Occasional Paper 2
Groundnut Improvement Program

Screening for Resistance to *Aspergillus flavus* Invasion and Aflatoxin Production in Groundnuts

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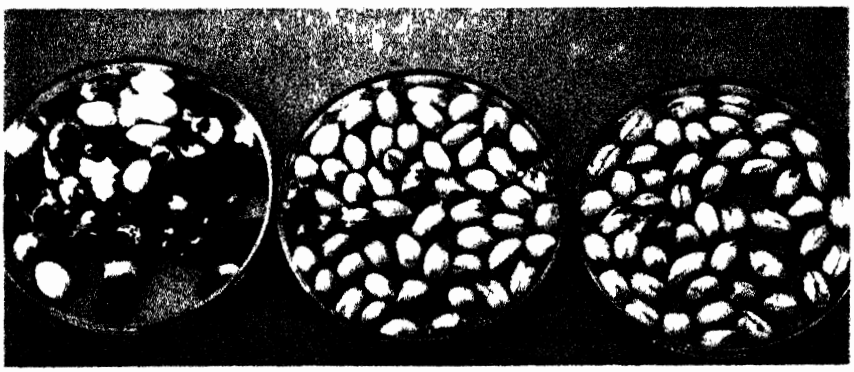

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SCREENING FOR RESISTANCE TO ASPERGILLUS FLAVUS INVASION AND AFLATOXIN PRODUCTION IN GROUNDNUTS ____'DRY SEED' RESISTANCE TESTS

Aflatoxins are highly toxic and carcinogenic substances which are produced by certain strains of fungi of the *Aspergillus flavus* and *Aspergillus parasiticus* groups when growing under favourable conditions upon suitable substrates. Groundnut seeds, and in particular mature dried seeds which are wetted, are considered to be favourable substrates for growth of the fungi and for production of aflatoxins It is not uncommon in the tropics to find groundnuts stored on the farm and in commercial units under very poor conditions with a high risk of accidental wetting. Spores of the fungi are almost universally present and if the stored seeds absorb sufficient moisture (from rain water seepage, condensation, or as a result of heavy insect infestation) to raise them above 8% water content, then invasion by *A. flavus* and production of aflatoxin can occur. Seeds with damaged testas are more easily and rapidly invaded by *A. flavus* than are seeds with intact testas and it was logical to consider the possibility that groundnut genotypes might be found which had high levels of resistance to penetration of the testa by *A. flavus*. Recent research has shown that a number of cultivars and breeding lines do possess high resistance to invasion and colonisation by *A. flavus* of intact, dried seeds when these are rehydrated and surface inoculated with spores of the fungus. Most researchers have used the screening technique devised by Mixon and Rogers (see Agron.J. 65: 560-562) or modifications of it. Such a modification is described in detail in this paper.

Another approach to solving the aflatoxin problem by selection and breeding for resistance is based on the possible existence of groundnut genotypes with seeds susceptible to invasion and colonisation by *A. flavus* but which are unfavourable substrates for aflatoxin production. Early work in this area produced promising results but further research failed to confirm the findings. However, there were definite indications of differences in the efficiency of various cultivars as substrates for aflatoxin production and it is considered useful that germplasm collections be screened for the ability of entries to support aflatoxin production. Such screening could well be integrated with screening for dry seed resistance to invasion and colonisation by *A. flavus* and this approach is taken in the method described in this paper.

Methods used for routine analysis of aflatoxin in groundnut samples by the 'minicolumn' technique, and for quantitative analysis of aflatoxin involving the use of spectrophotometer, are also described.

PREPARATION OF MATERIALS FOR DRY SEED RESISTANCE TESTING

For successful screening it is essential that test results should be accurate and reproducible. The laboratory test procedures described in this paper are fairly easy to standardise but the preparation and selection of the test groundnut seeds can present problems in that slight variations in growing conditions and post-harvest drying procedures may have significant effects upon the performance of

entries in the dry seed resistance test. If possible, sowing dates of test groundnuts should be so arranged that all entries reach maturity at the same time. However, if this cannot be done, comparisons should only be made between groups of entries which have set pods and matured under comparable conditions. Check cultivars should be included and these should match the season length of the test entries and should include dry seed resistant and susceptible types.

Well grown 'healthy' plants should be lifted at optimum maturity (determined by lifting and examining sample plants) and undamaged, mature pods carefully removed. The pods are then washed and dried in a forced draught oven operating at $8^{o}C$ above ambient temperature until the seeds are below 5% m.c. Seed moisture contents are determined using 5 replicate samples of 10 seeds each. The seeds are sliced into small pieces which are placed in a previously weighed container and this is then weighed to determine the weight of seeds. The containers with seeds are then dried in a forced draught oven at $105^{o}C$ for 5 hours afterwhich time they are placed in dessicators to cool. When cool they are re-weighed and the weight of water driven off is calculated. Moisture content of the initial seed samples is expressed on a wet weight basis.

Initial 'natural' infection of seeds with *A. flavus* and other fungi is of obvious importance as such infection could interfere with the inoculation test for resistance to *A. flavus* invasion and also with assessment of aflatoxins production. It is therefore necessary

to test seeds after post-harvest drying to determine the percentage of seeds infected by *A. flavus* and various other fungi. Pods are opened carefully by hand and seeds removed. For each entry 100 full-sized, undamaged seeds are taken. The seeds are placed in a sterile container and soaked for 1 minute in sterile distilled water. The water is then drained off and replaced by a 0.5% aqueous solution of sodium hypochlorite in which the seeds are soaked for 3 minutes. The solution is then drained off and the seeds rinsed in 3 changes of sterile water. Seeds are then plated out aseptically onto Czapek Dox Rose Bengal Streptomycin Agar medium in 9 cm diameter petri-dishes, 5 seeds to a dish. The plates are incubated at 25°C for about 8 days with observations being made of any development of fungal colonies from the seeds from day 3 onwards. All fungi should be identified to species if possible and particular attention should be paid to *A. flavus* and related species. If levels of infection in excess of 5% are recorded, the above test should be repeated but with the modification that the sodium hypochlorite solution is replaced by a 0.1% aqueous solution of mercuric chloride.

Dried pods should be stored in sealed containers at room temperature for 2 months before resistance testing.

PREPARATION OF INOCULUM OF *A. FLAVUS/A. PARASITICUS*

The inoculum of *A. flavus* or *A. parasiticus* is prepared as follows for inoculation of seeds for the dry seed resistance test. Known

aflatoxin producing strains of *A. flavus* and *A. parasiticus* should be selected and maintained on Czapek Dox Agar medium. For use in the screening tests the cultures should be 8-10 days old. It is preferable to have fresh cultures from the stock cultures and it should be arranged that at all the times fresh 8-10 day old cultures are available for the dry seed resistance testing. For preparation of the inoculum, cultures of 8-10 days in age are flooded with sterile distilled water containing 5% Tween 20 (v/v) and spores detached gently from the cultures and removed in the suspension which is adjusted to contain 4.0×10^6 conidia per ml. A haemocytometer is used in estimating spore concentration.

TEST FOR 'DRY SEED' RESISTANCE TO INVASION AND COLONISATION BY *A. FLAVUS*

1. Pods produced, selected, dried and stored as described above are removed from their containers, carefully hand shelled, and seeds selected for the test that are full-sized and free from visible damage to the testa.

2. Moisture content is determined on 50 seeds by the method already described.

3. Sound seeds of uniform size and shape with intact testas are taken in approximately 20 g seed lots with 3-5 replications.

4. Approximately 20 g of seed in replicates are surface-sterilized separately by soaking them for 2 minutes in a 0.1% aqueous solution of mercuric chloride. This is followed by 4 rinses in sterile distilled water.

5. The seeds are then hydrated to approximately 20% m.c. (on weight basis using data from 2. above) by soaking them for between 10 and 15 minutes in sterile distilled water. Time of soaking required is determined by initial seed moisture content and genotype.

6. The seeds are then placed aseptically in a sterile 9 cm diameter petri-dish and 1 ml of the spore suspension (prepared as described above) added to them. The spore suspension is spread over the seeds by gently swirling them around within the dish.

7. The plates are placed over water in semi-rigid plastic boxes which have tight fitting lids and these are then placed in an incubator running at 25^{o}C and incubated for 8 days.

8. Remove boxes containing plates to an examination chamber fitted with an air extraction system voiding to the outside of the laboratory. Remove the plates and record results of test. STAFF DOING THE RECORDING SHOULD WEAR PROTECTIVE RUBBER GLOVES AND ALSO FACE MASKS AND ALL HANDLING OF TEST MATERIALS MUST BE DONE WITHIN THE EXAMINATION HOOD (A SEPARATE 'SAFETY' LEAFLET -- "SAFETY MEASURES WHEN HANDLING *A. FLAVUS* GROUP FUNGI AND AFLATOXINS" -- HAS BEEN PREPARED).

9. Recording of results:-

a) record total number of seeds

b) record number of seeds with sporulating growth of *A. flavus* on their surfaces. This figure to be converted to a percentage, i.e., the percentage of test seeds on which sporulating growth of *A. flavus* occurred.

c) where sporulating growth occurs, classify this into the grades of: i) sparse ii) moderate iii) dense

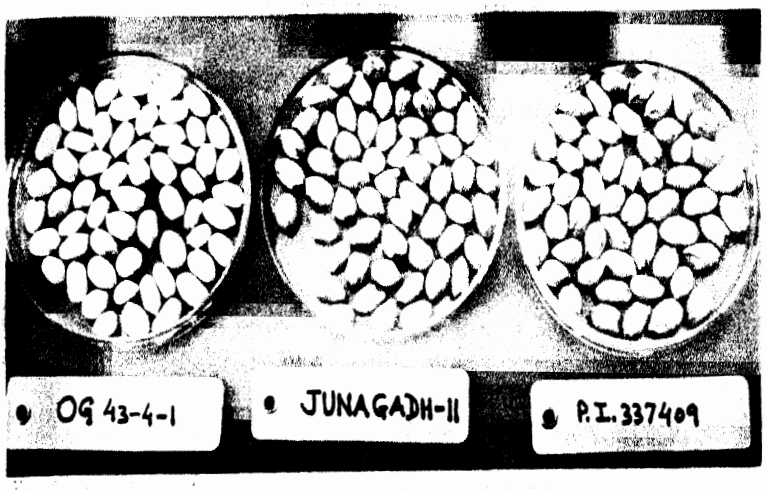
d) with a sharp blade cut open every seed (transverse section) and record whether or not the *A. flavus* has colonised the interior of the cotyledons. Record the numbers of seeds with and without external sporulating growth of *A. flavus* which have internal growth of the fungus. These figures can then be expressed as percentages.

In cases where sporulation is very sparse it may be necessary to examine the seeds under the stereo-microscope. It may also be necessary to examine in this way seeds on which sporulating growth is restricted to small areas of the seed surface in case this is linked with damage to the testa that may have escaped notice during initial selection of the seeds.

The following guide can be used to classify genotypes:

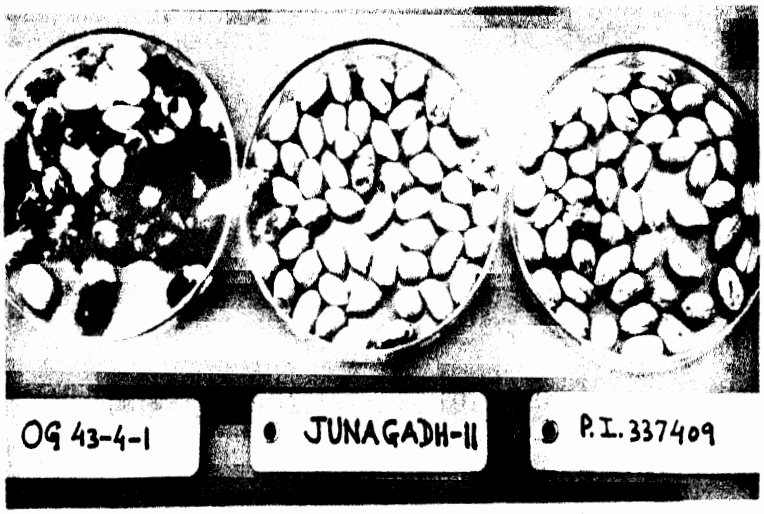
RESISTANT ——— sporulating growth of *A. flavus* present on less than 15% of seeds; growth and sporulation sparse.

DRY SEED RESISTANCE TESTING



SAMPLES BEFORE TEST

Uninoculated seeds of groundnut cultivars



SAMPLES AFTER TEST

(a) Highly susceptible cultivar (OG 43-4-1);

RESISTANT CULTIVARS

(JUNAGADH-11 and PI 337409)

(b) Seeds cut open to show internal infection by *A. flavus*

MODERATELY RESISTANT	———	sporulating growth of *A. flavus* present on 16-30% of seeds; sporulation moderate to dense.
SUSCEPTIBLE	———	sporulating growth of *A. flavus* present on 31-50% of seeds; sporulation dense.
HIGHLY SUSCEPTIBLE	———	sporulating growth of *A. flavus* present on over 50% of seeds; dense growth and sporulation.

10. Checks: It is essential that uninoculated but otherwise similarly treated plates of each test entry be included. It is also required that resistant and susceptible check cultivars/lines be included at each time of testing to monitor the test conditions.

TEST FOR CAPACITY OF SEEDS TO SUPPORT PRODUCTION OF AFLATOXINS FOLLOWING COLONISATION BY TOXIGENIC STRAINS OF *A. FLAVUS/A. PARASITICUS*

The starting point for this test is the end of the test described above for dry seed resistance to invasion and colonisation by *A. flavus*. The test could be carried out independantly of the previous one in which case the seeds would be surface scarified to facilitate fungal invasion before carrying out stages 4 to 7 of the dry seed resistance test.

1. Take sample of seeds that have been:

   (a) scarified and inoculated then incubated as per stages 4 to 7 of the dry seed test.

or (b) seeds from stage 9 of dry seed test where most of seeds have been successfully colonised by the *A. flavus* strains. Where colonisation has been less successful or growth of the fungus over the seeds has been sparse, seeds should be surface scarified, a further 1 ml of spore suspension (prepared as described above) added, and the plate incubated at 25°C for a further 8 days. Proceed to detection of aflatoxins.

Minicolumn Detection Method For Aflatoxins:

For convenience in large scale screening a 'minicolumn' technique for the detection of aflatoxins has considerable advantages and the method described is that of Romer using the velasco minicolumn (Romer, T.R. 1975. JAOAC 58:500-506). In the screening method, aflatoxins are extracted with acetone-water (85 : 15), and interferences are removed by adding cupric carbonate and ferric chloride gel. The aflatoxins are extracted from the aqueous phase with chloroform and the chloroform extract is washed with a basic aqueous solution. A velasco-type minicolumn is used to further purify the extract and capture the aflatoxins in a tight band. In addition, the presence of aflatoxins in the positive samples can be confirmed and the toxins can be quantitatively measured, using the same extract as that used for the minicolumn.

The following steps are involved in the minicolumn detection method for aflatoxins:

1. Extraction:

(a) Place 50 g sample in a clean stainless steel blender jar, add 250 ml of an acetone : deionized water (85:15) mixture, and blend for 3 minutes. It is essential that the jar has a close fitting lid.

(b) Filter the extract through No.4 Whatman paper.

(c) Transfer 150 ml of filtrate to a 250 ml capacity beaker.

2. Purification:

(a) Prepare a mixture of 170 ml of 0.2 N NaoH and 30 ml $FeCl_3$ in a 600 ml capacity beaker, mix well.

(b) To the sample extract (1.c.) add 3 g basic $CuCo_3$, mix well, then add to mixture 2.a. in the 600 ml beaker. Add to the mixture 150 ml of diatomaceous earth and mix well. Filter the mixture through No.4 Whatman paper.

(c) Transfer 150 ml of the filtrate to a 500 ml capacity separating funnel; add 150 ml of 0.03% H_2So_4 and then 10 ml of $CHCl_3$. Shake vigorously for 2 minutes then allow to separate out.

(d) Transfer the lower $CHCl_3$ layer (13-14-ml) to a 125 ml capacity separating funnel. Add 100 ml of KOH wash solution, swirl gently for 30 seconds then allow to separate out. (-if emulsion is formed, drain this into a 10 ml g-s graduate, add 1 g of anhydrous Na_2SO_4, shake for 30 seconds then allow separation to take place.

The $CHCl_3$ phase need not be completely clear. If emulsion is not broken, transfer it to a 125 ml separating funnel and wash with 50 ml of 0.03% H_2SO_4). Collect 3 ml of the $CHCl_3$ layer for test.

3. Preparation of Minicolumns:

The minicolumn is prepared using a thin-walled glass tube 200 mm in length by 5 mm outside diameter.

(a) Block one end of the tube with a plug of glass wool of about 2-3 mm long.

(b) Insert the tip of a small plastic funnel into the open end of the tube.

(c) Pour drierite, non-indicating, 20-40 mesh (calcium sulfate, anhydrous) into the column to a depth of 8-10 mm.

(d) Add florisil (Fisher F-101), also to a depth of 8-10 mm.

(e) Add a layer of silica gel to a depth of 16-20 mm.

(f) Add a layer of neutral alumina to a depth of 8-10 mm.

(g) Add a second layer of drierite to a depth of 8-10 mm.

(h) Finally, insert a small plug of glass wool on top of column.

N.B.: Tap column after each addition and apply pressure to the glass wool at top of column with thin rod after completion.

Preparation of Reference Minicolumns:

To prepare reference column containing 10 ng each aflatoxins B_1

and G_1, place 1 ml $CHCl_3$ in small vial and add 5 µl standard solution. Transfer entire solution to packed minicolumn and let drain. Add 3 ml elution solvent, $CHCl_3$ - acetone (9+1) and allow to drain.

Reference columns prepared by using chloroform extracts from a uncontaminated sample, spiked with a suitable ratio and level of aflatoxins, will give the best reference columns for quantitative approximation. The reference column is not necessary for the screening test; however, it aids in locating and becoming familiar with the aflatoxin band. Any quantitative estimation performed by using reference columns containing pure aflatoxins is at best an approximation.

4. Development of Aflatoxins on Minicolumns:

Add 1 ml of the $CHCl_3$ sample solution (stage 2.d.) to the prepared column and allow to drain; this should take 15-30 minutes. This process may be speeded up by applying a slight air pressure (rubber bulb attached to top of minicolumn) to force the solution down through the top layers of the column at a rate not exceeding 10 cm per minute. After the solvent reaches the adsorbent florisil layer all further draining to be by gravity alone. Add a further 3 ml of elution solvent (chloroform : acetone, 9:1) and allow to drain by gravity until solvent reaches top of the adsorbent.

Detection of Aflatoxins:

Place sample column on bench top adjacent to reference column,

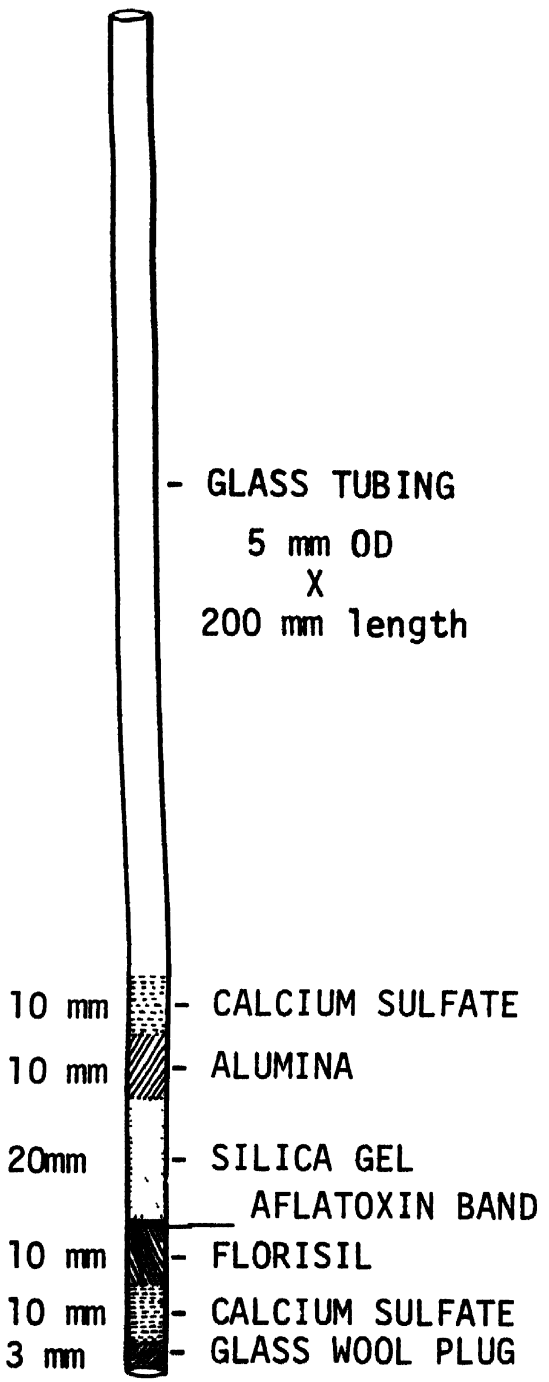


Fig.1. VELASCO MINICOLUMN

aligning packing interfaces. Turn off room lights and shine UV light on columns. Note blue fluorescent band 2.5 cm from bottom of reference column at top of florisil layer due to presence of aflatoxin. If a similar blue fluorescent band is visible on the sample column the sample contains aflatoxin. Some uncontaminated samples show faint white, yellow, or brown fluorescence bands towards the top of the florisil layer. If the bands have no definite bluish tint, samples are negative.

Estimation of the aflatoxin content of extracts by thin-layer Chromatography:

If sample is positive by minicolumn screening test, collect 4 ml alkali-washed first $CHCl_3$ extract in 10 ml graduate. Discard any of first $CHCl_3$ extracts remaining in separatory funnel. Add 10 ml $CHCl_3$ to acetone-water in separatory funnel and repeat steps in $CHCl_3$ extraction section. Combine 4 ml KOH-washed $CHCl_3$ from second $CHCl_3$ extraction with 4 ml from first $CHCl_3$ extraction and transfer to 125 ml separatory funnel. Add 50 ml 0.03% H_2SO_4 and swirl gently for 30 seconds. Collect 6 ml $CHCl_3$ layer in 10 ml vial and evaporate to dryness in 40°C water bath. Add 100 µl of the same solvent. Dissolve residue in vial by shaking vigorously for 1 min. Perform TLC quantitation.

Preparation of TLC plates:

Kieselgel 'G-HR' Chromatoplates:

Weigh Kieselgel 'G-HR' (30 g) into a stoppered flask and shake for 1 min with distilled water (60 ml). The resulting slurry is sufficient

to coat five 20 x 20 cm plates. Coat the plates with a layer of slurry 250 μ thick and leave the plates in a dust-free atmosphere until gelled (10-15 min.). Heat the plates for one hour in a forced draught oven at 110°C. Cool the plates in a dust-free atmosphere for 30 min and store them in a plate cabinet.

Development of the plates:

Apply 20 μl extract samples to the chromatoplates using a micropipette. Wash the micropipette thoroughly after application of each extract. The micropipettes may be reused after washing in 5 percent sodium hypochlorite solution followed by water and finally acetone rinses. Spot the extract samples in a line 2 cm from the bottom of the chromatoplate and at least 2 cm from either side. They should be applied as quickly as possible and in subdued light. When the spots are being applied to the coated surface of the chromatoplate, the solvent be allowed to spread over an area of not more than 5 mm in diameter. Also spot a qualitative standard of aflatoxin. This helps to distinguish aflatoxin spots from other fluorescent spots which may be present and to ensure that plates are developed correctly.

Development of TLC plates:

Add Chloroform-Methanol (97:3) to the chromatography tank to a depth of not more than 1 cm. Line the tank with chromatography paper and leave for a short time (15 min) before use. Develop the chromatoplates until the solvent front has run 10 cm from the base line. Remove the plates from the developing tank and allow them to dry in air in

subdued light. Examine plates in a dark-room, at a distance of 30 cm from a ultraviolet lamp (peak emission 365 mμ) and observe the presence or absence of blue or green fluorescent spots corresponding to aflatoxin B at 0.43-0.47 and G at 0.33 to 0.38. Compare the fluorescent spots from the sample extracts with those from the standards which have similar R_f values.

Quantitative Analysis:

Spectrophotometric Method for Determining Aflatoxin Levels (see Nabney and Nesbitt, 1965, Analyst 90 : 155-160).

A method is described for determining the aflatoxins, particularly aflatoxin B_1, based on the intensity of the ultraviolet absorption at 363 mμ, after purification by thin-layer chromatography.

Scrape the Kieselgel 'G' containing the aflatoxin B_1 from the plate and extract with cold methanol for 3 minutes. Filter off the methanol in a 50 ml beaker and wash the Kieselgel 'G' three times with methanol, making the combined filtrates upto 5 ml.

Record the ultraviolet absorption spectrum of the methanolic solution in a 2 cm cell and calculate the amount of aflatoxin B_1 present in the sample. The optical density at 363 mμ, minus that at 420 mμ, is used for calculations.

The aflatoxin B_1 concentration is given by $\frac{D \times M \times 10^6}{e \times 200 \times 2}$ μg per 5 ml where D is the corrected optical density at 363 mμ, M is the molecular weight (312) of aflatoxin B_1, and e is the molar extinction coefficient (22,000).