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Analysing Aflatoxin Production Conditions in Feed Samples Using a Preparative Thin Layer Chromatography (TLC) Method

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Abstract

In this research, aflatoxins were produced, extracted, isolated, and purified in order to optimize the storage conditions of feed. Using a preparative thin layer chromatography (TLC) method, with commercially available plates of 1.5 to 2.0 mm, B1 aflatoxin was isolated from the feed samples of whole wheat, maize, and crushed rice, and the procedure was repeated four times. A purity value of 99 % for B1 aflatoxin was achieved and tested using spectrophotometric and chromatographic methods. As solvents, acetone:water (85:15) was used for aflatoxin extraction from the feed sample, whereas methanol: water (50: 50) was used for trichothecenes extraction. The primary findings of this research indicate that B1 aflatoxin reached a maximum yield when coarsely crushed rice was inoculated with *Aspergillus parasiticus* at a temperature of 30C°, moisture level of 35%, and incubation duration of 7 days. The observations obtained from aflatoxins production process provide an insight regarding storage conditions that need to be avoided in order to reduce the incidents of feed contamination.

Keywords: B1 aflatoxin, *F.sporotrichoides*, *A.parasiticus*, TLC method

تحليل ظروف انتاج الافلاتوكسين في عينة العلف باستخدام طريقة كروماتوغرافيا التحضيرية ذات الطبقة الرقيقة (TLC)

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الخلاصة

في هذا البحث تم إنتاج الأفلاتوكسينات واستخلاصها وعزلها وتنقيتها لغرض تحسين ظروف خزن العلف. باستخدام طريقة كروماتوغرافيا الطبقة الرقيقة التحضيرية (TLC) والألواح المتاحة تجارياً من 1.5 إلى 2.0 مم، تم عزل سموم B1 من عينات العلف التي شملت القمح والذرة والرز، وتكرر الإجراء أربع مرات. تم تحقيق (99%) من نقاء B1 aflatoxin واختباره باستخدام طرق القياس الطيفي والكروماتوغرافي. المذيب المستخدم في هذا البحث لاستخلاص السموم الفطرية هو الأسيتون: ماء (85: 15 لتر)، وبالنسبة لمركبات الترايكوثيسين، تم استخدام مذيب ميثانول: ماء (50:50). أشارت النتائج الأولية لهذا البحث إلى أن B1 aflatoxin وصل إلى الحد الأقصى من المحصول عند تلقيح *Aspergillus parasiticus* على أرز مطحون بشكل خشن عند درجة حرارة 30 درجة مئوية، ومستوى رطوبة 35%، ولمدة حضانة 7 أيام. ان الملاحظات التي تم الحصول عليها من عملية إنتاج aflatoxins توفر رؤية عن ظروف الخزن التي يجب

تجنبها لتقليل فرص حدوث تلوث العلف. ان الملاحظات التي تم الحصول عليها من عملية انتاج aflatoxins توفر رؤية عن ظروف الخزن التي يجب تجنبها لتقليل فرص حدوث تلوث العلف.

1. Introduction

Agricultural and food industries have been scaling up the techniques needed to meet the market demands for food products [1]. For instance, poultry relies on innovative modifications in food processing to provide the consumers' needs and the industry's demands [1, 2]. Therefore, there is a need to produce quality feed in terms of its nutrient's composition and absence of toxic substances [3, 4]. Nonetheless, due to their moisture content, compound feeds and feed ingredients are susceptible to the growth of fungus. Hence, there is a need to improve the storage conditions in order to avoid the formation of mycotoxins [5-6].

Fungus can form on feed at different phases of handling, production, processing, and harvesting [7]. It can also be damaging to feed in storage. Feeds co-contamination with various mycotoxins is a very common situation. Some of these mycotoxins include aflatoxins, T-2 toxin, and ochratoxin A, which can have harmful effects on poultry, livestock, and consequently, human beings [8, 9, 10]. B1 aflatoxin is the most potent hepatotoxin among aflatoxins (AF), because it has severe and carcinogenic effects on poultry [7, 11]. Ingestion of aflatoxin can lead to aflatoxicosis, which is an acute and chronic condition that can cause reduction in feed efficiency, and feed consumption, as well as increased bruising, immunosuppression, and mortality [4]. Due to its residual toxic effect, Aflatoxin B1 is the most dangerous contaminant [10]. Feeds contamination with AF has been well documented. It was found that its level ranged from 10 to 1500 ppb in commonly used ingredients and from 34 to 115 ppb in mixed feed samples. Such elevated load of contamination may not necessarily cause intense aflatoxicosis; yet, in terms of health and productivity, it may lead to high economic loss [1]. In addition, *Fusarium sporotrichoides* can produce a toxin known as T-2 which can be categorized under mycotoxins [12, 13]. This toxin can cause damages in the reticuloendothelial system in broilers, and decrease their body weight [13]. In order to reduce such incidents, this research aims to shed light on the storage conditions which lead to feed contamination by analysing the conditions of aflatoxins production in feed samples.

2. Materials and Methods

2.1 Subculture Production

Agar media of oat-meal and potato dextrose were prepared and 20 ml of each was divided between test tubes in order to produce subcultures. The media were autoclaved for 20 minutes at 121 C° (15 psi). The tubes remained slanted until the medium was solidified. Under aseptic conditions, slants were inoculated with *A. parasiticus* to merge with the parent culture spores of fungi. The subcultures remained at room temperature for 2 weeks in the dark in order to help fungus sporulate. The resulting subcultures were used to produce culture material (B1 aflatoxin).

2.2 B1 Aflatoxin Culture Material Production

B1 aflatoxin was obtained using rice and relying on the method of subculture production described by Shotwell *et al.* (1966) [14]. Cold water was added to crushed rice (30 grams) in conical flasks (250 ml) in order to reach a moisture of 35%. Aluminium foil and non-absorbent cotton plug were used to seal the flasks which were left to soak overnight, followed by autoclaving for 20 minutes at 121 C° (15 psi). They were then left to cool and shaken in order not to allow the formation of clumps. The fungal spores (*A. parasiticus*) were gathered from the previously prepared slants by adding Triton-X (0.05%) to sterile distilled water (5 ml) to help recover spores. Spore suspension (1.0 ml) was added to the rice flasks, which were shaken to uniformly distribute the spores and left for 7 days at 30 C° in a biological oxygen demand (BOD) incubator. By the 2nd day of incubation, white mycelial growth started to show on the rice. By the 7th day, it gradually turned to dark green. These flasks were

autoclaved for 20 minutes at 121 C° and 15 psi. The culture material was collected on aluminium foil and left to dry overnight for 12 hours. It was then grounded to fine powder and stored in a dim room. Later, the concentration of B1 aflatoxin was examined using TLC based on AOAC (1995) [15].

2.3 Effects of Different Substrates on the Production of B1 Aflatoxin

In order to evaluate the impact of various feed types on B1 aflatoxin production, coarsely crushed wheat and maize were inoculated with *A. parasiticus* to test their effects. The flasks were transferred into the BOD incubator at moisture of 35% and temperature of 30 C°. They were harvested after seven days and examined to find the exact concentration of B1 aflatoxin using TLC, as described by AOAC (1995) [15].

2.4 Incubation Period Effect on the Production of B1 Aflatoxin

To examine the way by which the incubation period could affect the production of B1 aflatoxin on coarsely crushed rice, conical flasks (250 ml) filled with rice (30 gm) were inoculated with spore suspension (1.0 ml) and incubated at 30 C° in the BOD incubator. Moisture was kept at 35% using tap water. These flasks were harvested on the third, seventh, and ninth days and examined to find the exact concentration of B1 aflatoxin using TLC.

2.5 Moisture Effect on the Production of B1 Aflatoxin

The percentages of moisture used in this experiment were 25, 35, and 45 % for the rice in the conical flasks. The effects of changing moisture content on the production of B1 aflatoxin were tested by following the same steps described above, with changing the percentage of moisture.

2.6 Extraction, Purification, and Isolation of B1 Aflatoxin

The extraction of B1 aflatoxin relied on Romer (1975) [16]. The isolation and purification processes were conducted using the chromatographic method for a preparative thin layer. A volume of 200 µl of the extracted B1 was added in spots, 2 cm apart, on the plates of preparative TLC. These plates were developed using (85:15) chloroform:acetone in an unequilibrated chamber and left to dry. They were viewed using 365 nm UV-light. Aflatoxins were categorized according to molecular weight and R_f value. At 0.31 R_f value, B1 aflatoxin showed bluish fluorescence. It was carefully scrapped out using silica gel. The toxin in the silica gel was dissolved for 30 minutes in chloroform solvent (10 ml for each 2 g of silica gel). Using sintered glass filters, the resulting gel was filtered. A filtrate was noticed for the second time on the TLC preparative plates during the purification. In order to get the maximum purification, these steps were repeated 4 times.

2.7 B1 Aflatoxin Quantification

B1 aflatoxin concentration after purification was examined using the TLC method at different volumes, starting from 5 µl to 40 µl, and visually compared to authentic B1 aflatoxin from Sigma Standard, Merck, India. The purified aflatoxin solution was developed using (85:15) chloroform:acetone and analyzed based on AOAC, (1995) [15].

2.8 Purity Determination

The chromatographic method was used to determine the purity of the solution based on Rodricks and Stoloff (1970) using Merck® TLC plate which is commercially available with a Sigma standard of 5µl resolution reference [17]. The resolution reference standard (5µl) and B1 aflatoxin solution (5µl) were observed at intervals of 2 centimetres.

3. Results and Discussion

The findings obtained after producing, isolating, extracting, and purifying B1 aflatoxin are specified in Figures 1 and 2.

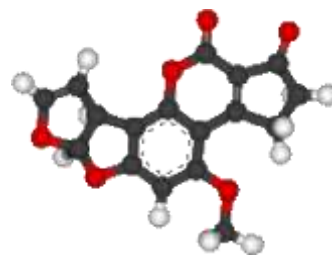
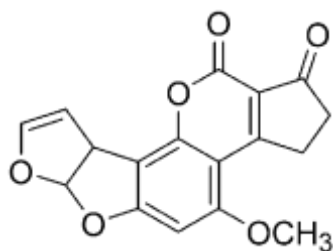


Figure 1-2D structure of the purified B1 aflatoxin. **Figure 2**-3D structure of the purified B1 aflatoxin.

3.1 Parent Culture Maintenance

F. sporotrichoides and *A. parasiticus* parent cultures were obtained from an agar of oatmeal and agar of potato dextrose at refrigerated conditions below 7 °C (figure -3).



Figure 3-Subcultures and parent cultures (middle) of *Fusarium sporotrichoides* (right) and *Aspergillus parasiticus* (left)

3.2 *F. Sporotrichoides* and *A. Parasiticus* subcultures Production

F. sporotrichoides was subcultured on the agar of oatmeal under aseptic conditions at room temperature in a dark space, using the parent culture. No growth was spotted on the first day. On the second day, white mycelial growth was noticed. It turned pink by the fourth day. Sporulation was also noticed. It turned dark red by the end of the seventh day, when sporulation was easily spotted (figure -3). As for *A. parasiticus*. It was subcultured on the agar of potato dextrose in similar conditions to those used for *F. sporotrichoides*. It also had similar results (figure 3).

3.3 Culture Material of Aflatoxin: Production in Relation to Incubation Period

The effects of variation in the type of substrate, moisture, temperature, and incubation period during the production of B1 aflatoxin were examined in this research. It was observed that there was an increase in aflatoxin production based on incubation period. On the third day, the toxin average yield was 280 ppm. On the fifth day, it became 533 ppm. By the seventh day, it increased to 786 ppm. On the ninth day, it decreased to 764 ppm. The yield of B1 aflatoxin seems to increase in parallel with the increase in the incubation period. Yet, it decreased after the seventh day (Tables 1 and 2). The reason could be the inter-conversions of aflatoxins.

Table 1- Impact of incubation period on the production of B1 aflatoxin by *A. parasiticus* on coarsely crushed rice.

Period of incubation	Yield of B1 aflatoxin (ppm)
3 DAYS	280.33 ± 9.64 ^c
5 DAYS	533.66 ± 5.78 ^b
7 DAYS	786.33 ± 20.33 ^a
9 DAYS	764.00 ± 8.25 ^a

Means are (P < 0.05).

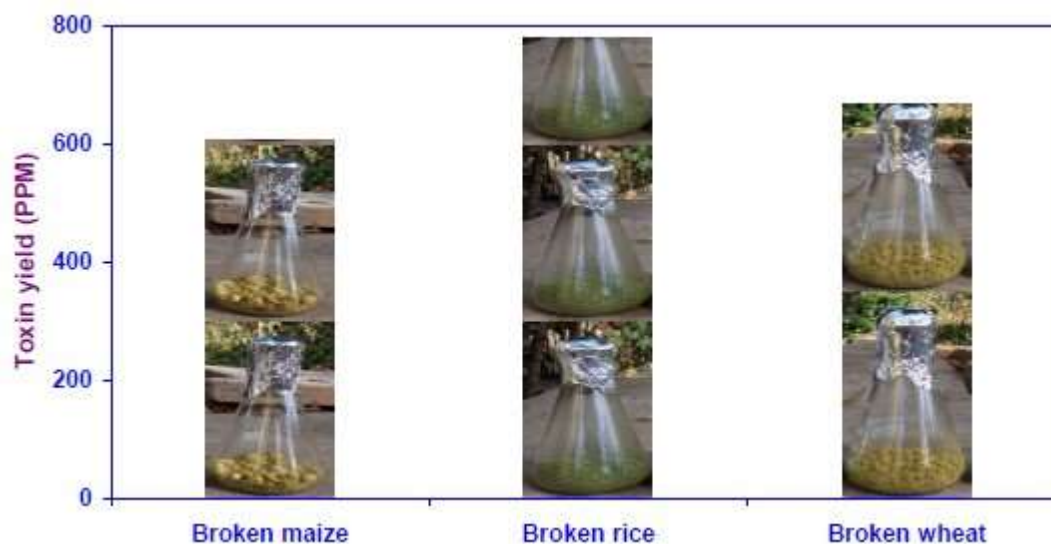
Table 2- Analyzing the impact of incubation period on the production of B1 aflatoxin by *A. parasiticus* on coarsely crushed rice.

Source- Df	Pr > F	Mean square
Day 3	0.0001	168193
Error 8	-	446

B1 aflatoxin reached a maximum yield when coarsely crushed rice was inoculated at a temperature of 30C°, moisture level of 35%, and incubation duration of 7 days. The results obtained in this research are in parallel with the findings of Diener and Davis (1966) in terms of the increase in the yield until the seventh day [18, 19]. This change in the yield level was highly affected by temperature and moisture content [3, 21]. Therefore, B1 aflatoxin production can be affected by other factors in addition to time, such as incubation temperature and moisture.

3.4 Different Substrates Impact

Substrates of whole wheat, maize, and crushed rice were examined. B1 aflatoxin yield showed the highest value when coarsely crushed rice was utilized, as compared to other substrates (Tables 3 and 4; Figures 4 and 5).

**Figure 4-** Different substrates impact on the production of B1 aflatoxin

The results of this experiment are in parallel with those of Goldblatt (1968) [20]. He found that aflatoxins yield in crushed rice was higher than the yield in peanuts, soybeans, and sorghum. Similar results were also obtained by Shotwell *et al.* (1966), who found that crushed rice achieved the highest yield when compared to wheat and corn [14]. In a laboratory environment, various natural substrates have been employed in research related to aflatoxin production. Most of these substrates succeeded when experiments were conducted in suitable

conditions in terms of temperature and moisture. Nonetheless, the yield value varied based on the substrate type.



Figure 5-Rice, maize and wheat injected with *Aspergillus parasiticus*

3.5 Moisture Impact

After 7 days of incubation, yield level of B1 aflatoxin on crushed rice at moisture level of 35 % was higher than that at moisture levels of 25% and 45 % (Tables 5 and Table 6; Figure 6).

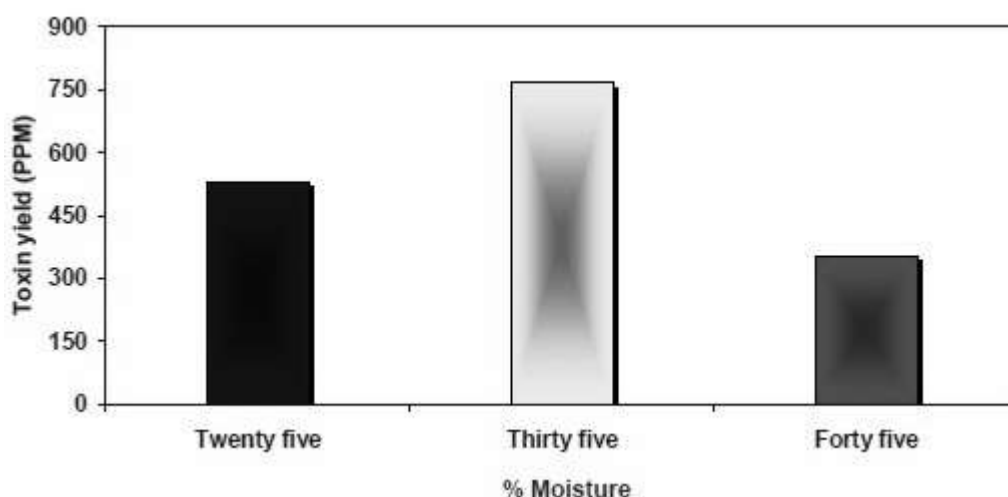


Figure 6-Moisture impact on the production of B1 aflatoxin

Table 3-Different substrates impact on the production of B1 aflatoxin by *A.parasiticus*

Feed type	Yield of B1 aflatoxin
Crushed rice	780.66 ± 2.08 ^a ppm
Crushed maize	605.66 ± 5.60 ^b ppm
Crushed whole wheat	667.66 ± 6.11 ^c ppm

Means are (P < 0.05).

Table 4- Analyzing several types of substrates impact on the production of B1 aflatoxin by *A. parasiticus*

Source - Df	Mean square	Pr > F
Substrate 2	23506	0.0001
Error 6	73	-

Table 5-Moisture effect on B1 aflatoxin production on crushed rice by *A. parasiticus*

Moisture percentage	Yield of B1 aflatoxin
25 %	531.66 ± 6.08 ^a ppm
35 %	768.66 ± 6.96 ^a ppm
45 %	352.00 ± 11.37 ^c ppm

Means are ($P < 0.05$).

Table 6- Analyzing several types of substrates impact on the production of B1 aflatoxin by *A. parasiticus*

Source - Df	Mean square	Pr > F
Substrate 2	131030	0.0001
Error 6	213	-

It was found that aflatoxins yield in crushed rice was higher than the yield in crushed maize and crushed whole wheat. The findings above are in parallel with the results of Tuason and Madamba (1980) who found that at 35 % moisture and 30 °C, aflatoxin content in copra was the highest [21]. Calderwood and Schroeder (1968) found that at 24% to 26 % moisture content, rough rice introduced large aflatoxins amounts in a period of seven days at relatively high-temperature in storage room [22]. Therefore, one of the factors influencing aflatoxin production by *A. parasiticus* was moisture content. Yet, as argued above, moisture content is not the only factor influencing B1 aflatoxin production, but the period of incubation and incubation temperature also have influences on this process.

3.6 Temperature Impact

The effects of temperature (20, 30, and 40 C°) on B1 aflatoxin production were examined in this research on crushed rice as a substrate. After 7 days of incubation at 30 C°, the yield was higher than that at 20 and 40 C° (Tables 7 and 8, figure 7, and figure 8).

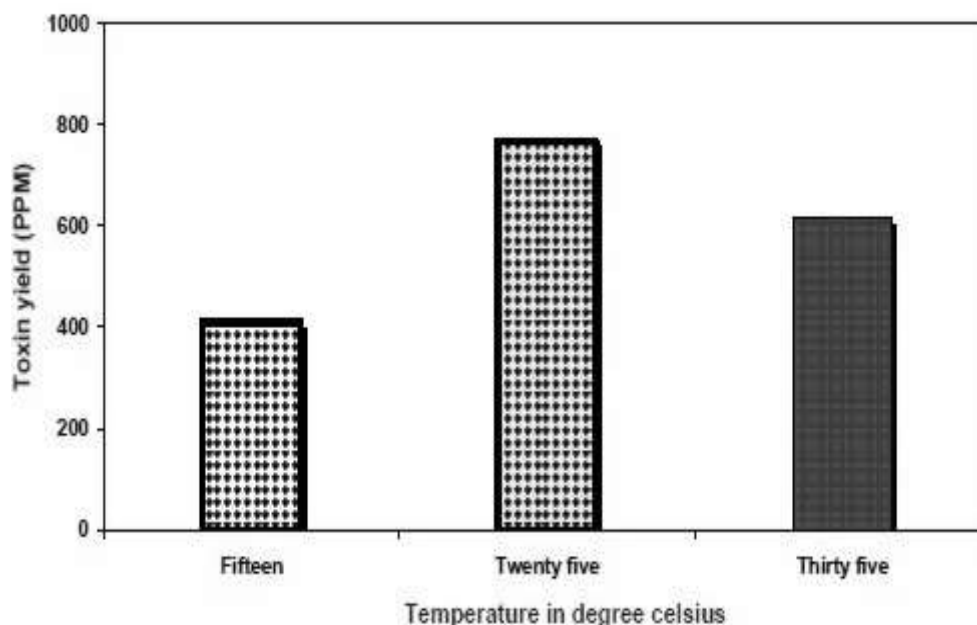
**Figure 7 -** Temperature impact on the production of B1 aflatoxin.



Figure-8 Crushed rice mixed with *Aspergillus parasiticus*

These findings are in parallel with those of Schroeder and Hein (1967) who examined temperature impact on aflatoxin production on rough rice, Spanish peanuts, and moistened cottonseed inoculated with *A. parasiticus* [23]. At 25 C°, the peak of production was reached on all three substrates. It was almost similar at 30 C°. Yet, it was slightly lower at 20 and 25 C°. Yields decreased as temperature increased, on all types of feed used. The peak was reached at 30 C° in peanuts and crushed rice and at 35 C° in cottonseed.

Table 7 -Temperature impact on the production of B1 aflatoxin on crushed rice by *A. parasiticus*.

Temp.	Yield of B1 aflatoxin
15 C°	410.66 ± 1.2 ppm
25 C°	768.66 ± 6.96 ^a ppm
35 C°	614.33 ± 8.68 ^b ppm

Means are (P < 0.05).

Table 8-Analyzing temperature impact on the production of B1 aflatoxin on crushed rice by *A. parasiticus*

Source - Df	Mean square	Pr > F
Error 6	125	-
Temperature - 2	96731	0.0001

Similar findings were obtained by Sorenson *et al.* (1967) [24]. He found that 28 C° is the optimum level of temperature for aflatoxin B1 and aflatoxin G1 to form on crushed rice. A lower temperature than 28 down to 15 C° can result in less aflatoxin. At 11C°, only 1ppm B1 aflatoxin was detected, whereas at 8 C°, no aflatoxin was detected. Nonetheless, when the temperature was increased to 40 C°, aflatoxin yield also decreased to 3-8 ppm, despite the increase in fungus growth.

3.7 Aflatoxins Extraction

The extraction of aflatoxins was based on the Annexure II method, in which culture material (25 g) was mixed with 125 ml of aqueous acetone (85 acetone:15 water) and shaken for an hour. 90% of aflatoxin was recovered and extracted.

3.8 B1 Aflatoxin Isolation and Purification

B1 aflatoxin was isolated and purified from a crude mixture composed of B1, B2, G1 and G2 aflatoxins. This process was conducted using the preparative TLC method with preparative TLC plates and a solvent containing chloroform:acetone (85:15). Using unequilibrated chamber, this experiment relied on visual observation with long-wave UV-light (365nm). Various aflatoxins as well as other impurities were observed. B1 aflatoxin was

carefully scraped, isolated, and compared with B1 aflatoxin of Sigma standard, with a matching Rf value of 0.31 (figure 9).

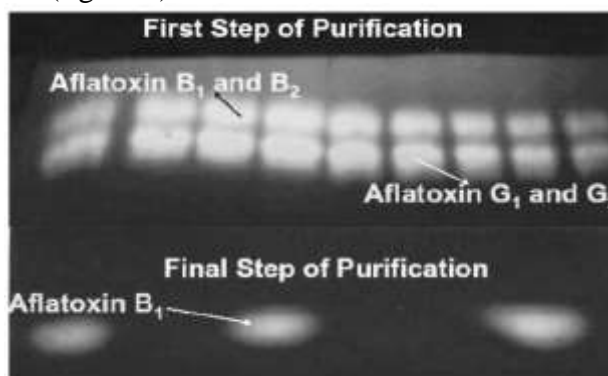


Figure-9 Aflatoxins isolation and purification using preparative TLC

Acetonitrile: benzene (98:2) was used to dissolve the silica gel scraped with B1 aflatoxin. When tested in UV spectroscopy, a purity of 58 to 60% was detected. To achieve 98% purity, the same steps were repeated 4 times. The purity reached 65 – 70, 90, and 98 %, respectively, after each repetition.

Relying on a method developed by Rodricks *et al.* (1970) [18], a UV-spectroscopy was used to test the purity in each step.

3.9 B1 Aflatoxin Standardization

A reference solution was standardized, after purifying B1 aflatoxin, to a 2 µg/ml concentration by using the methods described below.

3.9.1 Thin layer chromatography

This method was used to compare the fluorescence of the developed solution with that of the Sigma aflatoxin standard solution of the concentration of 2µg/ml. The concentration of the developed solution was decreased, reaching 2µg/ml after adding benzene: acetonitrile (98:2).

3.9.2 UV-spectrophotometry

At a wavelength of 348nm, the OD values of Sigma B1 aflatoxin standard were compared with those of the developed reference standard. The values were calculated relying on a molar attenuation absorptivity of 19,800 in benzene: acetonitrile (98:2).

3.10 Determination of B1 Aflatoxin Purity in the Reference Standard

Using TLC plate, a developed reference solution was noticed in association with Sigma B1 aflatoxin standard. After developing the plate, it was noticed that B1 aflatoxin had the only visible fluorescence (figure 10).

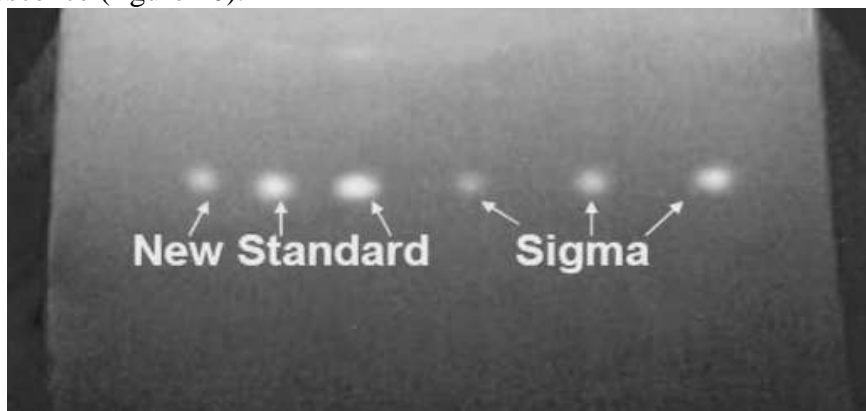


Figure 10-Spectrophotometric fluorescence intensities showing a new standard compared with that of the pure Sigma standard.

Several concentrations, in terms of the values of molar absorptivity, of aflatoxin mixed with benzene: acetonitrile (98:2 at wavelength of 333 nm were obtained and compared to the values provided by Rodricks *et al.* (1970) [17]. They reported that, at 348 nm wavelength, aflatoxin was found to be 100% pure when the value of molar absorptivity was 19,800. The findings of this research are in parallel with those reported by Rodricks *et al.* The purity was found to be 99% (Table 9).

3.11 UV-Spectra of B1 Aflatoxin and Sigma Standard

Figure 11 demonstrates that the peaks are overlapped, and are shown by the spectrum. These include the peak of the developed B1 aflatoxin and the peak of Sigma reference standard. Variations of wavelengths of 220 - 270 nm were included.

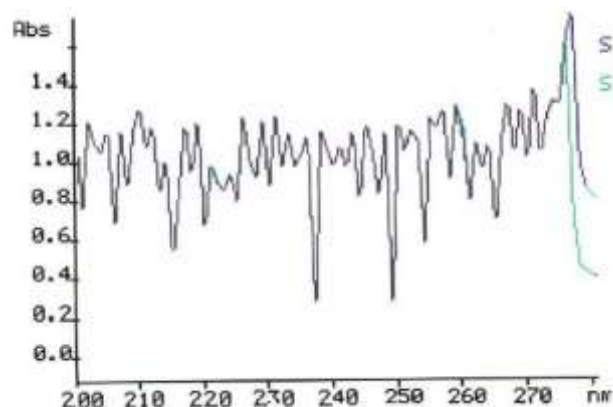


Figure 11- UV-spectra of the developed B1 aflatoxin in benzene: acetonitrile (98:2) compared to the Sigma aflatoxin standard.

Table 9 -Five different concentrations of B1 aflatoxin with respective values of purity and molar absorptivity.

Concentration value	Molar absorptive value	Purity percentage
5 µg / ml	19556.6 ± 8.45	98.77 ± 0.04 %
4 µg / ml	19545.2 ± 9.65	98.71 ± 0.04 %
3 µg / ml	19569.8 ± 7.35	98.83 ± 0.03 %
2 µg / ml	19608.8 ± 9.17	99.21 ± 0.15 %
1 µg / ml	19,699.2 ± 30.29	99.48 ± 0.15 %
Mean value	19595.92 ± 13.90	99.00 ± 0.08 %

The value in each column represents the average of five measurements per concentration.

4. Conclusions

This research included various procedures encompassing the production, isolation, and purification of B1 aflatoxin at different temperature levels, moisture levels, and incubation periods using various substrates such as crushed rice, crushed wheat, and crushed maize. The substrates were inoculated with *A. parasiticus* in a BOD incubator. Yield of B1 aflatoxin reached its maximum value by applying an incubation period of seven days, temperature of 30 °C, moisture level of 35%, using coarsely crushed rice. After the extraction of aflatoxins using acetone:water (85:15), trichothecenes were obtained by using 50:50 methanol:water. The isolation of B1 aflatoxin was performed using the preparative TLC. After repeating this process for 4 times, purity was analyzed by spectrophotometric and chromatographic methods. A purity value of 99% of B1 aflatoxin was gained. The UV-spectrum showed the maximum absorbance of B1 aflatoxin reference standard at 275 nm wavelength.

5. Acknowledgment

5.1 Funding and Financial Interest

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5.2 Conflict of Interest

The author would like to declare that there is no conflict of interest related to this work.

5.3 Availability of Data and Material

All data and materials described in the manuscript, including all relevant raw data, will be freely available to any researcher wishing to use them for non-commercial purposes.

5.4 Ethics Approval and Consent to Participate

This research was done using feed sample. No human participant and/or animals were involved.

6. References

- [1] V.M. Nunes, M. Moosavi, A.M. Khaneghah, and C.A. Oliveira, "Innovative modifications in food processing to reduce the levels of mycotoxins," *Current Opinion in Food Science*, 2020.
- [2] R.W. Yonis, K. J. K. Luti, and G.M. Aziz, "An Application of Response Surface Methodology for Optimizing the Production of Chitin Deacetylase Enzyme by *Aspergillus Flavus*," *Iraqi Journal of Science*, pp.1206-1220, 2019.
- [3] F.C., Muga, M.O. Marennya, and T.S. Workneh, "Effect of temperature, relative humidity and moisture on aflatoxin contamination of stored maize kernels," *Bulgarian Journal of Agricultural Science*, vol. 25, no. 2, pp.271-277, 2019.
- [4] D.K. Mahato, K.E. Lee, M. Kamle, S. Devi, K.N. Dewangan, P. Kumar, and S.G. Kang, "Aflatoxins in food and feed: an overview on prevalence, detection and control strategies." *Frontiers in microbiology*, vol.10, pp. 2266, 2019.
- [5] M. Tola, and B. Kebede, "Occurrence, importance and control of mycotoxins: A review," *Cogent Food & Agriculture* vol.2, no. 1, 1191103, 2016.
- [6] A.R., Hussein, Z.Z., Khalaf, Z. Samir, and R. Samir, "Antibacterial activity of crud Bacteriocin-like substance against food borne bacterial pathogens," *Iraqi journal of Science*, vol. 59, no. 1A, pp.16-24, 2018.
- [7] M.S., Monson, R.A. Coulombe, and K.M. Reed, "Aflatoxicosis: Lessons from toxicity and responses to aflatoxin B1 in poultry," *Agriculture*, vo. 5, no. 3, pp.742-777, 2015.
- [8] S., Puri, S. Shingh, and P. Tiwari, "Mycotoxins: A Threat to Food Security and Health," *International Journal of Applied Sciences and Biotechnology*, vol.7, no. 3, pp.298-303, 2019.
- [9] G.R., Murugesan, D.R. Ledoux, K. Naehrer, F. Berthiller, T.J. Applegate, B. Grenier, T.D. Phillips, and G. Schatzmayr, "Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies," *Poultry science*, vol. 94, no. 6, pp.1298-1315, 2015.
- [10] W., Hammami, S. Fiori, R. Al Thani, N.A. Kali, V. Balmas, Q. Migheli, and S. Jaoua, "Fungal and aflatoxin contamination of marketed spices," *Food Control*, vol. 37, pp.177-181, 2014.
- [11] F.F., Hassan, "Detection of aflatoxin b1 in some canned foods and reduction of toxin by ultraviolet radiation," *Iraqi Journal Of Science*, vol. 58, pp.2343-2349, 2018.
- [12] Li, Y., Wang, Z., Beier, R.C., Shen, J., Smet, D.D., De Saeger, S. and Zhang, S., "T-2 toxin, a trichothecene mycotoxin: review of toxicity, metabolism, and analytical methods," *Journal of agricultural and food chemistry*, vol. 59, no. 8, pp.3441-3453, 2011.
- [13] F., Soleimany, S. Jinap, A. Faridah, and A. Khatib, "A UPLC-MS/MS for simultaneous determination of aflatoxins, ochratoxin A, zearalenone, DON, fumonisins, T-2 toxin and HT-2 toxin, in cereals," *Food control*, vol. 25, no. 2, pp.647-653, 2012.
- [14] Odette L. Shotwell, C. W. Hesseltine, R. D. Stubblefield, and W. G. Sorenson, "'Production of aflatoxin on rice," *Applied microbiology*, vol.14, no. 3, pp. 425-428, 1966.
- [15] Association of Official Analytical Chemists, *Official methods of analysis of AOAC International*, 1995.

- [16] Thomas R. Romer, "Screening method for the detection of aflatoxins in mixed feeds and other agricultural commodities with subsequent confirmation and quantitative measurement of aflatoxins in positive samples," *Journal of the Association of Official Analytical Chemists*, vol.58, no. 3, pp.500-506, 1975.
- [17] J.V. Rodricks, Stoloff, L., "Determination of Concentration and Purity of Aflatoxin Standards," *Journal of Association of Official Analytical Chemists*, vol. 53, no.1, pp. 92-95, 1970. <https://doi.org/10.1093/jaoac/53.1.92>
- [18] N.D. Davis, Diener, U. L. and D. W. Eldridge, "Production of aflatoxins B1 and G1 by *Aspergillus flavus* in a semisynthetic medium," *Applied microbiology*, vol.14, no. 3, pp.378-380, 1966.
- [19] Urban L. Diener, and Norman D. Davis, "Aflatoxin production by isolates of *Aspergillus flavus*." *Phytopathology*, vol.56, no. 12 ,pp.1390-1393, 1966.
- [20] L. A. Goldblatt, "Aflatoxin and its control," *Economic Botany*, vol. 22, no. 1, pp. 51-62,1968.
- [21] M. A., Tuason, and L. S. P. Madamba, "Aflatoxin production in copra by *Aspergillus flavus* [study conducted at the University of the Philippines at Los Banos, College, Laguna]," *Philippine Agriculturist (Philippines)*, 1980.
- [22] D. L. Calderwood, and H. W. Schroeder, "Aflatoxin development and grade of undried rough rice following prolonged storage in aerated bins," *U.S. Agricultural Research Service publication*, no. ARS, pp. 52-26, 1968.
- [23] Harry W. Schroeder, and Hugo Hein, "Aflatoxins: production of the toxins in vitro in relation to temperature," *Applied microbiology*, vol.15, no. 2, pp. 441-445, 1967.
- [24] W. G. Sorenson, C. W. Hesseltine, and Odette L. Shotwell, "Effect of temperature on production of aflatoxin on rice by *Aspergillus flavus*," *Mycopathologia et mycologia applicate*, vol. 33, no. 1, pp. 49-55, 1967.