, AFG2 and OMST (0.5 µg/mL), OTA and ROQ C (1.0 µg/mL), STERIG (0.625 µg/mL), T-2, HT-2, NEO, 3-ADON and 15-ADON (2.5 µg/mL), DON, FB1, FB2, FB3, AME and CIT (5 µg/mL). The standard mixtures were prepared in MeOH, stored at (-20)$^{o}$C and renewed every 2 months.

**2.3. Fungal isolates, preparation of spore solution and inoculation**

The strains of *A. flavus* and *A. parasiticus* used in this study were isolated from black pepper samples (n=82) collected from various markets in Sri Lanka. More details on mould isolation and characterization can be found in Yogendrarajah et al. (2014). Species level identification of the moulds was confirmed at Mycothèque de l'Université Catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium) using molecular techniques and morphology (based on the identification keys of Samson et al., 2004).

Spore solutions of each fungal isolate were prepared at a concentration of $10^6$ CFU/mL as described below. Tween 80 solution (0.1 g/100 mL water), PBS (1 tablet/100 mL water) and PBS+Tween80 solution (0.1 g Tween 80 and 1 tablet PBS per 100 mL water), cotton plugs and pipette tips were autoclaved for 15 min at 121°C. To prepare the fungal inoculum, centrally inoculated MEA plates were incubated at 30°C for 10 days to enable sporulation to take place. Five mL of Tween 80 solution (wetting agent) were spread on the agar plate containing sporulated mould culture. After gently spreading the solution and scrapping off the spores, this solution was pipetted out from the agar plates and transferred to a sterile falcon tube containing a cotton plug on top for filtering out debris and mycelium. This extraction process was performed a second time on the same plate. Three agar plates were used for each fungal inoculum extraction. The extracts of three agar plates were collected in a single falcon tube. After removing the cotton plug, the falcon tubes were centrifuged at 8500 rpm for 15 min at 4°C. Supernatant was discarded out and 20 mL of PBS + Tween 80 solution was added to the sedimented spores. After vortexing for 30 seconds, the spore solution was centrifuged again at same conditions. After discarding the supernatant, 20 mL of the PBS solution were added to the sedimented

spores and vortexed again. The spores were counted in a 16 cell thoma chamber using an inverse microscope (Olympus, IX81, Tokyo, Japan) and CellF imaging software. Appropriate dilution was made to obtain a standardised spore solution concentration of $10^6$ CFU/mL in PBS. This spore suspension was stored at 4°C until further use.

The basic medium used in this study was malt extract agar (malt extract 30 g/L, mycological peptone, 5 g/L and agar 15 g/L) supplied by Oxoid Ltd, Hampshire, England. The medium was, supplemented with chloramphenicol (Oxoid Ltd, Hampshire, England) to inhibit bacterial growth, sterilized and poured in 90 mm Petri plates. MEA plate was centrally inoculated using 10 µL of the spore solution ($10^6$ CFU/mL) of the different *Aspergillus* isolates. The Petri plates were enclosed in a polyethylene bag (wet paper was placed inside to prevent drying of the medium) and incubated at three temperatures (22, 30 and 37°C). For each isolate, plates were prepared in triplicate.

### 2.4. Assessment of fungal growth and lag phase

Fungal colony growth was measured daily using an electronic digital calliper at orthogonal directions (x, y) until the colony reached the edge of the plate. The average of both the diameters (x, y) was recorded as the growth measurement for each isolate. Mean colony diameter (mm) of the triplicate experiments was plotted against incubation time (days) to develop growth curves for each fungal isolate. The colony growth rate ($\mu_{max}$, mm/day) was determined from the slope of the growth curve while the lag phase ($\lambda$, days) was estimated by extrapolating the linear regression equation to the time axis. Following the growth study, the mycotoxigenic potential of all the *Aspergillus* isolates at each temperature was determined at colony diameter ~80-90 mm, when it covered the plate completely.

### 2.5. Sample preparation for analysis of mycotoxins in malt extract agar

A straightforward sample preparation method was developed for several fungal metabolite analyses in malt extract agar (MEA). Finely ground and homogenized 2.0 ± 0.05 g of MEA was weighed in a 50 mL extraction tube (prior to weighing, agar was smashed in to fine pieces using a spatula). For method validation, MEA was

spiked with a mixture of mycotoxin standards at different concentrations. A fixed concentration (50 µg/kg) of ZAN internal standard (IS) was added. The samples were left for an hour in the dark for equilibration. Thereafter, 10 mL of the extraction solvent (MeCN/1% formic acid (v/v)) was added and after a brief shaking, samples were extracted using an end-over-end shaker (*Agitelec*, J. Toulemonde and Cie, Paris, France) at position 7 for an hour. The tubes were centrifuged at 4000x g for 15 min and the supernatant was filtered using a folded filter paper (Whatman® Schleicher & Schuell® qualitative filter paper, grade 595 ½: 4-7 µm) into a new extraction tube. The filtrate was evaporated under $N_2$ at 40°C. The residue was reconstituted in 200 µL of the injection solvent (mobile phase A/B, 60/40 (v/v) mL as described in section 2.6.1) and centrifuged at 4000x g for 7 min. The reconstituted residue was transferred to a centrifuge filter and centrifuged at 10000x g for 3 min. After filtration an aliquot was transferred to the vials for LC-MS/MS analysis. Appropriate dilutions were made whenever production of mycotoxins was found to be very high following the fungal growth. Mycotoxin analyses were performed in triplicates.

### 2.6. Instrumental conditions

#### 2.6.1. HPLC apparatus and conditions

Liquid chromatography was performed using a waters ACQUITY ultra-performance liquid chromatography (UPLC™) system. The conditions were the same as described in Yogendrarajah et al. (2013). Mobile phase A was MeOH/water (20/80 v/v) and mobile phase B was MeOH/water (90/10 v/v), both contained 5mM ammonium formate and 0.1% formic acid. A gradient elution programme starting with 50% B was maintained for 2 min. From 2 to 10 min it linearly increased to 100% B. Over further 5 min, the gradient was kept unchanged at 100% B. In 1 min the gradient switched to 50% B and was equilibrated at the initial mobile phase conditions for further 4 min before the start of the next injection. Total run time was 20 min.

#### 2.6.2. MS/MS apparatus and conditions

Mass spectrometry (MS/MS) was performed with a Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). The MS was operated at electrospray ionization in positive mode (ESI+). The instrumental conditions were the same as described in Yogendrarajah et al. (2013). Analysis of

the mycotoxins was performed in multiple reaction monitoring (MRM) mode. For each mycotoxin, at least one precursor ion and two fragment/product ions were monitored. The most abundant product ion was selected for quantification and the second intense one for qualification. The quantification and qualification ion transitions of the respective mycotoxins and the optimum cone voltages and collision energies were programmed (Yogendrarajah et al., 2013) (Table S1). In addition to those mycotoxins described earlier, additionally OMST was tuned, and the precursor (m/z 339), quantification (m/z 306) and qualification (m/z 324) ions were monitored (Fig. 1). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used.

**2.7. Matrix effect evaluation**

The matrix effect (ME) was evaluated by comparing the peak responses of the standard mycotoxins (n=3) spiked in the extraction solvent with the spiked agar extracts at five concentration levels for each analyte. A standard mixture of mycotoxins was prepared using the individual stock and working standard solutions at the following concentrations for determining the ME: AFB1, AFB2, AFG1, AFG2 and OMST (0.5 µg/mL), OTA and ROQ C (1.0 µg/mL), STERIG (0.625 µg/mL), T-2, HT-2, NEO, 3-ADON, 15-ADON, AME and CIT (2.5 µg/mL) and DON, FB1, FB2, FB3 (5 µg/mL). The ME was calculated using the formula: ME (%) = (A2-A1/A1)*100, where A1 is the average area of the mycotoxin standard in solvent (MeCN/1% formic acid (v/v)) at a specific concentration and A2 is the average area of the mycotoxin standard in blank MEA extract at the same concentration (Chambers et al., 2007). In this way it was possible to compare the positive or negative ME, that is an increase or decrease of the detector response, respectively.

**2.8. Method validation**

The multi-mycotoxin analytical method for MEA was validated using spiked blank MEA samples. MRM chromatograms obtained following the extraction of spiked MEA are shown in Fig. S1. A set of performance characteristics that were in compliance with the recommendations and guidelines defined by the Commission Decision 2002/657/EC (EC, 2002) and Regulation EC/401/2006 (EC, 2006) were evaluated. Validation parameters assessed were, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), intra-day repeatability (RSDr) and intermediate precision (RSDR). Calculations based on both peak area (absolute

response (AR)) and relative peak area (relative response (RR)) were used to evaluate or compare the performance criteria of the method developed. Relative response was calculated by dividing the absolute peak area of the analyte by the peak area of internal standard ZAN.

### 2.8.1. Calibration curves, linearity, LOD, LOQ and recovery

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank MEA at six concentration levels. Calibration curves were constructed by plotting the analyte response (absolute or relative response (y)) versus the concentration of analyte (x). The concentration ranges used for this validation study were*:* AFs and OMST (2.5-20 µg/kg); OTA and ROQ C (5-40 µg/kg); T-2, HT-2, NEO, 3-ADON, 15-ADON, ZEN and CIT (12.5-100 µg/kg), STERIG (3.125-25 µg/kg), AME, DON, FB1, FB2 and FB3 (25-200 µg/kg). Calculations were performed separately on absolute and relative peak responses (n=6). Linear regression was used to fit the calibration curve and lack of fit test was used to assess the fitting of the regression model.

In general, the LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily be quantified, under the stated conditions of the test. LOQ is the lowest amount of an analyte in a sample, which can be quantitatively determined with suitable precision and accuracy (ICH, 1996). LOD and LOQ were determined using the MMC curves. LODs were determined as the concentration corresponding to three times the standard error of the y-intercept divided by the slope. The linest function of the Microsoft Excel 2010 program was used. LOQ equaled the concentration corresponding to six times the standard error of the y-intercept divided by the slope; which is two times the LOD. For each of the analyte, the calculated LODs and LOQs were also verified by the S/N ratio, which should be more than 3 and 10, respectively (Vial and Jardy, 1999). The validation experiments that were used to calculate the LODs and LOQs were utilized also to calculate the recovery of the method. According to IUPAC, the apparent recovery is the ratio of the predicted value obtained from the MMC curve divided by the actual/theoretical value (Sulyok et al., 2006).

## 2.9. Intra-day repeatability and intermediate precision

Intra-day repeatability was calculated via relative standard deviations (RSD) to explain the same day variability. The intermediate precision, which could explain the total variability of the method, was calculated using the analysis of variance (ANOVA) approach.

### 2.10. Statistical analysis

One way analysis of variance (ANOVA), lack of fit test, non-parametric Mann-Whitney U test and Kruskal Wallis one way ANOVA were performed using the SPSS statistical software (IBM®, Version 22). Level of significance was 0.05, unless otherwise specified.

## 3. Results and Discussion

### 3.1. Method development and matrix effect evaluation

A straightforward extraction procedure using acidified acetonitrile (1% formic acid) was performed to extract multi-class secondary metabolites that could be produced by different fungal species (*Aspergillus, Penicillium, Fusarium* and *Alternaria* spp.) from MEA. All twenty analytes were simultaneously detected in a run time of 20 min using ESI (+)ive mode. All the metabolites however eluted before 10 min of run time, leaving sufficient time for column cleaning and regeneration for the start of next run. Having close retention times or co-elution was not a problem, since analyte specific ions were fixed for quantification and confirmation using MRM mode, thus selectivity was not compromised. Previous studies using rather complex extraction procedures for YES agar, failed to extract STERIG, one of the major metabolites of the aflatoxin biosynthetic pathway and reported very high LOQs for a number of metabolites (Van Pamel et al., 2011).

Matrix effects are common problems that occur when using LC-MS or MS/MS, and thus have an adverse effect on the analytical results. The response of the target compound can be enhanced or suppressed due to the interfering matrix components, which is commonly known as signal suppression/enhancement effect (SSE). The ME due to the co-extractives from MEA on different metabolites is shown in supplementary material Fig. S2. A comparison was made between the absolute response (AR) and relative response (RR) on matrix effect. The ME for most of the analytes ranged from (-15.6) to 34.7% and from (-28.3) to 1.71% based on AR and RR calculations, respectively. Strong signal suppression was observed with DON in both the approaches (AR:

-42.2%) and (RR: -51.2%). A range in between (-20) to +20% ME or SSE in between 0.8 to 1.2 was generally considered as tolerable (Frenich et al., 2011). Based on the AR, 90% of the analytes were within this acceptable ME range, while considering their RR 75% of the analytes were inside this range. Values outside this range indicate severe ME as it has been observed with DON and CIT when using AR and with DON, 3-ADON, AFG2, AFG1, AFB2 and AFB1 when using RR. This could explain the insignificant contribution of the used IS in compensating the ME of these analytes. However, significantly low ME was obtained for some other metabolites like T-2, AME and CIT when RR was used for ME evaluation (Fig. 3). Though ZAN is the most appropriate IS for ZEN (in terms of its similar chemical property and elution closer to the retention time of ZAN), the ME for ZEN is still rather high compared to AR. The best option to tackle matrix effects is the use of isotopically labeled IS, lacking those; structural analogues could be the second best option. However, this adds cost and finding structural analogues of each metabolite has never been easy. Hence, in this study MMC curves were used to compensate these variables ME and to improve the linearity, reliability and accuracy of the analytical results of the developed method.

### 3.2. Performance characteristics of the method

Method validation was performed in terms of linearity (lack of fit), LODs, LOQs, recovery, repeatability and intermediate precision. The performance characteristics were compared using both absolute and relative responses (Tables 1 and 2).

#### 3.2.1. Linearity, LOD, LOQ and recovery

MMC curves developed on different blank spice matrices were linear over the working concentration ranges in all of the studied mycotoxins. Calibration curves were fitted by linear regression and the linearity was assessed using the p-values of the lack of fit test (Table 1). P-values of lack of fit test were in the range of 0.083-0.858 and 0.063-0.747 based on the absolute and relative response calculations, respectively. P-values greater than 0.05 is considered, as there is no lack of fit, thus showing good fit of the model for all the studied analytes. There were no significant differences ($p=0.562$) in the mean lack of fit values of different analytes, in both calculations at 5% level of significance. This explains that there is no significant contribution of the IS used in “good” fitting of the linear regression of different analytes. Hence, either absolute or relative response

could be used in fitting the calibration curves in this method. Additionally, residual plots of each mycotoxins were assessed to ensure decent fit of the data to the linear model.

Moreover, mean apparent recoveries for all the tested mycotoxins were in the range of 86-113% (Table 1), within the acceptable range of required performance criteria (EC, 2006). There were no significant differences in apparent recoveries ($p=0.561$) between different analytes based on both absolute or relative response estimations. Associated variability is minimum probably because the sample preparation procedure applied is rather simple avoiding long clean-up steps; thus losses could be marginal.

The LOD and LOQs of different analytes ranged from 0.8-14.6 and 1.7-29.2 µg/kg, respectively (Table 2). The purpose of this analytical method is to quantify the production of several secondary metabolites following pure fungal culture inoculations (generally they produce in high concentrations in agar under optimal conditions). Most of the LOQs obtained with this method are quite low hence, it could be useful in studying the wide range of toxigenic variability of several fungal (*Aspergillus, Fusarium, Penicllium and Alternaria spp.*) metabolite production and also to identify the non-toxigenic ones for potential use in aflatoxins bio-control. Many different metabolites were extracted with this single solvent; therefore, this extraction procedure as it is or with slight modification (with some water) can be used also in untargeted analysis of other fungal metabolites.

### 3.2.2. Intra-day repeatability and intermediate precision

Relative standard deviations (RSD) were calculated at intra-day repeatability (RSDr) and intermediate precision (RSDR) conditions. The results are summarized in Table 2. RSDr values were within the acceptable range of <20%, matching with the performance criteria requirement of the EC (2006) except for few analytes (DON and ADONs (20-22.2%)). RSDr ranged from 6.8 to 22.2% and 8.6-23.5% based on absolute and relative response, respectively. The results obtained by both the approaches were very much comparable, except CIT and NEO that had much lower RSDr (CIT 6.8% Vs 20.7% and NEO 10.9% Vs 20.3%) values based on the absolute response compared to the relative ones.

Considering the intermediate precision, the RSDR values based on absolute and relative response ranged from 14.5-24% and 10.2-28.5%, respectively. There were no significant differences in RSDr ($p=0.195$) or RSDR ($p=0.951$) when comparing the values calculated either using AR or RR. Hence, statistical comparison of all the method performance parameters shows that the quantification of most of the analytes can be performed either using absolute or relative responses.

To assess the applicability of the developed method different *Aspergillus* isolates were used in this study. Moreover, aflatoxins have shown smaller signal suppression when using absolute response compared to the relative thus absolute response of the MMC curves were used for quantification of the metabolites produced by these fungi.

### 3.3. Growth assessment of the fungal isolates at different temperatures

Of the 105 isolates of *A. flavus* and/or *A. parasiticus* from black peppers, 38 (36%) of them were found to be capable of producing toxins at different extent and the remaining 67 (64%) were atoxigenic. They have been grouped as atoxigenic only based on their inability to produce aflatoxins, OMST and STERIG (cyclopiazonic acid (CPA) analysis was not performed). From the 38 toxigenic isolates, 29 of them were identified as *A. flavus* while, 11 were *A. parasiticus* (based on morphology, mycotoxin production and molecular level identification as mentioned). This study focuses on the growth and mycotoxin production potential of the selected (based on the sample origin (district) in Sri Lanka) eleven *A. flavus* (Kandy/Matale-5; Anurdhapura-2; Jaffna-4) and six *A. parasiticus* (Kandy/Matale-3; Anurdhapura-2; Jaffna-1) isolates. Information on the origin of each of the isolate is given in Table 3.

Different isolates of the same species (*A. flavus* or *A. parasiticus*) showed almost similar growth rates at a particular temperature (Table 3). A slightly higher growth rate was found only with one *A. flavus* isolate UG AF82 (4.50±2.07 mm/day) at 22°C. Moreover, comparing both the species, *A. flavus* and *A. parasiticus*, they were showing a similar growth response at a particular temperature (Table 3). For both species highest mean radial colony growth rates ($\mu_{max}$) were observed at 30°C (mean±SD 5.21±0.68 mm/day for *A. flavus* and 4.97±0.33 mm/day for *A. parasiticus*) for most of the isolates, followed by those at 37°C (4.00±0.86 mm/day for *A. flavus* and 4.50±1.00 mm/day for *A. parasiticus*). Generally, the lowest growth rate was observed at

22°C (2.79±0.70 mm/day for *A. flavus* and 2.48±0.33 mm/day for *A. parasiticus*) comparing different temperatures. The distributions of the medians of growth rates and lag phases between the different temperature groups were significantly different ($p<0.001$) in both species. The findings are consistent with other studies showing that the optimum temperature for the growth of *A. parasiticus* on MEA is 31°C (Garcia et al., 2011), while for *A. flavus* on other types of synthetic culture media it is 32-36°C depending on the substrate and the isolate (Astoreca et al., 2012).

### 3.4. Toxigenic potential of the fungal isolates at different temperatures

Number of isolates of each fungal species producing different secondary metabolites at different temperatures (22, 30 and 37°C) is shown in Table S2. Capability of toxic secondary metabolites production ("toxigenicity") by various isolates of *A. flavus* (AFB2, AFB1, OMST and STERIG) and *A. parasiticus* (AFG2, AFG1, AFB2, AFB1, OMST and STERIG) isolates incubated at different temperatures are shown in Table 4 and 5, respectively. MRM chromatograms showing the production of all the four aflatoxins, STERIG and OMST by an *A. parasiticus* isolate and the production of AFB2, AFB1, OMST and STERIG by an *A. flavus* isolate are shown in Fig. 2A and 2B, respectively. MRM chromatograms showing the production potential of different metabolites by *A. parasiticus* and *A. flavus* isolates in MEA at three temperatures are given as supplementary material (Fig. S3-S7).

#### 3.4.1. Toxigenicity of *A. flavus* isolates

All of the *A. flavus* isolates produced AFB1 and OMST while 91% of them produced AFB2 and 82% produced STERIG at 30°C (Table S2). STERIG, the carcinogenic polyketide is the penultimate intermediate in the aflatoxin biosynthetic pathway. It is converted to OMST prior to the production of AFB1 or AFG1 (Rank et al., 2011; Cleveland et al., 2009; Versilovskis & De Saeger, 2010).

A large variability in toxigenicity was observed among different isolates of the same species grown in MEA at a particular temperature. Marín et al. (2008) had emphasized that a high variability exist in mycotoxin production by a given strain in a given substrate. The fungal isolates were found to be highly temperature dependent in metabolite production; larger number of isolates of each fungal species produced higher

concentration of mycotoxins at 30°C. Number of isolates producing mycotoxins and the concentration of the mycotoxins produced were very small at 37°C compared to the other two temperatures (Tables S2 and 4). At 37°C only four isolates of *A. flavus* produced AFB1, but at very low concentrations (0-42.1 µg/kg) compared to the production at 30°C (up to 8004.5±1563.4 µg/kg). These findings are in agreement with other studies in which both *A. flavus* and *A. parasiticus* had optimum temperature for growth and mycotoxin production around 30°C however, different growth media were used in those studies (Mousa et al., 2013; Garcia et al., 2011; Schmidt-Heydt et al., 2010 and Lozano-Ojalvo et al., 2013). According to Bhatnagar et al. (2006), biosynthesis of aflatoxins by *A. flavus* is optimal at temperatures between 29 and 30°C, but it is significantly decreased at temperatures <25°C and >37°C (O'Brian et al., 2007).

Among the eleven *A. flavus* isolates studied, UG AF93, AF60, AF54, AF06 and AF35 were identified as high mycotoxin producers (Table 4). Exceptionally, some *A. flavus* isolates (UG AF60, AF06 and AF35) produced very high concentration of AFB1 at 22°C (max 16-40 mg/kg) than at 30°C (max 10 mg/kg) and the least production was observed at 37°C (max 42 µg/kg). Higher mycotoxin production at 22°C could be explained by the fact that under high $a_w$ conditions (agar) the optimum temperature for aflatoxin production can vary a lot, depending on the strain (Klich et al., 2007). Also the type of growth medium could play a role. In agreement with O'Brian et al. (2007), all the *A. flavus* isolates were producing significantly low concentration of metabolites (or no production) at 37°C. In our study, 64% of the *A. flavus* isolates did not produce any of the studied secondary metabolites at 37°C. Difference in intensity of sporulation (not quantified) was also observed between different temperatures though sporulation initiated at different periods. Previous studies have shown that temperature affects aflatoxin production and the transcriptional profile of *Aspergillus* (O'Brian et al., 2007). At elevated temperatures of 37°C, one or more pathway enzymes become non-functional due to their significant reduction in transcription, leading to failure of the strains to produce the toxins. Therefore, it can be concluded that mycotoxin production is very much temperature dependent, as well as strain specific.

Despite, the similar growth response and sporulation with other *A. flavus* isolates, the isolate UG AF861 was found to be the weakest isolate to produce any toxin in this medium followed by UG AF411. The isolates UG

AF861 and UG AF411 did not produce STERIG at any temperatures but both produced small quantities of OMST and AFB1 at levels lower than the detection limit of the method (1.3 µg/kg). Probably, the little amount of STERIG produced has been already bio-transformed to OMST.

Generally, growth rate of these isolates at 37°C was lower than at 30°C but higher than at 22°C. It was not possible to find an association between secondary metabolite production and growth rate of a particular isolate. Mostly, poor correlation was observed between growth and mycotoxin production and the relationship between the rates of primary and secondary metabolism is still not clear (Garcia et al., 2009). Moreover, production of mycotoxins by a particular fungal isolate varied between replicates even at the same growth conditions. This makes the prevention, control and regulation of fungal secondary metabolism a very challenging topic to understand till today.

### 3.4.2. Toxigenicity of *A. parasiticus* isolates

All of the *A. parasiticus* isolates produced AFG2, AFB1 and OMST at 30°C (Tables 4 and 6). At 22°C, 83% of the isolates produced AFBs, STERIG and OMST while only one isolate produced AFGs (Table S2). Similar to *A. flavus* species, only two *A. parasiticus* were able to produce mycotoxins at 37°C. Among all the isolates, the highest concentration of aflatoxins (185-6,500 µg/kg), STERIG (59±15 µg/kg) and OMST (821±1260 µg/kg) were produced by the isolate UG AP542 at 30°C (Table 5). The isolate UG AP61, AP631 and AP28 produced high concentrations of B aflatoxins (max 12 mg/kg) and OMST (max 1170 µg/kg) at 22°C than at 30°C. Other isolates were found to be very low AFG producers in this medium, however they produced considerably higher amount of AFBs, STERIG and OMST. UG AP821 was the weakest mycotoxin producer among all the *A. parasiticus* isolates. According to Rank et al. (2011) the chemical potential of filamentous fungi is highly influenced by the growth conditions, particularly by nutrients. Many different species required different media to produce high levels of mycotoxins. According to Rodrigues et al. (2009), *A. parasiticus* strains are uniform in their toxigenic ability and usually strong aflatoxigenic. Other authors have reported that non-toxigenic *A. parasiticus* isolates are extremely rare (Horn et al., 1996; Tran-Dinh et al., 1999). In a study by Vaamonde et al. (2003), only 2 were non-toxigenic of the 30 *A. parasiticus* strains isolated from peanuts.

Aflatoxin G production in this studied medium (malt extract agar) was found to be much lower compared to the AFB production of most of the isolates. It might be worth to mention here also that the production pathway of AFB2 and AFG2 is different from that of AFB1 and AFG1. The critical branch point leading to the formation of either AFB1/AFG1 or AFB2/AFG2 is versicolorin B. Production of OMST is only necessary for AFB1 and AFG1 production, while transformation of versicolorin B to dihydro-O-methyl sterigmatocystin (DHOMST) is necessary for AFB2 and AFG2 production (Yu, 2012; Cleveland et al., 2009). According to Georgianna and Payne, (2009) the regulation of STERIG and especially aflatoxin production by *Aspergillus* generally require simple sugars, low pH, reduced nitrogen source and mild oxidative stress. Recent studies have even shown that when *A. flavus* was grown in peptone-containing media, higher initial spore densities inhibited aflatoxin biosynthesis, but promoted mycelial growth (Yan et al., 2012). However, it is still hard to say that this could be the reason in our substrate for its observed low AFG production, since they still produced high level of B aflatoxins. May be genetically those isolates are low AFG producers. More research is necessary to elucidate this in detail. Moreover, aflatoxigenic ability in *A. flavus* seems to have a very unstable character. Its adaptation to carbon rich environments of certain agricultural commodities may be involved in losses of genes that in turn make it loose its toxigenic potential (Rodrigues et al., 2009). Substrate composition could have a significant influence on the toxigenic potential of the same fungal isolate even at similar growth conditions. Thus, it is very essential to report the toxigenicity of the fungal isolate together with the growth substrate and growth conditions.

### 3.5. Correlation between secondary metabolite productions among different isolates

The secondary metabolite production was found to be very much strain specific and highly temperature dependent among the *Aspergillus* isolates of black peppers. As mentioned, significant variability in their production was also found even between replicates of the same isolate at identical growth conditions, which is rather challenging to explain. Nevertheless, the pooled data set (using the mycotoxin production data of all the replicates of both *A. flavus* and *A. parasiticus* species) shows some notable correlations in mycotoxin production between the different isolates (Fig. 3).

A non-linear correlation (expressed as coefficient of determination $R^2$) was found between STERIG-AFB1 (0.81) and OMST-AFB1 (0.79), while a linear correlation was existent between OMST-STERIG (0.80) and AFB1-AFB2 (0.87) production. In fact, considering the biosynthesis, STERIG is converted to OMST to produce AFB1 thus high correlation between these metabolites could be conceivable. However, this higher correlation between AFB1 and AFB2 production has not been previously reported which makes it an important observation in view of their bio-synthesis. Though both AFB1 and AFB2 have similar precursors in the initial stages of their bio-synthesis, however conversion of versicolorin B to versicolorin A in later stages was necessary for bio-transformation to AFB2 (Cleveland et al., 2009). However, in natural contaminated samples generally AFB1 was found to occur more frequently and at higher concentration than AFB2. The growth media used in this study might have played a significant role to obtain this high correlation between these two mycotoxins. As previously mentioned, the simple sugars and peptone with complex sugars (lactose, mannose, xylose, galactose) have shown different effects in fungal growth and mycotoxin production (Georgianna and Payne, 2009; Calvo et al., 2002). Because of the small number of isolates and low production of AFG toxins, possible correlations of AFGs with other mycotoxin production was not investigated in this study.

Moreover, it was interesting to note that OMST was detected whenever there was AFB1 production; a 100% association in occurrence between OMST and AFB1 was found at 30°C for both fungal species, *A. flavus* and *A. parasiticus*. Besides, when there was high AFB1 production generally high OMST (and low STERIG) was produced by the isolates of both species regardless of the temperature. Hence, production of OMST (rather than STERIG) could be used as an indicator for the prediction of AFB1 production by *A. flavus* and/or *A. parasiticus* species. Moreover, the results showed that not all the OMST produced was bio-transformed to AFB1. Considering the whole dataset, STERIG (max 109.7 µg/kg) concentration was found to be very low compared to the OMST (10.9 mg/kg) or AFB1 (40 mg $kg^{-1}$) concentration for all the isolates of both *A. flavus* and *A. parasiticus* (Tables 4 and 5). This could be probably due to the rapid bio-transformation of STERIG to OMST (Rank et al., 2011) necessary for AFB1 production.

Aflatoxin biosynthesis requires a complex regulatory mechanism orchestrated by the pathway-specific regulatory genes, *aflR* and to a lesser extent *aflS* (Amare and Keller, 2014). Moreover, *aflQ* is the only gene involved in transforming OMST to AFB1, a unique step in aflatoxigenic species (Rodrigues et al., 2009). Thus, it appears that the toxigenic potential of any fungal isolate is all about their gene expression in different growth substrates and their growth conditions. A higher number of isolates of different fungal species need to be studied on their mycotoxigenic potentiality as well on their toxigenic stability in different growth medium and conditions. This could help to confront their complexity in secondary metabolism and to define possible measures to control the production of these toxic secondary metabolites in food and feed products, harmful to human and animal health.

## 4. Conclusions

An LC-MS/MS based multi-mycotoxin method was developed to determine the production of secondary metabolites by pure fungal cultures in malt extract agar and was successfully validated. The method was applied to assess the toxigenicity of *A. flavus* and *A. parasiticus* species isolated from black pepper. Secondary metabolite production was very much temperature dependent, as well as strain specific for both species. There was no correlation between the growth rate and any of the secondary metabolite production of both these fungal species. However, notable correlations were found between the concentrations of the different metabolites in the substrate used. A strong correlation between AFB2 and AFB1 concentration was observed despite differences in their biosynthetic pathways in later stages. Low STERIG level, high OMST and AFB1 concentration in this substrate suggest that OMST could be used as a predictor for AFB1 production in both fungal species. The developed method may be of great importance for chemotaxonomic research and to study the conditions, which could induce or suppress the complex secondary metabolism of various pure fungal isolates.

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**<u>Figures:</u> Toxigenicity of *A. flavus* and *A. parasiticus* isolates of black pepper**

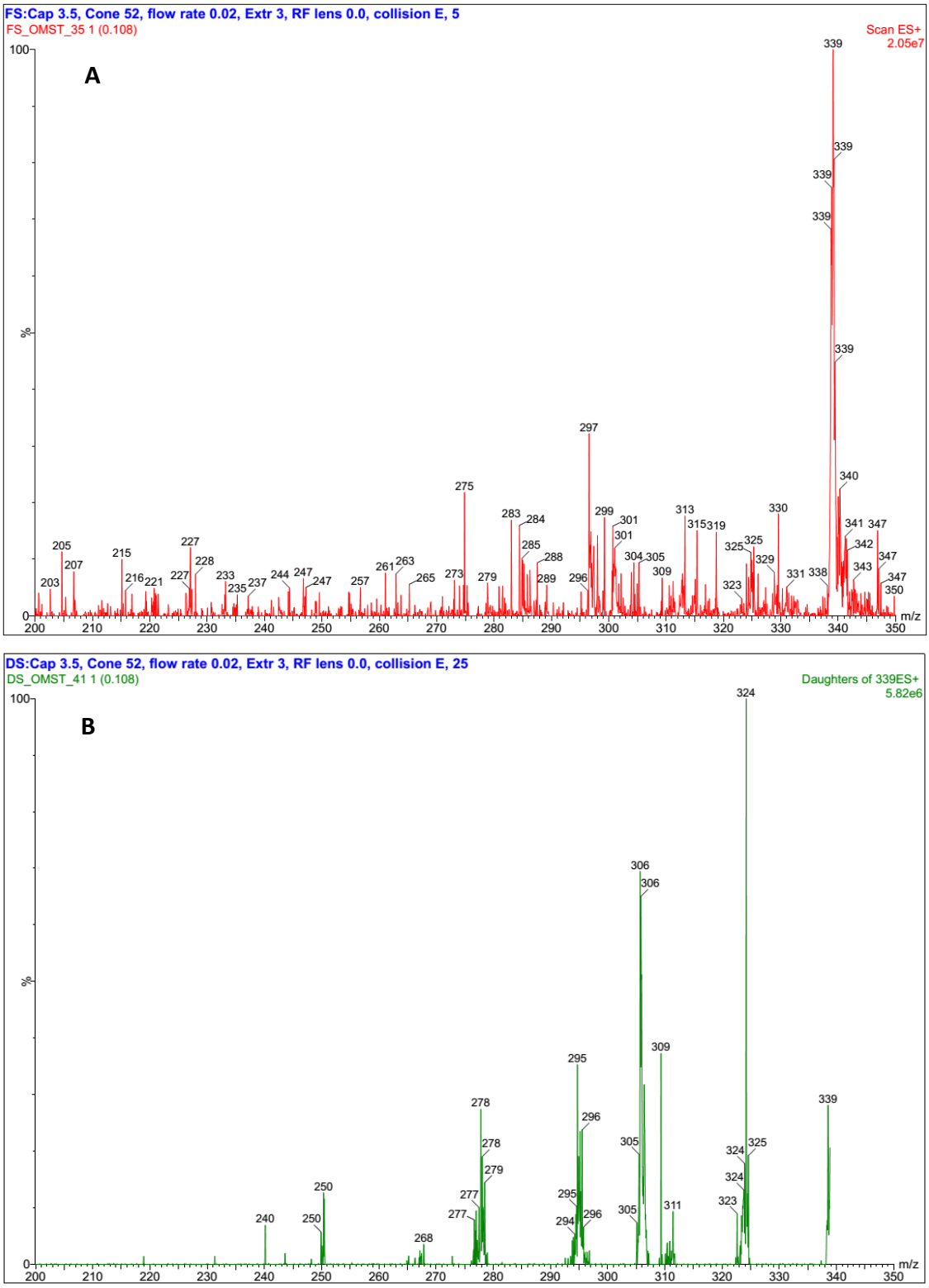
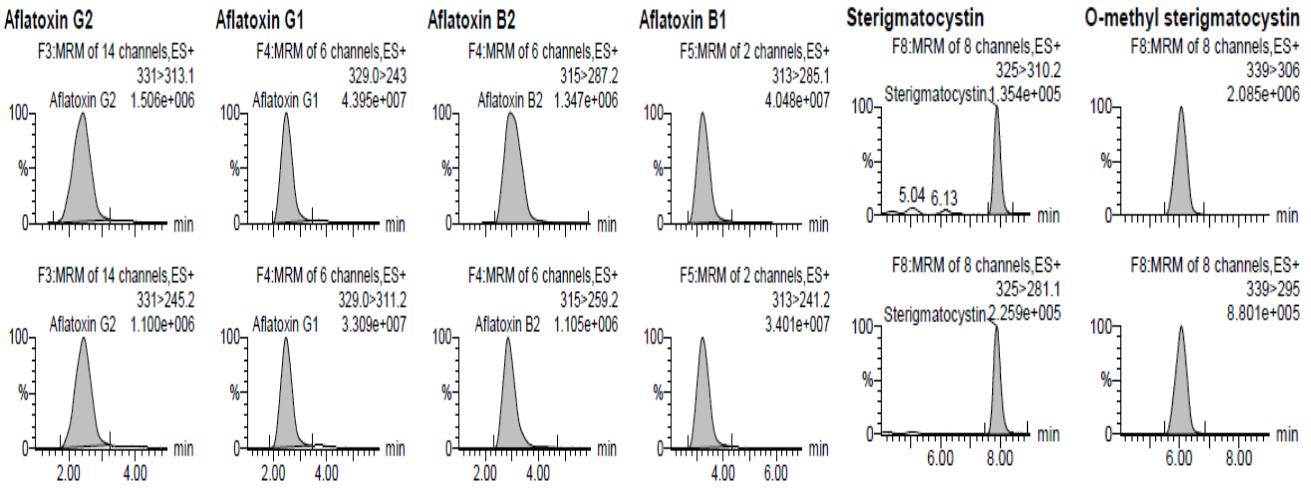


**Fig. 1.**

**A**


Aflatoxin G2
F3:MRM of 14 channels,ES+
331>313.1
Aflatoxin G2 1.506e+006
F3:MRM of 14 channels,ES+
331>245.2
Aflatoxin G2 1.100e+006
Aflatoxin G1
F4:MRM of 6 channels,ES+
329.0>243
Aflatoxin G1 4.395e+007
F4:MRM of 6 channels,ES+
329.0>311.2
Aflatoxin G1 3.309e+007
Aflatoxin B2
F4:MRM of 6 channels,ES+
315>287.2
Aflatoxin B2 1.347e+006
F4:MRM of 6 channels,ES+
315>259.2
Aflatoxin B2 1.105e+006
Aflatoxin B1
F5:MRM of 2 channels,ES+
313>285.1
4.048e+007
F5:MRM of 2 channels,ES+
313>241.2
3.401e+007
Sterigmatocystin
F8:MRM of 8 channels,ES+
325>310.2
Sterigmatocystin 1.354e+005
5.04
6.13
F8:MRM of 8 channels,ES+
325>281.1
Sterigmatocystin 2.259e+005
O-methyl sterigmatocystin
F8:MRM of 8 channels,ES+
339>306
2.085e+006
F8:MRM of 8 channels,ES+
339>295
8.801e+005
100
%
0
min
2.00
4.00
6.00
8.00

**B**

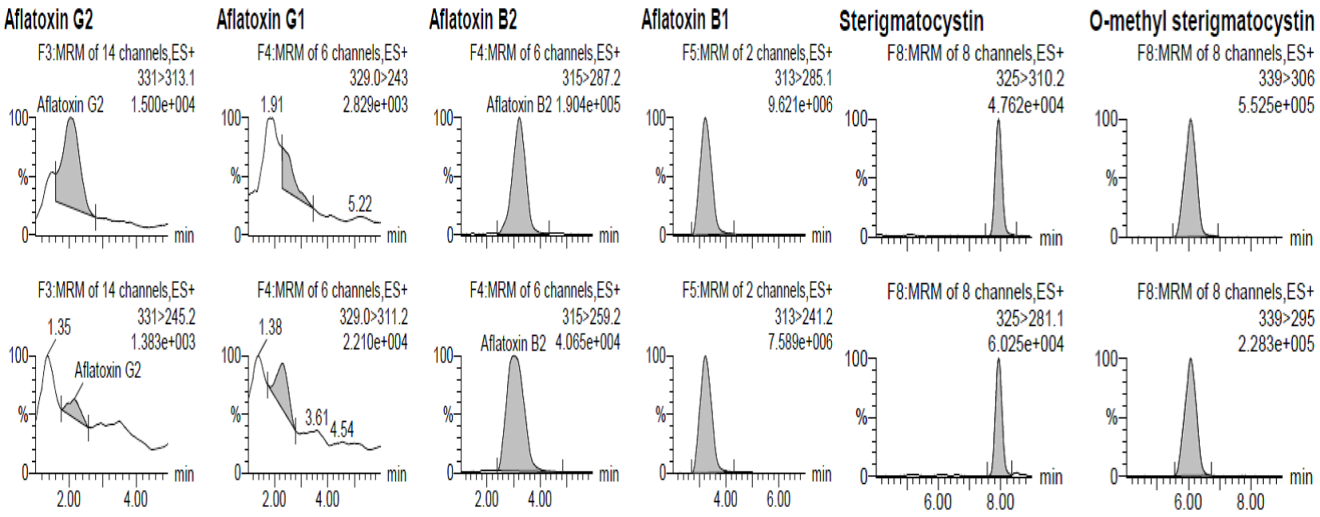
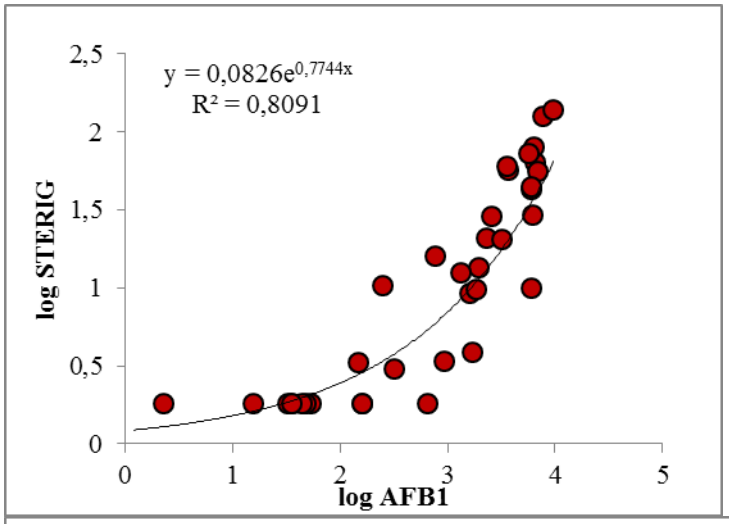
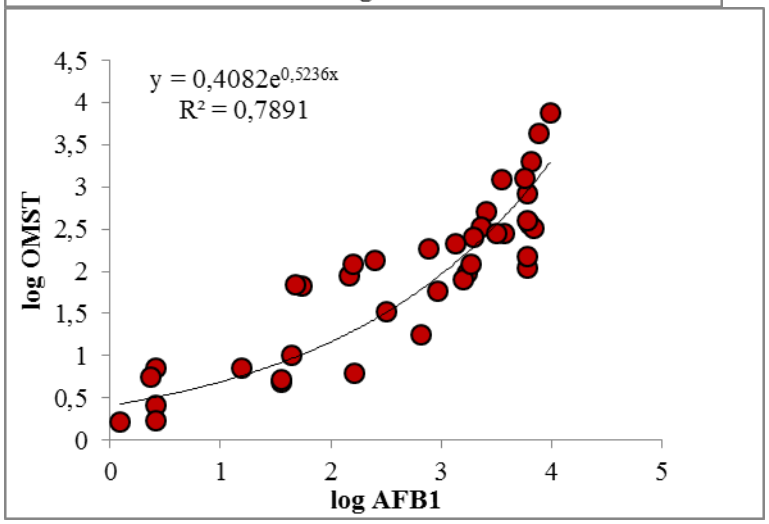

Aflatoxin G2
F3:MRM of 14 channels,ES+
331>313.1
Aflatoxin G2 1.500e+004
F3:MRM of 14 channels,ES+
331>245.2
1.35
1.383e+003
Aflatoxin G2
Aflatoxin G1
F4:MRM of 6 channels,ES+
329.0>243
1.91
2.829e+003
5.22
F4:MRM of 6 channels,ES+
329.0>311.2
1.38
2.210e+004
3.61
4.54
Aflatoxin B2
F4:MRM of 6 channels,ES+
315>287.2
Aflatoxin B2 1.904e+005
F4:MRM of 6 channels,ES+
315>259.2
Aflatoxin B2 4.065e+004
Aflatoxin B1
F5:MRM of 2 channels,ES+
313>285.1
9.621e+006
F5:MRM of 2 channels,ES+
313>241.2
7.589e+006
Sterigmatocystin
F8:MRM of 8 channels,ES+
325>310.2
4.762e+004
F8:MRM of 8 channels,ES+
325>281.1
6.025e+004
O-methyl sterigmatocystin
F8:MRM of 8 channels,ES+
339>306
5.525e+005
F8:MRM of 8 channels,ES+
339>295
2.283e+005
100
%
0
min
2.00
4.00
6.00
8.00

**Fig. 2.**


y = 0,0826e0,7744x
R² = 0,8091
log STERIG
log AFB1
0
0,5
1
1,5
2
2,5
0
1
2
3
4
5


y = 0,4082e0,5236x
R² = 0,7891
log OMST
log AFB1
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
0
1
2
3
4
5

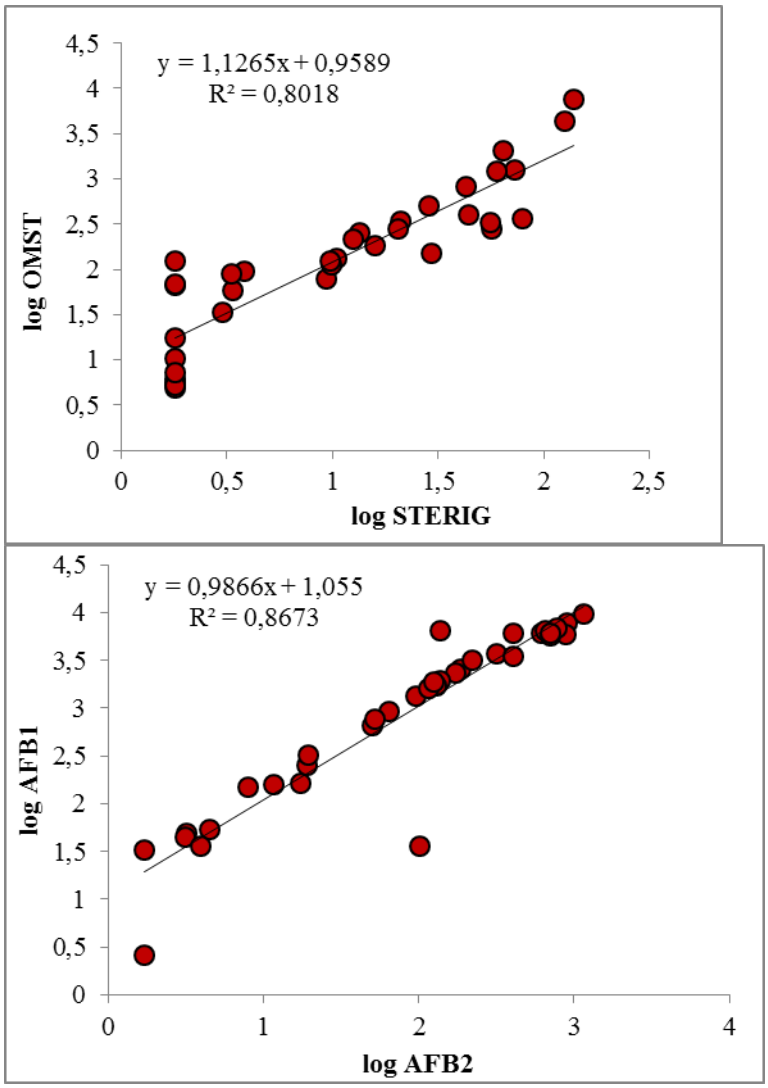



**Fig. 3.**

**<u>Supplementary material: Toxigenicity *A. flavus* and *A. parasiticus*</u>**

**Table S1. Parameters for the mass spectrometric detection of mycotoxins including analyte retention time (RT), precursor ions, molecular formula, cone voltage, quantification, confirmation ions and collision energy.**

| Mycotoxins | Retention time (min) | Precursor ion (m/z) | Molecular formula | Cone voltage (V) | Quantification ion (m/z) | Confirmation ion (m/z) | Collison energy (eV) |
|---|---|---|---|---|---|---|---|
| AFG2 | 2.19 | 331.0 | $[M+H]^+$ | 53 | 245.2 | 313.1 | 30<br>25 |

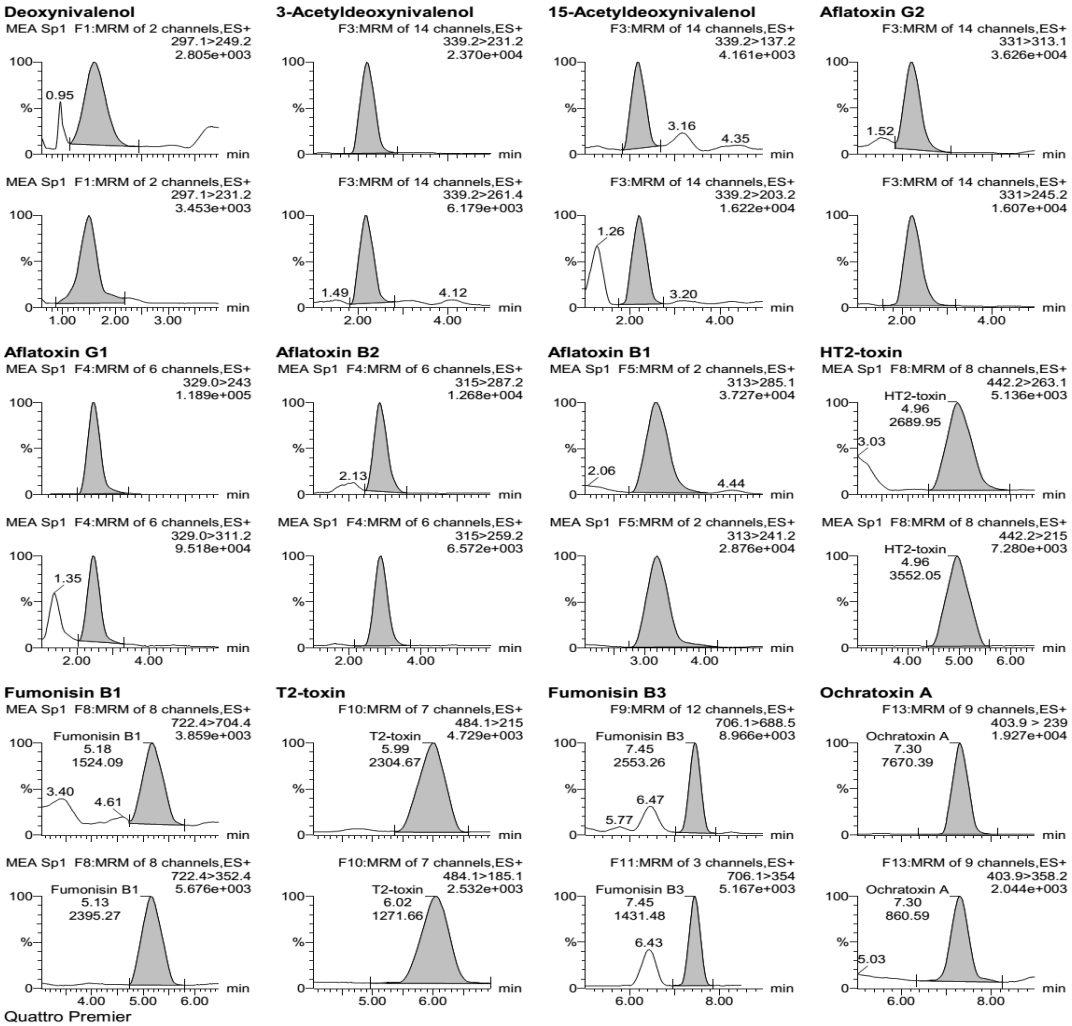
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AFG1 | 2.44 | 329.0 | $[M+H]^+$ | 45 | 243.0 | 311.2 | 25<br>20 |
| AFB2 | 2.85 | 315.0 | $[M+H]^+$ | 51 | 287.2 | 259.2 | 27<br>30 |
| AFB1 | 3.20 | 313.0 | $[M+H]^+$ | 51 | 285.1 | 241.2 | 24<br>36 |
| OTA | 7.33 | 403.9 | $[M+H]^+$ | 25 | 239.0 | 358.2 | 22<br>20 |
| T-2 | 6.02 | 484.1 | $[M+NH_4]^+$ | 30 | 215.0 | 185.1 | 20<br>18 |
| HT-2 | 4.92 | 442.2 | $[M+NH_4]^+$ | 20 | 263.1 | 215.0 | 13<br>13 |
| STERIG | 7.87 | 325.0 | $[M+H]^+$ | 47 | 310.2 | 281.1 | 25<br>36 |
| ROQ C | 4.29 | 390.0 | $[M+H]^+$ | 40 | 322.2 | 193.2 | 26<br>21 |
| FB1 | 5.18 | 722.4 | $[M+H]^+$ | 56 | 704.4 | 352.4 | 29<br>36 |
| FB2 | 7.47 | 706.0 | $[M+H]^+$ | 50 | 336.5 | 318.0 | 35<br>29 |
| FB3 | 6.61 | 706.1 | $[M+H]^+$ | 54 | 688.5 | 354.0 | 34<br>31 |
| CIT | 5.62 | 250.9 | $[M+H]^+$ | 32 | 233.2 | 205.4 | 17<br>26 |
| AME | 8.63 | 272.9 | $[M+H]^+$ | 57 | 258.2 | 199.3 | 26<br>30 |
| 3-ADON | 2.16 | 339.2 | $[M+H]^+$ | 24 | 231.2 | 261.4 | 12<br>10 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15-ADON | 2.16 | 339.2 | $[M+H]^+$ | 24 | 137.2 | 203.2 | 10<br>12 |
| NEO | 1.61 | 400.1 | $[M+NH_4]^+$ | 26 | 185.0 | 305.3 | 19<br>12 |
| OMST | 6.04 | 339 | $[M+H]^+$ | 52 | 306 | 324 | 28<br>25 |
| ZEN | 7.40 | 319.1 | $[M+H]^+$ | 27 | 187.2 | 283.3 | 15<br>20 |
| ZAN (IS) | 7.51 | 321.0 | $[M+H]^+$ | 27 | 303.3 | 189.2 | 13<br>19 |

Aflatoxin G2, AFG2; Aflatoxin G1, AFG1; aflatoxin B2, AFB2; Aflatoxins B1, AFB1; Ochratoxin A, OTA; T-2, T-2 toxin; HT-2, HT-2 toxin; Sterigmatocystin, STERIG; Roquefortine C, ROQ C; Fumonisin B1, FB1; Fumonisin B2, FB2; Fumonisin B3, FB3; Citrinin, CIT; Alternariol Methyl Ether, AME; 3-ADON, 3-Acetyl Deoxynivalenol; 15-ADON, 15-Acetyl Deoxynivalenol; NEO, Neosolaniol; OMST, O-methyl sterigmatocystin; ZEN, Zearalenone; ZAN (IS), Zearalanone (Internal Standard).

**Table S2. Number (percentage) of fungal isolates of each *Aspergillus* species producing mycotoxins at temperatures 22, 30 and 37°C, at colony diameter ~80-90 mm or when plate was completely covered by the colony.**

| Fungal species | Temperature (°C) | AFG2 | AFG1 | AFB2 | AFB1 | STERIG | OMST |
|---|---|---|---|---|---|---|---|
| ***A. flavus*** (n=11) | 22 | 0 | 0 | 7 (64) | 8 (73) | 7 (64) | 9 (82) |
| | 30 | 0 | 0 | 10 (91) | 11 (100) | 9 (82) | 11 (100) |
| | 37 | 0 | 0 | 1 (9) | 4 (36) | 3 (27) | 3 (27) |
| ***A. parasiticus*** (n=6) | 22 | 1 (17) | 1 (17) | 5 (83) | 5 (83) | 5 (83) | 5 (83) |
| | 30 | 6 (100) | 5 (83) | 5 (83) | 6 (100) | 5 (83) | 6 (100) |
| | 37 | 2 (33) | 1 (17) | 1 (17) | 1 (17) | 1 (17) | 1 (17) |


Deoxynivalenol
MEA Sp1 F1:MRM of 2 channels,ES+
297.1>249.2
2.805e+003
0.95
MEA Sp1 F1:MRM of 2 channels,ES+
297.1>231.2
3.453e+003
3-Acetyldeoxynivalenol
F3:MRM of 14 channels,ES+
339.2>231.2
2.370e+004
F3:MRM of 14 channels,ES+
339.2>261.4
6.179e+003
1.49
4.12
15-Acetyldeoxynivalenol
F3:MRM of 14 channels,ES+
339.2>137.2
4.161e+003
3.16
4.35
F3:MRM of 14 channels,ES+
339.2>203.2
1.622e+004
1.26
3.20
Aflatoxin G2
F3:MRM of 14 channels,ES+
331>313.1
3.626e+004
1.52
F3:MRM of 14 channels,ES+
331>245.2
1.607e+004
Aflatoxin G1
MEA Sp1 F4:MRM of 6 channels,ES+
329.0>243
1.189e+005
MEA Sp1 F4:MRM of 6 channels,ES+
329.0>311.2
9.518e+004
1.35
Aflatoxin B2
MEA Sp1 F4:MRM of 6 channels,ES+
315>287.2
1.268e+004
2.13
MEA Sp1 F4:MRM of 6 channels,ES+
315>259.2
6.572e+003
Aflatoxin B1
MEA Sp1 F5:MRM of 2 channels,ES+
313>285.1
3.727e+004
2.06
4.44
MEA Sp1 F5:MRM of 2 channels,ES+
313>241.2
2.876e+004
HT2-toxin
MEA Sp1 F8:MRM of 8 channels,ES+
442.2>263.1
5.136e+003
HT2-toxin
4.96
2689.95
3.03
MEA Sp1 F8:MRM of 8 channels,ES+
442.2>215
7.280e+003
HT2-toxin
4.96
3552.05
Fumonisin B1
MEA Sp1 F8:MRM of 8 channels,ES+
722.4>704.4
3.859e+003
Fumonisin B1
5.18
1524.09
3.40
4.61
MEA Sp1 F8:MRM of 8 channels,ES+
722.4>352.4
5.676e+003
Fumonisin B1
5.13
2395.27
T2-toxin
F10:MRM of 7 channels,ES+
484.1>215
4.729e+003
T2-toxin
5.99
2304.67
F10:MRM of 7 channels,ES+
484.1>185.1
2.532e+003
T2-toxin
6.02
1271.66
Fumonisin B3
F9:MRM of 12 channels,ES+
706.1>688.5
8.966e+003
Fumonisin B3
7.45
2553.26
6.47
5.77
F11:MRM of 3 channels,ES+
706.1>354
5.167e+003
Fumonisin B3
7.45
1431.48
6.43
Ochratoxin A
F13:MRM of 9 channels,ES+
403.9 > 239
1.927e+004
Ochratoxin A
7.30
7670.39
F13:MRM of 9 channels,ES+
403.9>358.2
2.044e+003
Ochratoxin A
7.30
860.59
5.03
min
%
100
0
Quattro Premier

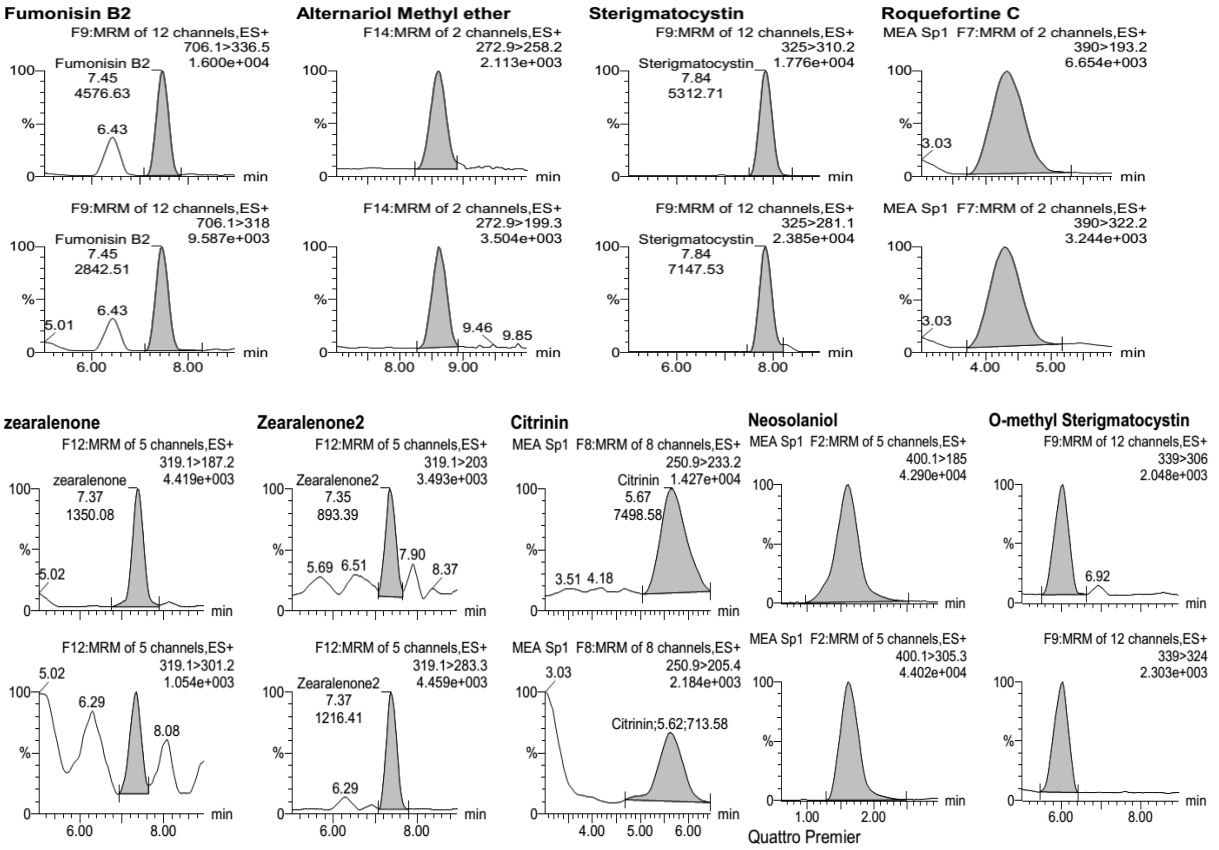



**Fig. S1. MRM chromatograms of the spiked malt extract agar at the lowest calibration concentration. Quantification and confirmation transitions are shown for each mycotoxin.**

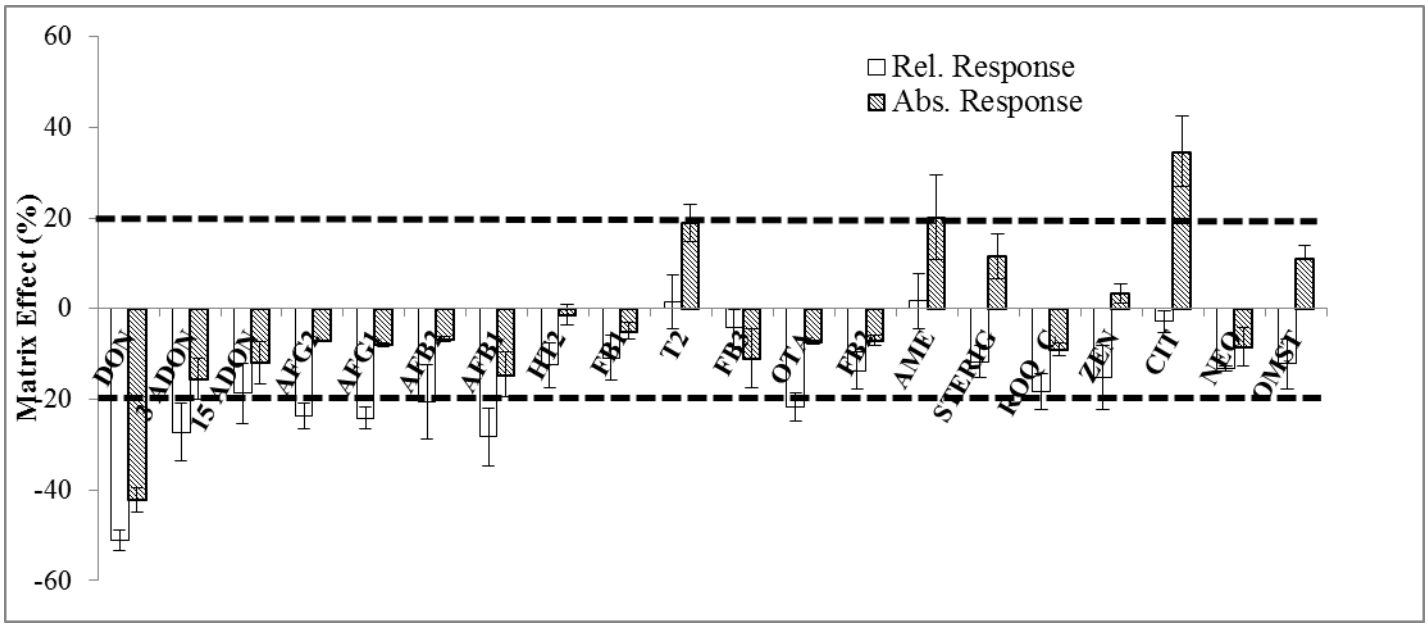



**Fig. S2. Matrix effect of different mycotoxins in malt extract agar; a comparison based on absolute versus relative analyte response. A tolerance level of matrix effect is shown between the two dashed lines.**

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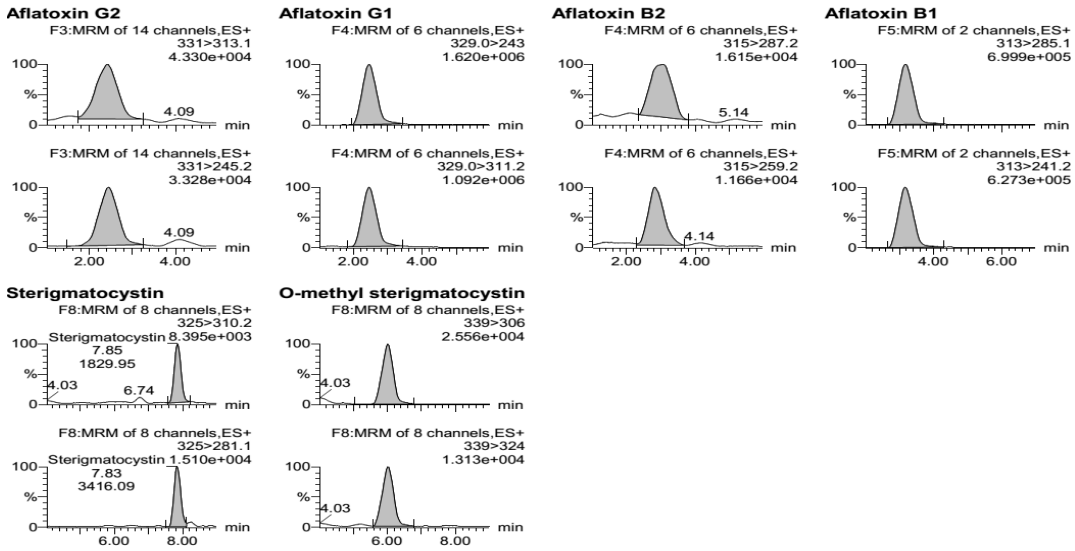
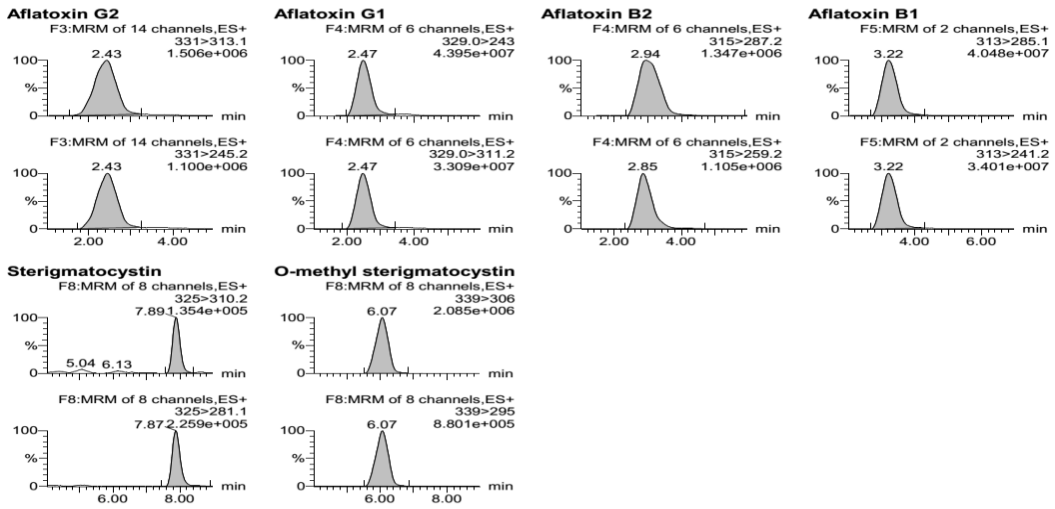



**Name: 15092013_03, Date: 15-Sep-2013, Time: 15:40:01, Description: AfAp 1104542 R1 30/MEA**



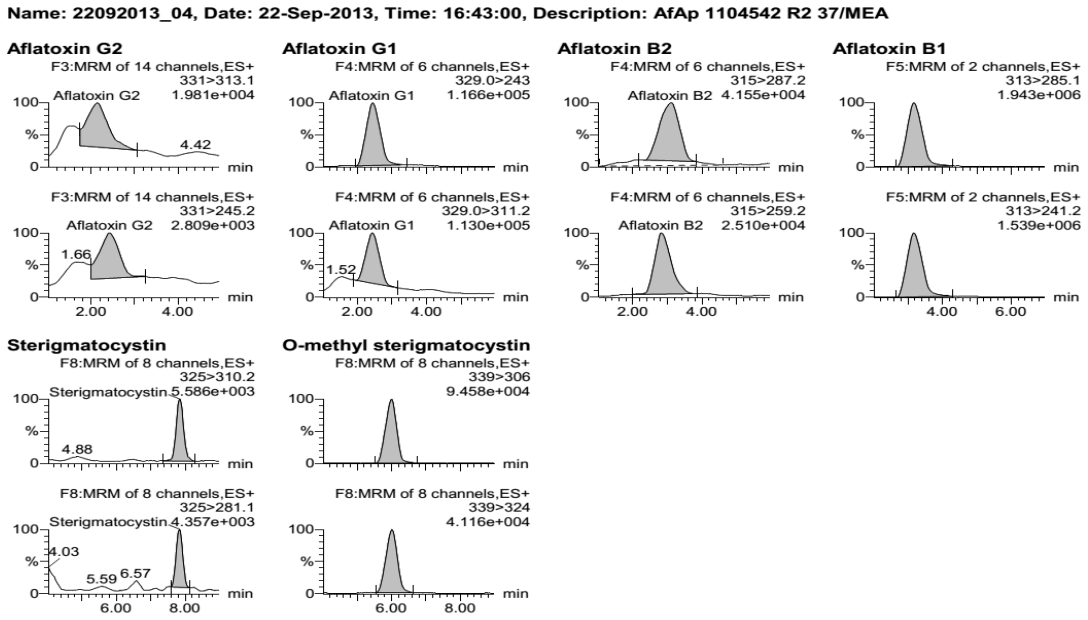
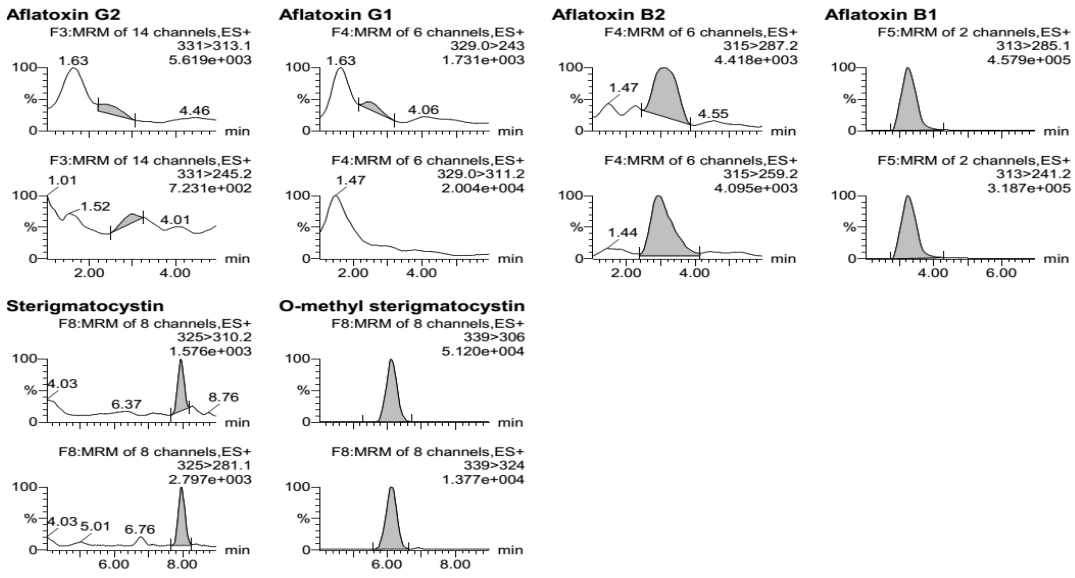



**Fig. S3. MRM chromatograms showing the production of AFG2, G1, B2, B1, STERIG and OMST by the *A. parasiticus* strain UG AP542 at 22, 30 and 37°C in malt extract agar.**



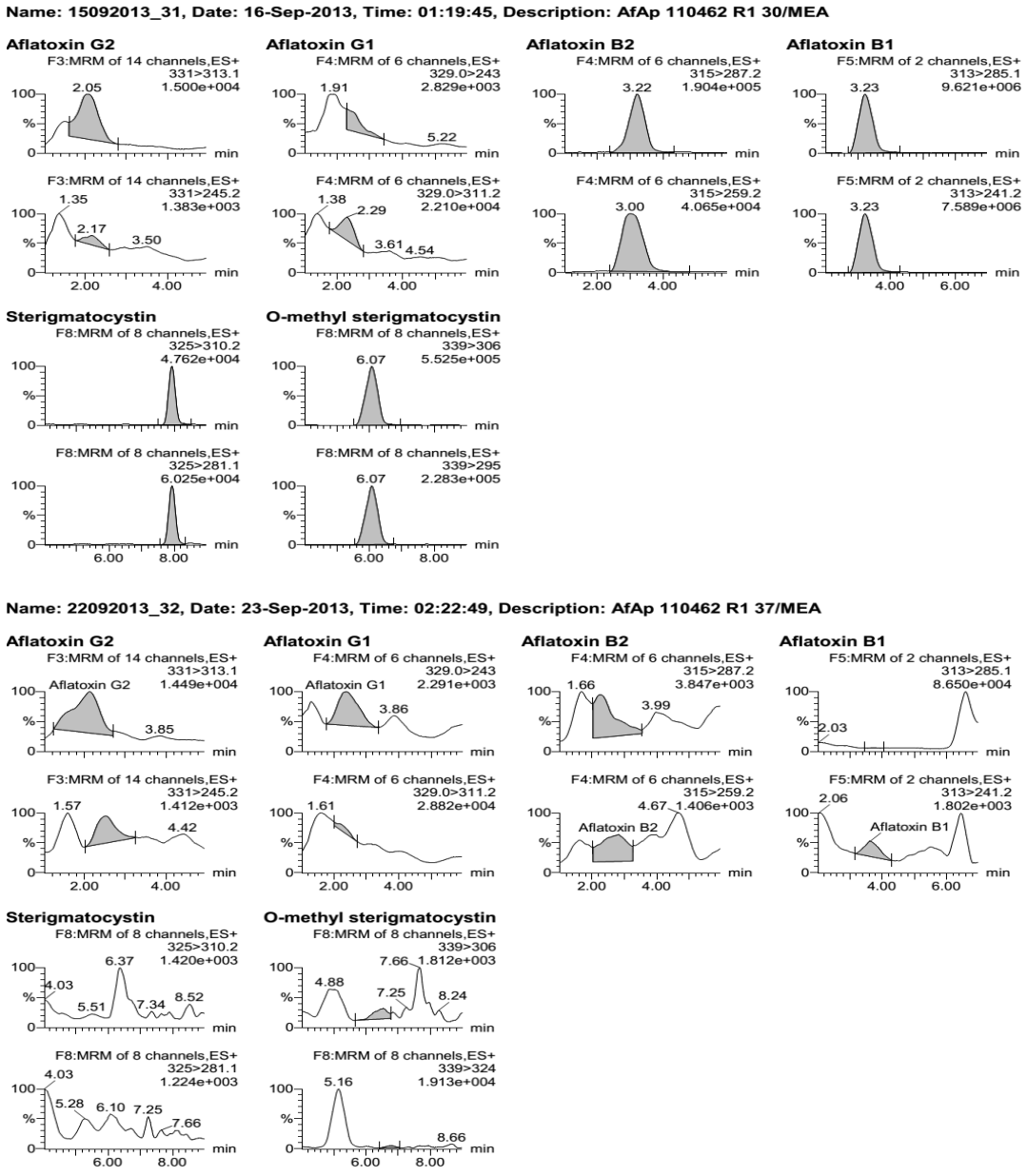
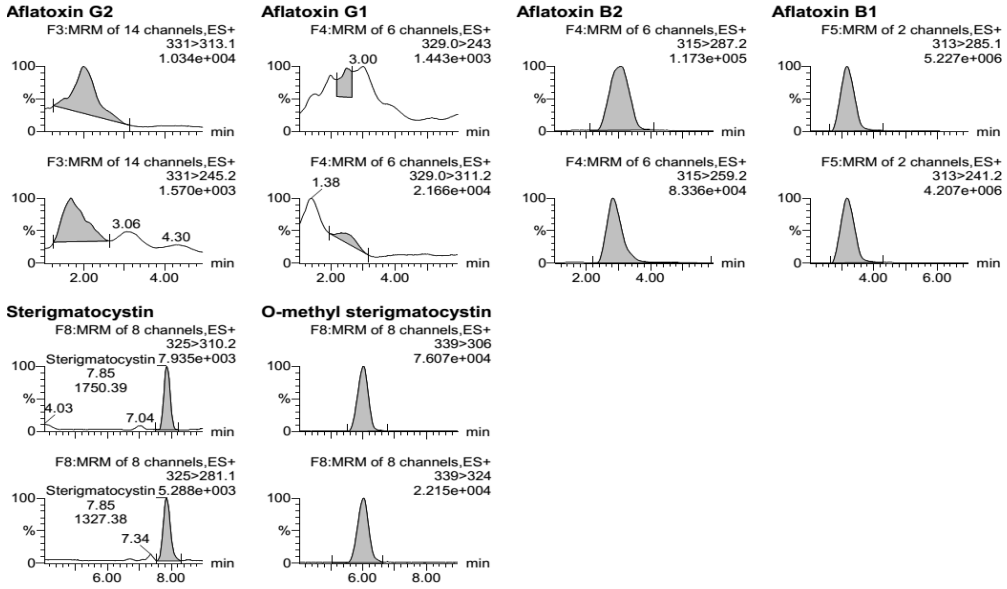




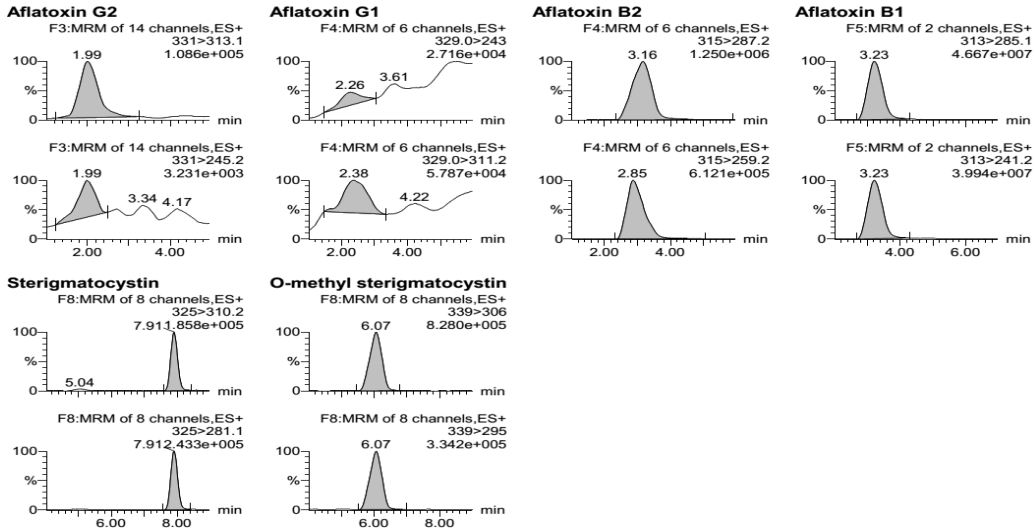


**Fig. S4. MRM chromatograms showing the production of all the mycotoxins at 22 and 30 and their absence at 37°C by the *A. flavus* strain UG AF62 grown in malt extract agar.**

**Name: 05102013_05, Date: 05-Oct-2013, Time: 17:08:16, Description: AfAp 110435b R1 22/MEA**




**Name: 15092013_05, Date: 15-Sep-2013, Time: 16:21:27, Description: AfAp 110435b R1 30/MEA**

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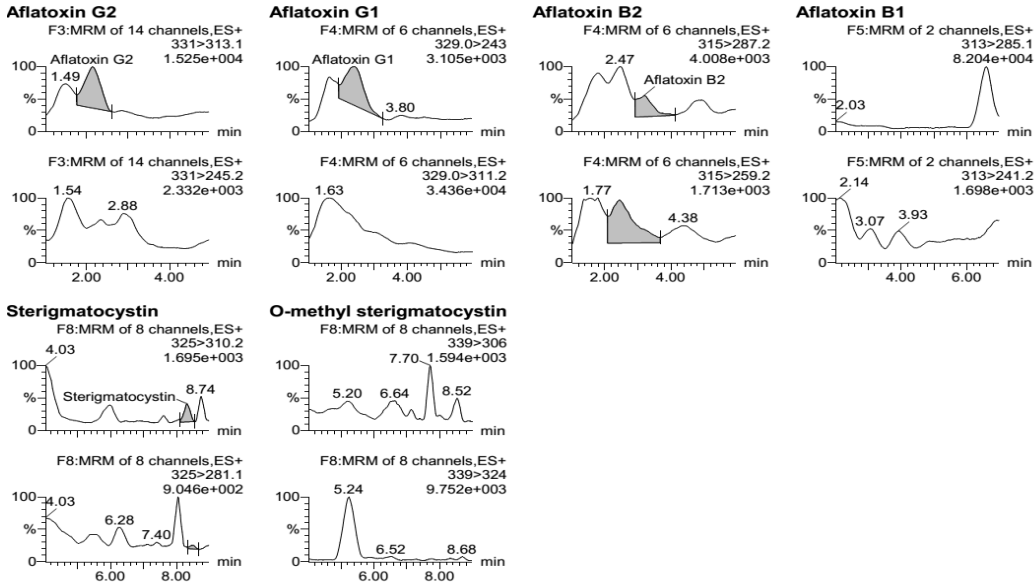
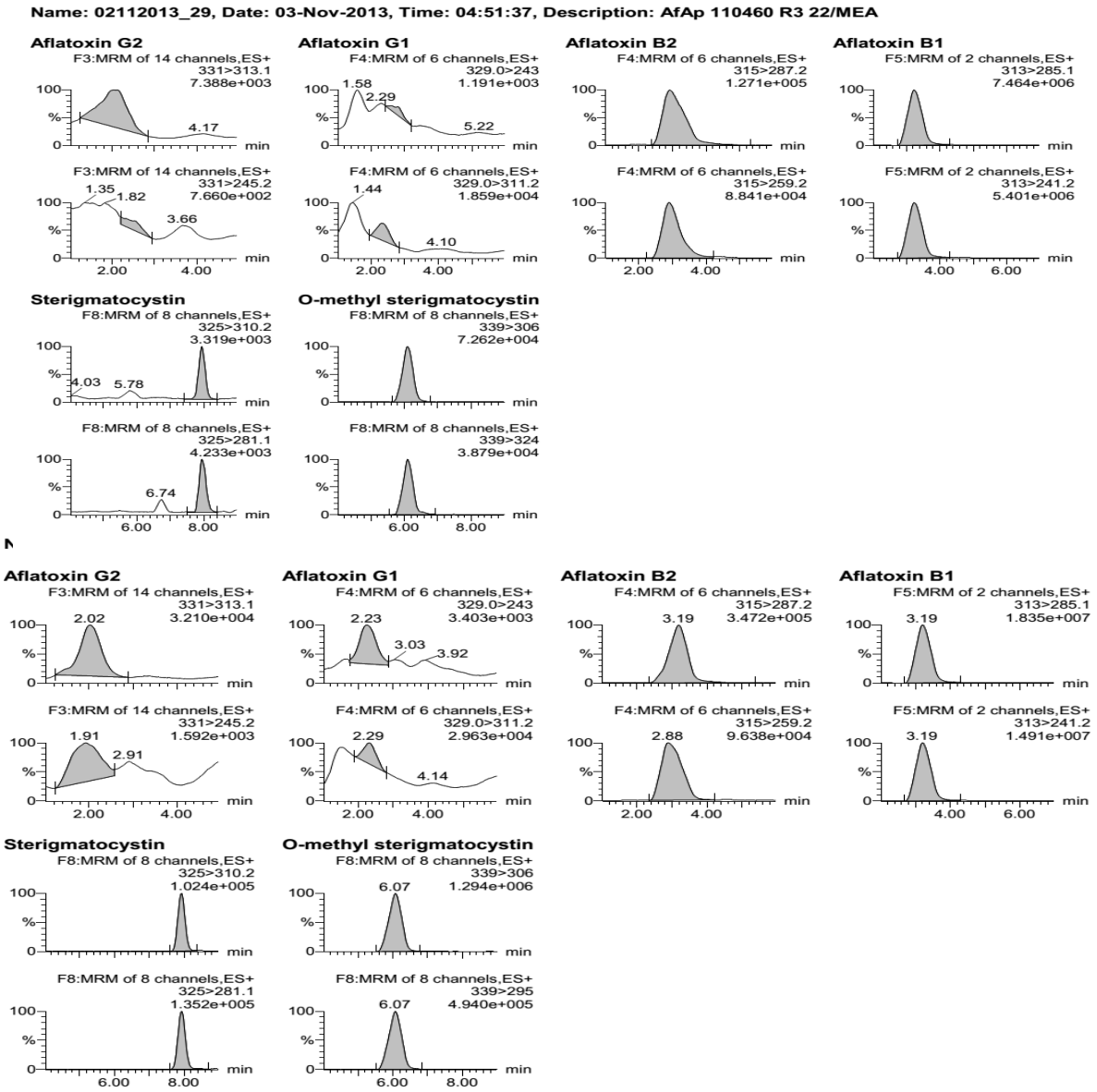


**Fig. S5. MRM chromatograms showing the production of all the mycotoxins at 22 and 30 and their absence at 37°C by the *A. flavus* strain UG AF35 grown in malt extract agar.**



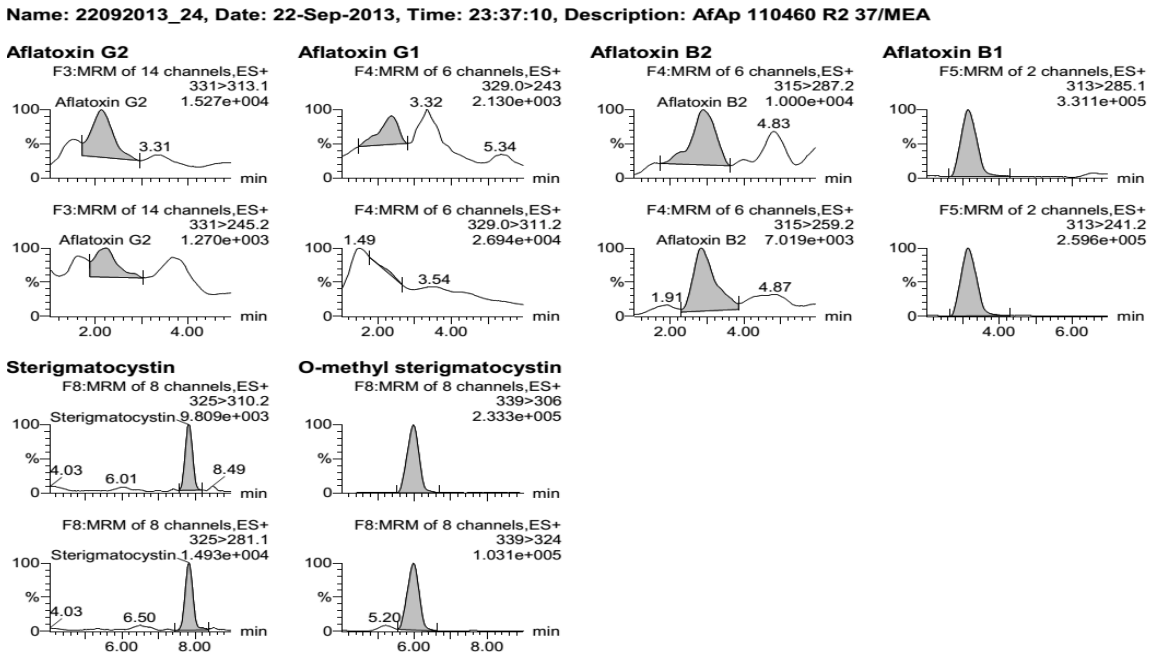
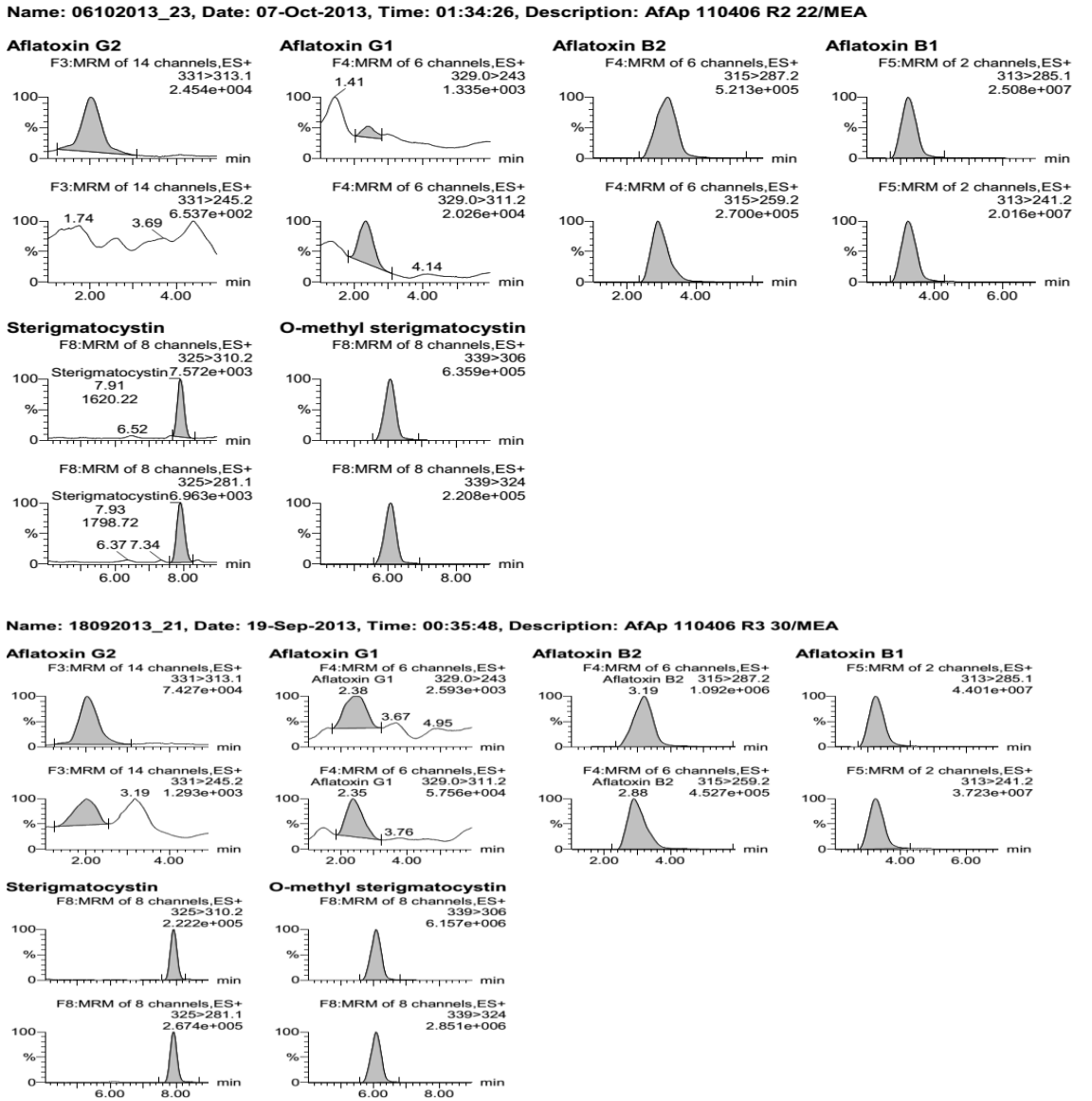



**Fig. S6. MRM chromatograms showing the production of mycotoxins at all the temperatures 22, 30 and 37°C by the *A. flavus* strain UG AF60 grown in malt extract agar.**



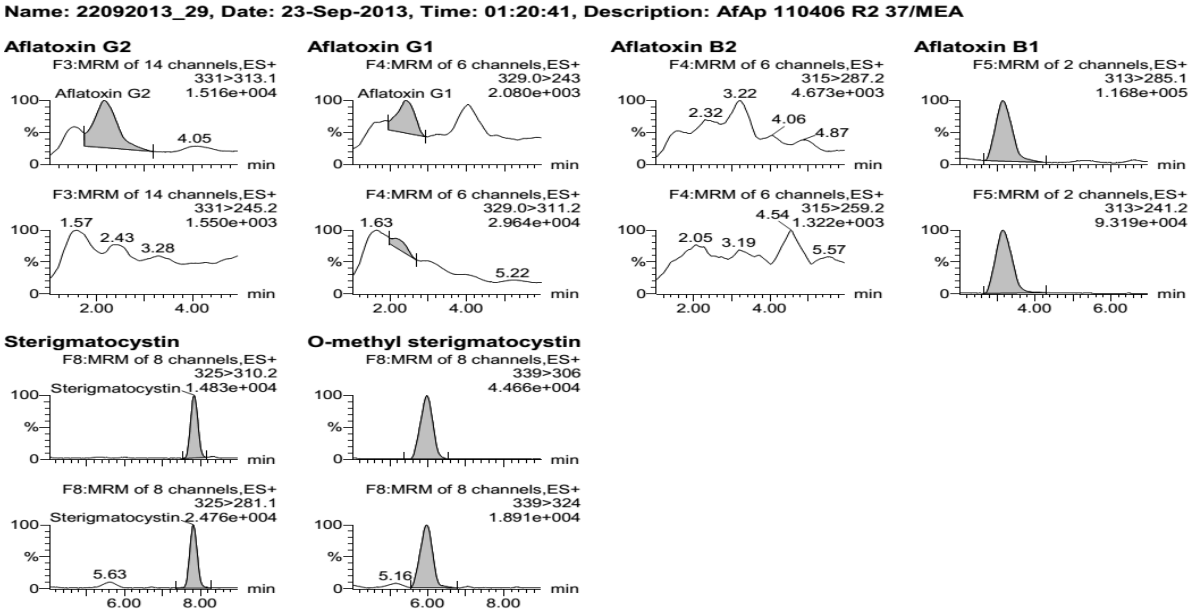



**Fig. S7. MRM chromatograms showing the production of all mycotoxins at all the temperatures 22, 30°C and 37°C (except B2) by the *A. flavus* strain UG AF06 grown in malt extract agar.**

**<u>Tables:</u> Toxigenicity of *A. flavus* and *A. parasiticus* isolates of black pepper**

**Table 1. Lack-of-fit and apparent recovery of the developed multi-analyte method; a comparison based on the calculations of absolute versus relative response.**

| Mycotoxin | Absolute Response | | Relative Response | |
|---|---|---|---|---|
| | Lack of fit (p-value) | Apparent recovery (%) | Lack of fit (p-value) | Apparent recovery (%) |
| DON | 0.125 | 95 | 0.215 | 92 |
| 3-ADON | 0.706 | 111 | 0.539 | 112 |
| 15-ADON | 0.244 | 113 | 0.722 | 111 |
| AFG2 | 0.078 | 104 | 0.166 | 110 |
| AFG1 | 0.289 | 95 | 0.600 | 98 |
| AFB2 | 0.29 | 106 | 0.212 | 107 |
| AFB1 | 0.397 | 97 | 0.747 | 94 |
| HT-2 | 0.282 | 109 | 0.755 | 106 |
| FB1 | 0.338 | 100 | 0.461 | 104 |
| T-2 | 0.536 | 102 | 0.201 | 113 |
| FB3 | 0.858 | 109 | 0.406 | 110 |
| OTA | 0.241 | 108 | 0.168 | 111 |
| FB2 | 0.083 | 110 | 0.187 | 108 |
| AME | 0.418 | 108 | 0.699 | 110 |
| STERIG | 0.441 | 93 | 0.331 | 101 |
| ROQ C | 0.711 | 113 | 0.627 | 100 |
| ZEN | 0.662 | 105 | 0.520 | 105 |
| CIT | 0.179 | 86 | 0.210 | 87 |
| NEO | 0.186 | 97 | 0.063 | 111 |
| OMST | 0.506 | 103 | 0.503 | 96 |

**Table 2. Limit of detection (LOD), limit of quantification (LOQ) (µg/kg), repeatability (RSDr) (%) and intermediate precision (RSDR) (%) of different mycotoxins in malt extract agar determined based on the absolute and relative response.**

| Mycotoxin | Absolute response | | | | Relative response | | | |
|---|---|---|---|---|---|---|---|---|
| | LOD | LOQ | RSDr | RSDR | LOD | LOQ | RSDr | RSDR |
| DON | 6.4 | 12.9 | 20.3 | 24.0 | 10.1 | 20.2 | 23.5 | 25.8 |
| 3-ADON | 3.5 | 7.0 | 19.4 | 22.6 | 6.8 | 13.6 | 18.9 | 22.8 |
| 15-ADON | 2.4 | 4.7 | 22.2 | 23.4 | 7.3 | 14.6 | 18.5 | 19.1 |
| AFG2 | 1.4 | 2.9 | 17.5 | 17.7 | 1.4 | 2.8 | 13.4 | 13.7 |
| AFG1 | 1.8 | 3.6 | 14.1 | 16.8 | 1.1 | 2.2 | 22.1 | 23.3 |
| AFB2 | 0.8 | 1.7 | 15.4 | 21.3 | 0.9 | 1.8 | 14.9 | 28.5 |
| AFB1 | 1.3 | 2.6 | 9.4 | 14.5 | 1.3 | 2.6 | 13.5 | 15.3 |
| HT-2 | 3.3 | 6.7 | 16.9 | 17.8 | 3.7 | 7.4 | 15.8 | 17.0 |
| FB1 | 5.7 | 11.5 | 17.6 | 19.7 | 3.7 | 7.3 | 15.1 | 22.4 |
| T-2 | 1.6 | 3.2 | 14.2 | 16.8 | 2.6 | 5.3 | 9.8 | 13.1 |
| FB3 | 7.4 | 14.7 | 19.8 | 19.7 | 8.8 | 17.7 | 12.6 | 23.1 |
| OTA | 1.8 | 3.6 | 19.0 | 15.9 | 37. | 7.4 | 8.6 | 13.0 |
| FB2 | 7.1 | 14.2 | 17.5 | 20.3 | 8.1 | 16.2 | 12.1 | 22.1 |
| AME | 5.7 | 11.5 | 14.4 | 14.6 | 2.6 | 5.3 | 9.2 | 15.1 |
| STERIG | 0.9 | 1.8 | 19.6 | 21.1 | 0.9 | 1.7 | 9.4 | 10.2 |
| ROQ C | 1.9 | 3.9 | 13.5 | 18.3 | 1.1 | 2.2 | 19.1 | 23.7 |
| ZEN | 3.3 | 6.6 | 14.8 | 15.4 | 3.1 | 6.2 | 13.1 | 16.0 |
| CIT | 14.6 | 29.2 | 6.8 | 19.7 | 5.9 | 11.8 | 20.7 | 21.4 |
| NEO | 6.2 | 12.5 | 10.9 | 23.4 | 2.4 | 4.9 | 20.3 | 24.9 |
| OMST | 1.3 | 2.6 | 13.0 | 18.2 | 1.6 | 3.2 | 12.6 | 16.4 |

**Table 3. Maximum radial growth rates ($\mu_{max}$) (mean±SD, mm/day) and lag phases (λ) (mean±SD, days) of *A. flavus (*A) and *A. parasiticus* (B) isolates of black pepper grown on malt extract agar and incubated at 22, 30 and 37°C.**

| Strain ID | Origin of the *Aspergillus* isolate[a] | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 22 | | 30 | | 37 | |
| | | $\mu_{max}$ | λ | $\mu_{max}$ | λ | $\mu_{max}$ | λ |
| | | ***A. Aspergillus flavus*** | | | | | |
| UG AF861 | Kandy/Matale | 2.67±0.48 | 1.23±0.34 | 5.20±0.14 | 0.87±0.02 | 3.68±0.81 | 3.13±0.06 |
| UG AF60 | Kandy/Matale | 2.75±0.45 | 1.32±0.37 | 5.00±0.04 | 0.85±0.08 | 3.43±0.47 | 2.65±0.95 |
| UG AF93 | Anuradhapura | 2.35±0.09 | 0.92±0.23 | 4.97±0.54 | 0.92±0.03 | 2.95±0.84 | 2.54±0.92 |
| UG AF36 | Jaffna | 2.71±0.20 | 1.32±0.11 | 5.77±0.36 | 0.89±0.03 | 3.57±0.92 | 2.94±0.25 |
| UG AF62 | Kandy/Matale | 2.74±0.17 | 1.16±0.04 | 5.30±0.64 | 0.80±0.14 | 4.79±0.28 | 3.13±0.03 |
| UG AF54 | Anuradhapura | 2.70±0.13 | 1.24±0.14 | 5.24±0.57 | 0.81±0.16 | 4.56±0.19 | 3.12±0.02 |
| UG AF411 | Jaffna | 3.08±2.14 | 0.73±0.33 | 5.53±0.94 | 0.83±0.06 | 4.26±2.37 | 2.90±0.46 |
| UG AF82 | Kandy/Matale | 4.50±2.07 | 0.91±0.42 | 4.07±1.30 | 0.74±0.17 | 2.57±1.77 | 2.90±0.44 |
| UG AF06 | Kandy/Matale | 2.87±0.36 | 1.18±0.22 | 5.79±0.16 | 0.90±0.05 | 3.95±0.32 | 3.14±0.03 |
| UG AF362 | Jaffna | 2.44±0.06 | 0.86±0.22 | 5.69±0.17 | 0.92±0.09 | 4.67±0.56 | 3.11±0.06 |
| UG AF35 | Jaffna | 2.69±0.17 | 1.21±0.05 | 5.49±0.38 | 0.86±0.07 | 4.38±0.64 | 3.08±0.04 |
| | | ***B. Aspergillus parasiticus*** | | | | | |
| UG AP821 | Kandy/Matale | 2.52±0.07 | 1.25±0.15 | 5.15±0.37 | 0.90±0.04 | 2.54±0.56 | 3.30±0.37 |
| UG AP631 | Kandy/Matale | 2.42±0.43 | 1.20±0.19 | 5.67±0.40 | 0.87±0.04 | 4.33±0.06 | 3.08±0.05 |
| UG AP61 | Kandy/Matale | 2.16±0.40 | 0.61±0.64 | 5.67±0.23 | 0.90±0.02 | 4.20±1.77 | 2.97±0.22 |
| UG AP542 | Anuradhapura | 2.10±0.38 | 0.54±0.63 | 5.21±0.16 | 0.85±0.05 | 2.15±1.75 | 3.68±1.52 |
| UG AP46 | Anuradhapura | 2.47±0.11 | 1.19±0.05 | 5.22±0.04 | 0.85±0.02 | 3.88±0.50 | 3.07±0.05 |
| UG AP28 | Jaffna | 2.57±0.19 | 1.08±0.16 | 5.90±0.28 | 0.86±0.09 | 4.93±0.38 | 3.00±0.12 |

[a] Origin of each *Aspergillus* isolate; sampling district in Sri Lanka.

**Table 4. Mycotoxin production (mean±SD µg/kg) of *A. flavus* strains isolated from black pepper following the growth in malt extract agar at different temperatures.**

| Mycotoxin[a] | Temperature | UG AF861 | UG AF60 | UG AF93 | UG AF36 | UG AF62 | UG AF54 | UG AF411 | UG AF82 | UG AF06 | UG AF362 | UG AF35 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AFB2 | 22°C | ND[b] | 1742.7, 1785.6[d] | 352.9, 784.9 | 50.2 | 60.3, 83.3 | 133.6, 193.5 | ND | ND | 1611.9, 4964.9 | ND | 365.0, 3728.8 |
| | | (0/2)[c] | (2/2) | (2/2) | (1/2) | (2/2) | (2/2) | (0/2) | (0/2) | (2/2) | (0/2) | (2/2) |
| | 30°C | <LOQ[d] | 166±25.8 | 70.3±56.2 | 3.2-4.5 | 40.9±47.7 | 78.9±52.1 | ND | 4.0-11.7 | 728.9±526.2 | <LOQ | 624.5±193.7 |
| | | (1/3) | (3/3) | (3/3) | (2/3) | (3/3) | (3/3) | (0/3) | (2/3) | (3/3) | (1/3) | (3/3) |
| | 37°C | ND | 5.18 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| | | (0/3) | (1/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) |
| AFB1 | 22°C | ND | 14400, 16460 | 3655.5, 9627.7 | 598.5 | 516.7, 823.6 | 889.7, 1466.4 | ND | ND | 17870, 40350 | <LOQ | 3627.2, 31749 |
| | | (0/2) | (2/2) | (2/2) | (1/2) | (2/2) | (2/2) | (0/2) | (0/2) | (2/2) | (2/2) | (2/2) |
| | 30°C | <LOQ | 2287.8±308.8 | 936.4±780.5 | 35.0±28.5 | 579.0±660.8 | 653.4±836.9 | <LOQ | 35.8-160.3 | 8004.5±1563.4 | 15.7 | 6347.2±471.7 |
| | | (2/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (2/3) | (2/3) | (3/3) | (1/3) | (3/3) |
| | 37°C | ND | 42.1 | 23.6 | ND | ND | 1.37 | ND | ND | 12.6 | ND | ND |
| | | (0/3) | (1/3) | (1/3) | (0/3) | (0/3) | (1/3) | (0/3) | (0/3) | (1/3) | (0/3) | (0/3) |
| STERIG | 22°C | ND | 2.4, 2.7 | 2.7, 5.8 | 2.0 | 2.2, 2.6 | 2.1 | ND | ND | 4.5, 21.7 | ND | 4.7, 19.1 |
| | | (0/2) | (2/2) | (2/2) | (1/2) | (2/2) | (1/2) | (0/2) | (0/2) | (2/2) | (0/2) | (2/2) |
| | 30°C | ND | 21.0±7.6 | 3.4-3.8 | <LOQ | 8.8±4.9 | 3-9.2 | ND | <LOQ | 109.7±40.1 | <LOQ | 43.2±13.2 |
| | | (0/3) | (3/3) | (2/3) | (3/3) | (3/3) | (2/3) | (0/3) | (2/3) | (3/3) | (1/3) | (3/3) |
| | 37°C | ND | 1.06 | 0.61 | ND | ND | ND | ND | ND | 1.4 | ND | ND |
| | | (0/3) | (1/3) | (1/3) | (0/3) | (0/3) | (0/3) | (0/3) | (0/3) | (1/3) | (0/3) | (0/3) |
| OMST | 22°C | ND | 177.7±44.5 | 214.5±109.5 | 64.3, 681.4 | 156.4±156.2 | 76.5±62.7 | 177.9 | ND | 6282±4939.5 | 6.1, 6.4 | 677.8±840 |
| | | (0/3) | (3/3) | (3/3) | (2/3) | (3/3) | (3/3) | (1/3) | (0/3) | (3/3) | (2/3) | (3/3) |
| | 30°C | <LOQ | 366±126.5 | 53.7±45.2 | 41.2±35.9 | 146.1±64.2 | 39.1±37.6 | <LOQ | 5.2, 15.7 | 4674.9±2816.7 | 125.6 | 292.1±129.6 |
| | | (1/3) | (3/3) | (3/3) | (3/3) | (3/3) | (3/3) | (2/3) | (2/3) | (3/3) | (1/3) | (3/3) |
| | 37°C | ND | 80.7 | 204.5 | ND | ND | ND | ND | ND | 16.5 | ND | ND |
| | | (0/2) | (1/2) | (1/2) | (0/2) | (0/2) | (0/2) | (0/2) | (0/2) | (1/2) | (0/2) | (0/2) |

[a] Mycotoxin production determined at colony diameter ~80-90 mm, when the plate was completely covered by the colony; [b] Not detected; [c] Number of positives per total number of replicates; [d] Limit of quantification; [d] Both values are presented because of duplicate analysis.

**Table 5 Mycotoxin production (mean±SD µg/kg) of different *A. parasiticus* strains isolated from black pepper following the growth in malt extract agar at different temperatures.**

| Mycotoxin[a] | Temperature | UG AP821 | UG AP631 | UG AP61 | UG AP542 | UG AP46 | UG AP28 |
|---|---|---|---|---|---|---|---|
| AFG2 | 22°C | ND[b] (0/2)[c] | ND (0/2) | ND (0/2) | 33.1, 48.3[d] (2/2) | ND (0/2) | ND (0/2) |
| | 30°C | 1.4±0.3 (3/3) | 2.5, 2.5 (2/3) | 10.8±6.9 (3/3) | 301.6±155.8 (3/3) | 1.6, 3.0 (2/3) | 3.2±1.3 (3/3) |
| | 37°C | ND (0/3) | ND (0/3) | ND (0/3) | 5.79, 6.36 (2/3) | ND (0/3) | 4.6, 5.3 (2/3) |
| AFG1 | 22°C | ND (0/2) | ND (0/2) | ND (0/2) | 376.6, 447.4 (2/2) | ND (0/2) | ND (0/2) |
| | 30°C | ND (0/3) | 9.5 (1/3) | <LOQ[e] (2/3) | 185±708.5 (3/3) | <LOQ (1/3) | <LOQ (2/3) |
| | 37°C | ND (0/3) | ND (0/3) | ND (0/3) | 5.20 (1/3) | ND (0/3) | ND (0/3) |
| AFB2 | 22°C | ND (0/2) | 259.6 (1/2) | 652.0, 1281.5 (2/2) | 44.0, 44.2 (2/2) | 7.7, 8.5 (2/2) | 165.0, 1007.2 (2/2) |
| | 30°C | ND (0/3) | 3.14 (1/3) | 529.3±184.0 (3/3) | 667.7±246.2 (3/3) | 50.1 (1/3) | 133.6±85.5 (3/3) |
| | 37°C | ND (0/3) | ND (0/3) | ND (0/3) | 3.0, 21.7 (2/3) | ND (0/3) | ND (0/3) |
| AFB1 | 22°C | ND (0/2) | 2698.5 (1/2) | 3716.2-12002.1 (2/2) | 475.6, 637.6 (2/2) | 35.1, 98.9 (2/2) | 1022.3,8697.8 (2/2) |
| | 30°C | <LOQ (1/3) | 33.0-43.9 (2/3) | 5403.2±1475.5 (3/3) | 5103.3±1359.6 (3/3) | 1.2, 653.4 (2/3) | 1947.1±1231.6 (3/3) |
| | 37°C | ND (0/3) | ND (0/3) | ND (0/3) | 114.3, 307.7 (2/3) | ND (0/3) | ND (0/3) |
| STERIG | 22°C | ND (0/2) | 5.6 (1/2) | 1.8-23.1 (2/2) | 3.7, 4.8 (2/2) | 1.9-2.0 (2/2) | 4.3, 24.5 (2/2) |
| | 30°C | ND (0/3) | <LOQ (2/3) | 49.0±35.7 (3/3) | 58.5±15.0 (3/3) | <LOQ (1/3) | 15.4±5.4 (3/3) |
| | 37°C | ND (0/3) | ND (0/3) | ND (0/3) | 0.42, 0.80 (2/3) | ND (0/3) | ND (0/3) |
| OMST | 22°C | ND (0/3) | 110.6, 188.9 (2/3) | 725.1±548.9 (3/3) | 49.4±2.5 (3/3) | 20.0±17.7 (3/3) | 204.8±265.0 (3/3) |
| | 30°C | 0.3, 2.7 (2/3) | 0.9, 10.2 (2/3) | 251.1±127.4 (3/3) | 1096.2±239.9 (3/3) | 1.7, 17.6 (2/3) | 195.7±79.3 (3/3) |
| | 37°C | ND (0/2) | ND (0/2) | ND (0/2) | 33.1 (1/2) | ND (0/2) | ND (0/2) |

[a] Mycotoxin production determined at colony diameter ~80-90 mm, when the plate was completely covered by the colony;

[b] Not detected; [c] Number of positives per total number of replicates; [d] Both values are presented because of duplicate experiments

or two positive replicates; [e] Limit of quantification.