Detection of Aflatoxigenic Fungi and Toxins from Export Grade Peanut Samples: Comparison of qPCR and LC/MS Techniques

Application Note

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Abstract

Aflatoxins are toxic secondary metabolites produced by various species of *Aspergillus* fungi and have gained global significance as a result of their deleterious effects on human and animal health. In this study, we detected both the toxin pathway gene, O-Methyltransferase (*omt-1*), of *A. flavus* as well as the toxin, aflatoxin B1, produced on peanut seeds by using probe-based qPCR and LC/MS assays, respectively, by conducting a time-course experiment. The peanut samples were artificially infected with fungal spores, and sampling was performed at different time points. The results revealed that the aflatoxin amount was well within the minimum residue level limit (MRL) specified by EU regulation up to Day 1 after fungal inoculation, but exceeded the MRL from Day 2 onwards. Similarly, the quantification cycles (Cq) for the *omt-1* gene also decreased in a time-dependent manner, indicating the fungal growth on peanut. A time-dependent increase of DNA copies and the amount of secondary metabolite was evident. These results suggest that combination of the qPCR and LC/MS methods provide confidence for critical evaluation of peanut consignments.

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Introduction

Agricultural crops such as peanuts, corn, figs, and cereals are subject to fungal infection during pre- and post-harvest stages under certain conditions[1]. The fungi belong to the genera *Aspergillus*, which produce toxic secondary metabolites known as aflatoxins. These aflatoxins have received considerable global attention because they cause severe health hazards at trace levels and pose significant difficulties for international trade. Early detection of these toxic metabolites is important to prevent food contamination during storage or transport, and thus reduce the economic impact caused by rejection of consignments[2]. Conventional methods are time-consuming, require mycological expertise, and may result in false positives. Several methods have been developed by targeting the aflatoxin pathway genes using qPCR[3,4]. Similarly, LC/MS-based detection of the toxic metabolites has also been well established[5]. However, it has frequently been observed that export-quality peanut samples that tested negative for aflatoxins at the exporting port by certifying agencies using LC/MS were found to be positive for aflatoxins by the importing authorities, resulting in consignment rejections. This might be attributed to the presence of aflatoxins in quantities lower than threshold levels due to trace numbers of fungal spores, which establish and produce higher levels of the toxins later. To address this issue, we performed a time-course study analyzing both the *A. flavus* aflatoxin-producing pathway gene O-methyltransferse (*omt-1*) and the secondary metabolite of interest (aflatoxin B1) by using probe-based qPCR and LC/MS techniques, respectively, as a more effective strategy compared to a single technique.

Experimental

Reagents and materials

The aflatoxin-producing strain of *A. flavus* was obtained from University of Mysore, Mysore, India. The qPCR probes and primers were purchased from Sigma. The Brilliant III Ultra-Fast QPCR Master Mix for qPCR experiments was procured from Agilent Technologies. The LC/MS grade methanol was purchased from Fluka (Germany). Milli-Q water (Millipore Elix 10 model, USA) was used for mobile phase preparation. Ammonium formate was procured from Fluka (Germany). The standard, aflatoxin B1, was purchased from Sigma.

Fungal treatment to seed sample

Healthy peanut seeds were inoculated with *A. flavus* at a spore load of 1×10^8 cfu/g, and incubated at 24 ± 2 °C for 5 days. The experiment was conducted in triplicate. Samples were harvested at different time points of 0, 1, 2, 4, and 5 days post-inoculation with the fungus, and stored at –80 °C until further analysis.

gDNA extraction and qPCR assay

DNA from the infected peanuts was extracted using Qiagen DNeasy plant mini kit with slight modifications as described earlier[4]. Briefly, the seeds were powdered using a mortar-pestle in the presence of liquid nitrogen. The powder was dissolved in digestion buffer AP1 (Qiagen) and subjected to zymolase (Zymo Research Corp) treatment for 1 hour. Genomic DNA was isolated as per the manufacturer's instructions. Purified DNA was quantified using NanoDrop 1000 (Thermo Scientific) and Qubit (Invitrogen). The probe-based qPCR protocol targeting the conserved regions of the *omt-1* gene was used for detecting the *A. flavus* gDNA, as described earlier[6]. The sequences of the primers and probe used for qPCR amplification are provided in Table 1. Real-time quantitative PCR was performed by adding 10 µL of 2x Brilliant III ultra-fast QPCR Master Mix (p/n 600880) to each well containing 10 ng of infected peanut template DNA, 600 nM forward and reverse primers, and 375 nM *omt-1* probe in a 20-µL reaction volume using an Agilent Mx3005P qPCR system. After an initial polymerase activation step at 95 °C for 3 minutes, the cycling profile was 95 °C for 20 seconds, 52 °C for 20 seconds, and 60 °C for 20 seconds for 40 cycles of amplification. The measurement was performed in triplicate and the experiment was repeated three times. No Template Controls (NTC) were maintained in each run to detect DNA contaminations. The amplicon sizes of the resulting PCR products were analyzed using the Agilent 2100 Bioanalyzer system and a DNA 1000 assay, according to the manufacturer's instructions.

Table 1. Primers and probe sequences used for qPCR experiment.

Primers and probes	Nucleotide sequences (5′–3′)
Foward-*omt-1*	GATCGTAGGTAATCCACTC
Reverse-*omt-1*	GGTAGACTGCTGAAATCA
omt-1 probe	[HEX]-CCACTGGTAGAGGAGATGT-[BHQ1]

Aflatoxin extraction procedure for LC/MS experiments

One gram of infected peanut samples was crushed in 10 mL of acetonitrile/water (84:16 v/v) solvent. The solution was shaken for 90 minutes at 50 rpm at ambient temperature. Later, the solution was subjected to solid phase dispersive cleanup. Eight hundred microliters of the supernatant were transferred to a 1.5-mL microfuge tube and 200 mg of C18 ODS bulk sorbent (p/n 5982-1182) was added. The solution was vortexed for 1 minute and centrifuged at 14,000 rpm for 5 minutes. One microliter of the supernatant was injected onto an Agilent 1290 Infinity LC System interfaced to an Agilent 6460 Triple Quad LC/MS system with Agilent Jet Stream Source (AJS). Water with 8 mM ammonium formate and methanol were used as mobile phases A and B, respectively. The MS acquisition and chromatographic parameters followed for LC/MS runs are provided in Table 2. The aflatoxin B1 diluted with extraction solution served as standard.

Table 2. Instrument parameters for the LC/MS experiment.

Agilent 1290 Infinity Series LC System instrument parameters	
Column	Agilent ZORBAX Extended C18, 2.1 × 50 mm, 1.8 µm
Flow rate	0.2 mL/min
Mobile phase A	8 mM ammonium formate in water (53 %)
Mobile phase B	Methanol (47 %)
Injection volume	1 µL
Run time	3 minutes
Agilent 6460 Triple Quad MS parameters	
Ion source	AJS ESI
Ion polarity	Positive
Drying gas temperature	275 °C
Drying gas flow rate	10 L/min
Nebulizer pressure	45 psig
Sheath gas temperature	300 °C
Sheath gas flow rate	11 L/min
Capillary voltage	4,000 V
Nozzle voltage	0 V
Fragmentor	130 V
delta EMV	400 V
MRM parameters	
Precursor ion	313.2 (Quantifier)
Product ion	241.2 (Quantifier) 269.1 (Qualifier)
Collision energy	31 V
Dwell time	25 ms

Results and Discussion

The artificial inoculation of aflatoxigenic *A. flavus* onto peanuts followed by time-course assessment of gDNA by qPCR as well as its secondary metabolite, aflatoxin B1, by LC/MS was done in this study. The inoculated seed samples, when quantified by qPCR, resulted in a Cq of 35 immediately after inoculation. A drastic decrease in Cq to 29 was noticed on Day 1 after inoculation (Figures 1 and 2). The 2-day incubated samples showed Cq of 25.5, revealing the luxuriant growth of fungi on peanuts. The qPCR amplicons revealed a single band at the expected size on the 2100 Bioanalyzer instrument. The intensity of the product peak in the electropherograms showed a steady increase, with each day reflecting the increased amount of starting template found in the samples (Figure 3). A similar trend of increase in aflatoxin B1 from these samples was evident when detected by LC/MS. The amount of aflatoxin B1 was 4.4, 5.1, 16.8, 25.5, and 26.6 ppb at 0, 1, 2, 4, and 5 days after inoculation, respectively (Figure 4). The MRL for total aflatoxins specified by EU regulations is 10 ppb, and this limit was crossed on the second day of incubation. These results clearly show the correlation between the presence of increased amounts of fungal *omt-1* DNA and increased amounts of secondary metabolite aflatoxin B1 on peanuts upon incubation.

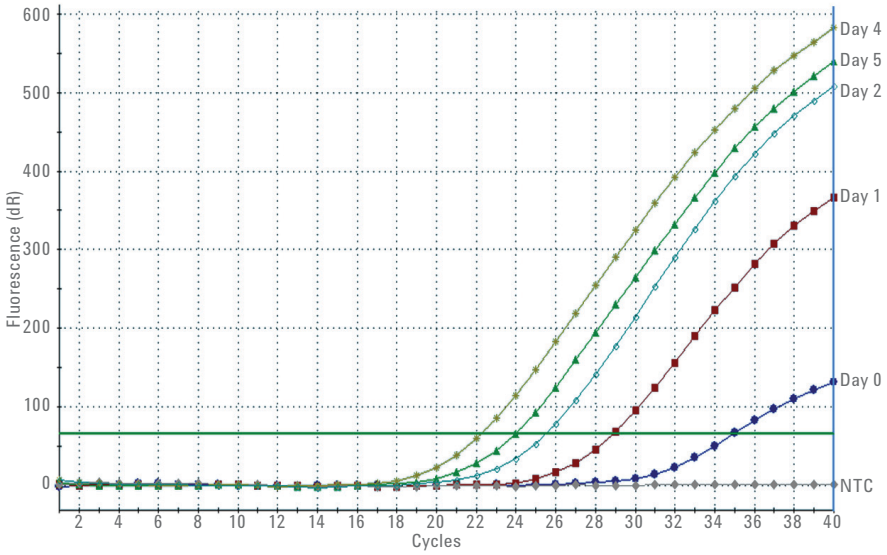

Figure 1. Amplification plot for infected peanut samples at different time intervals.

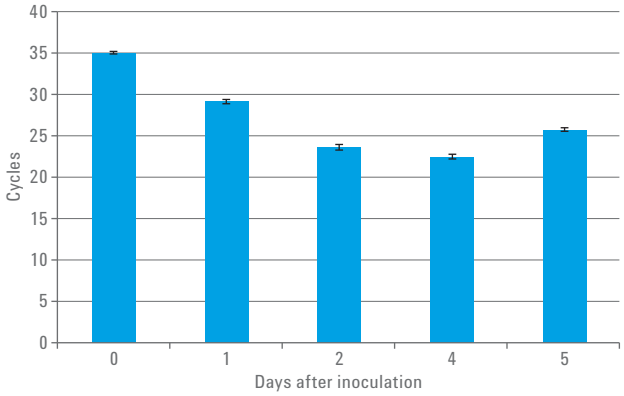

Figure 2. Cq values for *omt-1* gene amplification by qPCR for samples harvested at different time points. Error bars = standard deviation.

Conclusions

This study reveals that the presence of minor populations of aflatoxigenic fungi could grow on the surface of peanut seeds and produce toxins above the minimum residue limits during transit. Although initially, the load of aflatoxin B1 is below EU regulated thresholds, continued incubation of a shipment during transport ultimately results in rejection of the consignment at the port of entry in the EU. Real-time qPCR and LC/MS results for the time-course experiment show correlation between the detection of *omt-1* gDNA and aflatoxin B1, making qPCR-based detection a sensitive, potentially complementary method in monitoring food commodity shipments for aflatoxigenic fungi.

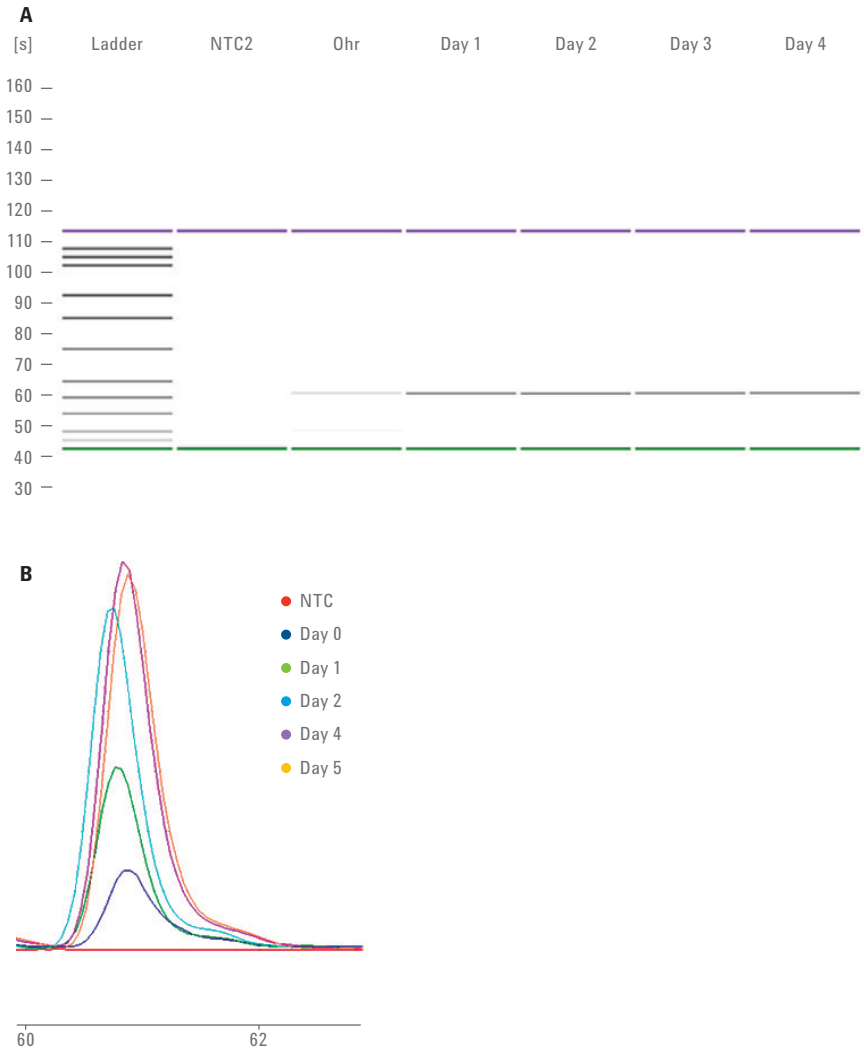

Figure 3. A) Gel-like image showing amplified *omt-1* gene products of size 185 bp from the Agilent 2100 Bioanalyzer system analysis. B) Electropherogram trace overlay of *omt-1* gene qPCR products from different time points on an Agilent 2100 Bioanalyzer system.

Acknowledgements

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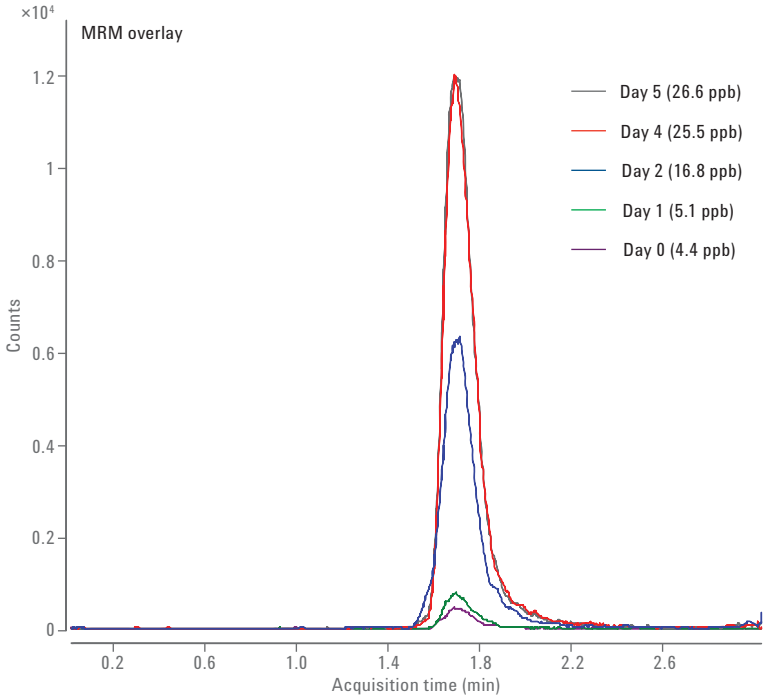

Figure 4. LC/MS analysis of aflatoxin B1 levels at different time points after inoculation of the toxigenic fungi into peanuts.

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