

Effect of Moisture on Aflatoxins Production of Isolated *Aspergillus Flavus* from Vietnamese Peanut

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Received: May 25, 2017; Accepted: May 25, 2018

Abstract

Aflatoxins are known as toxic chemical compounds having serious effect on animal's and human's health. Aflatoxins are prevalent in various foods in Vietnam. This study is conducted to demonstrate the effect of moisture on Aflatoxins production in peanut. Peanut kernel with 6%, 8%, 10%, 12% and 14% moisture are injected with 10^3 CFU/g of strain *A. flavus* TUHT115 then packaged in sealed PVC bag and kept at 25°C in 4 months. The mold growth and Aflatoxin (AF) production are checked every two weeks. As the results, peanut sample with 6% and 8% moisture are not contaminated with AF after 4 months study; the samples with moisture 10%, 12% and 14% are detected to be contaminated with AF after 2 weeks study and only AFB1 & AFB2 are detected in studied samples. AFB1 contaminated level is higher than maximum permissible level (8µg/kg) as regulated in QCVN 8-1:2011/BYT after 4th week and total AF is higher than maximum permissible level (15µg/kg) after 6th week. This study also revealed that peanut moisture content of level from 10% and more are accumulated AFB1 and total AF have linear relative with moisture and time.

Keywords: peanut, peanut moisture, peanut preservative, Aflatoxin, *A. flavus*

1. Introduction

Aflatoxins are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. *A. flavus* grows and produces Aflatoxins (AFs) mainly in cereals, grains and oil-seed crop, [1] in which peanut is the preferred substrate for aflatoxins production and growth of *A. flavus* [2] and produces most AFB1 [3]. Aflatoxins including AFB1, B2, G1, G2 and M1 are classified into group 1 carcinogenesis substances by International Agency for Research on Cancer (IARC). In naturally contaminated foods, aflatoxins B1 and B2 or aflatoxins G1 and G2 usually occur together; however, B2 and G2 are less biologically active and there is limited or inadequate evidence of their carcinogenicity in experimental animals, respectively. Aflatoxin G1 is less mutagenic than aflatoxin B1. Aflatoxin M1 occurs almost exclusively in milk and milk products and is less carcinogenic than aflatoxin B1 [4].

Moisture is an important factor having effect on *A. flavus*' growth and producing Aflatoxins in natural substrate. H.G Chang and P. Markakis have shown that aflatoxin traces at 16.5%, a maximum accumulation of aflatoxin was observed in cultivars of barley with moisture range 28-31% [5]. Maize

grains conditioned to an initial moisture content of 10.5 and 13.5% were inoculated with a known quantity (50:50) of *A. flavus* and *A. parasiticus* the study over a period of four weeks, the aflatoxin accumulation were quantified by HPLC. The findings have shown that, maize grains dried to moisture level of 11% (on wet weight basis) can be stored for longer periods of time under environment conditions 25±3°C, 70 - 75% RH (Relative humidity) devoid of fungal's growth and the accumulation of aflatoxin [6]. Moisture content for safe storage (no mold growth) of seeds and other substrates generally has been established at substrate moisture in equilibrium with 70%RH, vegetative growth of *A. flavus* was limited by 80%RH, which is equivalent to 9% kernel moisture content in peanuts [1].

The influence of moisture content on aflatoxin production by *A. flavus* carried out in four months to determine the best preservative moisture in peanut kernel and to seek for relationship between Aflatoxins producing with moisture content in peanut kernel in the present study.

2. Experimental

2.1. Materials and facilities

Clean kernel peanut collected from Bac Giang province of Vietnam and selected without molds infection for study.

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Aspergillus flavus TUHT115 strain isolated from aflatoxin infected peanut collected from Bac Giang province (the aflatoxin production strain confirmed by PCR, generation sequencing, and tested aflatoxin accumulation in peanut).

PVC bag 200x150mm dimension put 100g specimen.

Dryer oven temperature $105 \pm 2^\circ\text{C}$.

Incubator kept at 25°C .

Routine facilities in laboratory.

2.2. Chemicals and reagents

Aflatoxin standards B1, B2, G1, G2 purchased from Supelco (mix standards can be used)

HPLC-grade acetonitrile, methanol, benzene, chloroform, n-hexan, amoni acetate and acetone were purchased from Meck.

Analytical grade Sodium chloride

Purify demineralized water

2.3. Facilities

LC-MS/MS TRIPLE QUAD™ 5500 OF AB SCIEX with reverse phase column C18 (Water: 150 mm x 4.6 mm x 5 μm),

Solid phase extract (SPE) with vacuum pump, filter membrane 0.45 μm .

Balance, accuracy ± 0.1 mg.

Analytical balance, accuracy ± 0.01 g.

Sample homogeneous machine.

Horizontal vibrating machine.

Ultra sound

Vacuum evaporating machine

2.4. Methods

Moisture in the kernel peanut is determined by dryer in accordance with TCVN 2384 -93: Peanut in shell and peanut kernels - Method of test [7].

Aflatoxin determination by LC-MS/MS, in-house method of National Institute for Food Control which accredited in accordance with TCVN ISO/IEC 17025:2007.

Moisture judgement calculation:

$$\frac{m_1}{m_0} = \frac{w_2 - w_0}{w_1 - w_2}$$

In which:

m_1 : the water need to added (g/ mL)

m_0 : initial peanut weight (g)

w_0 : initial moisture of peanut (%)

w_1 : water moisture = 100 (%)

w_2 : target moisture need to adjust to (%)

Peanut samples are prepared to moisture level of 6, 8, 10, 12%, and infected with 10^3 CFU *A. flavus*/g.

Samples are kept in $25 \pm 1^\circ\text{C}$ incubator.

Every 2 week, samples are checked Aflatoxins accumulation and *A. flavus* growth.

Control sample 1 (C1): 100g/sealed sample bag without *A. flavus*, storage in ambient air.

Control sample 2 (C2): 100g/sealed sample bag which is infected 10^3 CFU *A. flavus*/g, storage in ambient air.

Samples are packaged 100g/bag. Samples are coded as M11, M12, M13, M14 and M15 with moisture level of 6%, 8%, 10%, 12% and 14% equivalence as Fig. 1.



Fig. 1. Sample packed for study

Maximum AFs content are compared with QCVN 8-1:2011/BYT [8].

The Aflatoxins data are used linear model for simple Linear Regression analysis by **R** software, version 3.3.3 [10]. Which dependent variables are Aflatoxin B1 (AFB1) and AF total; Moisture (Moi) and Time (Week) are independent variables.

3. Results and discussion

3.1. The growth of *Aspergillus flavus* in different moistures of peanut sample

The *A. flavus* mold growth is observed every 2 weeks. The mold color change from pale yellow, green yellow, green to dark green as Fig. 2.

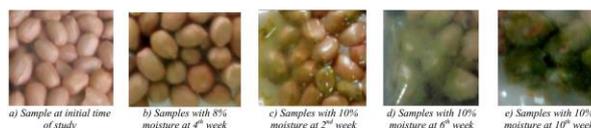


Fig. 2. *Aspergillus flavus* growth and its color changes on peanut sample

As the results:

- The control samples C1 & C2 with peanut moisture of 3.22%, the mold growth is not observed.
- The peanut sample with 6% moisture, the mold growth is not observed in first 2 weeks, there are some mold growths in pale yellow in the 4th week, then turn to green yellow in the following weeks.
- The peanut with 8% moisture, the mold growth is not observed in first 2 weeks, there are some mold growing in green yellow in the 4th week and not/becoming matured more in the following weeks.
- The peanut samples with moisture of 10%, 12% and 14%, the mold grows in pale yellow at the first 2 weeks, changes into green yellow in the 4th week and gradually changes to green then dark green in the following weeks.

3.2. The accumulation of Aflatoxins in peanut samples

In addition to *A. flavus* growth checking, AFs analysis is carried out every 2 weeks. The chromatogram of AFB1 standards and samples after 2 weeks is presented in Fig. 3 as follows:

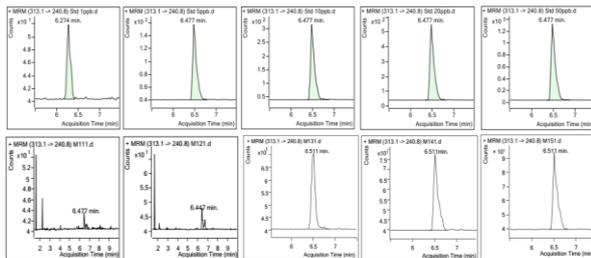


Fig. 3. AFB1 chromatogram of the samples after 2 weeks study

The chromatogram of AFB2 standards and samples after 6 weeks is presented in Fig. 4 following:

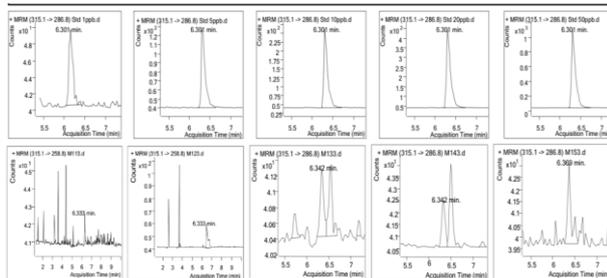


Fig. 4. AFB2 chromatogram of the samples after 6 weeks study

The *A. flavus* growth and AFs accumulation are summarized in Table 1.

The results have shown that:

- Control sample C1 & C2 are not detected AFs after 16 weeks study.
- Samples with 6% and 8% of moisture content are not detected AFs after 16 weeks study.
- Only detect AFB1 & AFB2 in samples with moisture of 10%, 12% and 14%. AFG1 & AFG2 are not detected in studied samples. AFB1 and AF total accumulation in samples with moisture of 10%, 12% and 14% are expressed in Fig. 5.

Table 1. Aflatoxins accumulation and color

Sample code	Parameter	Aflatoxins (µg/kg) at study time (Week)							
		2	4	6	8	10	12	14	16
C1/C2	AFB1	ND	ND	ND	ND	ND	ND	ND	ND
C1/C2	AFB2	ND	ND	ND	ND	ND	ND	ND	ND
C1/C2	Total AF	ND	ND	ND	ND	ND	ND	ND	ND
M11	AFB1	ND	ND	ND	ND	ND	ND	ND	ND
M11	AFB2	ND	ND	ND	ND	ND	ND	ND	ND
M11	Total AF	ND	ND	ND	ND	ND	ND	ND	ND
M12	AFB1	ND	ND	ND	ND	ND	ND	ND	ND
M12	AFB2	ND	ND	ND	ND	ND	ND	ND	ND
M12	Total AF	ND	ND	ND	ND	ND	ND	ND	ND
M13	AFB1	4.28	10.97	16.69	20.80	26.28	26.73	26.39	25.27
M13	AFB2	ND	ND	0.42	0.99	1.65	3.08	3.87	6.69
M13	Total AF	4.28	10.97	17.11	21.79	27.93	29.80	30.26	31.96
M14	AFB1	5.19	12.84	18.45	34.35	45.41	48.64	51.72	53.79
M14	AFB2	ND	ND	0.69	1.52	1.79	3.88	4.51	6.63
M14	Total AF	5.19	12.84	19.14	35.88	47.20	52.52	56.23	60.41
M15	AFB1	6.68	15.70	36.03	47.64	57.62	61.51	62.99	63.69
M15	AFB2	ND	0.30	0.76	1.60	1.85	3.91	5.98	7.51
M15	Total AF	6.68	16.00	36.79	49.25	59.47	65.42	68.97	71.19
ML-AFB1		8	8	8	8	8	8	8	8
ML-Total AF		15	15	15	15	15	15	15	15

Note: ML-AFB1 - Minimum limit of AFB1.

ML-Total AF - Minimum limit of Total aflatoxins

ND – Not detected

AFs analysis results revealed that:

- Control sample C1 & C2 are not detected AFs after 16 weeks study.
- Peanut samples with 6% and 10% moisture are not detected AFs after 16 weeks study.
- The samples with moisture level of 10%, 12% and 14% are produced AFB1 after 2 weeks, AFB2 is produced after 4 weeks with 14% moisture samples and 6 weeks with sample moisture level of 10% to

14%. Not detect AFG1 & AFG2 in the studied samples. AFB1 accumulation is higher than ML-AFB1 (>8µg/kg) in accordance to QCVN 8-1:2011/BYT.

- The samples with moisture level of 10%, AFB1 accumulation are over 2 times compare with ML-AFB1 after 6 to 8 weeks, then over 3 times after 10 to 16 weeks. AF total is higher than ML-Total AF (>15µg/kg) in accordance to QCVN 8-1:2011/BYT after 6th to 16th week.

- The samples with moisture level of 12%, AFB1 accumulation is over 2 times compare with ML-AFB1's after 6 to 8 weeks, then over 3 times after 10 to 16 weeks. AF total is higher than ML-Total AF (>15µg/kg) after 6th week, then over 2 times after 8th week, over 3 times after 10th to 14th week and over 4 times after 16th week.

- The samples with moisture level of 14%, AFB1's accumulation are over 2 times compared with ML-AFB1 after 4th week, then over 4 times after 6th week, then over 5 times after 8th week, then over 7 times after 10th to 16th week. AF total is higher than ML-Total AF (>15µg/kg) after 4th week, then over 2 times after 6th week, over 3 times after 8th to 10th week and over 4 times after 12th to 16th week.

- AFs' accumulation accelerate from 2 to 10 weeks then increases slowly in the following weeks, this may due to the lack of oxygen in the pack and the reduction of nutrition for mold.

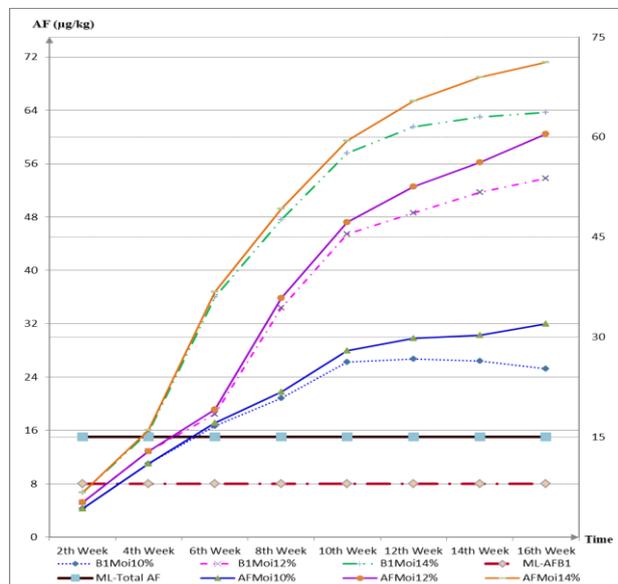


Fig. 5. Aflatoxins accumulation in different moisture content samples at every 2 weeks

Note: B1Moi10% - AFB1 in sample 10% moisture

AFMoi10% - AF total in sample 10% moisture

The results in Table 1 have shown that at 25°C, peanut samples moisture level of 10% and up to 14% starts to accumulate AFs after 2 weeks and *A. flavus*' color is green yellow after 2 weeks and became dark green from the 8th weeks.

Linear regression analysis the relationship between AFB1's and AF's total accumulations with moisture (%) and time (Week) by R program. AFB1 & AF total are dependent variables while moisture and time is independent variables. By using lm function, the p-value is <0.05 so the relationship is linear relative.

As the results, peanut samples with different moisture level and time are accumulated different AFB1 content, and linear regression equation (1) is:

$$y_{AFB1} = 6.08 * Moi + 3.21 * Week - 69.32 \quad (1)$$

This equation can predict when moisture increases 1%, the AFB1's accumulation increases 6.08 µg/kg, and the AFB1's accumulation increases 3.21 µg/kg when time is longer than 1 week.

Linear regression equation (2) expresses relationship between AF total with moisture and time as follows:

$$y_{AFtotal} = 6.24 * Moi + 3.69 * Week - 73.24 \quad (2)$$

Equation (1) is appropriate when peanut with atleast 10% of moisture content and infected *A. flavus* after 2 weeks. Equation (2) is appropriate when peanut with atleast 10% of moisture content and infected *A. flavus* after 3 weeks.

4. Conclusion

Peanut with moisture level of 6% and 8% can prevent *A. flavus* from growing and aflatoxin from producing in sealed package.

A. flavus TUHT115 strain in peanut substrate can produce AFB1 after 2 weeks and produce AFB2 from 4th week. No AFG1 & AFG2 is produced after 16 weeks study.

Peanut with moisture level of 10%, 12% and 14% storage at 25°C and *A. flavus* infected of 10³CFU/g, the AFB1 accumulation is higher Maximum permissible level of AFB1 after 4th week, total AFs level is higher than maximum permissible level after 4th with sample moisture of 14% and after week 6th with sample moisture of 10% & 12%.

Moisture and time have linear relative with AFB1 and AF total accumulation when peanut has moisture from 10% and more.

Peanut for storage shall be sorted and kept at moisture level less than 10% to inhibit the growth and aflatoxin production of *A. flavus*.

Acknowledgements

This work was supported by research program of the National Institute for Food Control on factors influencing *A. flavus* growth and Aflatoxins production.

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