Aflatoxin sampling and determination in bulk maize for export

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by Pin Pithaya-Acharlyakul

INTRODUCTION

Societe Generale de Surveillance (SGS), a worldwide independent inspection company with headquarters in Geneva, Switzerland, is represented in more than 140 countries by 155 affiliated companies, and has 152 laboratories and 1,000 offices.

SGS has been established in Thailand for more than 39 years. Here, there are over 800 staff with a wide range of sophisticated laboratory facilities to cope with the trade of the country.

SGS (Thailand) services cover:

- Agricultural and industrial fumigation through the Siam Control Company (SCC)
- Laboratory analysis

- Minerals/chemical products
- Consumer products
- Industrial non-destructive testing (NDT)
- Petroleum/petrochemicals
- Marine products
- SGS' maize export is presently in the form of bulk. If maize is exported in bags, it is usually packed during loading.

For practical reasons, determination as to the level of aflatoxin contamination in a maize consignment must be carried out before shipment. This will allow opportunity for selection of acceptable cargo for loading. To reject cargo after loading is economically impractical.

Sampling and screening process will enable exporters to segregate their stocks, according to the level of aflatoxin, for their own trade planning. Sampling is carried out during:

1. Intake from trucks into silo/godown
2. Transfer from dryer into the silo/godown.

In principle, sample increments are drawn at regular intervals during the transfer. From trucks of about 13 metric tonnes (t) per load, 3 increments are drawn during tipping - one from the rear, one from the middle, and one from the front. On the conveyor belt which transfers maize after drying from the dryer to the storage bin or godown, increments are drawn at every interval of about 10 t.

Sample increments are then combined to form a gross sample for every 100 t. Each gross sample is

mixed well and divided into the required number of representative samples each weighing about 2 kg. Analysis can be performed either on every 100 or 500 t basis depending on the reqirements.

Once the results of the analysis is known for each individual lot, the silo operator can then release or transfer the cargo from temporary storage and group the maize as per maximum aflatoxin level, i.e., 20 ppb, 30 ppb, 50 ppb, etc., for shipment.

The results of the aflatoxin analysis for each stock has validity for a maximum of 2 weeks prior to shipment, on condition that both the temperature and moisture are well maintained.

The elements of time/speed and expenses are of prime importance to traders and exporters. Usually, they would require sampling and aflatoxin determination on their accumulated stocks prior to loading. The maize is stored in bulk either in silo bins or in flat warehouses (godowns) as stock piles.

SCOPE OF AFLATOXIN DETERMINATION AND SAMPLING PROCEDURES FOR MAIZE SHIPMENT

Representative samples from stocks either in silo bins or stockpiles are examined for aflatoxin levels prior to loading. Results of aflatoxin levels in the stocks found within the contractual specification are valid within 2 weeks from the date of sampling and with the conditions that temperature and moisture of the stocks are well maintained and that SGS seals are intact.

GENERAL PROCEDURE

Increments are systematically drawn from the stock and composited on the basis of 200 t per subgross sample. Each sub-gross sample is thoroughly mixed and reduced by an appropriate device (a riffle divider) to a minimum of 2 kg representative subsamples. The number of sub-samples depends on the size of the stocks, i.e.:

- stock of 1,000 t/200 = 5 sub-samples
- stock of 1,800 t/200 = 9 sub-samples

SAMPLING METHODS

Ex-silo bins

In silo bins where sampling is feasible from top and bottom of the bins, 2/5 of the total number of samples is prepared from increments drawn from the top of the bin by a pneumatic probe. The rest, 315 of the total number of samples, is prepared from increments drawn from the bottom of the bin by a scoop during recycling of the contents of the bin.

For example, the calculation of the number of samples from a silo bin with a volume of 1,800 t maize follows:

1,800 t/200 = 9 sub-samples

Number of sub-samples to be prepared from the top of the bin:

9 x 2/5, or 4 sub-samples

Number of sub-samples to be prepared from the bottom:

9 x 3/5 = 5 sub-samples

Top Sampling. Increments are drawn by a pneumatic probe extracting sample material from surface down to 5-7 meters depth or a minimum 1/4 depth of the bin contents. Each sub-sample is prepared from increments drawn from one location during probing.

Therefore, the 4 sub-samples are to be prepared from increments drawn separately from 4 different locations.

Bottom Sampling. To recycle about 10% of the bin contents, calculation is:

1,800 t x 10% = 180 t

Increments are systematically drawn at regular intervals from the bottom of the bin during recycling and number of sub-samples to be prepared from each subgross sample composited for every 180 t is:

180 t/5 = 36 t

Thus:

5th sub-sample to be prepared from increments drawn from 1st 36 t recycling

6th sub-sample to be prepared from increments drawn from 2nd 36 t recycling

7th sub-sample to be prepared from increments drawn from 3rd 36 t recycling

8th sub-sample to be prepared from increments drawn from 4th 36 t recycling

9th sub-sample to be prepared from increments drawn from 5th 36 t recycling.

In silo bins where sampling is feasible only from the bottom, 20% of the bin contents is recycled. For example, in a silo bin with 1,000 t of maize:

Number of sub-samples = 1,000 t/200 = 5 sub-samples

Recycling 20% of 1,000 t = 200 t

Sub-samples to be prepared from increments drawn and composited for every 200 t/5 = 40 t.

Sampling procedures to be applied are similar to the previously mentioned bottom sampling.

Ex-stockpiles in bulk

Stockpiles may vary in size, tonnage, height, width and length. They may range from 1,000 t to over 10,000 t.

To sample a stockpile which is over 1,500 t in size, it should be divided into sectors or "sub-lots" of approximately 1,000 t each. Each sub-lot is to be treated separately for sampling and analysis.

Thus, number of samples to be prepared from a sub-lot:

1,000 t/200 = 5 sub-samples

Increments are to be drawn by a pneumatic probe extracting sample material from surface either perpendicularly down to the bottom of the pile or at right angles from the surface toward the centre of the pile, depending on the structure or shape of the stockpile.

Five sub-samples are to be individually prepared from each probing at a designated location.

SAMPLE PREPARATION FOR LABORATORY ANALYSIS

The second stage of the sampling process is to prepare final samples for analysis of aflatoxin content. It is essential that at all stages, the equipment involved in the sample preparation must be clean and free from previous sample residues to avoid any contamination.

The following procedures are designed to systematically treat all the sub-samples to constitute a

representative "final sample" for a silo bin or a stockpile sub-lot for aflatoxin analysis.

For each silo bin or stockpile sub-lot, mix all the sub-samples into a gross sample.

Divide the gross sample by a riffle divider to prepare 4 representative final samples of about 2 kg each:

1 sample for analysis

2 samples reserved for second and third analysis, if necessary

1 sample retained for reference

Crush the sample for analysis into an appropriate size so it can pass a 1-mm sieve. Mix and reduce the sample by a sample divider to a minimum weight of 500 grams (9). Mix and reduce the sample by "increment method" to a minimum weight of 50 9 for laboratory treatment and analysis of aflatoxin.

The Increment Method. After thoroughly mixing the sample, spread the sample into a rectangle with an appropriate even thickness. Divide the rectangle into 20 equal cells. Take sample material from each cell with an appropriate scoop to the bottom of the cell.

Following the principle of Gafta Rules, if aflatoxin analysis fails on the first sample (exceeding specification to a certain limit but not over 50%), a second analysis can be performed on the second sample. If the results of the second analysis are lower than specifications and when averaged with the first analysis produces a result within the specifications, then the lot is considered acceptable. On the

other hand, if the second analysis is still higher than the specifications but lower than the first analysis, a third analysis can be performed on the third sample. If the average of the three analyses is within specifications, the lot is still considered acceptable. Otherwise, it is rejected.

For example, aflatoxin analysis for a specification of 50 ppb maximum shows the following:

1st analysis = over 75 ppb, rejected and does not qualify for second analysis.= up to 75 ppb, allowed for second analysis

2nd analysis = less than 25 ppb, the lot is accepted = over 25 but less than 75 ppb, allowed for third analysis

3rd analysis = average with previous 2 analyses is 50 ppb or below, lot can be accepted = average with previous 2 analysis is over 50 ppb, lot is rejected

METHODS OF ANALYSIS

Rapid screening method

This is applicable to yellow corn and can be used outside the laboratory (AACC Method 45-14, AOAC Method 26.020/1984).

Aflatoxin is extracted by aqueous methanol followed by clarification with zinc acetate. Aflatoxin is

then trapped in the florisil layer in a minicolumn. Fluorescence in the column, indicating the presence of aflatoxin, is detected visually under ultraviolet (UV) lamp and compared with the standards for quantification.

This method only gives results indicating higher or lower amounts than the standard. The whole process takes only 10 minutes.

Velasco fluorotoxin meter

This is a rapid method, but is not recognised by standard organisations.

Aflatoxin is extracted by aqueous acetone followed by clarification with ferric gel. Aflatoxin is then trapped in the florisil layer in a minicolumn and is quantified by the Neotec Velasco Fluorotoxin Meter. Results from this method are reasonably accurate, taking about 1.5 hours to complete.

Thin layer chromatographic method (TLC)

This is applicable to corn (AACC Method 45-05, AOAC Method 26.049/1984).

Aflatoxin is extracted by aqueous methanol followed by column chromatography for clarification. Aflatoxin is the detected by TLC and quantified by a densitometer. Results from this method are accurate and most acceptable. This method will take 3-4 hours to complete.

High performance liquid chromatographic method (HPLC)

This is a highly sophisticated method used for umpire (?) analysis, research and development and trouble shooting purposes. The following sampling and sample preparation procedures are designed for shipment certification by aflatoxin analysis utilising the TLC method.

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Bright greenish-yellow fluorescence test for aflatoxin estimation

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by Prisnar Siriacha

There are three approaches to aflatoxin estimation in corn: (1) a presumptive test to identify corn lots that may contain the toxin and to determine whether a corn lot should be analysed for aflatoxin or not (2) rapid screening methods to establish the presence or absence of the toxin and to know the range of aflatoxin level; and (3) quantitative methods to determine types and, aflatoxin contents.

Since most samples do not contain a detectable amount of aflatoxin, there is a need for a method which correctly identifies the many negative samples with minimum expenditure of time and money. Such a

method is known as the BGYF test or black light test. Corn is inspected under the UV lamp (365 m.) for a characteristic bright greenish yellow fluorescence in broken and damaged kernels. The test takes 5 minutes or less.

The basis of the presumptive test for aflatoxin in corn known as the bright greenish-yellow fluorescence (BGYF) or the 'black light' test is the characteristic fluorescence under long-wave ultraviolet light (365 m.) associated with the presence of kojic acid produced from Aspergillus flavus or A. parasiticus, aflatoxin producing fungi, or possibly mycotoxin itself.

Marsh (1) postulated that BGYF resulted from the action of heat labile peroxidase in the living plant tissues (cotton fibers, corn, etc.) and from some microorganisms on kojic acid produced by many fungal species including A. flavus and A. parasiticus. Thus the BGYF test indicates the growth of the fungi that may have resulted in the production of aflatoxin.

Because the BGYF test is so easily done, attempts have been made to establish aflatoxin levels by numbers of BGYF particles or by the weight and by area of BGYF particles in whole kernel samples. Although a relationship existed between numbers of BGYF particles and kernels and aflatoxin levels, the correlation was not high enough to encourage use of the numbers as an indication of aflatoxin content (2). So BGYF test can not provide a satisfactory quantitative estimate for aflatoxin but should only be used to identify lots for further chemical analyses.

Previous studies indicate that the aflatoxin content of samples with BGYF kernels may range from none to very high concentrations (4). When there are no BGYF particles in 4.5 kg samples of cracked maize, the probability is very low that the sample contains aflatoxin (5). Therefore, an effective aflatoxin

screening programme may consist of accepting lots with no BGYF in the samples, and only analyzing those with BGYF kernels.

PROCEDURES FOR BGYF TEST

1. Usually, at least fresh 4.5 kg samples should be collected, but even larger ones would be better.

2. Sample should be cracked or coarsely ground in a straub disc mill or coffee grinder before BGYF inspection because the fluorescence can occur under the seed coat (3). Sometimes it can be detected as a dull gold color under the seed coat, usually in the germ area, and becomes fully visible when cracked. The grinding process creates dust and if the maize dust contains aflatoxin, it presents a potential hazard to workers inhaling it, thus efficient exhaust fume hoods are needed.

3. Ground maize is discharged in a monolayer onto a black tray.

4. Inspect under long-wave ultraviolet light (365 m.) or black light in a darkened chamber or room. A high-intensity light is recommended, but lower intensity lights may be used in complete darkness. Goggles that screen out UV light must be used to lessen eye strain and prevent possible eye damage from continuous exposure.

BGYF has a bright glow, sometimes called a firely glow, that differentiates it from other fluorescence materials in corn. False results may be reduced by use of the color standards Tinopal BHF (Ciba-Geigy Corporation, Greensboro, NC 27409) or a Black-Ray green fluorescing crayon (Ultra-Violet

Products, Inc., 5100 Walnut Grave Avenue, San Gabriel, CA 91778).

REFERENCES

1. MARSH, P.B., M.E. SIMPSON, R.J. FERRETT, G.V. MEROLA, J. DOSONO, G.O. CRAIG, M.W. TRUCKSESS, and P.S. WORK. 1969. Mechanism of formation of a fluorescence in cotton fiber associated with aflatoxins in the seeds at harvest. Agric. Food chem. 17: 468472.
2. SHOTWELL, O.L., M.L. GOULDEN, A.M. JEPSON, W.F. KWOLEX, and C.W. HESSELTINE. 1975. Aflatoxin occurrence in some white corn under loan, 1971,111 Association with bright greenish-yellow fluorescence in corn. Cereal Chem. 52: 670-677.
3. SHOTWELL, O.L., M.L. GOULDEN, and C.W. HESSELTINE.1974. Aflatoxin: Distribution in contaminated corn. Cereal Chem. 51: 492-499.
4. SHOTWELL, QL., and C.W. HESSELTINE. 1981. Use of bright greenish-yellow fluorescence as a presumptive test aflatoxins in corn. Cereal Chen,. 58: 124-127.
5. SHOTWELL, O.L. 1983. Aflatoxin detection and determination in corn, pp. 38-43. In Aflatoxin and Aspergilius flavus in corn, U.L. Diener, R.L. Asquith and J.W. Dickons, eds. Southern Cooperative Series Bulletin 179. Alabama Agricultural Experiment Station. Auburn, Alabama, USA.

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Plastic minicolumn for aflatoxin detection

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by Srisit Karunyavanij

A method for determination of aflatoxin in food and agricultural products suitable for a rural setting is needed for quality testing of the products. The method should require inexpensive equipment, easy to operate by inexperienced persons, and be sensitive enough to detect aflatoxin down to the tolerated limit of 20 ppb. (microgram/ kilogram). To fulfill this goal, a technique using plastic minicolumn, a kind of small chromatographic tube, was developed by the Department of Medical Sciences of Thailand. With this method, aflatoxin could be measured within two hours with an accuracy of lower than 10 ppb. The method is described in this paper.

The development of this method started since 1976 based on some minicolumn procedures of Holyday, dip column, Velasco et al, Romer etc. by using 5 ml. pipette.

In 1979, glass minicolumn with conical shape on the top part, 4 mm. diameter and 16 mm. length was developed. This minicolumn can contain 1 ml. of the extraction. Alumina, silica gel and florisil were

packed in the column. Sodium sulfate anhydrous was used to absorb moisture, Aflatoxin was examined as a blue fluorescent band at the top of the florisil layer under high intensity long weave UV lamp (365 m.). This procedure can be completed in 1 hour starting from extraction and has a limit of detection of 5 g/kg.

After this modified glass minicolumn was developed and used at the Regional Center of Medical Sciences for sometime, there was a problem of the changes of the packing materials in the glass column which led to more time consumed to finish the procedure.

In 1981, plastic minicolumn was investigated using clear polyethylene straw (without any UV fluoresing substances) instead of glass column. The diameter of this plastic minicolumn is 6 mm. and 25 cm. in length. Same kinds of materials were packed in the column. Moisture and water traces were absorped by calcium sulfate anhydrous. The precipitating solution was a mixture of zinc acetate and saturated ammonium sulfate which will increase the precipitation property in the interaction to reduce interfering substances in the extraction. Saturated NaCI was used as precipitating solvent that will precipitate more in higher acidity while aflatoxin will be extracted more easily.

This procedure can be completed within 15 minutes and has a limit of detection of less than 10 ppb. Final extract from this procedure can also be used for TLC.

MATERIALS AND METHODS

Solid samples such as grain or feed should be finely ground and mixed very thoroughly.

Apparatus

1. High speed blender(1 It-explosion-proof feature not required)
2. Ultraviolet light - Longwave UV with intensity of 430 watt/cm at 15 cm. at 365 m, or Chromatovue cabinet.
3. Plastic minicolumn - clear polyethylene straw (non fluorescent materials) 6.0 mm. in diameter and 250 mm. in length.
4. Glassware
   4.1 beaker (250 ml.)
   4.2 cylinder 25,50 and 100 ml.
   4.3 funnel (75 mm.)
   4.4 filter paper (Whatman No.1) 125 ml.
   4.5 Test tube 15 x 150 mm.
   4.6 Pipette pasteur or graduated pipes (5 cm)
   4.7 Glass rod 150-170 mm.
5. Reagents
   5.1 Organic solvents: Methanol, Acetone, Chloroform, Benzene, Ispropanol (Analytical Grade)
   5.2 Extracting solvents: Methanol: 4% KCI or 5% NaCI (60:40), Burning alcohol + 1% Salt solution 60+40
   5.3 Precipitating solution:

a. Zinc acetate solution: dissolve 125 9 Zn (OAc)2 and 62.5 9 (NH4)2SO4 in distilled water, add 1 ml. glacial acetic acid, add water and make up to 1000 ml.
b. 0.1 MH3PO4: dissolve 5.6 ml H3PO4 spgr. 1.750 in distilled water, make up to 1000 ml.

5.4 Diatomaceous earth: Celite 545 or Hyflo-Super Cel
5.5 Eluting solution: Chloroform: Acetone (90:10)
5.6 Packing materials:

a. Alumina Neutral: E. Merck 70-230 mesh Astm Brockman Activity I, Art 1077.
b. Calcium Sulfate anhydrous fine.
c. Florisil: E. Merck 60-100 mesh Astm Art 12518.
d. Silica gel 60: for c.c.F. Merck No. 7734.
e. Sodium sulfate anhydrous.
f. Defatted cotton wool. (The packing materials a, c and d should be dried at 110C for 1-2 hours twice a week and stored in a desiccator over silica-gel).

COLUMN PREPARATION

Tamp a small plug of cotton wool on the bottom of the column and then add the packing materials: calcium sulfate anhydrous (10 mm), florisil (10 mm), silica gel (20 mm), alumina neutral (15 mm) and

calcium sulfate anhydrous (10 mm) in a consecutive order. Tamp a small piece of cotton wool on the top (as shown in fig.1). Tap the columm between addition of the materials to ensure even packing. Then seal the column with alcohol lamp.

PURIFICATION OF THE EXTRACT

1. Weigh 50 9 of the prepared sample and put into a conical flask. Add 200 ml of extracting solvent and shake contents of the flask well by a shaker for 30 min. or blend with a medium speed blender for 3 min.
2. Filter contents through a folded paper filter into a graduated cylinder.
3. Take 40 ml of the filtrate and pour into a 250 ml beaker. Add 20 ml of zinc acetate solution, stir, and mix well. Leave for 1 min. Then add 40 ml of 0.1 MH3PO4, stir and mix well, and stand for 5 min. Add 5 9 of celite. Mix well.
4. Filter through a folded paper filter into a graduated cylinder.
5. Take 50 ml of filtrate and place the solution into a separating funnel. Add 4 ml of benzene, tighten the glass stopper and shake vigorously for 1 min. Leave until the solutions are separated. Collect benzene from the upper layer and discard the lower layer. Use 1-2 9 Na2 SO4 anhydrous to clear or lessen emulsion in benzene solution.

COLUMN CHROMATOGRAPHY BY A MINICOLUMN

This minicolumn screening technique falls in the category of semi-quantitative methods used in testing foodstuffs for aflatoxin content. It is very useful in screening and grading food products. The procedures are as follow:

Cut both ends of the column and hold the column in place with a clamp. Place a test tube under the column to collect eluate. Add 1 ml of benzene final extract to the column. Let the solvent run down to the surface of the sorbant layer. Add eluting solution 4 times at 1 ml each time. Let all the solvent run down into the test tube. To hasten the flow, use a small rubber bulb with gentle pressure.

Inspect the minicolumn in the dark with a high intensity, long wave UV light (365 m). Compare results with a standard minicolumn of 20, 50, and 100 ppb. If a bluish-green fluorescent band is detected at the proper height (as shown in Fig.1), the sample is judged to be positive to aflatoxin. The higher intensity shows high concentration of aflatoxin.

Good estimation by this method depends on the following factors: 1) skills of analyst, 2) UV light intensity, and 3) darkness of the place used in viewing the chromatogram.

When the sample's column shows higher intensity when compared with standard 20 ppb minicolumn, this means that the sample has aflatoxin higher than 4 ppb but less than 20 ppb. If the sample's column has a higher intensity than the 20 ppb standard but lower than the 100 ppb standard minicolumn, the sample has an aflatoxin concentration from 20-100 ppb.

When the sample's column has higher intensity than the 100 ppb standard, the sample has more than

100 ppb aflatoxin. Aflatoxin concentration in this sample can be estimated by diluting the final extract (1 ml of the extract eluded with 4 ml eluting solvent in another column) and comparing again with 100 ppb standard. If the diluted column has about the same intensity as the 100 ppb standard, the sample has an aflatoxin content of around 500 ppb.

REFERENCES

1. Barabolak, R., Colburn, C.R. and Smith, R.J.J. Assoc. Off. Anal. Chem. 1974, 57: 764-766.
2. Goldblatt, A.F., Pons, W.A. Jr., and Goldblatt. L.A.J. Assoc. Off. Anal. Chem. 1972, 55: 1114-1119.
3. Danteman, J. and Stoloff, L.J. Assoc. Off. Anal. Chem. 1972, 55: 139-141.
4. Hesseltine, C.W.C. and Shorwell, Q.L.Pure Appl. Chem. 1983, 35: 259-266.
5. Holaday, GE. and Lansden, J. Agric. Food Chem. 1975, 23: 1134-1136.
6. Holaday, C.E. J. Am. Oil Chem. Soc. 1976, 53: 603-605.
7. Jemmali, M.J.Assoc. Off. Anal. Chem. 1973, 56: 1512-1513.
8. Knake, R.P., Rao, C.S. and Deyoe, C.W. Feedstuffs. 1972, 44:32.
9. Mc Kinney, J.D.J. Am. Oil Chem. Soc. 1975, 52 : 213
10. Romer, T.R.J. Assoc. Off. Anal, Chem. 1975, 58: 500-506.
11. Romer, T.R. and Campbel, A.D.J. Assoc. Off. Anal. Chem. 1976, 59: 110-117.
12. Romer, T.R., Ghouri, N. and Boling, T.M. J.Am. Oil Chem. Soc. 1979, 56: 795-797.
13. Shannon, G.M., Stubblefield, S.D. and Shotwell, O.L.J. Assoc. Off Anal. Chem. 1973, 56: 1024-

1025.
14. Velasco, J.J. Am. Oil Chem. Soc. 1972, 49: 141-142.
15. Velasco, J. Assoc. Off. Anal, Chem. 1972, 55: 1357-1360.
16. Velasco, J. Assoc. Off. Anal. Chem. 1975, 58: 757-763.
17. Official Methods of Analysis. 1980. 3rd Ed., AOAC, Washington, DC.

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Minicolumn screening methods for detecting aflatoxin: state of the art

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T.R. ROMER, N. GHOURI, and T.M. BOLING, Ralston Purina Company,

ABSTRACT

The use of small chromatographic columns (minicolumns) for the detection of aflatoxin in food or feed extracts was introduced in 1968. Since then many different analytical methods for aflatoxin which involve a minicolumn detection step have been developed. Four of these have been adopted as official Association of Official Analytical Chemists (AOAC) procedures. The advantages and disadvantages of the types of minicolumns along with a comparison of the minicolumn technique to thin layer chromatography is discussed.

INTRODUCTION

Holaday (1) introduced the minicolumn technique in 1968. In this, the first analytical method of this kind, the minicolumn was used to detect aflatoxin in peanuts in a manner similar to that in which a thin layer chromatography (TLC) plate is traditionally used. The minicolumn consisted of a section of 4 mm (ID) glass tubing cat 75 mm in length, containing: (a) a 5.0 mm glass fiber plug to hold the packing material in place; (b) 45 mm of silica gel; and (c) another 5.0 mm glass fiber plug. The minicolumn was placed in a beaker containing a "developing solvent" which was drawn up the column by capillary action. After 10-15 min. the column was removed from the beaker and examined under longwave ultraviolet (UV) light for the characteristic blue or bluish - green color that the aflatoxins emit when exited by light of this (365 nm) wavelength. The two main advantages of this first minicolumn technique for detecting aflatoxins in peanuts over the TLC methods available at that time (24) were that the minicolumn method was both more rapid (25 min VS. 2 hr) and simpler to use. In order to distinguish between this first type of minicolumn and those that were developed later, we shall call this first type of minicolumn, because it was "dipped" into a solution containting aflatoxin, the Holaday

"dip" column.

Modification of the Holaday "Dip" Column

In 1972, Cucullu et al. (5) reported a screening method for the detection of aflatoxin in cottonseed products which used a slightly modified Holaday "dip" column. The modification involved the use of a small layer (15 mm) of acidic alumina beneath 90 mm of silica gel. Thus, in the development of this "dip" column, the sample extract would first pass through the alumina and then through the silica gel. The alumina would serve the purpose of removing certain pigments, etc., from the extract that would otherwise interfere with the detection of aflatoxins on the silica gel. This method was modified by Pons et al. (6) and then by Shannon et al. (7) until finally it became an official Association of Official Analytical Chemists (AOAC) screening method for aflatoxin in corn (8).

Limitations of the "Dip" Column

Although the use of a minicolumn was, as stated previously, more rapid and simpler than the available TLC methods, the "dip" column technique suffered from some serious limitations. These limitations were (a) the volume of sample extract that was drawn up the column packing varied from column to column; (b) the final height of the aflatoxin band varied from column to column; and (c) the aflatoxin band would spread soon after the column was removed from the sample extract. The first limitation impaired the quantitative aspect of this technique. The second limitation seriously hindered the qualitative accuracy of this technique, and the third limitation imposed a variability onto the sensitivity of the method, since the diffusion or spreading caused the fluorescent aflatoxin band to become dimmer with time.

Improvement on the "Dip" Column

In the same year (1972) that Cucullu (5) reported the use of alumina as a second adsorbent in the "dip" column of Holaday, Velasco (9) reported the optional use of alumina in a minicolumn in a method for detecting aflatoxin in cottonseed 25 products, but in this case the minicolumn was not a "dip" column, but rather was one to which a specified volume of sample extract added to the top of the column was allowed to drain through the packing material under the force of gravity. By incoroating into a minicolumn method the use of a constant volume of sample extract, Velasco had overcome the first "dip" column limitation listed above. The other two limitations were also overcome by Velasco in the novel use of florisil beneath the silica gel in a minicolumn. With the elusion solvent used (9:1, chloroform/acetone), aflatoxin will attach itself to the top of the florisil layer in a tight band. With the use of florisil in this manner, there is no doubt where the aflatoxin from standard solution or sample extract will be banded on the minicolumn, nor is there any problem with diffusion of the aflatoxin band after the band has been formed. Velasco also incorporated the use of a layer of sand immediately beneath the florisil to provide an even base for the florisil. Later, when Velasco developed a screening method for aflatoxin in corn which became an official AOAC method (10), a layer of alumina on top of the silica gel was incorporated as a permanent part of the minicolumn.

Other Methods That Use the Velasco Minicolumn

In 1975 Romer (11) modified the Velasco minicolumn and incorporated the modified column into a method for detecting aflatoxin in 24 agricultural commodities. This method has since become on official method for the AOAC (12). The two modifications that Romer incorporated into the Velasco minicolumn were: (a) calcium sulfate was added to both ends of the minicolumn, replacing the sand on

the lower end, and (b) the l.D. of the glass tubing was changed from 3 mm to 6 mm. The calcium sulfate at the top of the minicolumn removes any residual water from a solution added to the minicolumn; the calcium sulfate at the bottom provides an even base for the florisil and also, because of the relatively large granules used (20-40 mesh), allows for a more rapid flow of solution through the column than would the florisil if it were the bottom layer. Both the top and bottom layers of calcium sulfate keep the inner layers (florisil, silica gel and alumina) of the minicolumn packing free of moisture for a short period of time after the column is removed from a desiccator. Moisture on any or all of the three inner layers can render the minicolumn nonfunctional. The use of larger diameter glass tubing (6 mm vs. 3 mm l.D.) provides a minicolumn that is much easier to work with and can hold 3 ml of-solvent on top of the packing without becoming so long that it is unwieldy .Figure 1 (see Fig 1 Minicolumn with layers of packing materials) shows a sketch of this version of the Velasco column. This is the type of Velasco column that is commercially available (13).

In 1974 Barabolak (14) reported a method for aflatoxin in corn products which used a Velasco minicolumn. This method has since been shortened, and the shortened version has been successfully tested in an AOAC collaborative study. (O. Shotwell, personal communication, 1978, results to appear in future issue of JAOAC). Along with the modified Barabolak method, a method reported by Holaday in 1975 (15) was tested in this same collaborative study (O. Shotwell, personal communication, 1978, results to appear in future issue of JAOAC). The latter method uses another modification of the Velasco minicolumn which is referred to as the Holaday minicolumn. In this version, the activated (dry) silica gel and alumina, which had been packed on top of the florisil are replaced by alumnina that contains ca.15% water. This column is also commercially available. (MycoLab Co. provides two types of Holaday columns, one that requires a vacuum source and one that does not. Ag. Science Corp., PO Box 253, Shellman, GA, 31786, and Tudor Scientific Glass Co., 555 Edgefield Rd., Belvedere,

SC, 29841 also provide Holaday columns.) In the collaborative study, both the Velasco column and the Holaday column were tested as part of Holaday's method. When the Velasco column was used, the elusion solvent that was published in the original method (9:1, CHCl3/ Acetone) (7) was used. The results of this collaborative study show that, although both the Velasco and Holaday minicolumns can be used with Holaday's method, the Velasco minicolumn gives better results. Holaday's 1975 method with the Velasco minicolumn is now an official AOAC method.

Fig. 2 Analysis of Aflatoxin by minicolumn

The Use of Vacuum with Minicolumn

Any of the methods that use a Velasco column or a modification of it can be completed in less time if a small vacuum is used to drain the sample extract and elusion solvent through the column. In fact, since the Holaday columns are packed using paper pulp on both ends to hold the absorbents in place, a vacuum is essential to complete the method in fess then an hour. However, if too strong a vacuum is used, the solutions placed on the minicolumn will travel through the absorbents fast enough to cause interferences and/or aflatoxin to spread over the absorbents. As the aflatoxin spreads over more of the florisil layer, the bluish fluorescence becomes dimmer and dimmer until finally a band, which would be very intense if no vacuum were used, will not be detectable. If interferences on the alumnina or silica gel layers spread sufficiently to reach the florisil layer, they will cover any aflatoxin present. This, also, tends to dim the fluorescent intensity of the aflatoxin band. A vacuum should be used that drains a solution through the minicolumn no faster than 1.0 ml/min to ensure that spreading of the aflatoxin or interferring bands will not take place.

Major Uses of Minicolumn Tests

The two major uses of minicolumn tests for aflatoxin are: (a) as "go or no go" field tests to accept or reject a truckload or railroad car of peanuts or corn, and (b) as central laboratory screening tests to reduce the time necessary to test sample that do not contain a detectable amount of aflatoxin. The main reasons that minicolumn tests are so widely used at the plant or field level to accept or reject lots of peanuts and corn are the same reasons that the minicolumn test is preferred to TLC at the plant level; i.e., little time or expertise is required. The minicolumn method that is popular at the central laboratory level (11,12), where the elapsed time is often not as important as at the plant level, retains this popularity because of these characteristics: (a) it applies to virtually all commodities that one might wish to test for aflatoxin; (b) if aflatoxin is detected in a sample, some of the same solution that is used for the minicolumn test can be used for confirmation and quantitation of the aflatoxin present; and (c) this method is an official method of the Association of Official Analytical Chemists (AOAC), the American Association of Cereal Chemistry (AACC), and the International Union of Pure and Applied Chemists (IUPAC).

SOLVENTS

Some of the minicolumn methods use solvents that are known carcinogens, such as benzene and chloroform. In most methods, toluene can be substituted for benzene, and methylene chloride for chloroform. However, controlled experiments should be performed to demonstrate that the method performs well with the solvent substitute.

REFERENCES

1. Holaday, C.E., JAOCS 45:680 (1968).
2. Nesheim, S., D. Barnes, L. Stoloff, and A.D. Campbell 1974. JAOAC 47:586 (1970).
3. Robertson, J.A., Jr., L.S. Lee, A.F. Cucullu, and L.A. Goldblatt 1965 JAOCS 42:467 (1965).
4. Eppley, R.M., JAOAC 49:1218, (1966).
5. Cucullu, A.F., W.A. Pons, Jr., and L.A. Goldblatt, Ibid. 55:1114 (1972).
6. Pons, W.A., Jr., A.F. Cucullu, A.O. Franz, Jr., L.S. Lee, and L.A. Goldblatt, Ibid. 46:803 (1963).
7. Shannon, G.M., R.D. Stubblefield, and O.L. Shotwell, Ibid. 56:1024 (1973).
8. Official Methods of Analysis, 1975, 12th Ed., 26.040-16.053, AOAC, Washington, DC.
9. Velasco, J., JAOAC 49:141 (1972).
10. Official Methods of Analysis, 1975, 12th Ed., 26.044-26,047, AOAC, Washington, DC.
11. Romer, T.R., JAOAC 58:3, 500 (1975).
12. First supplement (March, 1975) to Official Methods of Analysis, 1975, 12th Edition, Chapter 26, Sec. 26.A01.
13. MycoLab Co., PO Box 321, Chesterfield, MO 63107.
14. Barabolak, R., C.R. Colburn, and R.J. Smith, JAOAC 57:764 (1974).
15. Holaday, C.E., J. Lansden, J. Agric. Food Chem. 23:1134 (1975).

Factors affecting the TLC of aflatoxins analysis

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Srisit Karunyavanij

Aflatoxins occur in foods and feeds as a result of mold growth and in products such as milk meat and eggs as a result small of ingesting moldy feed by animals. Aflatoxins are potent carcinogens. Their control requires quantitative method of analysis that are sensitive to concentrations of micrograms to picograms per kilogram of food or feed.

Since the first analytical methods for aflatoxins were published in 1964. Thin Layer Chromatography (TLC) has been the only technique cepable of detecting and quantitating aflatoxins at low levels. Now, methods based on higher performance liquid chromatography (HPLC) and radio immunoassay (RIA) or Enzymelinkaged Immuno Sorbent Assay (ELISA) have been developed, but as yet they have not been tested collaboratively.

The aflatoxins are well suited for analysis by TLC since most of the compounds fluoresce strongly under long-wave UV light. Approximately 0.5 ng-spot can be routinely detected either visually or

instrumentally. The TLC technique serve as both purification and quantitation step. Before TLC analysis, the aflatoxins are extracted from the sample, usually with an aqueous organic solvent, and the extract is initially purified by one or more techniques such as solvent partition, heavy metal precipitation, column filtration, or chromatography. These techniques affect the result of analysis published by the Association of Official Analytical Chemists (AGAC), the American Oil Chemist Society (AOCS), the European Economic Community (EEC), and the American Association of Cereal Chemists (AACC).

Successful analyses of any commodity depend on the selection of appropriate methods for preparation of extract. Also crucial to successful analysis is thin layer chromatography itself. It is not sufficiently appreciated that good quantitation requires efficient chromatography, i.e., separation of the analysis from each other and from other extractives. Many factors can adversely affect TLC separation and quantitation. These factors are:

THE TLC PLATE

Silica gel is used almost exclusively for TLC of the aflatoxins. The commercially available silica gel vary greatly in their chromatographic properties. These variation show up as difference in their ability to separate the four principal aflatoxins (B1, B2, G1 and G2) from each other and from interference.

Only some of the silica gels completely separate these four toxins with the widely used acetonechloroform as the mobile phase. Some products which have been found satisfactory are list in

Table 1, although lot-to-lot differences in their chromatographic properties. The Silica gels also differ in their retentionty for the aflatoxins. Thus with ethyl ether as the developer, migration does not occur on some silica gels but does occur on other. CaSot is the most widely used binder. It produces a soft layer that tends to flake off in the developing solvent. The hard plates produced with polysilicate or organic polymer binders are less prone to mechanic damage, these binders are finding increased use in ready-made or manufacturer prepared plates.

The silica gel can be coated on glass, aluminum or plastic. The advantage of aluminum and plastic is that they can be cut either before or after the chromatography to remove impurities after development or to extract spots for further analysis or identification.

Glass plate is window glass of 0.5 cm thickness and 20x20 cm plate, Glass of this thickness is capable of withstanding handling, clean and heating during repeated use. Thinner glass is too fragile and thicker glass tend to shatter on being heated to 100C or above. The glass must be clean and free of alkaline residue from cleaning agents, which tend to adhere to glass and which, if left, will cause the aflatoxins to decompose. The alkalirie residues are best removed by rinsing with dilute mineral acid and deimized water.

For small laboratory, laboratory-prepared plates have been preferred: they were cheaper, were generally clean and yielded the best resolution.

Now, commercially prepared TLC plates have improved in uniformity of layer thickness and hardness. They can separate the four aflatoxins (B1, B2, G1 and G2) quite well.

Optimum separation is achieved on plates 0.25 mm thick, sometime the plate up to 2 mm thick have been used for preparatory TLC isolation of the aflatoxin. For best resolution and quantitation, 20x20 cm plates are used but 10x10 cm and 10x10 cm plates have also been used to advantage particularly for preliminary screening. The so called high-performance TLC plate made from a narrow-range silica gel of small particle size in the latest to become available and has been evaluated for aflatoxin.

The hand-make or manufactured TLC plates are activated at 80-120c for cat 1 hour. They are stored in a desicator to avoid contamination with atmospheric moisture and pollutants, particularly acids.

TLC plate coating material other than silica gels have been tried but so far have not much use. They include alumine polyamide, formamide-impregnated, diatamaceous earth, or allulose and C18 hydrocarbon bond to TLC silica gel. This system creverse phase TLC of separation on an organic stationary phase using aqueous developing solvents, it works well for the HPLC of aflatoxins.

REFERENCE STANDARDS

Standard aflatoxin are obtained from many sources Table II. The standard is often a source of error in analysis. The accuracy and purity of the standard or standard solution is the responsibility of the individual analyst. The standard solution must be prepared with the same solvent that will be used for the sample extract. Acetonitrite-benzene (2+98) is used for the more soluble aflatoxins (B1, B2, G1 and G2) or Acetonitrite-Benzene (1+9) is used for the aflatoxins to the glass wall of container and (c) detection of deterioration or decomposition during storage of the solution. The aflatoxins are liable to

light or UV irradiation both as solid and in solution in such solvents as benzene, chloroform, ethanol methanol, or water. When not used, the standard solutions should be stored in the dark and at low temperature.

SPOTTING OF THE TLC PLATE

The extract is dissolved for convenient application of amounts equivalent to 0.01 9 of original sample for products expected to contain 5-100 ug/kg of aflatoxin B. and extract equivalent to 5 9 of sample for products such as meat, milk and eggs, that are expected to contain less aflatoxin, i.e., between 0.02-10 ug (B1 or M1)/kg. The use of microliter syringe and the application of 1-50 ul of solution per spot has become standard practice in aflatoxin methodology. Application loss than ul without special equipment may give rise to large errors, likewise, the use of large volumes can result in diffused spots and increased chance of damaging the silica gel [ayes.

Application of the sample should be done rapidly under subdued light without pricking or marring the surface, in an atmosphere of less than 60% relative humidity. At higher humidity the silica gel can be protected, (a) by heating the plate during spotting, (b) by covering the silica with a clean glass plate, (c) by spotting under innertgas in a spotting chamber, (d) by spotting the plate after it has been equilibrated with a solvent. The control of moisture is of utmost importance. Some moisture in the silica gel improves the resolution and eliminates tailing. Under very dry conditions, it is necessary to add water to the developing chamber or to the solvent. However, too much moisture results in poor resolution, slow development, and excresively high Rf's. Tecnniques to control humidity also reduce

chances that the plate will be contaminanted, such as fluorescent lint or pollutants, that can cause fading of aflatoxins.

For quantitation of the aflatoxin by visual estimation 1, 2, 3 and 5 ul portions of standard solution (1, 1, 0.2, 0.2 ng/ul B1, G1, B2 band G2, respectively) are used. The same volumes of the extract solution are spotted after the aflatoxin concentration in the sample extract has been adjusted to approximately that of standard solution, based on the preliminary TLC analysis. The amount of aflatoxin in the extract is then determined by visually matching the intensity of fluorescene of the sample spots with that of the standard spots. To avoid erroneous identification of unknown spot as aflatoxin, the sample should also be co-chromatographed with superimposed standard aflatoxins (internal standard). This procedure helps overcome the confusion that may arise from uneven development across the TLC plate and from the interaction of aflatoxin with constituents of the extract, particularly fatty materials.

DEVELOPMENT OF THE CHROMATOGRAM

The TLC should be developed as soon as possible with the enosen solvent. Only one chromatogram should be developed at a time in a standard chamber (size 25x25x10 cm). The chamber should be well insulated to minimize temperature gradients (not necessary if the chamber is glass) and sealed to prevent solvent loss. For most solvent combinations the best resolution is obtained in a chamber not equilibrated with solvent before the plate is developed. Equilibration, if used, speeds up the development and gives uniform Rf's across the plate, but at the expense of poorer resolution. Development (10-12 cm) should take 30-90 minutes depending on the solvent, the silica gel particle

size, and activity (moisture content). A fixed volume of freshly mixed solvent should be used and preferably placed in a solvent trough rather than in the bottom of the chamber. This procedure saves solvent and improves reproducibility and resolution of the chromatography.

MOBILE PHASES

In choosing solvents and solvent combinations the analyst can take advantage of the wide range of solvent selectives for the individual aflatoxins and interfering constituents of the extract and greatly improve the analytical results. Tables I and III list many of the mobile phases that have been used. The neutral acetonecnioroform mixture is recommended for testing the performance of silica gels and other conditions used for TLC. The acidic developers Benzene-acetic acidmethanol give excellent resolution of aflatoxins B1, B2, G1 and G2 from each other and from certain extract interferences, even though many acidic and most other materials are moved to higher Rf's relative to the aflatoxins than they are with acetone-chloroform. Benzene-ethanol-water and ether-methanol-water are examples of developers in which the separation may take place by partition. The moisture content of the silica gel has less of an effect on separation with aqueous solvents than with mixtures containing no water.

QUANTITATION

The estimation of aflatoxins visually or by densitometry by measuring the intensity of fluorescence of

the aflatoxin spots is the most widely used technique. Basically, in visual estimation two techniques have been used. One is the method of serial dilution of unknown extracts and standard to the point at which fluorescence is not detectable (about 0.1-0.5 ng for aflatoxin B. or G.). This point of extinction depends on the intensity of the UV light, the silica gel, and the residual solvent in the silica gel, as well as the compactness of the spot, the quantity and type of extract interferences, the darkness of the room and the visual acuity of the observer.

The second technique for visual estimation involves comparing the fluorescence intensity of extract spots with those of standard aflatoxin spots. Differences of abound 20% are detectable in the range of 0.2-10 ng aflatoxin. For viewing the TLC spots a Chromato Vue cabinet equipped with one or more 15W, longwave UV lights is adequate. The eyes should be allowed to adjust to subdued lights bofore the fluorescence intensity comparisons are made. If the intensity of the unknown spot is between those of two of the standard spots it is interpolated as half the difference of the volume or amounts in the two spots, i.e., 2.5 if between 2 and 3 ug ng. If the sample falls outside the range of the standard spots, it must be diluted or concentrated and rechromatographed; estimates must never be made by extrapolation from a series of the standard spots.

Densitometry by UV absorptions spectophotometry has been used but because it requires microgram-amount of toxin for detection, it is not practical for analysis of samples in the ug/kg range. Fluoredensitometry is the most widely used technique and is gradually supplanting visual estimation. Again, good chromatography with well-resolved, small spots is essential for good quantitation, even more than for visual estimation, and the same factors influence both humidity, silica gel, developing solvent, solvent residues, layer thickness and uniformity, contaminants, Rf, and type and mode of operation of instrument.

One of the basic problems in the instrumental measurement of the aflatoxin spots is minimizing the effect of extract interferences, a problem as yet only partially solved. One approach is that of standard addition, i.e., graded amounts of standard aflatoxin are added to the unknown extract. The observed responses are plotted against amounts added. The amount of aflatoxin originally present in the unknown is calculated from the observed response for the point at which no aflatoxin was added. This technique is more tedious than others and offers no compensating advantages. The second approach is to spot the standard at four concentrations, close to dried including the concentrations of the unknowns, construct a standard curve for each TLC plate, and make the determination of the unknown from the standard curve. This would appear to be the safest approach because the system is tested for linearity for each chromatogram. The third approach as described in AOAC method is the simplest, most efficient, and most widely used. The sample and standards are then spotted in duplicate at similar concentrations. In this procedue only an occasional check on the response linearity is done as an analytical quality control.

In the scanning of the chromatogram the following precautions may help improve the results, Because the amount and nature of the solvent remaining in the silica affects the fluorescence intensity of the aflatoxins, volatile developing solvent must be rigorously eliminated. For the solvents commonly used drying the plate 15 minutes at 25C or 5 minutes at 40-80C is sufficient. Fading of the aflatoxins can be prevented by covering the chromatogram while still hot with a clean glass plate. Although it is advisable to scan the plate after development as soon as possible, some delay can be tolerated. If delay is necessary, the chromatograms are best stored in the dark, protected from atmospheric reactants, prefesably in a cold place. Storage for as long as a week has shown no problems.

The spots should be scanned in direction parallel to the direction of development and from low to high

background. The duration and intensity of the UV and other light exposure must be kept to a minimum to avoid undesirable changes. Exposure of aflatoxin to light for 4 hours has resulted in a 40% reduction. If fading of the aflatoxin spots occurs, the standards usually fade faster than the aflatoxin spots in extract. The standards should therefore be scanned before the unknowns. The TLC and scanning results should be evaluated as they are being carried out. Duplicate spots should agree within 5% or else be rescanned.

The instrumental parameters to be optimized include the following: selecting excitation light filters or monochromator setting for maximum response; setting the gain for maximum signal - to noise ratio; selecting scanning and recorder speeds: selecting UV cutoff filter or monochromator setting before the detector for maximum response, minimum noise, and case and accuracy in measurement of instrument response; and selecting optimum slit widths and lengths for the best signal-to-noise ratio consistent with adequate resolution of adjacent spots. The output from the instrument can be a strip chart recording on which peak areas can be measured manually or mechanically, it can be electronically integrated using any of many types of integrators.

After the appropriate instrumental parameters are selected, the linearity of response to concentration must be determined by preparing a standard curve covering the range of concentrations of interest. For instruments such as the Schoeffel, peak areas appear best related to concentration, whereas the Zeiss spectrophotofluoro-densitometer accurately measures concentration by peak height as well.

MULTIPLE DEVELOPMENT AND TWO-DIMENSIONAL TLC

For many commodities, i.e., eggs, cheeses, tissue milk figs, spices, mixed feeds and fish-meals, one-dimensional TLC is not is not adequate for separating the aflatoxins from interfering constituents. It is also not adequate when levels lower than 1 ug/kg are to be detected.

The simplest technique for improving separation is multiple development with the same or different solvents (Table III). An extension of this technique is a procedure in which the sample and standard are spotted along a horizontal line midway on a silica gel coated alumina sheet. The sheet is predeveloped with ethyl ether which moves many interferences to the top of the chromatogram but leaves the aflatoxins near the origin. The top of the chromatogram is cut off and the sheet is rotated 180 and developed with a solvent appropiate for aflatoxins. An apparatus that automatically performs multiple development is commercially available. It has been applied to aflatoxin in a preliminary test.

The technique of two-dimensional is timeconsuming, because only one sample can be applied perTLC plate. Usually the standards are developed in only one direction, but this appears to be satisfactory as demonstated in studies room. Both visual methods and densitometry can be used for estimation.

CONFIRMATION OF IDENTITY

Because initial identification of unknowns in a chromatogram is based on the similarities of their Rf values with those of standard aflatoxins, additional proof of identification is needed. This need can hardly be over-emphasized, particularly for regulatory samples and for commodities with a limited history of analysis or incidence of contamination. Several techniques that have been used for

confirmation of identity (Table IV). Rechromatography with several different solvent such as in Table 1 and the use of the spray reagents in Table IV are not specific tests and do not give conclusive positive identification. They are conclusive only when they show that the unknown is not aflatoxin. The mineral acid sprays are widely used tests; the acid changes the fluorescence of the aflatoxins from blue to yellow. If an unknown remains blue fluorescent on spraying, one can safely conclude that it is not aflatoxin.

The preparation of chemical derivatives of the aflatoxins with changed chromatographic properties is the simplest method for positive confirmation of chemical identity. Widely used tests for aflatoxin B., G. and M, involve the formation of a water addition product, with trifluoroacetic acid as the catalyst, or formation of acetates. TLC derivatives of the unknowns are then compared by TLC with the derivatives of standard aflatoxins, the derivative are formed directly on the TLC either before or after it is developed.

Move specific than any to the above techniques is that of mass spectrometry. Recent improvements have greatly lowered its detection limits, now approaching nanogram amounts of aflatoxin B1 from crude extracts.

Table 1. Mobile Phase and Sorbents for Thin Layer Chromatography of Aflatoxins

Table II Source of Mycotoxin Reference Standard

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1. Aldrich Chemical Co., 940 St Paul Ave., W. Milwaukee, Wl 53233

2. Applied Science Laboratories, Inc., P.O. Box 440, State College, PA
3. Calbiochem, 10933 N. Torrey Pines, La Jolla, CA 92037
4. Makor Chemicals Ltd., Box 6570, Jerusalem, Israel
5. Dr. L. Leistner, Federal Meat Research Institute, 865 Kulmbach Germany, (standard only)

Table III Molecular weights, m, wavelengths of maximum absorption, max. and molar absorptivities of mycotoxins

DETECTION, ESTIMATION AND CONFIRMATION OF AFLATOXIN BY THIN LAYER CHROMATOGRAPHY (TLC)

Several TLC methods may be used to estimate the concentration of aflatoxins.

The method routinely used at TDRI is the "comparison-of-standards" technique, where known concentrations of pure aflatoxin standards are chromatographed alongside portions of the extract. The fluorescent intensities of the spots on the developed chromatograms are compared visually or instrumentally. This method has superseded the "dilution-to-extinction" technique where the minimum amount of aflatoxin required to produce a fluorescence is known, and used as a standard factor and the sample is diluted until the extinction point is reached.

APPLICATION OF EXTRACTS ONTO TLC PLATES

The method is the same for all TLC techniques. Apply the solutions to the chromatoplate using disposable micro-pipettes, renewing the micropipettes after application of each solution. The extracts should be applied such that the resultant spots are as compact as possible (less than 5 mm diameter). The volumes of extracts and standards applied to the plates vary according to the method of estimating and suspected concentration of the extract.

UNI-DIMENSIONAL CHROMATOGRAPHY

Development of Plates

Develop the plate by standing it in a chromatography tank containing the appropriate solvent to a depth of not more than 0.5 cm. For some solvent systems, the chromatography tank should be left for a short time to equilibrate before the plates are developed. The development time is approximately 20 minutes, depending on the environmental conditions, (the solvent - front must be at least 10 cm from the baseline). Examine the dry, developed plate under long wave (365 nm) ultra violet light, preferably in an enclosed viewing cabinet fitted with a protective filter.

Detection and Estimation

(a) "Comparison of standards" technique

Compare the fluorescence intensities of the spots at the Rf of B1 in the sample with those of the B1 standard spots and determine which of the sample spots matches one of the standards and record the corresponding aliquot volumes. If the sample spot intensity lies between two adjacent standard spots the average liquor volume of the standard spots is recorded. If the spots of the smallest volume of sample are too intense to match the standards the sample extract should be diluted and re-chromatographed.

The concentration of aflatoxin B1 in the sample in ug/kg, is calculated from the formula:

Aflatoxin B. content (ug/kg) = $\frac{S \times Y \times V}{W \times Z}$

Where

S = Volume, in ul of aflatoxin B1 standard of equivalent intensity to Z ul of sample.
Y = concentration of aflatoxin B1 standard in ug/ml
Z = Volume, in ul, of sample extract required, to give fluorescence intensity comparable to that of S ul of the B. standard.
V = Volume, in ul, of solvent required to dilute final extract.
W = Weight, in g, of original sample contained in final extract. At TDRI this is termed, final “effective weight".

Proof of the equation $\frac{S \times Y \times v}{W \times Z}$: -

From the TLC plate:

S ul standard = Z ul sample

The weight of aflatoxin in the sample (Z ul) and standard (S ul) spots are equal.

Weight of aflatoxin in the standard spot: (S ul)

The concentration of standard

$= Y$ ug/ml or $\frac{Y}{1000}$ ug/ul

Therefore weight of aflatoxin is S ul

$= \frac{Y \times S}{1000}$ ug

Weight of aflatoxin in sample spot: (Z ul)

This must also $= \frac{Y \times S}{1000} ug$

Therefore Z ul sample contains $\frac{Y \times S}{1000} ug$ aflatoxin

The V ul final sample extract must contain $\frac{Y \times S \times V}{1000 \times Z}$ ug and this is the weight of aflatoxin in the final "effective weight" (W) of starting material.

Thus the weight of aflatoxin in 19 starting material

$$= \frac{Y \times S \times V}{1000 \times Z \times W} ug$$

Therefore weight of aflatoxin in 1 kg of starting material

$$\frac{Y \times S \times V}{Z \times W} ug/kg \quad (ppb)$$

Example using "Comparison -fo - Standards" methods of estimation

20 g comminuted groundnuts were defatted to give 10.9 g material, 10 g of which was extracted with

100 ml chloroform and 10 ml water. The extract was filtered and 50 ml extract was dried and made up to 0.5 ml then, 3 ul 5 ul and 10 ul of this solution were spotted onto a chromatoplate together with 2 ul, 5 ul and 10 ul of standard B1 solution. After development, inspection of the chromatoplate under UV light showed that the 5 ul spot of the extract was equal in intensity to the 2 ul standard spot.

Using the equation: $\frac{Y \times S \times V}{Z \times W}$ ug/kg (ppb) - described above.

In this case:

S = 2 ul

Y = 0.66 ug/ml

V = 0.5 ml or 500 ul

Z = 5 ul

W = 9.18 g

The "Effective Weight" (W) for this example is calculated es follows:

20 g sample yielded 10.9 g defatted material. (It is assumed that all the aflatoxin remains in the meal).

Thus the "effective weight" at this point = $\frac{20\times10}{10.9}\text{g} = 18.35\text{g of}$ starting material

All the aflatoxin extracted from the 10 g sample will be homogeneously distributed in the 100 ml of chloroform. The 10 ml of water used in the extraction is absorbed by the sample and is therefore disregarded.

The "effective weight" (18.35 9) at this point is, therefore, contained in 100 ml of CHC13.

But after filtration, only 50 ml filtrate is used for further analysis and the "effective weight" at this point is therefore given by $18.35\times\frac{50}{100} = 9.18\text{g}$ of starting material

The 50 ml of extracted material is concentrated to 0.5 ml and, contains all the aflatoxins which were present in the 50 ml volume. Therefore, the "effective weight" does not change during the concentration step.

Therefore the aflatoxin content of the sample

$$= \frac{2\times0.66\times500}{5\times9.18} = 14.4\text{ ug/kg (or 0.014 ppm)}$$

(b) "Dilution-to-extinction" technique

Under UV (365 nm) light look for a blue fluorescence in the sample at an Rf corresponding to aflatoxin

B (B1 and B2 will not be resolved) in the standard reference marker. The object of this technique is to find a dilution of the extract, such that, a 15 ul or 20 ul aliquot applied to the TLC plate will give a blue fluorescence of aflatoxin B which is JUST VISIBLE. This means that the 5 ul and 10 ul aliquot will give no fluorescence but in the 25 ul aliquot the fluorescence will be easily visible. Consequently, if the chromatogram shows blue fluorescences in all the aliquots, then the extract has to be diluted sufficiently to give the above extinction point. Trial and error and eventually experience enables the correct dilution to be made and the extinction point determined efficiently.

It is essential that the conditions used for this method are standard, ie:

1. The plate is hand coated Kieselgel "G".
2. The thickness of the plate is 0.5 mm.
3. The plate is spotted exactly as described above.
4. The plate is developed to a distance of 10 cm between the solvent front and the baseline.
5. The plate is placed at a set distance (30 cm from the UV lamp).
6. The output of the UV lamp is 125 watt.

Under these conditions the smallest weight of aflatoxin B giving a fluorescence which is just visible is 0.4 ng (0.0004 ug), and for aflatoxin G is 0.3 ng (0.0003 ug).

This factor is used in calculating the aflatoxin content as follows,

$$\frac{0.4 \times D \text{ ug/g (ppm)}}{W \times v \text{ aflatoxin B}}$$

or $\frac{0.4 \times D}{W \times v} \times 1000 = \text{ug / kg (ppb)}$

and $\frac{0.3 \times D}{W \times v}$ ug/g aflatoxin G (ppm) or

$\frac{0.3 \times D}{W \times v} \times 1000 = \text{ug/kg (ppb)}$

Where

D = total volume, in ml, necessary to dilute the extract so that the fluorescence is just visible in V ul of sample extract
V = aliquot volume, in ul, at which the fluorescince is just observed on the developed chromatoplate
W = weight, in g, of original sample contained in the extract

Proof of the above equations:-

A "*just visible*" fluorescence at Rf B (or G) in the sample extract contains 0.4 ng aflatoxin B (or 0.3 ng aflatoxin G).

This was applied to the plate in V uls sample extract, taken from a final dilution volume of D mist

Thus if V uls aliquot contains 0.4 ng (or 0.3 ng) aflatoxin

D ml (D x 1000 ul) will contain $\frac{D \times 0.4}{V} \times 1000$ ng

aflatoxin B

D ml also contains the "effective weight" (W), so weight of aflatoxin in Wg is:

$\frac{D \times 0.4}{v} \times 1000$ ng in Wg or $\frac{D \times 0.4}{v}$ ug in Wg

Therefore I g of starting material contains

$\frac{D \times 0.4}{v} \times \frac{1}{w}$ ug aflatoxin B (ppm)

and 1 kg of starting material contains

$\frac{D \times 0.4}{v} \times \frac{1000}{W}$ ug aflatoxin B (ppb)

Example using dilution to extinction method of estimation

Assume that the defatting, extraction and filtration procedures are the same as in the sample on page 138. Take 50 ml filtrate and concentrate this to 5 ml. Spot 5, 10, 15, 20 and 25 ul volumes on to a Kieselgel "G" chromatoplate. After development of the plate the fluorescent spot of aflatoxin B is just visible (under the prescribed conditions of illumination) in the 15 ul volume but not in the 10 or 5 ul

volumes.

For this example the calculation is as follows:

$$\frac{0.4 \times D}{W \times V} \text{ ug/g aflatoxin B}$$

or

$$\frac{0.4 \times D \times 1000}{W \times v} \text{ ug/kg aflatoxin B}$$

Where

D = 5 ml

V = 15 ul

W = 9.18 g (calculating of the effective weight is as shown in the previous example).

Therefore: $$\text{aflatoxin B content} = \frac{400 \times 5}{9.18 \times 15} = 14.4 \text{ ug/kg}$$

TWO DIMENSIONAL TLC

Two-dimensional TLC can provide a means of incorporating a "clean-up" step into the quantification procedure ("comparison-of-standards" technique) of the analysis.

Figure

Standards are applied to the plate on the pencil lines in the reference channels and the sample is applied at the junction of these two lines (see diagram above). This means that the sample is in line with the standard when the plate is developed in both directions. Only one sample can be assessed at a time. The same solvent system can be used in both directions, or two different systems can be used. Another plate is scored and spotted, identical to that just spotted, with the exception that an extra 5 ul of standard solution is applied on top of the sample spot, which acts as an internal marker for the toxin on this so-called "spiked" plate. The former "unspiked" plate is developed in the same tank as the "spiked" plate, so that the development conditions are exactly the same for both plates. The plates are placed in the tanks such that their coated surfaces face towards the centre, but do not touch. The solvent is allowed to rise up the plate until score line A is reached. The plate is removed and thoroughly dried in the dark (no solvent residue should remain).

Diagram showing chromatograms and area of plate affected by solvent after development in direction 1

The plate is turned through 90 and developed in the second solvent system

After development in second direction

Final chromatoplate (viewed as spotted) showing pathway of the toxin fluorescences

It can be seen that the Rf values of the toxin fluorescence in the "sample" and "spiked-sample" do not correspond exactly to the standard Rf values in the reference channels but are slightly further advanced in direction (2) towards the right of the plate. This is because the coated surface of the plate within the score lines A and B is deactivated by the first developing solvent prior to the second development whereas the coating in the reference channels is not affected. Obviously these differences will depend on solvent systems and coating material used.

INSTRUMENTAL EVALUATION OF TLC PLATES

The methods described above for estimating the concentration of the mycotoxin extract, either by "dilution-to-extinction" or by "camparison-of-standards" techniques, are subjective measurements. Due to the difficulty of estimating small differences in fluorescent intensity with the eye, visual estimating has a coefficient of variation of about 30% even under ideal conditions. These limits of precision are, however, sufficient for most purposes.

A number of reports have appeared which describe the use of any one of a number densitometers available for measuring the fluorescent intensity of mycotoxins on TLC plates. The results indicate that both the accuracy and provision of mycotoxin analysis can sometimes be enhanced when objective instrumental measurements of the intensity of the fluorescence are made, but this is influenced by factors such as errors in spotting, poor resolution with some batches of silica gel etc.

Collaborative studies have indicated that the lack of reproducibility arising from these factors negates

any advantage obtained by utilising the more accurate detector within the densitometer. A coefficient of variation of 30% was obtained irrespective of whether visual or densitometric estimation was employed.

CONFIRMATION

After the identification and estimation steps of the analytical procedure, it is ESSENTIAL to include an additional step which confirms, unambiguously, the authenticity of the mycotoxins. There are a number of confirmatory procedures available and many are based on the formation of coloured or fluorescent mycotoxin derivatives. The following methods are those used routinely at TDRI for confirming the presence of the aflatoxins.

SAFETY PRECAUTIONS

Laboratory Facilities Required

1. A laboratory or part of a laboratory should be reserved for mycotoxin analysis only, and the work "confined to that area.
2. The bench top should be of a non-absorbent material, eg formica (Whatman "Benchkote" can also be used but it must be removed and destroyed after use), and should be screened from direct

sunlight.

3. Analyses should be performed in a well ventilated laboratory, preferably under an efficient extraction hood, and fume cupboard facilities should be available.
4. Eating, smoking or drinking should not be permitted in laboratories where mycotoxin analyses are carried out.
5. Many of the solvents used are highly flammable and have low flash points. Bunsen burners, electric fires, and sparking apparatus such as centrifuges should not be used in the same laboratory.
6. The amount of flammable solvents in the laboratory should be kept to a minimum and stored in a fire-resistant cupboard or bin.
7. Warning notices should be posted on mycotoxin laboratory doors.

Precautions During Analysis

1. During the grinding and weighing of samples there is a risk of absorbing toxin either through the skin or by the inhalation of dust. There is also the risk of developing allergic reactions due to spores and organic material. These risks should be minimised by working under an extraction hood, by good hygiene and also wearing protective clothing and masks.
2. Acetone used in the extraction of the toxin is highly flammable and every care should be taken to prevent a fire, eg by using flame-proof blenders and drying or washing filter papers soaked in acetone before incineration.
3. A number of solvents used during these analyses, particularly benzene, are toxic and care should

be taken to avoid inhalation and skin contact. Since benzene is a particular problem as far as skin adsorption is concerned gloves resistant to benzene absorption should be used.

4. Waste organic solvent should not be put down the sink but should be collected and disposed of into an appropriate waste solvent bottle. CARE, acetone or acetone/organic solvent mixtures must never be added to the waste solvent bottles as explosive mixtures with chloroform are possible in the presence of base. Instead, evaporate all acetone containing solvents on a water bath in a fume cupboard.
5. Gloves, masks and protective clothing must be worn whenever necessary.
6. Safety spectacles must be worn at all times when doing practical work.

Precautions During TLC

1. Toxin solutions for TLC, should be of the minimum strength necessary, and stored in small vials. All spotting of TLC plates should be carried out in a shallow tray that can contain any spillage of standard solutions.
2. Spillages should be mopped-up with absorbent material which is disposed of by incineration. The infected area should then be treated with a 4% solution of sodium hypochlorite. Extracts, samples, sample residues and filter papers should also be incinerated.
3. Glassware and TLC plates should be decontaminated by soaking for 2 hours in a 1% sodium hypochiorite solution. After this time an amount of acetone equal to 5% of the total volume of the bleach bath should be added, and the glassware soaked for a further 30 minutes (Anon., 1980).
4. Spraying of TLC plates must be carried out in an efficient fume cupboard or spray cabinet.

Always ensure that this equipment is working before commencing use.

5. When viewing chromatograms under UV light the eyes should be protected by UV filter (as in the "Chromatovue" cabinet) or by wearing protective spectacles.

REFERENCES

Anon. (1980) Laboratory Decontamination and Destruction of Aflatoxins B1, B2, G1, G2 in Laboratory Wastes (ed Castegnaro et al.) International Agency for Research on Cancer (IARC) Publication No. 37.

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Assessment of insect nfestation in stored maize and their relationship to aspergillus flavus contamination

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by Valerie F. Wright

ABSTRACT

An entomological assessment of Thai maize in storage was conducted in relation to Aspergillus flavus infection, aflatoxin contamination and association of stored - product insects. The pattern of insect infestation and A. flavus infection showed strong correlation to high densities of weevils (live or dead) and other secondary species. Fumigations when weevil populations were high produced many cadavers on which A. flavus propagated supplying great quantities of inoculum. Training in sanitation and pest control are needed. The National Training program should be implemented by upgrading Department of Agriculture staff for teaching broad-based storage management concepts. A training approach is suggested. Future research by the Stored-Product Insects Research Section was suggested in the following areas:

- Surveys of Thai grain for aflatoxin should include collection and quantification of insects in maize samples from various storage conditions;
- Maize weevil population densities should be correlated to the presence or absence of aflatoxin;
- Dispersion of insects from highly infested warehouses or silos can be documented by the use of various kinds of traps;
- Methods for treating maize with a grain protectant after drying need to be developed for extension use;
- A survey for resistance of insects to grain protestants is needed for baseline data and documentation of later changes during insecticide usage.

1. INTRODUCTION

Within the objectives of the joint project of the Government of Thailand and the United Nations Development Program (UNDP) to reduce postharvest losses in stored grains is an investigation of the entomological factors affecting grain damage especially in their relation to the spread of aflatoxin-producing fungi. The role of certain field and storage pests is known in the inoculation of maize before harvest and in the production of conditions which promote A. flavus growth and proliferation during storage. Climatic conditions in Thailand maize growing regions are especially conducive to both A. flavus growth and stored-grain insect population increase.

Maize is Thailand's second largest grain crop after rice. Dry maize is not for human consumption but for animal feed and export. Because of problems with aflatoxin contamination and insect infestation, recent crops have met with reduced prices and refusals at foreign ports (Walters 1987). Whether or not Thailand's maize crop contains permissible aflatoxin concentrations for export may depend on controlling stored grain insect populations as well as drying and handling the maize properly. The triple effect of losses due to insects, fungi and mycotoxins can be devastating in years when conditions are favorable for grain deterioration.

ASSESSMENT OF INSECT INFESTATIONS IN MAIZE AND THEIR RELATIONSHIPS TO ASPERGILLUS FLAVUS CONTAMINATION

The presence of insects associated with both field and storage damage of maize in Thailand has been documented by other consultants in this project. One of the objectives of Eugene Smalley's consultancy (December 1985 - February 1986) was to assess the role of insects and arthropods as vectors of mycotoxin-producing fungi. He sampled first crop maize in the field and found the maize weevil, Sitophilus zeamais Motsch, in freshly picked ears. The infestations were usually at the tip of otherwise sound ears and were rarely extensive. No weevil infestation was observed in second crop at harvest. However, first crop maize at middleman warehouses (stored for 3-4 months were heavily infested. Dead weevils were yellowgreen with conidia of A. flavus. Large silos also harbored tremendous weevil populations. Dilution plate counts of ground weevils revealed the presence of A. flavus and many other storage fungi. Weevil-bored kernels (first crop) from middleman warehouses were 100% infected with A. flavus while sound kernels were 40-50% infected. Second crop whole kernels without weevil damage from the drying floor contained no A. flavus. When weevil tunneling was taken into consideration as well as exit holes, 13-18% of the kernels sampled were damaged in first crop stored at middleman warehouses.

Field Studies

During this consultancy (November 1986), little maize was in storage. Small amounts of second crop maize were found but most farmers' cribs were empty. Middleman warehouses visited by this consultant contained 1 or 2 bags of second crop maize but mainly mung bean was in storage. First crop maize was found only at one port facility. The other two visited were exporting rice and other commodities. Samples of maize and insects taken at the above sites were not representative of the 1986 crops. However, the pattern of insect infestation and A. flavus infection is similar to previous findings. Maize from cobs collected at farms showed no growth of A. flavus after surface disinfection

with 2% sodium hypochlorite for 1 min. and 4-5 days on malt-salt (4%) agar plates. No storedproduct insects were found at the 3 farms visited. Cribs were essentially empty. Cobs present generally were low quality and immature. These were not sampled. They appeared to have been sorted from grain sold or gleaned from the field. One farm had good quality maize, taken as samples. No A. flavus was present (Table 1).

Of the two middleman warehouses visited, the first had only one bag of maize, a small seeded variety that at some time had been treated with an unknown pesticide. The rest of the storage was mung beans which also had been treated with pesticide. The maize sample contained no insects. A. flavus infected 45% of the kernels (Table 1, 3c).

The second middleman warehouse had new second crop maize on the drying floor and 1-2 month old second crop maize in 5 stacks. The Lopburi warehouse is the largest buyest buyer of maize in the province. They have a quota on maize export. Farmers know that they can sell here. The second crop of maize is dried on the concrete drying floor to about 16% MC (Steinlite Moisture Meter). The maize is cleaned (?), bagged and placed in stacks 10-15 bags high and 1015 bags wide (minimum). The maize is dried to 15% MC in these stacks by natural air currents over a 2-3 month period. The dryer is used only when the price of maize allows. Dryer bins are sometimes used for storage. Unused bags are piled behind the warehouse under a roof. These are fumigated as needed. Fumigations are undertaken by their personnel using methyl bromide under tarps. Rolled bags are used to "seal" the tarp to the floor. One stack was under fumigation with methyl bromide.

Table 1. Storage fungi counts from maize kernels collected at four sites in Thailand, November 1986, expressed as percent of total kernels plated (10 kernels/plate)*

	Site	No. of plates	Aspergillus flavus (%)	Aspergillus niger (%)	Penicillium spp. (%)	Others (%)
1.	Farm	5	0	8	2	48
2.	Godown					
	Stack a (no insects)	5	8	4	0	14
	Stack b (few insects)	5	18	18	4	26
	Stack c (many dead weevils)	4	90	15	3	3
3.	Middleman Storage					
	sample a	5	24	10	6	18
	sample b	5	24	16	0	44
	sample c	6	45	18	3	13
4.	Silo (cleaned for export)	6	17	3	53	12

*** Malt-salt (4%) agar for 4 days after surface disinfection (Seed and Postharvest Pathology Section, DOA). Data is not representative because of small sample size.**

Maize was sampled from three stacks. There were many insect present in the samples and wandering about the facility. The maize samples from areas in the stacks where no insects were found had low A. flavus counts (8%, Table 1, 2a). Samples with few insects had somewhat higher counts (18%) and samples with many dead weevils had a high degree A. flavus infection (90% of kernels, Table 1, 2c). Insects (both live and dead) from these samples reflected the same pattern (Table 2). The stack with lower infestation levels (shorter time in storage) had insects with lower contamination rates. Dead insects are an excellent substrate for A flavus. This shows well in the high A. flavus counts (76 and 100%) from dead insects of all kinds. Smalley (1986) observed piles of dead weevils in warehouses that were yellow-green with A. flavus spores. The spore load is likely to increase after death because the fungus is actively growing on the cadavers. This is an important source of inoculum for maize on the drying floor and insects walking over it, or within fumigated stacks. This silo generally kept maize 2-3 months.

The export silo in Bangkok was heavily infested with several species of stored-product insects. Samples taken at the bin tops were composed mainly of red flour beetle, flat grain beetle, and maize weevil. Samples from the cleaning floor were almost entirely maize weevil. All insects were carrying heavy spore loads of A. flavus after surface disinfection. Dead insects were as contaminated as live insects or more so (Table 2, a, b, c).

Inspection of maize samples with a black light showed presumptive evidence of aflatoxin only in one kernel of a maize cob from a farm but in many pieces of maize screenings below the cleaners in the export silo. Personnel at the silo were monitoring the grain as it was loaded on the ship and said that aflatoxin concentrations were less than 20 ppm in their whole kernel samples. The

screenings were allowed to accumulate for several months. Mounds of screenings (5 ft. high) beneath the cleaning apparatus were black with millions of live and dead weevils.

Table 2. Microbial counts from stored-product Insects collected at two sites in Thailand, November 1986, expressed as percent of total insects plated..

Table 3. Black light indication of bright green-yellow fluorescence in maize samples from three sites.

Site	BGYF
Farm Sample	
Maize cobs with damaged kernels	yes (1 Kernel)
Godown	
Kernels	yes
Screenings	no
Silo	
Cleaned for export	no
Sweepings on bin to	no
Screening from cleaner	yes (many pieces)

Discussion

Field infestation of maize before harvest has been strongly associated with A. flavus infection and aflatoxin contamination (many authors, see references-. The main correlation is with field pests, such as European corn borer, because they are capable of initial damage to the cob and sheath. Kernel damage by European corn borer opens sites for infection by A. flavus from any inoculum source. Stored-product insects found in the field before harvest generally require previous damage to the husk, or ears that protrude from the sheath (some hybrids), in order to gain entry. They may carry A. flavus spores into the ear and inoculate kernels. However, the maize weevil is considered a poor vector of A. flavus in the field (LaPrade and Manwiller, 1977; McMillian 1980). The main role of maize weevil in A. flavus infection and subsequent aflatoxin contamination occurs during storage. Large populations of weevils can change conditions within bagged or bulk grain to encourage the growth of storage fungi (Christensen and Kaufmann 1969). They can also carry inocolum from the warehouse or other storage areas back to field prior to harvest.

In Thailand the maize weevils and other storedproduct insects are a complicating factor in the production of aflatoxin in maize. The main reason for A. flavus growth and aflatoxin production seems to be high moisture maize at harvest. The need for drying the grain has been recognized and steps are being taken to promote rapid drying after harvest at the farm level and first buyer stations (several projects in Ag. Engineering and Extension). Maize weevil populations left unchecked in godowns and warehouses pick up a heavy spore load of A. flavus and transmit it to both high moisture and dry grain. They are a continuous source of A. flavus inoculum throughout

the year. As the population increases, weevils migrate to other areas, such as new maize brought into the warehouse. Even if this maize is dry (15% moisture content), the weevils can change the condition of the grain within the stack or bulk by increasing temperature and moisture through their metabolic activities until the limit for A. flavus growth is reached. That is why stacks that seem to have good storage characteristics can become heavily infected with storage fungi within a short period.

The second crop maize observed at the silo in Lopburi province was a good example. At 15% moisture, maize may be considered dry and storable by some. Actually, it is at its lower limit for growth of storage fungi. Any factor, such as weevil infestation. That can change the condition of the maize and raise the moisture content will cause deterioration sooner than expected (Christensen and Kaufmann 1969). Maize stored at 15% moisture has a somewhat predictable safe storage time as long as other factors do not intercede to change the quality of the grain. The damage to kernels by feeding weevils are primary sites for fungal growth. Storage fungi invade kernels more quickly through breaks in the pericarp.

Stored-grain insects other than maize weevil harbor storage fungi also (Table 2). These insects may dispense A. flavus spores throughout a grain mass as readily as the maize weevil. A further consequence of mouldy grain in storage is the rapid increase in populations of these "secondary" insects which can feed on damaged grains but not on intact kernels. These insects are aided in their growth by additional nutritional value from storage fungi growing on the kernels (Wright et al. 1980a). "Blooms" of these insects are often found in association with deteriorating grain. The effects of known mycotoxins on these insects are minimal (Wright et al. 1980b, 1982).

General Recommendations

Maize at "safe" moisture contents for storage or above is particularly vulnerable to maize weevil attack with subsequent rapid increases in weevil numbers, moisture content, temperature, growth of storage fungi and secondary insects. Controlling storage pests is a necessity for reducting losses in stored grain and maintaining grain quality. Fumigations of stacks or bulks already heavily infested -may control the insect population momentarily, but a great deal of damage has already been done. Kernels have been damaged by insects and opened to fungal attack. Fungi already in the kernels are not affected by fumigation. Dead insects are invaded by A. flavus and large. amounts of inoculum are produced. The consequences are obvious. Warehouses and silos that allow insects to build large populations are producing problems for themselves, for farmers and other storage facilities in their area. Migrating insects infest nearby farm fields and cribs and invade other facilities which may have an adequate control program. Insect populations should be controlled while the numbers are still low. Fumigating heavily damaged, highly infested, stacks or bulks may lead to control failures because the gas cannot penetrate dustpacked, insect-damaged areas. Highly infested warehouses act as harborage for insects in the structure itself so that stacks are rapidly reinfested after a fumigation. Other stacks that are not fumigated (usually not all are done at one time) also act as sources of infestation for recently fumigated stacks.

Fumigation of stacks heavily infested with weevils may induce the massive inoculation of the stack with A. flavus spores produced on the cadavers of insects. If populations are kept low, the amount of inoculum is also minimal.

Fumigation should be carried out by trained personnel whether commercial operators or in-house employees. Using the proper techniques are important for both economic and safety reasons. Fumigation failures cost money in wastage of chemicals, continued damage to grain and possible long term effects on resistance of insects to fumigants. With so few "safe" fumigants available to us, we must protect their effectiveness by using proper techniques. This will also insure minimal exposure of personnel to these dangerous gases. Initial training should be followed by refresher courses and updating on new techniques and safety measures.

Sanitation in the warehouses is essential. Cleaning up grain and debris that can harbor insects is part of any good control program. Bags of old grain, screenings from previous harvests or loads should be disposed of immediately. Leaving screenings in a facility for longer than one insect life cycle (a month or less for most insects under tropical conditions) allows exponential increase of insects in subsequent generations. Good housekeeping practices, including the value of sanitation as a control measure and the importance of cleaning schedules, should be taught along with fumigation techniques and other control practices.

INSECT CONTROL RESEARCH

Research on insecticides and other control measures for seed have been developed into recommendations published in the Manual on Insecticide Usage (partial copy in Annex 111). Extension personnel recommend pesticides based on this manual. A project on insecticide usage

on seed maize was underway. Four insecticides were being tested as sack sprays and by direct application on seed. Pirimiphos methyl was the only treatment still effective at 10 months. Fenitrothion, chlorpyriphos methyl and methacriphos gave only short term protection.

Direct application of insecticides to foodgrains is not a common practice in Thailand. Rice, the staple grain, is held in high regard, and therefore, is not "adulterated" with pesticides. This practice carries over to other grains to some degree. Maize may be treated by some farmers, but the most likely crop for direct application is mung bean. Educating farmers and middlemen to the safe and proper use of grain protestants will be an important part of the National Training Program. Extension personnel are obvious sources of information to the farmers. At this point in time, they require training themselves.

Fumigation with methyl bromide is a common practice in Thailand. Middlemen, especially larger regional buyers with quotas for maize export, routinely fumigate under tarpaulins. They may or may not contract with a private pest control operator to do the job. Often they purchase the methyl bromide and dispense the gas themselves. Many have had no training in the handling of fumigants. Misuse and unsafe practices are common. SPIRS has worked with phosphine and was planning future projects. More equipment was needed for safe and effective research on fumigants. A project developed with the Australian Centre for International Agricultural Reserch (ACIAR) was underway using phosphine under plastic covers and CO2 in rice storage.

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REFERENCES

Anderson, H. W., E. W. Nehring and W. R. Wichser. 1975. Aflatoxin contamination in corn in the field. J. Agric. Food Chem. 23(4):775-782.

Christensen, C M. and H. H. Kaufmann.1969. Granin Storage: The role of fungi in quality loss. University of Minnesota Press, Minneapolis.153 PP.

Dobie, P: 1983. Rural maize storage under humid conditions: possible techniques for coastal

regions of Mexico. Memorias del Coloquio Internacional sobre Conservacion de Semillas y Granos Almacenados, Insituto de Biologia, UNAM, Mexico. October, 1980. pp. 147-155.

Fennel, D. l., F. B. Lillehoj and W. D. Guthrie. 1978. Insect larval activity on developing corn ears and subsequent aflatoxin contamination of seed. Journai of Economic Entomology 71:624628.

G ray, F. A., W. F. Faw and J. L. Boutwell. 1982. The 1977 corn-aflatoxin epiphytotic in Alabama. Plant Disease 66:221-222.

Guthrie, W. D.1982. Aflatoxin contamination of preharvest corn: interaction of European corn borer larvae and Aspergillus flavus - group isolates. Journal of Economic Entomology 75(2):265-269.

Guthrie, W. D., E. B. Lillehoj, W. W. McMillian, D. Barry, W. F. Kwolek, A. O. Fray, E. A. Catalano, W. A. Russell and N. W. Widstrom. 1981. Effect of hybrids with different levels of susceptibility to secondgeneration European corn borers on aflatoxin contamination in corn. J. Agricultural and Food Chemistry 291170-1172.

Hemsuchi, P., A. Wongu-Rai and D. Buangsuwon. 1982. Incidence of aflatoxin in pre and post harvested corn. Regional Grains Postharvest Workshop, Chiang Mai, Thailand. Paper No. 8225. 7 pp.

Isbagijo, P. S. 1981. The effect of storage period on grain quality especially for maize. Biotrop

Special Publication No. 9. Proceedings of Biotrop Symposium on Pests of Stored Products. Bogor, Indonesia April 1978. pp. 155-160

Kyle, G. H.1981. Shuck protection for ear corn. USDA Bulletin No. 708. Washington, D.C. 16 pp.

LaPrade, J.G and A. Manwiller. 1977. Relation of insect damage, vector, and hybrid reaction to aflatoxin B1 recovery from field corn. Phytopathology 67:544547.

Lillehoj, E. B. and D. l. Fennel. 1978. Aflatoxin contamination of corn before harvest: Aspergillus flavus association with insects collected from developing ears. Crop. Science 18:921-924.

Lillehoj, E. B., W. F. Kwolek, D. l. Fennel and M. S. Milburn.1975. Aflatoxin incidence and association with bright greenish=yellow fluorescence and insect damage in a limited survey of freshly harvested high =moisture corn. Cereal Chemistry 52:403-412.

Sukprakarn, G 1984. Control of stored grain sect pests in maize. Proceedings of the 7th ASEAN Technical Seminal on Grain Post=harvest Technology. Kuala Lumpur, Malaysia pp. 95-99.

Tyler, P. S., R. W. Taylor and D. P. Rees. 1983. Insect resistance to phosphine fumigation in food warehouses in Bangladesh. International Pest Control 25:10-13, 21.

Ware, G. M. 1986. Mission Report: Inspection, Sampling and Analyses of Maize and Groundnuts for Aflatoxin. UNDP-FAO-THA-82-004 Reduction of Postharvest Losses in Stored Grains. 27 pp. + attachments.

Widstom, N. W. 1979. The role of insects and other plant pests in aflatoxin contamination of corn, cotton, and peanuts: A review. Journal of Environmental Quality 8(1): 5-11.

Widstrom, N. W., E. B. Lillehoj, A. N. Sparks and W. F. Kwolek. 1976. Corn earworm damage and aflatoxin B' on corn ears protected with insecticide. J. Econ. Entomol. 69(5):677-679.

Wright, V. F. and R. Burroughs.1983. Mold-damaged grain as a diet for three stored-grain beetles. Environ. Entomol. 12:558-560.

Wright, F. F., E. de las Casas and P. K. Harein.1976. The response of Tribolium confusum to the mycotoxins zearalenone (F-2) and T-2 toxin. Environ. Entomol. 5:371-374.

Wright, V. F., E de las Casas and P. K. Harein.1980b. The nutritional value and toxicity of Penicillium isolates for Tribolium confusum. Environ. Entomol. 9:204-212.

Wright, V.F., E. de las Casas and P. K. Harein.1980b. Evaluation of Penicillium mycotoxins for activity in stored-product Coleoptera. Environ. Entomol. 9:271-221.

Wright, V. F., R. F. Vesonder and A. Ciegler. 1982. Mycotoxins and other fungal metabolises as insecticides. In Microbial and Viral Pesticides, E. Kurstak, ea., Chap.17. Marcel Dekker, New York, pp. 559-583.

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Identification of mycotoxin producing fungi and conditions leading to aflatoxin contamination of stored foodgrains

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Eugene B. Smalley

ABSTRACT

A mycological assessment of Thai maize was carried out with DOA specialists. Aspergillus flavus in the air was detected on and around stored maize, in fields near piles of ears, but not in harvested fields. A. flavus contamination was higher in maize weevil (Sitophilus zeamais) infested maize kernels than in noninfested kernels. Weevils carried a significant body burden of A. flavus and Fusarium moniliforme spores. Diploidia zeae (not A. flavus) was the major pathogen attacking Thai maize. F. moniliforme, F. semitectum and Penicillium citrimum were found in all maize samples and may present a greater potential contamination problem than A. flavus. Recommendations include: (1) Mycological analysis should accompany all aflatoxin surveys. (2) Priority should be given to recruitment of an analytical chemist in mycotoxicology.

(3) Advanced staff training abroad should be programmed into future UNDP/FAO projects. (4) Develop an in-depth research program on the role of sclerotia, insects and arthropods on the development of aflatoxin in maize. (5) Schedule informed research discussions among international teams cooperating with DOA, and publish results of aflatoxin investigations in international scientific journals.

AFLATOXIN IN MAIZE: CURRENT STATUS AND INTERNATIONAL COOPERATION

Aflatoxin contamination in maize is the cause of great concern in Thailand. This was expressed dramatically at a recent (12119186) symposium on "Aflatoxin in Maize" sponsored by the Thai Maize and Product Traders Association (2). Farmers, traders, silo managers, feed association, Department of Agriculture (DOA), University, Ministry of Agriculture and Ministry of Trade representatives discussed the problem and defined present guidelines for prevention and control. An intense question and answer session followed each speaker. It was apparent that present levels of contamination are unacceptable and that integrated, economic control measures are needed.

Various groups in Thailand produce estimates of aflatoxin contamination in the maize crop (DOA, OMIC, SGS-FAr East Ltd., etc.). Importing countries determine and set standards for aflatoxin concentrations in the incoming maize. Mechanisms for national data collection, collation and assessment of sampling accuracy and analytical methods are not presently available.

Divergent estimates of crop contamination and the resultant uncertainty can and are used to manipulate and control maize prices in export trade. Because the DOA has only limited staff, expertise and facilities to address the problem, it has entered into several joint International Cooperative Projects to solve problems and improve the national capability.

RESULTS

Field observation. Farmers field observations were only made on the second crop which was harvested under hot, dry rain-free conditions. As such, these observations and conditions may differ from the situation in the first crop of maize grown and harvested in the rainy season. A high incidence of ear rot was common in all fields examined. The brown dry-rotted ears were very light in weight and cob and kernels offered little resistance to breakage. Frequently in moist ears, white mycelium could be seen massed between the kernels. The incidence of rot in one field we examined near Mauklek involved at least 20% of the crop. Pure cultures of Diploidia zeae (= Stenocarpella maydis) were isolated from all ears exhibiting these symptoms. The identity was confirmed from pycnidia forming in the isolation plates on some of the decayed kernels. We concluded that the variety Suwan 1 was probably extremely susceptible to this pathogen. We also saw evidence of this fungus with more limited rot in association with corn earworm (Heliothis sea.).

Early maize weevil (Sitophilus zeamais) infestations were observed in ears being picked. These

infestations were usually at the tips of otherwise sound ears but were rearly extensive. No weevil infestations were observed in good quality second crop shelled kernels on the drying floor, but 3 to 4 month stored first crop kernels were heavily infested at all middleman warehouses visited. In one instance fumigated bags of shelled maize were literally black with killed weevils. Piles of dead weevils 8 to 10 cm deep surrounded the stacks of bags. Upon closer inspection, we found that the dead weevils were yellow-green with conidia of A. flavus. At the silo, maize is regularly migated 2 times in 4 months with CELPHOS (cost 2.5 baht/ton), but it was not an exaggeration to say that in and around the silo builidings and elevators weevils were everywhere and we carried them away on our clothes.

Weevil infestations were heavy in all samples of stored first crop maize we examined with levels ranging from 1% (30/430) in Phetchabun to almost 18% (371248) at Lopburi. Maize at the silo level often had infestations of 20% or more, and it was apparent that these infestations were difficult to estimate when the larval stage is still inside the kernel.

Dilution plate counts from Pakchang first crop maize weevils reveal 133 propagules of A. flavus/ weevil and 576 propagules of Fusarium moniliforme. Maize weevils carried a great collection of other fungi including A. niger, A. glaucus, A. candidus, Penicillium islandicum, P. citrinum, Paecilomyces, Acremonium, Epicoccum, F. semitectum, yeasts and many others.

Air samples at Phetchabun revealed 4.7 propagules A. flavus/10 min. exposure per plate outside the merchants warehouse. Inside the same warehouse A. flavus counts were 4 times greater (19.0 propagules/10 min). A. flavus counts at the hill farm drying shed were 11.0 propagules/plate

and increased to 21/plate near the recently shelled cob pile. No A. flavus was detected in the maize fields. The highest A. flavus air counts in the Mauklek area were recorded in a maize field near a workers loading picked ears into bags (16 propagules/10 minj. No A. flavus was detected in the undisturbed maize field after picking. All plates at all locations trapped uncountable numbers of F. moniliforme conidia and the Mauklek plates also trapped additional large numbers of F. semitectum propagules. Maize cobs, stalks and debris apparently were not good hosts for A. flavus survival. No A. flavus was detected in any of the limited number of samples cultured, but moniliforme was omnipresent. Time did not permit a total assessment of A. flavus contamination of all the debris sample collected.

Many different fungi were isolated from surface sterilized maize kernels from the various sources. Forty to 50% of sound first crop kernels from local middlemens warehouses contained A. flavus, but weeviled kernels from the same sources were 100% infected. In contrast no A. flavus could be detected in second crop kernels (without weevil infestation) taken directly from the drying floor, although 30% and quality control of postharvest maize-aflatoxin). The cost of the program (63 million baht = $2.4 million) was shared jointly by Thailand and Japan (1). Aflatoxin survey aspects of this project were reviewed by Nesheim (17).

The project included studies which followed the progress of aflatoxin development throughout the various stages from the field to the middleman's warehouse and finally at the silo. Although experimental designs and statistical validity of comparisons left something to be desired, in most instances the conclusions seemed valid and have been borne out by later work (2,12). Their work indicated that production of aflatoxin begins at harvest and increases in storage until moisture

concentrations have been reduced to 15% or less. They concluded that grain precontamination with aflatoxin is transferred directly to the silo company with little further change. They recommended introduction of more effective drying facilities at both farmer and middleman levels, and shifting the crops harvest time to the dry season through the use of delayed planting and short maturing varieties. (Heated discussions on this point took place at the Bangkok Aflatoxin Symposium.) Early crop harvest (4 weeks before maturity) resulted in severe aflatoxin contamination (50 ppb after 20 days storage), but none developed under similar storage conditions in maize picked 2 weeks before maturity (2). Aflatoxin concentrations declined after 20 days storage, and reached levels of 11 ppb in 60 days. No explanation was given for this interesting result.

The Thai-Japanese project also studied the microbiology of fungi in harvested corn from farm through middlemans storage to the silo, Aspergillus flavus was detected 9% of kernels in the farmers field, and had increased to 40% by the time it reached the silo. Fusarium moniliforme was omnipresent in the grain from the farmers field (70% of the kernels) but declined to 21% by the time it reached the silo. The percent of other commonly detected fungi (Penicillium citrinum, P. islandicum, Fusarium semitectum, Botryodiplodia) varied but did not change remarkably between farm and silo. A. flavus, P. citrinum, P. islandicum, F. moniliforme and F. semitectum are all well known mycotoxin, producing fungi (5,15)..

All kernels from the ear rot yield Diploidia zeae. Overall, F. moniliforme and Penicillium citrinum were the second and third most commonly encountered fungi in maize kernels. In these studies F. moniliforme and F. subglutinans could not be distinguished with certainty in the isolation plates

and required the preparation of single spore culture on carnation leaf agar (16) for positive identification. For purposes of these preliminary studies both were classified as F. moniliforme (sensu Snyder and Hanson) (16).

In our brief studies on the mycology of Thai maize a number of the fungi we commonly detected have been reported to produce mycotoxins in association with various mycotoxicoses. Aside from Aspergillus flavus (aflatoxin) these included Diploidia zeae (Diploidiosis), F. moniliforme (Equine Leukoencephalomalacea, Esophageal Cancer, Moniliformin), F. subglutinans (Fusaric acid, Moniliformin, Esophageal Cancer), F. semitectum (Degnala resease of water buffalo and cattle, Esophageal Cancer, various Trichothecens and Zearalenone). The various Penicillia and Aspergilli are known to produce a large number of toxins and can always be considered potentially hazardous.

Conclusions

It was apparent that A. flavus was not the major fungal contaminant of the Thai maize. Diploidia zeae was detected in all of the maize fields. In addition it caused losses of 20% or more of harvested ears and although less rotted kernels survived sorting, shelling and cleaning it no doubt becomes a significant comparent of the exported commodity. The heavily infected ears can be removed by on-farm ear sorting but in terms of aflatoxin it presents no hazard since A. flavus was never isolated from Diploidia infected ears.

Neither the Thai-Japanese or Thai-British projects recognized the significant Diploidia

contamination. It may have gone unrecognised because the fungus rarely produces pycnidia on isolation media, although we found pycnidia fruiting on old rotted kernels. This pathogen has been reported to product a condition in farm animals called diploidiosis, but the identity of the toxin responsible is unclear.

Fusarium moniliforme (with F. subglutinans) may present the greatest threat of all the maize contaminants. Although it appeared not to cause serious ear rot it could be isolated from most kernels. Both fungi are well known toxin producers and have been associated with several serious diseases (15). While F. semitectum was encountered less frequently, it should not be overlooked because of its well known ability to produce the very toxic trichothecenes including 4-acetoxyscirpeneidol, diacetoxyscirpend, monoacetoxyscirpenol, soirpenetriol and T-2 toxin (15). It has also been reported to produce the fungal estrogen zearalenone. This fungus has been associated with the Degnala Disease and sore leg disease of cattle and water buffalo. These diseases are characterized by swelling of the legs with necrosis and gangrene following ingestion of contaminated rice straw.

A. flavus spores in low concentration were present in the air on and around stored maize; they were also present in the fields near piles of ears but were not generally in the air of the maize field. We did not isolate the fungus from maize stalks or maize debris, but only a few samples were studied. A. flavus contamination was almost non-existent in second crop maize in the field, but quickly built up in storage. This contamination was very much higher in weeviled kernels suggestion an important role may be played by the maize weevil in the aflatoxin intensification process. Weevils were shown to carry significant A. flavus contamination, as well as F.

moniliforme and P. islandicum and others. Because of the extremely high maize weevil populations in all stages of maize production from harvest and storage to export, its role needs further study. Its role as an important vector of A. flavus has been reported in North America and elsewhere (6,11).

RECOMMENDATIONS

Mycological Surveys

Mycological analysis should accompany and be companion to aflatoxin determinations. Survey and sampling procedures as recommended by Nesheim (17) for aflatoxin could be utilized for mycological assessment as well. Sampling methods might require modification to take into account the unique and complex marketing systems. It is apparent that the quality of the grain exported depends upon the particular exporter and his buyer. It may not be fair to state that Thailand maize contains 100 ppb aflatoxin when 10 ppb second crop maize ("supergrade") is being sold at premium prices.

Some method of centralized aflatoxin data collection and analysis should be developed in which mycotoxin analytical data from both government and private sector sources can be compiled for general use by the National Aflatoxin Director in DOA, as suggested by Nesheim (17).

Role of Sclerotia, Insects and Arthropods

At its best A. flavus is a weak plant pathogen but is a competitive saprophyte with a large capacity to produce conidia on nutritious substrates. However the factors that allow A. flavus to colonize and produce aflatoxin in maize are not well understood. Sclerotia have been reported to play a major role in producing airborne inoculum, but in our brief survey, we failed to find sclerotia in infected maize or in debris. The large weevil populations with natural A. flavus contamination appear to provide airborne capability and wounds for entry. I recommend the development of in depth research on the role of sclerotia, insects and arthropods on the development of aflatoxin in maize.

Mycotoxin production of Fungi from Maize and Groundnuts

The mycotoxin producing capability of fungi associated with Thai maize is largely unknown. I recommend that studies be initiated to determine the toxicity of culture filtrates from the predominant fungal associates of maize. These should include Diploidia zeae, Fusarium moniliforme, Fusarium subglutinans, fusarium semitectum, Penicillium islandicum, Penicillium citrinum and others. Experimental animals for test feeding might include ducklings, thickens or rats. Toxic strains as indicated in feeding trials should be examined chemically for the production of known mycotoxins or possible undescribed new toxins.

Integration of Projects and Publication of Research

The several cooperative international aflatoxin projects now ongoing at the DOA tend to have overlapping goals. These projects in the past have carried out data processing mainly in their

home countries with considerable time before final publication of reports. It might be desirable for the various groups working with DOA to have regularly scheduled research discussions where on-going research could be discussed. A considerable body of information and experience has been accumulated by the DOA on the aflatoxin problem in Thai stored food grain. I encourage publication of this information possibly as a feature article with colored photographs in Plant Disease.

NATIONAL GUIDELINES FOR PREVENTION AND CONTROL OF AFLATOXIN IN MAIZE

- **Harvest fully matured maize on a sunny day.**
- **Separate the sound cobs from damaged or rotted cobs.**
- **Never shell high moisture or wet grain.**
- **Dry cobs on raised bamboo floors.**
- **Keep maize on the cob during farm storage.**
- **Dry shelled maize on concrete drying floors (for merchants).**
- **Use a waterproof sheet (not plastic) to cover drying maize for rain protection.**
- **Use mechanical drying if possible.**
- **Keep grain dry in storage.**
- **Warehouse should have good ventilation (for silos).**

These guidelines in the form of large colored posters have been distributed by government

organizations and the private sector throughout the maize growing areas of Thailand. Twenty thousand were distributed in 1984 and an additional 50,000 in 1985. The following additional practices are suggested for possible addition to the current guidelines.

1. **Sanitation practices should be intensified! Destroy discarded ears, shattered kernels and shelled cobs which can harbor insects (especially maize weevil) and A. flavus. These should be sought out and destroyed in the field, on the farm and at the merchants warehouse.**
2. **Fumigation for insects (especially maize weevil) should begin at the earliest practical time after shelling. Allowing the build-up of infestations during temporary storage (prior to transport to the silo) may be a major contributor to A. flavus and aflatoxin build-up.**
3. **Drying should take place as rapidly as possible! Several days of wet storage (25% moisture) will be sufficient to allow the build-up of unacceptable aflatoxin concentrations.**

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REFERENCES

1. **Anon. 1984. The Report for the Technical Cooperation Project on Maize Development in Thailand. 1977-1984 Japan Interantional Cooperation Agency Printing Press. Ltd., Bangkok.**
2. **Adnon, 1985. Proc. Thailand National Aflatoxin Symposium--December 19, 1985. Hyatt-Bangkok Covention Center (In Thai).**
3. **Adnon.1985. Thai-British. Maize Aflatoxin Project. October 1985. Victoria Power Point Corp. Ltd. London Lep.**
4. **Carmichael, J. W., W. B. Kendrick, l. L. Conners and L. Sigler. 1980. Genera of Hyphomycetes. The University of Alberta Press, Edmonton. 386 pp.**
5. **Diener, U. L., et al. 1978. Aflatoxin and other mycotoxins: In agricultural perspective. Council for Agricultural Science and Technology Report No. 80.56 pp.**
6. **Diener, U. L., R. L. Asquith and J. W. Dickens (eds.). 1983. Aflatoxin and Aspergillus flavus in Corn. Southern Cooperation Series Bulletin 279. February 1983. Craftmaster Printers, Inc., Opelika, Alabama. 112 pp.**

7. **Fennel, D. J., E. B. Lillehoj and W. F. Kwolek. 1975. Aspergillus flavus and other fungi associated with insect damaged field corn. Cereal Chem. 52(3):314-321.**
8. **Ellis, M. B. 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew Surrey.**
9. **Ellis, M. B. 1976. More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey.**
10. **Ainsworth, G. C. 1983. Ainsworth and Bisby's dictionary of the fungi. th Edition 1983. Commonwealth Mycological Institute, Kew, Surrey. 412 pp.**
11. **Hesseltine, G. W., O. L. Shotwell, W. F. Kwalek, E. B. Lillehoj, W. K. Jackson and R. J. Bothost. Aflatoxin occurrence in 1973 corn at harvest. 11. Mycological Studies. Mycologica 64:341-353.**
12. **Kenniford, S., J. Meadley, J. Morris, and M. Nagler. 1985. Report on Aflatoxin in Maize in Thailand. March 1985. Vol. 2: Main Report. Overseas Development Administration, Government of the United Kingdom, London.**
13. **Lillehoj, E. B., W. T. Kwolek, G. M. Shannon, O. L. Shotwell and G. W. Hesseltine. 1975. Aflatoxin occurrence in 1973 corn at harvest. l. A limited survey in the southwestern U.S. Cereal Chem. 52(5):603-611.**
14. **Lindblad, C. and L. Druben. 1976. Small Farm Grain Storage. Vol.1 Preparing Grain for Storage. Vol. 2 Enemies of Stored Grain. Action/Peace Corps. Program and training Journal. Manual Series 2. Vita Publications-Manual Series No.35E.**
15. **Marasas, W. F. O., P. E. Nelson and T. A. Toussound. 1984. Toxigenic Fusarium Species. The Pennsylvania State University Press, University Park and London. 328 pp.**

16. **Nelson, P. E., T. A. Toussound and W. F. O. Marasas. 1983. Fusarium Specis: An Illustrated Manual for identification. The Pennsylvania state university Press, University Park and London. 193 pp.**
17. **Nesheim, S. 1985. Mission Report: Inspection, Sampling and Analysis of Corn and Peanuts for Aflatoxin. UNDP/FAO/THA/82/004 Reduction of Post-Harvest Losses in Stored Grains. 32 pp.**
18. **Prawat Tanboon-Ek.1986. Personal Communication.**
19. **Presina Siriarcha 1985. Personal Communication.**

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Aflatoxin analytical methods for groundnuts

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D.M. Wilson

Abstract

Aflatoxin determination in groundnuts can be approached in several ways. Groundnuts are often contaminated with aflatoxins B1 and B2, less often with aflatoxins B1, B2, G1, and G2 so it is important to have analytical values that represent the total aflatoxin content. Some countries are only interested in B1 content and others are interested in the total aflatoxin content. It is essential to safely handle all experimental materials associated with aflatoxin analyses or the aflatoxigenic fungi. Visual screening of suspect groundnut lots, based on the presence of conidial heads of the Aspergillus flavus group, is not a chemical test and may allow aflatoxin-contaminated lots into commerce. Minicolumn screening techniques can be useful but they should always be used in conjunction with a quantitative method. Several thin layer chromatographic (TLC) and high performance liquid chromatographic (HPLC) methods are suitable for quantification and are in general use. The newer immunochemical methods such as the enzyme - linked immunosorbent assay (ELISA) or affinity column methods are being rapidly developed. ELISA methods are available for screening as well as quantification, but these methods are temperature - sensitive and they should only be used with proper controls. The affinity column method is less temperature - sensitive and can be used for either screening or quantification. The chemical and immunochemical methods are reliable if care is taken and personnel are well trained. All analytical laboratories should stress safety and include suitable analytical validation procedures.

Introduction

The study of alfatoxin contamination of foods and feeds is important because aflatoxins are toxic and carcinogenic to humans and animals. This paper reviews the sampling plans and analytical methods for alfatoxins in groundnuts and groundnut products, and also refers to the safety aspects of such methods.

SAFETY

Chemical safety

Guidelines for mycotoxin safety precautions are given by the Association of Official Analytical' Chemists (AOAC) in Official Methods of Analysis Chapter 26 (1984). The mycotoxin analysis publication from the International Agency for Research on Cancer (IARC) also has a good discussion of safety precautions (Stoloff et al. 1982). The safety guidelines discussed in these books are appropriate for both crude and pure aflatoxin preparations. The chemicals should only be handled with gloves and used only in properly ventilated hoods or glove boxes.

Biological safety

Spores and other viable propagules of Aspergillus flavus, A. parasiticus, and other fungi can cause three types of disease in humans: allergy, poisoning, and infection (Hill et al. 1985). A. flavus infection in humans is uncommon but possible. Airborne spores and dust containing propagules of the A. flavus group may cause allergies in some people and the inhaled particles

may contain aflatoxins (Shotwell et al. 1981). Two thin layer chromatography (TLC) methods have been developed to measure aflatoxins in maize and grain dust (Ehrlich and Lee 1984, Shotwell et al. 1981).

Hill and co-workers (1984) found between to and 109 viable fungal propagules per m of air containing maize dust; air containing groundnut dust is probably equivalent. The majority of the A. flavus progagules in air samples were deposited on the stages of the Andersen sampler corresponding to the trachea, primary bronchi, and secondary bronchi in the human respiratory system (Hill et al. 1984). A. flavus spores and propagules in dust associated with inoculation, shelling, grinding, and extraction procedures are sufficiently hazardous to require safe handling procedures including gloves, masks, protective clothing, and efficient dust collection mechanisms.

Sampling

Sampling is the most important contributor to the variability of analyses for aflatoxin in agricultural commodities, particularly groundnuts, because of the nonhomogenous nature of aflatoxin distribution. The first consideration in any experimental or regulatory protocol should be the sampling method. Protocols have been published on sampling techniques (Dickens and Whitaker 1986). Schuller et al. (1976) published an excellent review of sampling plans and collaboratively studied methods for aflatoxin analysis.

Alfatoxin contamination in groundnuts generally is more variable in single fields, single test plots, or single lots than aflatoxin contamination in maize and some other crops. Therefore a 22 kg

sample is needed for groundnut whereas a 4.54 kg sample is usually sufficient for maize, especially when several analytical samples are averaged to approximate the true mean (Whitaker and Dickens (1983). In groundnut lots composed of mixed loads from different sources, a larger initial sample should be taken. In the United States three 22 - kg samples are taken from each groundnut lot (Dickens and Whitaker 1986). The total sample should be ground so that it passes through a 0.85 - mm sieve, thoroughly mixed or divided, and properly subsampled before analytical samples are taken. Sampling protocols for test plots must be part of the experimental design and should be tailored to meet the experimental objectives.

Aflatoxin Standards

Criteria for aflatoxin standards (Rodricks 1973) and procedures for checking the concentration and purity of aflatoxin standards can be found in AOAC Official Methods of Analysis(1984). The use of calibrated standards in all analytical laboratories is essential. Prepared standards are available from several commercial companies at reasonable prices and analytical laboratories should, if possible, routinely use these standards. Velasco (1981) found that cyclohexane, heptane, and toluene could be substituted for benzene in standards if the solutions were not exposed to light. Analysts should take solvent composition into consideration when standards are prepared for high performance liquid chromatography (HPLC) as it has been found that solvent composition affects aflatoxin fluorescence (Chang - Yen et al. 1984).

Presumptive and Screening Methods

Some applications require only presumptive or screening tests while others require the quantification of only B. or several of the aflatoxins. Groundnuts at the buying point are visually inspected in the United States for evidence of A. flavus conidial heads and if present the suspect lots are not allowed into commerce for human consumption (Dickens and Whitaker 1986). This visual examination is not a chemical test and may result in family acceptances or rejections. The other commonly used screening technique is the application of one of several minicolumn procedures to detect aflatoxin contamination above a predetermined level (Holaday 1981, Romer et al. 1979). Shannon and Shotwell (1979) conducted a collaborative study of two minicolumn methods and found that a combination method using the Holaday extraction and the Velasco minicolumn was the most satisfactory method. Minicolumn techniques should not be used for quantitative purposes where accurate quantitative data are required. Madhyastha and Bhat (1984) recently developed a minicolumn confirmation method for aflatoxins. These workers confirmed the identity of aflatoxins on the developed minicolumn by applying 20% H2SO4, 20% HCI, or trifluoroacetic acid (TFA) in 20% HNO3. All acids changed the fluorescence from blue to yellow, with the TFA in 20% HNO3 having the lowest detection limit.

Quantitative Methods

Many of the methods adopted by scientific groups and government agencies are based on TLC detection and quantification procedures that have been evaluated in collaborative studies. The HPLC methods are not more often recommended only because very few collaborative studies have been conducted on them as yet. The AOAC (1984) recommends the contamination branch (CB) and the best foods (BF) methods for aflatoxin analysis in groundnuts. Nesheim (1979) and

Shotwell (1983) have also reviewed methods on aflatoxin analysis.

Thin layer chromatography (TLC) methods

The CB method (AOAC 1984) is the standard by which other methods are judged. Details can be found in Stoloff et al. (1982). Shotwell and Goulden (1977) compared the AOAC BF groundnut method and the AOAC cottonseed method with the CB method for analysis of aflatoxins in maize. The BF method for uses a methanol + water (55 + 45) extraction solution, while the cottonseed method uses an acetone+water(85+15) extraction solution. Neither of these solvents extracted aflatoxins from maize as efficiently as did the chloroform+water(250+15) extraction of the CB method. The BF method is suitable for groundnuts with aflatoxin contents below 50 g kg-1. Lee and Catalano (1981) developed a scaled-down cleanup column as a solventsaving modification of the CB method. Laboratories which use fluorodensitometry for quantitative measurements need to be careful to avoid fading of aflatoxin spots on TLC plates; fading could be delayed by covering the layer on the TLC plate with another glass plate (Nesheim 1971).

The CB method is an excellent TLC method, but it has two major disadvantages: (1) it is expensive because it uses large amounts of solvents which create a disposal problem, and (2) the major solvent used is chloroform which may be a hazard to workers. Dantzman and Stoloff (1972) developed a modified a modified screening method in which they omitted the column chromatography step of the CB method and directly spotted the residual oil from maize extracted with CHCI3 water. Spilman (1985) modified this screening method for maize by adding benzene+acetonitrile (98+2) to the residual oil and measuring the volume to obtain quantitative

TLC results. Groundnuts would have to be defatted with hexane before the CHCI3 water extraction for these screening methods.

Kamimura et al. (1985) recently described a simple rapid HPTLC method which compared favorably with the CB method. Davis et al. (1981) used a novel approach by devising a method using the fluorescence of the iodine derivative of aflatoxin B. for quantification and TLC confirmation. Josefesson and Moller (1977) developed a multi-mycotoxin screening method for detection of aflatoxins, ochratoxin, patulim, sterigmatocystin, and zearalenone, while Seitz and Mohr (1976) and Thomas et al. (1975) developed methods for aflatoxin and zearalenone determination.

The AOAC aflatoxin confirmation method is based on the TFA reaction with B1, G1, or M1 (Przybylski 1975). The TFA procedure or direct acetylation (Cauderay 1979) can be carried out on a TLC plate before development. Trucksess et al. (1984) recently published a rapid TLC method using a disposable silicagel column for cleanup and confirmation by gas chromatography-mass spectroscopy. No matter which TLC method is used the aflatoxin identified needs to be confirmed. A review of confirmation methods has been written by Nesheim and Brumley (1981).

High performance liquid chromatography (HPLC) methods

Aflatoxin analysis using HPLC for separation and detection is quite similar to TLC because similar sampling and extraction procedures are used. The major advantages of HPLC over TLC are speed, automation, improved accuray, and precision. Both normal-phase and reverse-phase

HPLC separations have been developed for aflatoxin analyses. Early experimental work by Seitz (1975) and Garner (1975) on HPLC separations revealed that aflatoxins could be separated on nominal-phase columns and detected with either a UV detector or a fluorescence detector. Seitz (1975) noted that the fluorescence detector had limited usefulness for aflatoxin B1 and B2 with normal phase separations.

Panalaks and Scott (1977) developed a silica-gel packed flow cell for fluorometric detection of B1, B2, G1, and G2 with normal phase aflatoxin separations. A silica-gel packed cell was used by Pons (1979) and Thean et al. (1980) in two different HPLC methods for determination of aflatoxins. The major disadvantage of the packed cell is lack of stability. The cell needs to be repacked often and the detector signal weakens with time. The advantages of a packed cell method are that no derivative is necessary for detection and the mobile phase can be recycled.

Reverse-phase HPLC separations of aflatoxins are more widely used than normal-phase HPLC separations. However, the fluoresence intensities of B1 and G1 are diminished in reverse-phase solvent mixtures so the derivatives B and G are diminished in reverse-phase solvent mixtures so the derivatives B and G are generally prepared before injection. Analysts should be aware that derivatives B and G are not stable in methanol, which should be used with caution, especially in the injection solvent. Acetonitrile-water mixtures do not degrade B and G rapidly and are preferred to methanol-water mobile phases.

Several reverse-phase methods have been published (Cohen and Lapointe 1981, Stubblefield and Shotwell 1977) including three with comparisons to the CB method (De Vries and Chang 1982,

Hutchins and Hagler 1983, Tarter et al. 1984). Stubblefield and Shotwell (1977) found that M, and M2 as well as B1, B1, B2, G1, and G2 could be resolved and detected with a UV detector at 350 nm using reverse-phase chromatography. The methods developed by Hutchins and Hagler (1983), De Vries and Chang (1982) and Tarter et al. (1984) all use TFA derivatization and apparently compare favorably with other methods. Diebold et al. (1979) used laser fluorometry to detect aflatoxin B after reverse-phase chromatography.

Davis and Diener (1980) found that the iodine derivative of B1 could be used for confirmation and developed a reverse-phase method with fluorescence detection for this derivative. This work led to the development of a postcolumn iodination method to enhance B. and G. fluorescence after reversephase chromatography. Shepard and Gilbert (1984) investigated the conditions needed for the postcolumn iodination reaction to enhance fluorescence of aflatoxins B1 and G1.

Immunochemical methods

Aflatoxin B1 in groundnuts can be determined using solid-phase radio-immunoassay (RIA). (Langone and van Vunakis 1976, Sun and Chu 1977), monoclonal affinity column immunoassay (Groopman et al. 1984), or enzyme-linked immunosorbent assay (ELISA) techniques (Chu and Ueno 1977, El-Nakib et al. 1981, Lawellin et al. 1977, Pestka et al. 1980). ELISA or affinity column techniques are more suited to field use than RIA techniques and will probably be extensively developed and utilized. The major advantages of the ELISA and affinity column methods include speed, ease of sample preparation, ease of use, and a potentially low cost per anylysis. The disadvantages include different a antibody specificities for B1 and cross reactivity

with other aflatoxins. ELISA procedures are qualitative or semi-quantitative at best and are temperature sensitive. The major application for ELISA procedures at present is screening for aflatoxin B1 below a predetermined concentration. The color developed by the enzyme-mediated reaction gives an indication of the amount of B1 present. More development is needed before immunochemical techniques will be generally useful for applicatications where quantification is critical. Methods also need to be developed that will distinguish between B1, B2, G1, and G2 individually or collectively.

SELECTION OF ANALYTICAL APPROACH

Regulatory and experimental applications of methods for aflatoxin analysis do not always need to be the same. Regulatory applications need to be quantitative and legally acceptable, but acceptable methods may vary within a country or between countries. However, it is important to use validated methods for regulatory applications.

Aflatoxin analysis in experimentral work must be tailored to the objectives and method selection should be a part of the experimental design. Inexpensive minicolumn data may be sufficient for some experimental purposes whereas quantitative data on B1, B2, G1, and G2, may be required for other purposes. Costs and data requirements can sometimes seem to be at odds when quantitative data are necessary. The TLC and immunochemical methods may not always be cheaper than HPLC in the long run because HPLC requires a single large initial investment, and

TLC and ELISA both use expensive disposable plates. HPLC is possibly more suitable for large analytical laboratories while TLC is more suitable for laboratories with only a few samples to be analyzed. With further development, immunochemical methods will probably become more versatile and suited to a wider variety of applications.

REFERENCES

Alexander, R.J., and Baur, M.C.1977. Note on a twodimensional TLC procedure for determining aflatoxins in corn. Cereal Chemistry 54:699-704.

AOAC (Association of Official Analytical Chemists). 1984. Official methods of analysis of the Association of Official Analytical Chemists 14th edn. Arlington. VA 22209 USA:AOAC.

Cauderay, P.1979. Rapid chemical confirmation method for aflatoxins B1 and G1 by direct acetylation on a thin layer plate before chromatography. Journal of the Association of Official Analytical Chemists 62:197.

Chang-Yen, I., Stoute, V.A., and Felmine, J.B. 1984. Effect of solvent composition on aflatoxin fluorescence. Journal of the Association of Official Analytical Chemists 67:306-308.

Chu, F.S., and Ueno, I.1977. Production of antibody against aflatoxin B1. Applied and Environmental Microbiology 33:1125-1128.

Cohen, H., and Lapointe, M. 1981. High pressure liquid chromatographic determination and fluorescence detection of aflatoxins in corn and dairy feeds. Journal of the Association of Official Analytical Chemists 64:1372-1376.

Pages 29-51 in Modern methods in the analysis and structural elucidation of mycotoxins (Cole, R.J.ed.). Orlando, Florida, USA: Academic Press.

Diebold, G.J., Karny, N., Zare, R.N., and Seitz, L.M. 1979. Laser fluorometric determination of aflatoxin B. in corn. Journal of the Association of Official Analytical Chemists 62:564-569.

Ehrlich, K.C., and Lee, LS.1984. Mycotoxins in grain dust: method for analysis of aflatoxins, ochratoxin A, zearalenone, vomitoxin, and secalonic acid. Journal of the Association of Official Analytical Chemists 67:963-967.

El-Nakib, O., Pestka, J.J., and Chu, F.S. 1981. Determination of aflatoxin B. in corn, wheat and peanut butter by enzyme-linked immunosorbent assay and solid phase radioimmunoassay. Journal of the Association of Official Analytical Chemists 64:1077-1082.

Garner, R.C. 1975. Aflatoxin separation by highpressure liquid chromatography. Journal of Chromatography 103:186-188.

Groopman, J.D., Trudel, L.J., Donahue, P.R., MarshakRothstein, A., and Wogan, G.N. 1984. High affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays. Proceedings of the National Academy of Sciences of the United States of

America 81:7728-7731.

Hill, R.A., Wilson, D.M., Burg, W.R., and Shotwell, O.L. 1984. Viable fungi in corn dust. Applied and Environmentral Microbiology 47:84-87.

Association of Official Analytical Chemists 66: 4581465.

Josefesson, B.G.E., and Moller, T.E.1977. Screening method for the detection of aflatoxins, ochratoxin, patulin, sterigmatocystin, and zearlenone in cereals. Journal of the Association of Official Analytical Chemists 60:1369-1371.

Kamimura, H., Nishijima, M., Yasuda, K., Ushlyama, H., Tabata, S., Matsumoto, S., and Nishima, T. 1985. Simple rapid cleanup method for analysis of aflatoxins and comparison with various methods. Journal of the Association of Official Analytical Chemists 68:458-461.

Langone, J.J., and van Vunakis, H. 1976. Aflatoxin B1: specific antibodies and their use in radioimmunoassay. Journal of the National Cancer Institute 56:591-595.

Lawellin, D.W., Grant, D.W., and Joyce, B.K. 1977. Enzyme-linked immunsorbent analysis for aflatoxin B1, Applied and Environmental Microbiology 34:9496.

Lee, L.S., and Catalano, E.A.1981. Pons scaled-down clean-up column adapted for use in solvent-saving modification of the CB method for aflatoxin. Journal of the American Oil Chemists' Society 58:949A951A.

Madhyastha, M.S., and Bhat, R.V. 1984. Application of TLC chemical confirmatory tests to minicolumn chromatography of aflatoxins. Journal of the American Oil Chemists 'Society 61:907-908.

Nesheim, S. 1971. Fading of aflatoxin spots on TLC lates during fluorescence densitometry. Journal of the Association of Official Analytical Chemists 54:1444-1445.

Nesheim, S.1979. Method of aflatoxin anlysis. Pages 355-372 in Trace organic analysis:a new frontier in analytical chemistry:proceedings of the 9th Materials Research Symposium. Special Publication no. 519:355-372. USA:National Bureau of Standards.

Nesheim, S.1979. Method of aflatoxin analysis. Pages identity of aflatoxins. Journal of the American Oil Chemists 'Society 58:945A-948A.

Panalaks, T.,and Scott, P.M.1977. Sensitive silica gelpacked flow cell for fluorometric detection of aflatoxins by high pressure liquid chromatography. Journal of the Association of Official Analytical Chemists 60(3):583-589.

Pestka, J.J., Gaur, P.K., and Chu, F.S. 1980. Quantitation of aflatoxin B1 and B1 antibody by an enzyme-inked immunosorbent microassay. Applied and Environmental Microbiology 40(6): 1027-1031.

Pons, W.A., Jr. 1979. High pressure liquid chromatographic determination of aflatoxins in corn. Journal of the Association of Official Analytical Chemists 62:586-594.

Przybylski, W. 1975. Formation of aflatoxin derivatives on thin layer chromatographic plates. Journal of the Association of Official Analytical Chemists 58:163164.

Rodricks, J.V. 1973. Criteria for mycotoxin standards. Journal of the Association of Official Analytical Chemists 56:1290-1291.

Romer, T.R., Ghouri, N., and Boling, T.M.1979. Minicolumn screening methods for detecting aflatoxin: state of the art. Journal of the American Oil Chemists 'Society 56:795-797.

Schuller, P.L., Horowitz, W., and Stoloff, L. 1976. A review of sampling plans and collaboratively studied methods of analysis for aflatoxins. Journal of the Association of Official Analytical Chemists 59:1315-1343.

Seitz, L.M. 1975. Comparison of methods for aflotoxin analysis by high pressure liquid chromatography. Journal of Chromatography 104:81-89.

Seitz, L.M., and Mohr, H.E. 1976. Simple method for simultaneous detection of aflatoxin and zearalenone in corn. Journal of the Association of Official Analytical Chemists 59:106-109.

Shannon, G.M. and Shotwell, O.L. 1979. Minicolumn detection for aflatoxin in yellow corn: collaborative study. Journal of the Association of Official Analytical Chemists 62:1070-1075.

Shepard, M.J., and Gilbert, J. 1984. An investigation of HPLC post-column iodination conditions for the enhancement of aflatoxin B. fluorescence. Food and Additives Contaminants 1:325-335.

Shotwell, O.L. 1983. Aflatoxin detection and determination in corn. Pages 38-45 in Aflatoxin and Aspergillus flavus in corn (Diener, U.L., and Asquith, R.L. eds.). Cooperative Bulletin 279. Auburn, Alabama, USA:Auburn University.

Shotwell, O.L., Burg, W.R., and Diller, T. 1981. Thin layer chromatographic determination of aflatoxin in corn dust. Journal of the Association of Official Analytical Chemists 64:1060-1063.

Shotwell, O.L., and Goulden, M.L. 1977. Aflatxoin: comparison of analysis of corn by various methods. Journal of the Association of Official Analytical Chemists 60:83-88.

Spilman, J.R., Jr. 1985. Modification of the rapid screening method for aflatoxin in corn for quantitative use. Journal of the Association of Official Analytical Chemists 68:453458.

Stoloff, L., Castegnaro, M.,Scott, P., O'Neill, I.K., and Bartsch, H. (eds.). 1982. Environment carcinogens. Selected methods of analysis. vol.5. Some mycotoxins. Scientific Publication no. 44. Lyon, Cedex, France: International Agency for Research on Cancer.

Stubblefield, R.D., and Shotwell, O.L. 1977. Reverse phase analytical and preparative high pressure liquid chromatography of aflatoxins. Journal of the Association of Official Analytical Chemists 60:784790.

Sun, P., and Chu, F.S. 1977. A simple solid-phase radioimmunoassay for aflatoxin B1. Journal of Food Safety 1:67-75.

Trater, E.J., Hanchay, J.P., and Scott, P.M. 1984. Improved liquid chromatographic method for determination of aflatoxins in peanut butter and other commodities. Journal of the Association of Official Analytical Chemists 67:597-600.

Thean, J.E., Lorenz, D.R., Wilson, D.M., Rodgers, K., and Gueldner, R.G 1980. Extraction, cleanup, and quantitative determination of aflatoxins in corn. Journal of the Association of Official Analytical Chemists 63:631-633.

Thomas, F., Eppley, R.M., and Trucksess, M.W. 1975. Rapid screening method for aflatoxins and zearalenone in corn. Journal of the Association of Official Analytical Chemists 58:114-116.

Trucksess, M.W., Brumley, W.C., and Nesheim, S. 1984. Rapid quantitation and confirmation of aflatoxins in corn and peanut butter, using a disposable silica gel column, thin layer chromatography, and gas chromatograph/mass spectrometry. Journal of the Association of Official Analytical Chemists 67:973-975.

Velasco, J. 1981. Replacement of benzene as a solvent for aflatoxin standards. Journal of the American Oil Chemists 'Society 58:938A-939A.

Whitaker, T.B., and Dickens, J.W. 1983. Evaluation of a testing program for aflatoxin in corn. Journal of the Association of Official Analytical Chemists 66:1055-1058.

Table 1. Major physico-chemical properties of aflatoxins.

Figure 1. Molecular structure of aflatoxins.

Extracted from Goto and Marabe (1989).
Citation: ICRISAT (International Crops Research Institute for the Semi-Arid Tropics).1989. Aflatoxin contamination of groundnut: proceedings of the International Workshop, 6-9 Oct 1987, ICRISAT Center, India. Patancheru, A.P. 502324, India: lCRISAT.

Figure 2. Chromatograms of aflatoxins obtained by HPLC mobile phase, 1duene-ethylacetate-formic acid-methanol (89:7:2:2) Source: IBIP, p. 177

Table 2. Comparison of performance of Enzyme-Linked Immunosorbent Assay (ELISA) kits for aflatoxin analysis

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Training in mould isolation, identification, handling, and evaluation of conditions leading to mycotoxin production

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ABSTRACT

In-depth training was provided to Thai personnel in the proper methods for the isolation, enumeration, handling, and disposal of food borne moulds, and in their identification. Mould species were isolated from several Thai foodstuffs including corn, beans, vegetables, flour, and from Thai soil. More than 24 species of Aspergillus, 12 species of Penicillium and at least 10 other mould genera were detected. Stressed in the identification process were the ability to recognise the macroscopic, the microscopic, and the diagnostic characteristics of each species. Several toxic mould species were found including A. flavus A. parasiticus, A. versicolor, A. nidulans, A. ochraceus, A. terreus, P. citrinum, P. islandicum, and 1:? cyclopium. A number of non-toxic but closely related species were also detected. Limited training was also provided to the employees in the areas of laboratory quality assurance and safety. Regarding Thai corn, investigations were limited since there was virtually no 1986 first crop due to drought. The following recommendations have been made:

1. **The new mycotoxin wing should be finished and become operable as soon as possible.**

2. **The professional mycology staff should be given additional training abroad in a well equipped mycology laboratory.**
3. **Proper methodology should be stressed. Proper laboratory quality assurance-safety measures should be drawn up and followed in all the laboratories.**
4. **A mycological "clean room" should be constructed.**
5. **Regarding Thai corn, it should be dried down and kept dry and clean during handling, storage and transit.**

ISOLATION - ENUMERATION

The trainees were quite familiar with the direct plating technique. However, a few refinements on their methodology were suggested and were incorporated. One such was the addition of 7.5% sodium chloride to the agar medium, in this case, potato dextrose agar. NaCI is very effective in the inhibition of fast-growing "spreader" moulds such as Mucor, Phizopus, and Trichoderma. Yet, it does not inhibit the growth of other mould species, including the mycotoxin producers. Nor does it change their macroscopic-microscopic morphology, as does some other "spreader" inhibitors such as Rose Bengal. Thus, identification is not compromised. Another suggested refinement to the direct plating technique was the addition to the medium of an antibiotic to inhibit bacterial growth. We used 40 ppm of chlortetracyline-HCL, which I brought with me from the U.S.A. It was needed and it worked. A third suggested refinement was to hold all seeds, including corn, mungbeans, and groundnuts in a freezer for 72 hours prior to direct plating in

order to kill possible mites and their eggs. The trainees found that it worked.

With regard to the dilution plating technique, the trainees were not familiar with it, so I introduced it using samples of flour. However, many modifications of the standard method were necessary due to the lack of enough proper glassware, e.g., dilution bottles and pipettes. Yet, the results turned out to be quite informative to the trainees although not quantitatively accurate.

IDENTIFICATION

During the first 4 weeks of my consultancy, at least 24 species of Aspergillus, 12 of Penicillium, and 10 other mould genera were detected and studied by the trainees in depth. These species were isolated from Thai foodstuffs and soil and included several toxin producers, i.e., Aspergillus flavus and A. parasiticus (aflatoxins), A. versicolor and A. nidulans (sterigmatocystin) A. terreus and A. clavatus (patulin), Penicillium citrinum (penicillin acid, cyclopiazonic acid, tremorgens, etc.) and P. islandicum (luteoskyrin, etc.). Also detected were several species which are morphologically very similar to toxin producers. For instance, to the untrained eye, Aspergillus oryzee, A. tamarii, and even A. wentii, could be mistaken for A. flavus Likewise, several species of Penicillium are morphologically quite similar to - but diagnostically different from - P. citrinum, e.g., P. chrysogenum and P. oxalicum. The same is true for P. islandlcum, eg., P. variabile, P. aculeatum, P. verruculosum. I am now satisfied that the trainees can now differentiate between these toxic and non-toxic species, and, thus, will be capable of

independently determining mould flora profiles of various Thai foodstuffs in the future. The possibility of a refresher course in mould identification for the trainees does bear merit, of course. After 6-12 months of even intensive work in this area, problems still can exist. Mould identification is not easy. It requires patience and determination. Often times it ends in frustration.

Of interest, the mould species detected in the Thai foodstuffs-soils are, except for the genus Aspergillus different from what I have encountered in similar American foodstuffs-soils. This was unexpected. Of particular interest was the general lack of the genera Alternaria and Fusarium and the Penicillium species detected are not the same as those that we find regularly in America.

Listed briefly below are the diagnostic characteristics of a number of mould species Aspergillus flavus: colonies bright green often with black scierotia; conidiophores with usually 2 sets of sterigmata and with spiny stalks. Conidia globose and spiny.

***Aspergillus parasiticus*: same color as A. flavus but without sclerotia. Condiophore has only one set of sterigmata and stalk is spiny only at the top. Globose spiny conidia are produced.**

***Aspergillus oryzae:* In the A. flavus group but colony color is yellow green. Just one set of sterigmata with smooth conidiophore stalks. Conidia are usually smooth and elliptical and larger than those of A. flavus.**

***Aspergillus tamarii:* In the A. flavus group, but colonies quickly become deep chocolate brown. Conidiophores are the same as A. flavus but conidia are unique, having a double wall and color**

bars rather than spines.

Aspergillus wentii: **Similar in color to A. tamarii, but colonies produce much floccose mycelium and conidiophores are quite long with large vesicles. Conidia are similar to A. tamarii, but with a single wall.**

Aspergillus ochraceus: **Colonies consistently remain bright yellow and sometimes produce purple sclerotia Conidiophores have 2 sets of sterigmata, the stalk is spiny with definite yellow pigment in the walls. Conidia are usually smooth walled and globose to slightly ellipitical.**

Aspergillus versicolor **Colonies are dark green but with often highly and variously colored mycelial sectors. Colony reverse is purple. Conidiophores with 2 sets of sterigmata, smooth and colorless stalk, and small vesicles. Conidia slightly roughened.**

Aspergillus sydowi: **In the A. versicolor group but colonies are consistently blue and produce no highly colored mycelial sectors. Reverse is red. Conidiophores similar to A. versicolor, but conidia are very definitely spiny.**

Aspergillus nidulans: **Colonies are dark green and produce long columns of conidia that resemble sticks or "cigarettes". Conidiophores and conidia similar to A. versicolor but stalk and vesicle are pigmented brown. The species has a sexual stage and produces cleistothecia surrounded by hulle cells plus bright red ascospores.**

Aspergillus fumigates: **An important species because it causes human lung infections. Colonies**

are blue green and produce columns of conidia similar to A. nidulans. However, conidiophores have but one set of sterigmata and conidiophore vesicles and sterigmata are pigmented blue. Colony reverse is also blue to black.

Aspergillus terreus: **This species, apparently the most common in Thai soil, is like A. nidulans and A. fumigatus In producing long columns of conidia. However, colony color is tan to beige. Conidiophores are colorless and smooth, have a small vesicle and 2 sets of sterigmata.**

Aspergillus glaucus **group. Worldwide, perhaps the most common group of aspergilli in stored foodstuffs, the group consists of more than 12 species. However, the species are morphologically quite similar. No need to go beyond the group concept. Species colonies are blue-grey and contain numerous bright shiny yellow balls, which are the cleistothecla, or the sexual stage. Conidiophores are smooth with slight blue pigmentation and one set of sterigmata. Conidia are spiny. The colorless ascospores separate the individual species based on size, shape, and wall adornment.**

Penicillium oxalicum: **The most common Penicillium in American field corn, and apparently common in Thai field corn, this species should be unmistakeable. Colonies are dark green-black, reverse yellow-green. Conidia are formed in long thin chains which shine like threads of silk under illumination. When an agar plate containing P. oxalicum is tapped, the conidia fall away in crusts or clumps. The conidiophore is assymetrical. The conidia are relatively large, definitely elliptical and smooth.**

Penicillium citrinum: **Apparently the most common Penicillium in Thailand, it is relatively simple to identify although tremendous variation exists among isolates of this species. Colonies are blue gray, often producing yellow droplets on the surface and yellow in reverse. Conidiophores are bi-verticillate, having only metulae and flask-shaped sterigmata The metulae are not compressed to each other. Rather, there is space between them. Conidia are globose and usually smooth.**

Penicillium islandicum: **Another common Thai species, it is bi-verticillate, but unlike P. citrinum, the metulae are closely compressed but bear flask shaped sterigmata Conidia are elliptical and smooth. Colonies are orange with green areas of sporulation and produce abundent orange liquid droplets. Reverse is orangebrown.**

Pencicillium variabile: **This species is dark green with abundant areas of yellow mycelium, especially along colony margins. Its conidiophore differs from that of P. islandicum only in that the sterigmata are long and tapered, not flask shaped.**

Penicillium verruculosum: **Another biverticillate species, it is quite similar to P. variabile except that it does not produce abundant yellow mycelium and the conidia are globose and spiny.**

Penicillium funiculosum **Another bi-verticallate species (most Penicillium species in Thailand appear to be bi-verticillate), the colonies are gray and produce definite ropes or funicles of sponulating mycelium on the surface No other Penicillium species does this. Reverse is usually purple. Conidiophores are identical with those of P. variabile.**

Penicillium purpurogenum: **Another bi-verticillate species, it produces dark green colonies with abundant red droplets and a bright red reverse with the pigment diffusing into the surrounding agar. Its conidiophore is similar to that of P. verruculosum with the spiny globose conidia.**

Penicillium cyclopium: **This species was only detected once here, but I have drilled on this species since it is the most important Pencillium in storage in America. Colonies are bright blue, granular and usually produce maroon surface droplets and reverse. The colony produces a strong mouldy odor. Conidiophores as assymetrical with a usually spiny stalk. Conidia are globose and slightly roughened.**

HANDLING, MAINTENANCE AND DISPOSAL

This area was also stressed. Trainees were taught how to make proper microscope mounts and how to dispose of the slides after use. Also taught were proper ways of inoculating plates and of observing the plates on a daily basis. Dish and lids should never be removed unless one is certain that the mould is harmless. Regarding maintenance, the trainees are routinely keeping the cultures on agar slants. However, with time, many mould species change morphologically when maintained this way and suddenly become unidentifiable. I am recommending that a freeze-dryer be purchased for purposes of proper culture maintenance. Such an appliance is relatively inexpensive and once a culture has been freeze-dried it remains viable for years with little or no morphological change. Another alternative method of maintenance is preservation of spores in

sterile soil. In soil, cultures remain relatively stable. However, depending on the species, periods of viability may be relatively short. Another maintenance method would be to immerse the mould propaguies in liquid nitrogen. This is perhaps the most acceptable method, but is also the most costly. Regarding the proper disposal of mould. the only acceptable way is by auto-craving. But I pointed out that if the mold or molds were mycotoxin producers, in addition to growing they undoubtedly were also producing mycotoxins. So after autoclaving, decontamination, with e.g., NaOCI is in order.

Laboratory Safely-Quality Assurance

Although not a "term of reference' upon arrival at the laboratory I noticed a number of safety-quality assurance violations and attempted to point them out to the laboratory leaders. I obtained copies of the FDA, Bureau of Foods, Safety Regulations and of the FDA Division of Microbiology Quality Assurance Regulations (see attachment B). I discussed these documents with the laboratory leaders. They agreed that many violations existed but were in hopes that when the new wing for mycotoxin work became operable, that most of the violations could be corrected. The new wing is finished but has not been occupied due to the lack of air conditioning needed where volatile solvents are kept, laboratory hoods, benches, etc., and equipment, and a needed electronic transformer. I was then violations with the Director General of the Department of Agriculture. He decided that the new Department of Agriculture. He decided that the new wing should become operable as soon as possible. Within 5 days, the air conditioning units arrived and have been installed. We are still waiting the benches, equipment, and transformer.

A primary reason for the violations is the overcrowding that exists in the present facility. The added new space would sharply alleviate the crowded conditions. Yet, additional items will need to be purchased such as fire extinguishers, flame-proof solvent cabinets, clamps for compressed gas cylinders, fire blankets, etc. Also, additional electrical outlets are needed in the present facility. Too many extension cords are used. And special areas for eating lunch should be designated and employees should be urged to wear lab coats and hardtoe shoes when doing bench work.

The most serious quality assurance problem in the laboratory area where the mould identification training is being conducted is severe insect and mite infestation. I brought 11 standard Aspergillus Penicillium cultures with me. We plated them out. But within 72 hours, 6 of the cultures were overrun with bacteria and other mould species due to infestation with ants. We were able to construct a temporary structure that has, so far, prevented additional infestation, stackable shelves whose bottom legs are immersed in dishes of glycerine, thus trapping insects and mites. But this is only temporary. What is urgently needed once space becomes available is the construction of a mycological "clean room". That is, a room that is physically isolated from the rest of the laboratory facility, that is sealed to prevent insect-mite infestation, and that is used only for culturing, identifying, and storing mould species. The room should be sprayed weekly with insecticide and should be restricted to selected personnel. Plans are under way for the construction of such a room.

Moulds Detected In Thai Corn

Although corn samples were in limited supply, we had access to a few samples. Table 3 lists our results from 5 samples of "excellent quality" shelled corn that had been stored for 8 months at 10C. When placed in storage, this corn was of high quality with very low levels of aflatoxin. Initial mould floras had not been determine for this corn. Table 3 shows, however, that the corn had become extremely mouldy, 100% before surface disinfection and 87% after. In addition, A. flavus was the predominant species. This was unexpected since A. flavus grows poorly below 12C But I was informed that the 10C room sometimes loses power and warms up, thus, resulting in water condensation on the corn. In addition, the corn was infested with mites which are capable of mechanically transmitting mould propagules, from seed to seed and from plate to plate.

Table 4 lists results of the few samples of freshly harvested, wet season, corn that we were able to obtain. Of interest, although A. flavus was present, its levels were lower Rather, the A. glaucus group seemed to predominate. But, mites were also detected in these "fresh" samples. Thus, the mould flora can be expected to change.

ASSISTANCE IN DEVELOPING CAPABILITIES AND GUIDELINES FOR CONTROL OF MYCOLOGICAL CONTAMINATION OF FOODGRAINS, AND PREVEN TION AND CONTROL OF AFLATOXIN IN MAIZE AND GROUNDNUTS IN THAILAND

Introduction

Mould proliferation and subsequent metabolise (mycotoxin) production is dependent upon 3

necessary and inter-related factors, namely: 1. the physical presence of the generating organism; 2. a substrate suitable for growth; 3. an environment suitable for growth. All 3 factors must be met in order for growth and toxin production to occur. These 3 factors are discussed below and include some comments, based upon my 6-week stay in Thailand.

Physical Presence of the Generating Organism

As tables 1 and 2 show, many toxic mould species are present in Thailand. Although their sources are numerous, the chief source of the organisms would appear to be the soil, the air, former crop organic material, and contaminated storage facilities. If present, these moulds can be spread by the air, by insects, mites, higher animals, and even facility personnel. For instance, upon one field trip here, I observed that a number of animals were present in the areas of the drying corn, including dogs, cats, and chickens. I also noted dried organic material, possibly from an earlier crop, lying nearby. In my laboratory studies, large populations of mites were detected along with lower levels of insects. And on my field trip I watched personnel indiscriminantly walking over the drying corn with shoes on. All the above are potential sources of the spread of the mould. Granted, there is no way to eliminate the presence of these toxic mould species in Thailand, but ways do exist to lessen their spread and contamination potential. For instance, where corn is being dried there should be no freewalking animals around nor any indiscriminatingly walking humans. Nor should there be any residues of earlier crops. And after drying, just prior to placing in storage, the corn should be fumigated to eliminate mites and insects.

Regarding storage facilities, although I did not visit any here since most were empty due to this

season's crop failure in the field, they can be an Important source of mould presence if not kept clean. Thus, these facilities should routinely be cleaned up between storage loads, including fumigation. In addition, vehicles used to transport these foodcrops should also be kept clean. There is nothing to be done about the presence of toxic moulds in Thailand, but things can be done to alleviate their spread.

Substrate Suitable for Growth

Although most of the toxic moulds I detected in Thailand could conceivably grow on any Thai foodstuff, I doubt that this really is the case. We know that A. flavus thrives on corn and groundnuts. But it was rare on fresh vegetable crops. However, A. versicolor was the principal toxic species detected on black beans. Thus, it should be within the interests of my trainees to begin determining mould floras of Thai foodstuffs other than corn and groundnuts. For instance, A. versicolor may indeed be the chief toxic mould in black beans. Thus, the possibility of sterigmatocystin. Determining mould floras of individual substrates is indeed important when considering the overall picture of mycotoxin contamination.

Environment Suitable for Growth

The most effective means to control and prevent aflatoxin contamination of corn and groundnuts, and the mycotoxin contamination of any foodstuff, is the ability to control the environment so as to prevent mould growth. Several environmental parameters are involved, including:

1. Atmosphere All mold species are obligate aerobes, and cannot grow in the absence of free

oxygen. Although probably not economically feasible, one sure way to prevent mycotoxin contamination of corn, groundout, and small grains is to store them under anaerobic conditions, e.g. CO2 or nitrogen. For instance, this could be done in large airtight silos. High moisture would be no problem. The moulds just would not grow But for the farmer and the middleman, this type of environment control is understandably unrealistic.

2. Temperature In my experience, virtually all of the mycotoxin producing species I detected in Thailand grow poorly, it at all, at 10C. Thus, another environmental way to control or prevent mycotoxin production is a storage temperature of 10C or less. However, low temperature facilities are virtually nonexistent. Thus, temperature control is not the answer.

3. Moisture Without question, moisture control is the best and most economical means to control the environment to prevent mould growth and mycotoxin production. To reiterate the comments of previous consultants, Mr. Nesheim, Mr. Ware, and Dr. Smalley, corn must be dried down to 14.5% moisture, wet weight basis, to avoid aflatoxin contamination. The same for groundnuts. Certain of the small grains require even lower moisture contents, cat 13%. The problem in Thailand is that these desired moistures are not achieved, or at least, not quickly enough. Regarding corn, the farmer often harvests the crop when moisture content to too high, e.g., 25-35% or more, wet weight basis. Upon harvest, the farmers may attempt to sundry the corn on plastic sheets or concrete slabs-which may be effective with the dry season second harvest or they may deliver it directly to the buyer at its original field moisture, if for no other reason than need of the money. If the farmer could be convinced to delay harvesting of the corn, that is, if the ears were allowed to remain on the stalks in the field for an additional 1, 2, or even 3 weeks

(especially the first, wet season, crop), the moisture content of the corn would dry down naturally, maybe to as low as 18-22%. And rain would not significantly effect this natural drying in the field. Sundrying then could effectively bring the corn moisture close to the needed 14.5%. However, even after acceptable drying, the corn must be handled-by the farmer, the buyer, the exporter - in a manner that will not allow the moisture to return. It should never be exposed to free water and manner of transportation of the corn should be such as to minimize actual water condensation. For instance, corn transportation in trucks, railcars, and even water going vessels may be subject to moisture build-up due to condensation if transport time is lengthy. Hot day time temperatures followed by cooler nightime temperatures invariably will cause the corn to "sweat", thus, free water available for mould growth. And once a mould has begun to grow, it does not dissipate the water. It just transfers it. Thus, proper handling and shipment of the corn, even after acceptable drying, is essential in order to avoid moisture build-up, mould growth and toxin formation. To the farmer, to the buyer, to the exporter, to the importer, transport must be proper and rapid. And as referred to in the section, "Physical Presence of the Generating organism", transportation vehicles should be clean. No leftover mould or bacteria, no excrete, no unusual amounts of soil or organic debris.

REFERENCES

1. **Barnett, H. L.1972. Illustrated Manual of Imperfect Fungi. Burgess Publishing Co., Minneapolis.**

2. **Eillis, M. B.1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute. Kew, Surrey, England.**
3. **Gilman, J. C. 1959. A Manual of the Soil Fungi. Iowa State University Press, Ames, Iowa.**
4. **Mislivec, P. B. 1978. The Genus Penicillium. In T. Wyllie and H. Morehouse (ED). Mycotoxic Fungi, Mycotoxins, and Mycotoxicoses. Marcel Dekker, NY.**
5. **Nelson, P., T.A. Toussoun, and W. Marasas. 1984. Illustrated Manual of the Genus Fusarium. Pennsylvania State University Press, State College, Pennsylvania.**
6. **Raper, K. and D. Fennell. 1965. The Genus Aspergillus. Williams and Wilkins Press. Baltimore, MD.**
7. **Raper, K., and C. Thom. 1949. A Manual of the Penicillia. Williams and Wilkins Press, Baltimore, MD.**
8. **U. S. Food and Rrug Administration. 1984. Bacteriological Analytical Manual. Association of Official Analytical Chemists, Arlington, VA.**

Table 1. Mould species Detected In Various Thai Foodstuffs (maize, groundauts, mungbeans, fresh vegetables)

The Genus Aspergillus The Genus Penicillium

A. aculeatus	P. aculeatum
A. amstelodami	P. chrysogenum
A. cadidus	P. citrinum

A. chevalier)	P. cyclopium
A. flavus	P. funiculosum
A. flavus var. co/umnaris	P. implicatum
A. fumigatus	P. islandicum
A. niger	P. oxalicum
A. ochraceus	P. purpurogenum
A. oryzae	P. variabile
A. parasiticus	P. verruculosum
A. repens	
A. restrictus	
A. sydowi	
A. tamarii	
A. terreus	
A. versicolor	
A. wentii	
Other Mould Genera:	
Alternaria a/temata	
Cephalosporium spp.	

Cladosporium spp.	
Curvularia spp.	
Fusarium spp.	
Paecilomyces varioti	
Pestalotia spp.	
Rhizactonia spp.	
Rhizopus nigricans	
Syncephalastrum spp.	
Trichoderma viride	

Table 2. Mould Species Detected in 16 Soil Samples Collected from Thai Maize Farms

Mould Species	Number of Samples Positive
Aspergillus aculeatus	5
A. candidus	8
A. clavatus	1

A. flavus	12
A. flavipes	7
A. fumigatus	7
A. glaucus group	3
A. nidulans	13
A. niger	15
A. ochraceus	1
A. tamarii	2
A. terreus	14
A. terrcus var. africanus	2
A. ustus	3
A. versicolor	1
Chaetomium spp.	1
Cladosporium herbarum	1
Mucor spp.	2
Penicillium citrinum	14
P. funiculosum	1
P. luteum	1
P. variabile	3

P. verruculosum	2
Rhizopus nigricans	4
Syncephalastrum spp.	2
Trichoderma viride	2

Table 3. Mould Flora of Thai Corn Kernels after 8 Month's Storage at 10C

Non-Surface-Disinfected (% Mouldiness = 100%)	Surface Disinfected (% Mouldiness = 87%)
Species Detected	Species Detected(a)
Aspergillus flavus: 100%	Aspergillus flavus: 52%
A. glaucus group: 3%	A. glaucus group: 19%
A. niger: 15%	A. niger: 8%
Penicillium citrinum: 9%	A. tamarii: 2%
	A. wentii: 3%
	Cephalosporium spp.: 3%
	Mucor spp.: 3%

	Penicillium citrinum: 10%
	Phizopus nigficans: 1%

(a) The following species were detected at a rate of less than 1%: *Aspergillus aculeatus*; *A. candidus*; *A. fumigates*; *A. nidulans*; *A. restrictus*; *A. terreus*; *Penicillium chrysogenum*.

Table 4. Mould Flora of Freshly Harvested 1986 Thai Corn Kernels

Non-Surface-Disinfected (% Mouldiness = 100%)	Surface Disinfected (% Mouldiness = 88%)
Species Detected	Species Detected
Aspergillus flavus: 88%	Aspergillus flavus: 29%
A. glaucus group: 39%	A. glaucus group: 55%
A. niger 24%	A. niger 8%
A. fumigates: 2%	A. fumigates: 1%
A. wentii: 2%	A. wentii: 5%
	A. tamarii: 2%
	A. aculeatus: 1%
	A. terreus: 1%

	Penicillium citrinum: 5% P. oxalicum: 1%
	Rhizopus nigricans: 3%
	Cephalosporium spp.: 1%
	Syncephalastrum spp.: 1%

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Enumeration of yeasts and moulds and production of toxins

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Philip B. Mislivec and Michael E. Stack

The large and diverse group of microscopic foodborne yeasts and moulds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile, environmental requirements. Although all yeasts and moulds are obligate aerobes (require free oxygen for growth), their acid/alkaline requirement for growth is quite

broad, ranging from pH 2 to above pH 9. Their temperature range (10-35C) is also broad, with a few species capable of growth below or above this range. Moisture requirements of foodborne moulds are relatively low; most species grow at a water activity (aw) of 0.85 or less, although yeasts generally require a higher water activity.

Both yeasts and moulds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time, e.g., they invade field crops such as small grains, nuts, beans, tomatoes, and apples both in the field before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on food type, organisms involved, and degree of invasion, i.e., the contaminated food may be slightly blemished, severely blemished, or completely decomposed, with the actual yeast or mould growth manifested by rot spots of various sizes and colors, unslightly scabs, slime, white cottony mycelium, or highly colored sporulating mould. Abnormal flavors and odors may also be produced. Occasionally a food appears to be mould-free but upon mycological examination, is found to be contaminated. Contamination of foods by yeasts and moulds can also result in substantial economic losses to producer, processor, and consumer.

Several foodborne moulds, and possibly yeasts, may also be a potential hazard to human or animal health because of their ability to produce toxic metabolities known as mycotoxins. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though the generating organisms may not survive food preparation, the preformed toxin may still be present. Certain foodborne moulds and yeasts may also be a hazard because of their ability to elicit allergic reactions or even cause infection. Although most foodborne fungi are not

infectious, some species can cause infection, especially to vulnerable population groups, e.g., the aged and debilitated and individuals who are receiving chemotherapy or antibiotic treatment.

The dilution plating and the direct plating methods may be used to detect fungi in foods. The dilution plating method, which is the traditional method used in examining foods, is given here. It varies only slightly from the method described in previous editions of the Bacteriological Analytical Manual. The direct plating method is included here because we have found it to be more efficient than the dilution plating method for detecting individual mould species, including most of the toxin producers. (Note: It is less effective in detecting yeasts.) It can also be used to determine whether the presence of mould is due to external contamination or internal invasion. Methodology for testing the ability of isolates of toxigenic mould species to produce mycotoxins on sterile rice water substrate is also included.

Enumeration of Yeasts and Moulds in Foods-Dilution Plating Technique

A. Equipment and materials

1. **Basic equipment (and appropriate techniques) for preparation of a food sample homogenate as described in Chapter 2**
2. **Equipment for plating samples as described in Chapter 4**
3. **Incubator set at 22-25C**

4. **Arnold steam chest**
5. **pH meter**

B. Media and reagents

1. **Potato dextrose agar (M114), commercially available in dehydrated form**
2. **Potato dextrose-salt agar (M114). Same medium as above, amended with 75 9 NaCI. This medium requires 20 9 agar rather than 15 g agar per lifer.**
3. **Malt extract agar (M78), commercially available in dehydrated form**
4. **Plate count agar (standard methods) (M112)**
5. **Tartaric acid solution, 10%, sterile**
6. **Antibiotic solution (s), see C-2a, below**

C. Analysis of samples

1. **Prepare sterile agar medium (250 ml portions in prescription bottles or flasks, autoclaved 15 min at 121C and 15 psi). Temper to 45 1C in water bath. Prepare medium well in advance and let solidify before remelting and tempering. Do not re-melt solidified medium more than once or under pressure. An Arnold steam chest is recommended. Once medium has been tempered, it can be held for 2-3 h before use, provided water level of water bath is 2-3 cm above surface of agar in aliquot container. Medium of choice is potato dextrose agar, although other media listed above may be used. Potato dextrose-salt agar is especially useful for analyzing samples containing "spreader" moulds (Mucor, Rhizopus, etc.) since the**

added NaCI effectively inhibits their growth but readily allows detection of other yeast-mould propagules.

2. **To inhibit bacterial growth, amend agar medium with either antibiotics or sterile 10% tartaric acid solution (to be done after agar has been tempered and immediately before pouring plates) as follows:**

   a. **Antibiotics. Use of antibiotics is preferred to tartaric acid solution because stock solutions are relatively easy to prepare yeast and mould species, does not result. Chiortetracycline-HCl, at agar medium concentration of 40 ppm, is recommended. Other antibiotics may be used (e.g., chloramphenicol, streptomycin) but should always be used at the same concentration as chlortetracycline-HCl and in addition to it. Prepare stock solutions by dissolving 1 9 of antibiotic in 100 ml of sterile distilled water and filtering through a 0.45 m membrane (Nalge Sybron Corp., Rochester, NY). Store stock solutions in dark at 4-8C. Shelf life should exceed 1 month. Equilibrate stock solutions to room temperature immediately before use. If agar medium is in 250 ml aliquots, add 1 ml of 100 ml stock solution to obtain 40 ppm concentration. If medium aliquots are greater or less, adjustments will be necessary.**
   b. **Tartaric acid solution. A 10% solution may be used to adjust agar medium to pH 3 5 0.1 Sterilize solution by filtering through 0.45 m membrane. Titrate to determine amount of solution needed to adjust pH to 3.5. Type and aliquot volume of medium will affect amount of solution needed. After adding solution to medium, verify pH**

by letting a portion of medium solidify and checking with pH meter. Do this for every new lot of medium prepared.

3. **Prepare food homogenate (Chapter 2) and make appropriate dilutions (Chapter 4). Dilutions of 10(-6) should suffice.**
4. **Use sterile cotton-plugged pipes to place 1 ml portions of sample dilutions into prelabled 15 x 100 mm petri plates (plastic or glass), and immediately add 20-25 ml tempered agar medium containing either antibiotic (s) or tartaric acid solution. Mix contents by gently swirling plates clockwise then counterclockwise, taking care to avoid spillage on dish lid. Add agar within 1-2 min after adding dilution. Otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate, using wide bore pipets. From preparation of first sample dilution to pouring of final plate, no more than 20 min. preferably 10 min. should elapse.**
5. **Incubate plates in dark at 22-25C. Do not stack plates higher than 3 and do not invert. Let plates remain undisturbed until time for counting.**
6. **Count plates after 5 days of incubation. Do not count plates after 3 days since handling of plates could result in secondary growth from dislodged spores, making 5 day counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mould are present, depending on the type of mould, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colonies (col)/g or (col)/ml based on an average**

count of the triplicate set. Round off counts to 2 significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460).

ENUMERATION OF MOULDS IN FOODS-DIRECT PLATING TECHNIQUE FOR FOODS (SUCH AS DRIED BEANS, NUTS, WHOLE SPICES, COFFEE AND COCOA BEANS) THAT CAN BE HANDLED WITH FORCEPS

A. Equipment and materials

1. **Freezer, -20C**
2. **Beakers, sterile, 150 ml**
3. **Forceps, sterile**
4. **Arnold steam chest**
5. **Water bath, 45 1C**
6. **Incubator, 22-25C**

B. Media and reagents

1. **Potato dextrose-salt agar (M114)**

2. **Antibiotic solution**
3. **NaOCI solution, 5%**
4. **Sterile distilled water**

C. Analysis of non-surface-disinfected (NSD) foods

1. **Before plating. Hold samples at -20C for 72 h to kill mites and other insects that might interfere with analysis.**
2. **Preparation of agar plates. Use potato dextrosesalt agar (containing 75 9 Nacl/liter). NaCl inhibits growth of mould "spreaders" and prevents germination of viable seeds which otherwise could cause petri dish lid and stack disorientation. To tempered agar, add 40 ppm chlortetracycline-HCI (C-2a, above). Into 15 x 100 mm petri plates (plastic or glass) pour about 30 ml medium and let solidify. Because of prolonged incubation time, more medium is needed for direct plating than for dilution plating in each petri dish. Prepare 10 plates for each sample to be analyzed. Plates may be prepared in advance, but period between preparation and use should not exceed 24 h.**
3. **Plating of sample. From each sample, transfer about 50 9 into sterile 150 ml beaker. Using 95% ethanol-flamed forceps, place intact food items on surface of solidified agar, 5 items per plate (1 in plate center and 1 in each quadrant), 50 items total per sample. Flame forceps between plating of each item. Use several forceps alternately to avoid overheating. Do not plate visibly mouldy or otherwise blemished items.**
4. **Incubation of plates. Align plates in stacks of 10; identify top and bottom plate of each stack**

with sample number plus date of plating. Incubate stacks, undisturbed, in dark at 22-25C for 1421 days.

5. **Reading of plates. Determine occurrence of mould in percentages (e.g., if mould emerged from all 50 food items, mouldiness is 100%, if from 32 items, mouldiness is 64%). Determine percent occurrence of individual mould genera and species in like manner. Several Asperigillus species (or species complexes) plus most other foodborne mould genera may be identified directly on above medium by experienced analysts with low power (10-30X) magnification.**

D. Analysis of surface-disinfected (SD) foods

Perform disinfection in clean laboratory sink, not stainless steel, free from any acid residues, with tap water running (precautions against chlorine gas generation). Using rubber gloves, transfer about 50 9 of sample into sterile 150 ml beaker. Cover with 5% NaOCI solution for 1 min. swirling beaker contents gently but constantly in clockwisecounterclockwise motion Decant 5% NaOCI solution and give beaker contents three 1 min sterile distilled water rinses. Prepare plates, plate sample, incubate, and read plates as in C, 2-5, above. Comparison of NSD and SD results from same sample will indicate whether mouldiness was due mainly to surface contamination or to internal invasion and growth.

METHODS FOR DETERMINING TOXIN PRODUCTION BY MOULDS

A. Equipment and materials

1. **Erlenmeyer flasks, 300 ml, wide-mouth**
2. **Cotton, nonabsorbent**
3. **Funnels, short-stem glass, 90-100 mm diameter**
4. **Filter paper, 18 cm diameter, folded (S & S No. 588)**
5. **Boiling chips, silicon carbide**
6. **Fume hood equipped with steam bath; airflow rate, 100 cubic ft/min**
7. **Blender, high speed, explosion-proof**
8. **Thin layer chromatographic apparatus or high performance liquid chromatograph**
9. **Incubator, 22-25C**

B. Media and reagents

1. **Long or short grain polished rice**
2. **Chloroform for extraction of aflatoxins, ochratoxins, sterigmatocystin, xanthomegnin, luteoskyrin, patulin, penicillic acid, citrinin, T-2 toxin, zearalenone**
3. **Methanol for extraction of deoxynivalenol**
4. **Appropriate mycotoxin standards**
5. **NaOCI solution, 5%**

C. Toxin production

Into 300 ml wide-mouth Erlenmeyer flask, add 50 9 rice and 50 ml distilled water. Cotton plug flasks and autoclave 20 min at 121C and 15 psi. Aseptically multispore-inoculate separate cooled flasks with individual mould isolates. Incubate inoculated flasks at 22-25C until entire surface is covered with growth and mycelium has penetrated to bottom of flask (15-20 days). To each flask, add 150 ml chloroform (150 ml methanol if toxin in question is deoxynivalenol), using short-stem glass funnel inserted alongside unremoved cotton plug (to minimize mould spore dissemination). Hear flask contents in fume hood on steam bath until solvent begins to boil. (Conduct all subsequent steps in fume hood.) With spatula, break up mouldy rice cake and transfer flask contents into explosionproof blender and blend at high speed for 1 min. Filter blender contents through filter paper inserted into short-stem glass funnel. Collect filtrate in 300 ml Erlenmeyer flask. Return rice cakes to blender, add 100 ml unheated solvent and blend 1 min at high speed. Filter as above and combine filtrates. Add boiling chips to flask containing filtrates and steam evaporate to 20-25 ml. If analysis is not to follow immediately, evaporate to dryness and store flask in dark. Rinse all glassware, etc., used for extraction in 5% NaOCI solution before soap and water cleansing. Rice cake should be submerged in 5% NaOCI solution for 72 h before autoclaving and disposal.

D. Toxin analysis

Toxin analysis requires use of appropriate mycotoxin standards for both qualitative and quantitative evaluation. Use either thin layer chromatography or high performance liquid chromatography, as described in Chapter 26, of Official Methods of Analysis 14th ea., 1984 (Association of Official Analytical Chemists, Arlington, VA), for determination of most

foodborne mycotoxins.

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Fungal damage in durable foodstuffs with special reference to storage in the tropics

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1. What are fungi?

Fungi are micro-organisms generally classified as plants, although there is growing support for the separation of the fungi and other micro-organisms into an entirely new kingdom. For this reason Fungi are perhaps best described as plant like micro-organisms that do not possess chlorophyll.

Fungi may be simple in structure, as are the yeasts which consist of a single cell or chains of cells that reproduce by "budding" and which generally give rise to rather slimy, pink, pale brown or cream coloured colonies. Typical moulds on the other hand, are more complex, multicellular

fungi, of variable appearance and colour. Most reproduce by means of microscopic asexual spores which vary considerably in size and shape and are formed either on stalks or in special vesicles which grow from, or are embedded in, the vegetative part of the fungus (mycelium). This consists of many strands (hyphae) which typically grow together to form a 'mycelial mat'. It is this mycelium that is largely responsible for the formation of exzymes, fungal toxins, etc. Many fungi also have a sexual stage in their life cycle. (Note: Except in certain very high moisture content situations yeasts are relatively unimportant and this supplement refers mainly to the mould fungi).

Whereas fungi that attack growing plants are fairly restricted in number and are true pathogens, fungi in stored products are much more numerous and are saprophytic, or only weakly pathogenic by nature. This also applies to the 'field fungi'. The A/ternaria and Cladosporium groups are common examples causing a blackening or weathering effect on unharvested, mature crops of maize, sorghum and other grains. The Fusarium group is also important at this stage. Once in store the field fungi tend to decline, their place being taken by such typical storage groups as Aspergillus and Penicillium. These and other storage moulds often fall within the 'fungi imperfect)', so called because they reproduce only by asexual means. For descriptive details of the fungi and other micro-organisms that cause damage in stored produce please see T.S.T.S. OLL.

In common with other living things, when fungi grow they breathe (a process known as respiration) using oxygen from the air which reacts with the substrate on which they are growing to produce carbon dioxide, moisture and heat. By-products of the normal growth process are

often of a complex chemical nature and may be beneficial but are sometimes undesirable.

2. Where do fungi occur?

Fungi are found everywhere. Spores occur in dust and are therefore present in the air we breathe. All stored commodities are contaminated with fungal spores and these, unlike insects, cannot be excluded by careful handling and treatment. Even processed foods that been subjected to high temperatures during their preparation (e.g. oil-seed cake) though perhaps significantly free of living moulds immediately following processing, are rapidly recontaminated during packaging and storage. It is, therefore, most important to prevent fungal spores from germinating and growing into visible colonies.

3. When do fungi grow?

Fungal spores require moisture to germinate and if this is excluded (i.e. if grain is stored at its safe moisture content or below) moulds generally will not grow. The maximum safe storage moisture content may be defined as the amount of absorbed water held within a commodity which is in equilibrium with an atmospheric relative humidity of 70%. Moisture content is closely bound to temperature. Under certain circumstances, temperature differences can cause the re-distribution of moisture leading to local mould growth. Other factors that affect fungal development and the production of spores are availability of oxygen, light, acidity, and salt/sugar content.

4. Why is it important to prevent fungal growth?

Fungi do a tremendous amount of damage by causing:

1. **Direct loss when the grain is too mouldy to eat.**
2. **Caking in grain and flour, rendering the material difficult to handle.**
3. **Changes in colour, texture and flavour rendering the produce unacceptable by:**

    i. **fermentation (carbohydrates converted to acids and gas)**
    ii. **putrifaction (protein breakdown), and**
    iii. **rancidity (fats converted to acids).**

    The latter is especially important in oilseeds (e.g. groundnuts, oil palm kernels) and oily products (rice-bran, copra etc) where the enzymatic conversion of oil to free fatty acid results in uneconomic processing or in financial penalties on world markets.

4. **Charring or spontaneous combustion if mould growth is unchecked as this releases heat and grain is a poor conductor of heat. Quite dramatic temperature rises can occur especially within large bulks or stacks. Normally the temperature rise Is restricted to a maximum level of about 60C at which point moulds are generally inhibited. However, in oilseeds, enzymatic heating can continue even leading eventually to spontaneous combustion.**
5. **Reduction in germination capacity of grain.**
6. **Poisoning in man and animals due to the production of mycotoxins (poisonous fungal breakdown products) by certain species. Aflatoxin, produced by the common storage fungus Aspergillus flavus is known, for example, to cause liver collapse in certain domestic animals.**

7. **Lung diseases such as asthma and skin allergies if spores are present in the atmosphere in very high concentrations (e.g. handling mouldy straw or prolonged exposure when emptying underground pits).**
8. **Deterioration and weakening of fibres used for packaging or protecting grain (e.g. jute sacks, tarpaulins). This can lead to spillage or water entry. Container sealing materials such as stitching and adhesives are also liable to mould attack.**
9. **Deterioration of store fabrics, especially wood, causing rotting and disintegration.**

5. In what practical situations can fungal damage occur?

Fungi can develop if conditions favour their growth at any of the following times:

1. **In the field, prior to harvest, when the crop is maturing.**
2. **After harvest during the drying period.**
3. **In store. This can occur for two unrelated reasons and it is important to distinguish between them:**

    a. **due to the premature storage of inadequately dried material, and**
    b. **due to the re-absorption of water while in store (especially during the wet season), as follows:**

        i. **exposure to high relative humidity,**
        ii. **through a leaking store roof, cover or container,**

iii. **water uptake through the floor or walls of a store,**
iv. **development of temperature gradients in grain leading to condensation.**

4. **In transit.**
5. **During processing.**

6. Hints on preventing fungal damage

1. **Harvest the crop at maturity. Grain harvested prematurely takes longer to dry and is therefore more susceptible to mould damage. It is also likely to shrivel. The actively growing crop has a natural resistance to invasion by storage fungi (e.g. Aspergillus and Penicillium spp.) but this is to some extent lost at maturity or if the grains are damaged by rodents, insects, etc.**
2. **Dry the produce as quickly as possible bearing in mind the need for a 'curing' period in some crops such as groundnuts. Remember that hot sunlight is not always the best. A method that protects the commodity from rain, dew and damp soil but allows dry air to pass freely over the produce is ideal. In certain extreme climates where the air is continuously humid artificial drying may be essential.**
3. **Avoid physical damage to the produce at all stages of handling. Harvesting and shelling are two occasions when damage is likely to occur. Broken groundnut pods, for example, are more easily invaded by moulds than undamaged pods. The grain skin is also resistant to fungal invasion and should be kept intact if possible.**
4. **Check the moisture content of the commodity before it is stored. Make sure that it is quite**

dry first. if there is any doubt, or if drying has proved to be a problem, do not store the produce in solid walled containers. 'Pigeon hole' stacking can be used for bagged produce suspected of being damp as it allows air to circulate through the stack.

5. **Avoid storing warm grain as the heat will be retained and encourage rapid mould and insect multiplication. Also, if the store structure is cool, condensation may occur where the warm grain touches the cool surfaces.**
6. **Ensure that all stores, silos, etc. are in good repair before use.**
7. **When storing bagged produce, keep it well away from the walls of the store and use dunnage to raise the sacks away from the floor.**
8. **Allow newly constructed concrete stores or floors dry out throughly before use. If possible build a water vapour barrier (e.g. polyethylene sheet, bitumenastic layer) into the floor and walls during construction.**
9. **Allow for controlled ventilation around the produce within a store so that air of high relative humidity can be excluded during the wet season. Ventilate only if the internal air is moist and the outside air is dry, or if the produce needs cooling.**
10. **Ideally, produce and store should be maintained at an even temperature.**
11. **If produce is thoroughly dry, polyethyelene bags or sheets can be used to exclude moisture during storage. However, care must be taken to avoid exposure to direct sunlight or condensation will occur beneath the plastic surface. This is also likely to occur in a store where the internal temperature fluctuates excessively. To prevent condensation under these circumstances the top and sides of the stack should be insulated with a layer (several if possible) of sacks or similar material, placed outside the plastic sheet.**

12. **If practical, cover all metal silos to prevent direct sunlight from falling on the walls. If this is not possible a coat of white paint will help to reflect the heat and keep the produce cool.**
13. **Apply adequate pest control measures to prevent insect 'hot spots' from developing.**
14. **Do not load or unload grain in the open if it is raining and avoid placing sacks of produce on wet ground.**
15. **Ensure that all railway wagons, lorries, small boats and other mobile containers are adequately covered and in good repair, especially if movement during the rainy season is likely.**
16. **It is almost impossible to avoid some fungal damage in traditional underground pit stores. However, any form of lining which prevents grain from coming into direct contact with the soil will help. Also, pits should be completely filled; a little grain in a large pit will usually become very mouldy.**

7. The chemical control of storage fungi.

Chemicals for the prevention of mould growth in grain stored for human consumption are not at present available. The very broad spectrum of moulds found in stored products is one problem and toxicity to man another. However, propionic and other organic acids have been successfully used in temperate countries to protect highs moisture content barley and other grains destined for animal feed. While it may be possible in the future to use propionic acid in hot climates where drying is a problem, considerable research under tropical conditions is needed first. Taint and smell are two aspects requiring investigation if propionic acid is to be applied to grain for human consumption. Furthermore, the application rate is critical and complete coverage of the grain is

necessary before mould growth commences if adequate control is to be achieved.

8. What should be done with mouldy foodstuffs?

This is a vexed question as no-one wishes to waste food. Indeed, some foods like 'blue' cheese are purposely infected with fungi. However, since the discovery over the last decade that a wide range of storage fungi can produce substances that cause disease when fed to animals there has been considerable speculation as to whether man is at risk through eating badly stored foodstuffs which have been accidentally contaminated with fungi. There is strong circumstantial evidence to support such a suggestion, and fungal invaded grain, pulses and especially oilseeds should therefore be avoided if at all possible. It is worth noting here that cleaning grain to remove surface fungal growth (this is, for example, practised in some rural communities with mouldy pit-stored grain), is unlikely to remove any toxin present within the grain and therefore is not recommended. Similarly, cooking does not necessarily destroy mould toxins and aflatoxin is a good example of this. Careful sorting to remove visibly damaged grain is therefore recommended, and in certain commodities, especially oilseeds such as groundnuts, the seeds should be cut open and examined for hidden fungal growth within the grain.

The old maxim that mouldy grain can be safely fed to animals therefore no longer applies. If mouldy grain is used for animal feed it may cause death and at best is likely to give poor results (e.g. a reduction in expected weight increase). If used it should be considerably diluted with fungus free material. Extreme care is needed with oilseed cake in particular, as this is very readily contaminated with aflatoxin, a substance that is toxic in very small quantities.

Appearance is not a good criterion when judging oilseed cake, for although aflatoxin may have been present in the original seeds, visible mould damage will not be seen in the finished produce unless re-wetting has occurred.

AFLATOXIN ANALYSIS: OVERVIEW

Aflatoxin is one of the most well-known mycotoxin in tropical and sub-tropical areas. Crop in these regions or more subject to contamination than those in temperate regions, since optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. Toxin-producing fungi can infect growing crops. As a consequence of insect or other damage, and may produce toxins prior to harvest, or during harvesting and storage.

The ingestion of food containing aflatoxin may have serious adverse health effects in man. Aflatoxins are demonstrated liver toxin and liver carcinogens in some animals including non-human primates. Dose response relationships have been established in studies on rat and rainbow trout, with 10/tumour incidence estimated to occour at feed level of AF B1 of 1 /kg and 0.1 /kg respectively. In some studies, carcinoma of the colon and kidney have been observed in rats treated with aflatoxin. AF 3(1) causes chromosomal aberrations and DNA breakage in plant and animal cells after microsomal activation, gene mulation in several bacterial test systems. In high doses, it may be teratogenic.

The acute toxicity and carcinogenicity of aflatoxin are greater in male than in female rats;

hormonal involvement may be responsible for this sex-linked difference. Nutritional status in animals, particularly with respect to lipotropes, proteins, vitamin A, and lipids (including cyclopropenoid fatty acid) can modify the expression of acute toxicity or carcinogenicity or both.

Liver cancer is more common in some regions of Africa and Southeast Asia than other parts of the world when local epidemiological information is considered together with experimental animal data. It appears that increased exposure to aflatoxins may increase the risk of primary liver cancer.

In view of the evidence concerning the effects, particularly the carcinogenic effects of aflatoxin in several animal species, and in view of the association between aflatoxin exposure levels and human liver cancer, incidence observed in some parts of the world, exposure to anatoxins should be kept as low as practically achievable. The tolerance levels for food products established in several countries should be understood as management tools, intended to facilitate the implementation of aflatoxin control programmes, and not as exposure limits that necessarily ensure health protection.

Aflatoxins are now recognized to be involed in the aetiology of certain human and animal diseases. An awareness of the level of contamination of Aflatoxin in natural products can only be obtained by developing good analytical methodologies for detecting aflatoxin in foods, mixed feeds and Ingredients, animal tissue, blood, urine and milk.

Aflatoxin detection methods can be divided into three categories

1. **rapid presumptive tests to identify samples from agriculture products such as corn, peanut lots that may contain toxin,**
2. **rapid screening procedures to determine the presence or absence of toxin,**
3. **quantitative methods to determine aflatoxin levels.**

The presumptive test for aflatoxin in corn in the black light test or Bright Greenish-Yellow fluorescent test (BOY) based on the fluorescence under ultraviolet light (365 nm.) associated with Aspergillus flavus and A. parasiticus.

Rapid screening tests have included mini column methods that can be done in a laboratory with minimal facilities, and thin layer chromatography (TLC).

Quantitative methods to determine aflatoxin levels involves extraction, purification of extract, and measurement of the toxin by TLC using visual comparisons with a standard or densitometry or high pressure liquid chromatogaphy.

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Prevention and control of mycotoxins

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Maitree Suttajit, Ph.D

INTRODUCTION

Serveral mycotoxins in agricultural products cause health hazards to people and animals and economical problem. Dangerous mycotoxins are naturally present in foods, feeds and our environment. They are pathologically classified as hepatotoxins, nephrotoxins, vomitoxin and neuro-musculotoxin, some of which are potentially carcinogenic and mutagenic (Table 1). Aflatoxin, for example, is the most potent hepatocarcinogen and mutagen among mycotoxins. Therefore, the contamination of mycotoxins should be minimized by designing a series of measures of prevention and control.

STRATEGIES FOR PREVENTION AND CONTROL OF MYCOTOXINS

To design strategies for the reduction or elimination of mycotoxins, knowledge about their fungal sources are needed. The growth of fungi in crops and agricultural products is the main cause of toxin formation and related to the concentration of the toxic substances. Many factors are involved in enhancing the formation of mycotoxins. They are plant susceptibility to fungi

infestation, suitability of fungal substrate, temperate climate, moisture content and physical damage of seeds due to insects and pests.

Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest grains, toxinogenic fungi can be divided into three groups: (a) field fungi; (b) storage fungi; and (c) advanced deterioration fungi. The first category includes species of plant pathogenic fungi, namely, genus Fusarium, e.g. F. moniliforme, F roseus, F. tricinctum and F. nivale. The "storage fungi" are principally the general Aspergillius and Penicillium, e.g. A. flavus and A. parasiticus. The "advanced deterioration fungi" normally do not infest intact grains but easily attack damaged ones and require high moisure content. The examples of the third group are A. clavatus, A. fumigatus, Chaetomium, Scopulariopsis, Rhizopus, Mucor, and Absidia.

The prevention of mycotoxins in our environment is a big task. In general, prevention of the contamination of fungi and their mycotoxins in agricultural commodities can be divided into these following three levels.

1. Primary prevention

The step of prevention should be initially carried out before the fungal infestation and mycotoxin contamination. This level of prevention is the most important and effective plan for reducing fungal growth and mycotoxin production. Several practices have been recommended to keep the conditions unfavorable for any fungal growth. These include:

- **development of fungal resistant varieties of growing plants;**
- **control field infection by fungi of planting crops;**
- **making schedule for suitable pre-harvest, harvest and post-harvest;**
- **lowering moisture content of plant seeds, after post harvesting and during storage;**
- **Store commodities at low temperature whenever possible;**
- **Using fungicides and preservatives against fungal growth;**
- **Control insect infestation in stored bulk grains with approved insecticides.**

Table 1: Some mycotoxins, their sources and potential toxicities (1).

Toxins	Producing fungi	Toxicities
Aflatoxin	*Aspergillus flavus*	Hepatocarcinogen
	Aspergillus parasiticus	and fatty liver
Citreoviridin	*Penicillium viridicatum*	Cardiac beri-beri
Citrinin	*Penicillium vindicatum*	Nephrotoxin
	Penicillium citrinum	
Cyclochlorotine	*Penicillium islandicum*	Hepatotoxin

Cytochalasin E	*Aspergillus clavatus*	Cytotoxicity
Maltoryzine	*Aspergillus oryzae*	
Ochratoxins	*Aspergillus ochraceus*	Hepatotoxin
Patulin	*Penicilliumc-expansum*	Brain & lung hemmorrhage
	Penicillium patulum	and carcinogenicity
PR Toxin	*Penicillium requeforti*	
Rubratoxin	*Penicillium rubrum*	Liver hemmorrhage and fatty infiltration
Rugulosin	*Penicillium islandicum*	Nephrosis & liver damage
Sterigmatocystin	*Aspergillus flavus*	Hepatocarcinogen
	Aspergillus versicolor	
Tremorgens	*Penicillium and Aspergillus*	
Trichothecenes	*Fusarium graminearum*	Cytotoxicity
Vomitoxin (Deoxynivalenol)	*Fusarium graminearum*	Vomiting

Zearalenone	*Fusarium*	Hyper-estrogenic effect

2. Secondary prevention

If the invasion of some fungi begins in commodities at early phase, this level of prevention will then be required. The existing toxigenic-fungi should be eliminated or its growth to be stopped to prevent further deterioration and mycotoxin contamination. Several measures are suggested as follows:

- **Stop growth of infested fungi by re-drying the products;**
- **Removal of contaminated seeds;**
- **Inactivation or detoxification of mycotoxins contaminated;**
- **Protect stored products from any conditions which favour continuing fungal growth.**

3. Tertiary prevention

Once the products are heavily infested by toxic fungi, the primary and secondary preventions would not be then feasible. Any action would not be as effective as the practices mentioned above, since it will be quite late to completely stop toxic fungi and reduce their toxin formation. However, some measures should be done to prevent the transfer of fungi and their health hazardous toxins highly contaminated in products into our daily foods and environment. For example, peanut oil extracted from poor-graded peanut seeds always contains very high levels of aflatoxins and the oil-soluble toxin has to be eliminated by absorption and alkalinization during oilrefining process. Only a few practices are recommended:

- **Complete destruction of the contaminated products;**
- **Detoxification or destruction of mycotoxins to the minimal level.**

Since aflatoxin is the most well-known mycotoxin ever throughly studied and its prevention and control has been most successfully practiced in various countries, therefore, this paper will focus on such practices in certain detail for the prevention and control of aflatoxins mycotoxin contamination. Successful development will bring a great impact for the increased production of crops and safe and nutritious foods around the world. A number of researchers have been working on A. flavus-resistant or tolerant varieties of corn (2-3) and peanut (4-6)

It has been clear that the fungal-resistance of each variety is genotypic. However, the resistance to invasion of A. flavus has been attributed to several biochemical, environmental and physical factors. Uncontrollable factors could bring the failure in the utilization of selected fungal-resistant variety, as shown by laboratory screening, in the field.

Davis and his co-workers (7) reported the survey and comparison of aflatoxin contamination in upto 215 corn hybrids grown in Alabama, USA during 1976-81. Unfortunately, they could not find any hybrid tested resistant to aflatoxin formation. They were convinced that significant aflatoxin levels generally accompanied stress caused by high temperature, low rainfall, low moisture-holding capacity of sandy soils and insect infestation.

A differential pathogenic capacity of various toxigenic strains of A. flavus have been observed (8). Some strains would require physical damage for their infestation and others would not. The

association of mycotoxin production and physical damage to grain and drought during grain ripening indicates that Aspergillus spp. are weak pathogens. During long grain storage, the biochemical activity of grain is much reduced, while invasion of storage fungi and mycotoxin contamination would increase. More data is needed on the biochemistry and pathogenesis of toxigenic fungi to understand and evaluate their genotype.

The germination and viability of maize seeds could be affected by attack of Aspergillus and Penicillium species and their fungal infestation have been found to be different among maize genotypes (9-10).

Similarly, genotypes of peanut and biochemical properties of its seed such as tannin content (11), thin pericarp (12), small amount of cuticular wax (13) and chemical composition of the pericarps and embryos (14) have been shown to inhibit fungal invasion by A. flavus and aflatoxin formation.

Recently, antifungal enzymes, chitinase (15) and B-1, 3-glucanase (16), found in a number of plant seeds, may act as defense against pathogenic fungi, since chitin and glucan are major polymeric components of many fungal cell walls. Such polysaccharides in fungal cell wall could be enzymically hydrolysed into smaller products resulting the damage or killing of fungal mycelia or spores. The role of these enzymes for genotype evaluation is now being studied. It is foreseen that seeds rich in such antifungal enzymes likely resist the infestation of fungi. If so, the seeds for breeding would be easily screened out and used a stock one.

Even there are many technical problems in searching for the "super" plant against pathogenicity,

the development of fungal-resistant plant varieties utilizing genetic resistance to mycotoxin contamination is still possible and encouraged.

FUNGAL GROWTH INHIBITION

How to prevent growth and invasion of pathogenic fungi in agricultural commodities is very important in preventing mycotoxin contamination. The inhibition of fungal growth can be achieved by physical, chemical and biological treatments (17).

- **Physical treatment. After the crops have been harvested, drying and proper storage and suitable transportation of the commodities are of prime importance. Several flavourable factors contribute to the growth of fungi and aflatoxin production, namely high moisture content, humid climate, warm temperature (2540C), insect infestation and pes damage. Many means and measures to prevention of fungal contamination have been emphasized and practically done.**
- **Drying seeds and commodities to the safe moisture levels (<9% for peanut kernel, and < 13.5% for corn) (17).**
- **maintenance of the container or warehouse at low temperature and humidity.**
- **keep out insects and pests from the storage**
- **Gamma-irradiation of large-scale commodities (18).**
- **Chemical treatment with synthetic fungicides**

- **organic acids: acetic acid (19), propionic acid and butyric acid (20), malonic acid (21), benzoic acid (22, 23), sorbic acid (24), lactic acid (25), citric acid (25) and their sodium salts**
- **sodium chloride (26)**
- **Benzoic acid derivatives (27): Onitrobenzoate, O-aminobenzoate, paminobenzoate, benzocain (ethly aminobenzoate), ethyl benzoate, methyl benzoate and aspirin (O-acetoxy benzoic acid)**
- **potassium sulfite and potassium fluoride (27)**
- **dichorvos (28)**
- **fumigant: ammonia and phosphine (29).**
- **treatment with natural products from plants or herbs.**
- **allicin and related substances from garlic and onion extracts (30)**
- **chitosan or derivative of chitin isolated from crustacean shells (31)**
- **cinnamon extract: trans-cinnamic acid, trans-cinnamaldehyde, and ferulic acid (phydroxy-3-methyl cinnamic acid) (32)**
- **clove oil (32)**
- **other herbs: thyme, star anise seeds (33), black and white peper (34). plumbago indica (35).**

DECONTAMINATION OF MYCOTOXINS

Contaminated mycotoxins in foods and feeds should be removed, inactivated or detoxified by physical, chemical and biological means depending on the conditions. However, the treatment has

its own limitations, since the treated products should be healthsafe from the chemicals used and their essential nutritive value should not be deteriorated. The following methods are suggested to be applied for effective decontamination of some mycotoxins.

Physically, fungi-contaminated seeds can be removed by hand picking or photoelectric detecting machines. The method would consume time and Iabor or expensive.

Organic solvents (chloroform, acetone, hexane and methanol) have been used to extract aflatoxins for agricultural products, but mainly in vegetable oil refining process (36).

Heating and cooking under pressure can destroy nearly 70% of aflatoxin in rice compared to under atmospheric pressure only 50% destroyed (37). Dry and oil roastings can reduce about 50-70% of aflatoxin B1 (38). We could show that only about 10% of total 1242 ppb of aflatoxin B. decreased in naturally contaminated peanut by heating at upto 100C (39). Since aflatoxin resists to higher temperature upto 260C, long-time cooking and overheating would destruct essential vitamins and amino acids in treated foods.

Ionizing radiation such as gamma-rays can stop growth of food spoilage organisms, including bacteria, molds and yeasts. It also inactivates pathogenic organisms including parasitic worms and insect pests. It has been reported that gammairradiation (5-10 M-rad) caused reduction of aflatoxin (40). The irradiation, however, could not completely destroy the toxin and its mutagenicity. In our laboratory, only about 30% of total 600 ppb at aflatoxin B1, either pure toxin or in contaminated peanut, was destroyed by 1 and 5 Mrad or gamma irradiation (23). The

treatment combination of gamma irradiation and ammoniation should be therefore attempted for more aflatoxin decontamination.

Chemical treatment has been used as the most effective means for the removal of mycotoxins from contaminated commodities. The method should be sure that the detoxification system is capable of converting the toxin to a nontoxic derivative (s) without deleterious change in the raw product. Mutagenicity of the treated products should be assessed. The toxicity may be checked by feeding animals including bouts, egg embryos, chicken, ducklings and rats. Many common chemicals have been brought to test the effectiveness in detoxification of aflatoxin. These chemicals include the followings:

- **acetic acid (C2H5OH) (41)**
- **ammonia gas (NH3) or NH4OH (42,49) or ammonium salts, 3-5% (42)**
- **calcium hydroxide (Ca(OH)2) (43)**
- **formaldehyde (43, 47)**
- **hydrogen peroxide (H2O2 (44)**
- **methylamine (CH3-NH2) (45)**
- **ozone gas (03) (46)**
- **phosphoric acid (H3PO4) (47)**
- **phosphine gas (PH3), very highly toxic!**
- **sodium bicarbonate (NaHCO3) (48)**
- **sodium bisulfite (NaHSO3) (49)**
- **sodium bisulfite (NaOH) (48,49)**

- **sodium hypochlorite (NaOCI) (50)**

The chemical reactions of detoxification of aflatoxin are primary addition of the double bond of the furan ring and oxidation involving phenol formation and opening of the lactone ring. In the presence of acid, aflatoxins B. and G. will be converted into their 2-hydroxy derivatives, aflatoxins B2a respectively.

Figure 1 Proposed formation of aflatoxin-related reaction products following exposure to ammonia

Figure 2 Comparison detoxification efficiency of 3% ammonium bicarbonate and 3% ammonium carbonate at different length

Figure 3 Comparison of reduction of AFB1 content in different treatment periods and their mutagenicities

Figure 4 Effect of gamma irradiation with and without ammonium blbdrbondte (3%) on AFB1 in peanut.

Other mycotoxins which are like aflatoxin and have a lactone grouping in the molecule can be similarly destroyed by alkaline condition using ammonia, sodium hydroxide and sodium bicarbonate. These toxins are patulin, penicillin acid, citreoviridin, citrinin, cyclochlorotin, ochratoxin A, rubratoxin, trichothecenes and zearalenone.

Certain conditions such as moisture content, heat, ultraviolet or gamma irradiation, sunlight and pressure at different treatment-periods have been simultaneously combined with the chemicals for the enhancement of detoxification.

Inactivation methods can be achieved by mixing, packing, fumigation and immersion with the chemical used.

CONTROL OF MYCOTOXINS

Careful control of mycotoxins should be started and administered by the government of each country through ministries and organizations such as the Ministry of Health, the Ministry of Agriculture, Food and Drug Administration, National Environment Committee Board and Consumer Protection Committee Board. The control program may be set up by a special administrative committee and the legislative body who regulate the national policy of food safety and the maximum tolerance limits for mycotoxins. Farmers, middlemen, food and feed factories and exporters will be well educated about mycotoxins and encouraged to prevent and control the contamination of microflora and their health-hazardous mycotoxins in their commodities as much as possible.

International cooperation for the mycotoxin regulation in trading products or commodities is also needed. The countries should establish quality control limits for certain commodities intended for export or import. The producer countries would be stimulated to be aware of mycotoxin

contamination in their exported susceptible commodities. For example, the European Economic Community (EEC) has already established certain maximum tolerance limits of aflatoxins for animal feeds, i.e. not more than 20 ppb for complete feed for gigs, poultry and feed supplements for dairy animal; 50 ppb for produce to be processed into mixed feed and complete feed for cattle, sheep and goats.

International organizations such as FAO, WHO and UNEP in the UN system are engaged in providing essential information on various aspects of prevention and control of mycotoxin problems to all the countries. Guidelines for international trade include: a) procedure of sampling and analysis, b) surveillance and food control inspection systems, c) use of contaminated produce in feeding of different animals, d) protocols for detoxification and the quality control of the products. Conferences, symposiums, trainings and workshops on current informations of mycotoxins should be promoted. Low-cost technology for assessment, prevention and control of environmental mycotoxins could be then transferred from developed countries to developing ones.

CONCLUSION

Several effective ways for prevention and control hazardous fungi and their dangerous mycotoxins have been presented. The methods include biological control and physical and chemical treatments. Selection of fungal resistant hybrids of crops are recommended and further

experimented. Pre-harvesting preparation of the field and environments should be aware of. Drying of commodities after post harvest is the most economical and effective means for farmers or layment, but sometimes is not suitable during rainy season or wet condition. Thermal treatment or gamma irradiation is not effective or practically used by villagers. Chemical treatments such as alkalinization and ammoniation are well-recognized and industrially used. Some modifications of the application of effective chemicals to the detoxification of mycotoxins should be developed. International cooperations through authorized organizations should be promoted and supported aiming the benefits for the economics and health of people of all the nations.

REFERENCES

1. **Food Agriculture Organization of the United nations, Recommended Practices for the Prevention of Mycotoxins, Rome, 1979, 53-55**
2. **Widstrom, N.W., Wilson, D.M., - McMillan, W.W., Ear resistance of maize inbreds to field aflatoxin contamination, Crop-Sci., 24, 1984, 11545-1157.**
3. **Zuber, M.S., Clavert, O.H., Kwolek, W.F., Lillehoj, E.B> & Kang, M.S. Aflatoxin production in an eight-line dialler of Zea mays infected with Aspergillus flavus, Phytopathology 68, 1978, 1346-1349.**
4. **Mixon, A.C. and Rogers, K.M., Peanut accessations resistant to seed infection by Aspergillus, Agron J. 65, 1973, 560-562**

5. **Mixon, A.G, Reducing aflatoxin contamination in peanut genotypes by selection and breeding, J. Am. Oil. Chem. Soc., 58, 1981, 961A-966A.**
6. **Davidson, J.l., Hill, R.A., Cole, R.J., Mixon, A.GC. and Hennings, R.J., Peanut Science, 10, 1983, 43-47.**
7. **Davis N.D., Currier C.G., and Diener U. L., Response of corn hybrids to aflatoxin formation by Aspergillus flavus, Alabama Agricultural EXPERIMENT STATION, E.V.. Smith Research Center, Shorter, Auburn, Alabama, 3-22, 1984**
8. **Jones, R.K., Duncan, H.E., & Hamilton, P.B., Planting date, harvest date, and irrigation effects on infection and aflatoxin production by Aspergillus flavus in field corn, Phytopathology, 71, 1981, 810-816.**
9. **Sanders, T.H., Hill, R.A,. Cole, R.J. & Blankenship, P.D., Effect of drought on occurrence to Aspergillus flavus in maturing peanuts, J. Am. Oil. Chem. Soc., 58, 1981, 966A969A.**
10. **Sauer, D.B. & Christensen, GM., Germination percentage, storage fungi isolated from, and fat acidity values of export corn, Phytopathology, 58, 1968, 1356-1359.**
11. **Mixon, A.C., Differences among lines and varieties of corn in susceptibility to damage from invasion by storage fungi, Phytopathhology, 61, 1971, 1498-1500.**
12. **Rao, K.S, & Tupule, P.G., Varietal differences of groundnut in the production of aflatoxins, Nature, 214, 1967, 738-739**
13. **La Prade, J.C., Bartz, J.A., Norden, A.J. & Demunyk, T.J., Correlation of peanut seed coat wax accumulation with tolerance to colonizaion by Aspergillys flavus, Proc. Am. Peanut Res. Educ. Assn. 5, 1973, 89-94.**
14. **Lindsey, D.L. & Turner, R.B., Inhibition of growth of Aspergillus flavus and Trichoderma**

viride by peanut embryos, Mycopathologia, 55, 1975, 149-152.
15. Roberts, W.K. & Selitrennikoff, C.P., Isolation and partial charcterization to two antifungal proteins from barley, Biochimica et Biophysica Acta, 880, 1986, 161-170.
16. Nelson, T.E., Johnson J., Jantzen E. & Kirkwood, S., Action pattern and specificity of an exo-B- > (1 --3) D-g l ucanase f rom Basidiomycetes species QM 806, J. Biol. Chem., 244, 1969, 59725980.
17. Food and Agriculture Organization of the United Nations, Recommended practice for prevention of mycotoxins in food, feed and their products, Rome, 1979, 4-36.
18. World Health Organization, Food irradiation: A technique for preserving and improving the safety of food, Geneva, 1988.
19. Buchanan, R.L. & Ayres, J.G, Effect of sodium acetate on growth and aflatoxin producition by Aspergillus parasiticus NRRL 2999, J. Food Sci., 1979, 128-132.
20. Gosh, J. & Haggblom, P., Effect of sublethal concentration of propionic or butyric acid on growth and aflatoxin production by Aspergillus flavus, Interm. J. Food Microbylol, 2, 1985, 323330.
21. Megalla, S.E., and Hafez, A.H., Detoxification of aflatoxin B1 by acidogenous yoghurt, Mycopathologia, 77, 1982, 89-91.
22. Uraih, N. & Offonre, S., Inhibition of aflatoxin production in groundnut with benzoic acid derivatives and possible toxic efffect of their aromatic residues, Microbios,31, 1981, 93-102.
23. Chipley, J.R., and Uraih, N., Inhibition of Aspergillus growth and aflatoxin release by derivatives of benzoic acid. Appl. Environ. Microbiol. 40, 1980, 352-357.
24. Youssef, A.E. & Marth, E.H., Growth and synthesis of aflatoxin by Aspergillus parasiticus in

the presence of sorbic acid, J. Food Prot., 44, 1984, 736-741.
25. Reiss, J. Prevention of the formation of mycotoxins in whole wheat bread by citric acid and lactic acid, Experientia, 32, 1979, 168-169.
26. El-Gazzzar, F.E., Rusul, G. & Marth, E.H., Growth and aflatoxin production in meats. ll aged dry salamis and aged country cured hams, Appl. Microbiol., 18, 1969, 718-722.
27. Davis, N.D. and Diener, U.L., Inhibition aflatoxin synthesis by paminobenzoic acid, potassium sulfite and potassium fluoride, Appl. Microbiol. 15, 1967, 1517.
28. Yao, R.G & Hsieh, D.P.H., Step of dichlorvos inhibition in the pathway of aflatoxin biosynthesis, Appl. Microbiol., 28, 1967, 52.
29. Vandergraft, E.E., Hesseltine C.W. & Shotwell, O.L., Grain preservatives. Effect on aflatoxin and ochratoxin production, Cereal Chem. 52, 1975, 79=84.
30. Appleton, J.A. & Tansey, M.R., Inhibition of growth of zoopathologenic fungi by garlic extract, mycologia, 687, 1977, 882-885.
31. Cuero, R.G., Lillehoj, E.B., Cleveland, T.E., & Reine, A.H., Chitosan as a control agent of toxigenic fungal growth and aflatoxin production, proc. Jpn. Assoc. Mycotoxicol. (suppl.) 1, 1988, 194-198.
32. Bullerman, L.B., Lieu, F.Y. & Seiser, A.S., Inhibition of growth and aflatoxin production by cinnamon and clove oils, cinnamic aldehyde and eugenol, J. Food Sci., 42, 1977, 1107-1108.
33. Hitokoto, H., Morozumi, S. Wauke, T., Sakai, S. and Ueno, l, Inhibitory effects of condiments and herbal drugs on the growth and toxin production of toxigenic fungi, Mycopathologia, 66, 1978, 161165.
34. Madhyastha, M.S. & Bhat, R.V., Aspergillus parasiticus growth and aflatoxin production on

black and white pepper and the inhibitory action of their chemical constituents, Appl. Environ. Microbiol., 48, 1984, 376-379.

35. **Unpublished data.**
36. **Vorster, L.J., E'tudes sur la de' detoxification des arachides contaminees par l'aflatoxine et destinees a l'huilerie, Rev. Franc. Corps. Res., 13, 1985, 7.**
37. **Coomes, T.J., Crowther, P.C., Feuell, A.J. & Francis, B.J., Experimental detoxification of groundnut meals containing aglatoxin, Nature, 290, 1966, 406.**
38. **Feuell, A.J., Aflatoxin in groundnuts IX, Problems of detoxification, Trop. Sci., 8,1966, 61.**
39. **Songpan Wangjaisuk, Detoxification of aflatoxin B1 in peanut by ammonium bicarbonate and gamma irradiation, M. Sc. Thesis, 1989.**
40. **Sommer, N.F. & Fortlage, R.J., lonizing radiation for control of postharvest diseases of fruits and vegetables, Adv. Food. Res., 15, 1969, 147.**
41. **Pons, W.A. jr., Cucullu, A.F., Lee L.S., Janssen, H.J., & Goldblatt, L.A., Kinetic study of acid catalyzed conyersion of aflatoxins B1 and G1 to B2a and G2a, J. Am. Oil Chem, Soc.58, 1981, 995A-1002A.**
42. **Brekke, O.L., Sinnhuder, R.O., Peplinski, A.J., Wales, J.H., Putnam, G.B., Lee, D.J. & Ciegler, A., Aflatoxin in corn, Ammonia inactivation and bioassay with rainbow trout, Appl. Environ. Microbiol. 34, 1977, 34-37.**
43. **Codifier, L.P., Mann, G.E. & Dollear, F.G., Aflatoxin inactivation. Tratment of peanut meal with. formaldehyde and calcium hydroxide, J. Am. Oil Chem. Soc., 53, 1976, 204206. '**
44. **Spreenivasamurthy, V., Parpia, H.A.B., Srikanta, S7 and Shankarmurti, A., Detoxifiation of aflatoxin in peanut meal by hydrogen peroxide, J. Assoc. Off. Aal. Chem. 50, 1967, 350.**

45. **Park, D.L., Jemmali, M., Frayssinet C., Frayssinet L. & Vvon, M., Decontamination of Aflatoxincontaminated peanut meal, using the monomethylamine:Ca (OH) 2 method, Proc. Int. Symp. Mycotoxins, Sept. 6-8th, 1981 Cairo, Egypt, The General Organization for Govt. Cairo, 1983, 257-266.**
46. **Dwaratanath, CT., Rayner, E.T., Mann, G.E. & Dollear, F.G., Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization, J. Am. 0il Chem. Soc., 45, 1968, 93.**
47. **Mann, G.E., Codifier, L.P.Jr., Gardner, H.K. Jr., Kolton. S.P. & Dollear, F.G., Chemical inactivation of aflatoxins in peanut and cottonseed meals, J. Am. oil. Chem. soc. 47, 1970, 173.**
48. **Mashaly, El-Deeb, Ismail. A.A. & Youssef, A., Effect of some chemical treatments on detoxification of aflatoxins in cottonseed meal, proc. Int. Symp. Mycotoxins, Proc. Int. Symp. Mycotoxins, Sept. 6-8th, 1981, Cairo, Egypt, The General Organization for Govt, 1983, 515-522.**
49. **Moerch, K.E., McElfresh, P., Wohlman, A., & Hilton, B., Aflatoxin destruction in corn using sodium bisulfite, sodium hydroxide and aqueous ammonia, J. Food Prot. 43, 1980, 571-574.**
50. **Yang, C.Y., Comparative studies on the detoxification of aflatoxins by sodium hypochlorite and commercial bleachers, Microbiol. 24, 1972, 885.**
51. **Cucuilu, A.F., Lee, L.S., Pons, W.A., Jr. & Stanley, J.B., Ammoniation of aflatoxin B1: Isolation and characterization of a product with molecular weight 206, J. Agric. food Chem., 24, 1976, 408-410.**
52. **Duttin, M.F. & Heatcote, J.G., Two new hydroxyaflatoxins, Biochem. J., 101, 1966, 21.**
53. **Muller, H.M., A survey of methods of decontaminating mycotoxins, II. Chemical mthods and**

reactions with components of feed stuffs, Animal Research and development, Vol. 19, 1984, 7-37.

54. Slein, M.W. & Sansome, E.B., Degradation of Chemical Carcinogens, An Annotated Bibliography, Van Nostrand, Reinhold Co., N.Y., 1980.

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Fumigation

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Chamlong Chettanachitara

INTRODUCTION TO FUMIGATION

Fumigation is a treatment measure which is intended to eliminate 100 percent of pests infesting grains and other commodities as well with toxic chemicals or fumigants. The fumigants are generally preferred because they form lethal concentration within enclosures and inside

commodities while other insecticides penetrate with difficulty or not at all. A pesticide that is effective as a fumigant, it must be volatile enough to produce a toxic concentration in a close space in a short period of time. So, fumigation is a method of insect control that can effectively eliminate pests from agricultural commodities providing, if follows established practices and procedure. A fumigation that is conducted improperly will not kill the pests. Such fumigations are meaningless from grain storage point of view end' they may be hazardous.

CONDITIONS OF FUMIGATIONS

There are two conditions of fumigations. One of them is vacuum fumigation which is usually done in very strong enclosures such as thick iron chambers or concrete rooms. Fumigations may be performed under different levels of vacuum according to kind of commodities and target pests. Living plants or perishable commodities cannot be fumigated under much vacuum conditions while grains and other dry produces are not effected. Another one is normal atmospheric pressure, NAP, fumigation. The NAP fumigations are performed in various temporary enclosures, for example a stack under tarpaulin, silo bin under values and vents closed, cargo container during the door locked, barge or ship holds under sealed conditions. Those NAP fumigations are popular among local dealers and exporters because they can be done commercially.

PRINCIPLES OF FUMIGATIONS

Basic knowledge that must be made understanding prior to start performing fumigations are as follows.

Sealed enclosures

During a fumigation, the enclosure must be completely sealed otherwise the fumigant used will leak causing the fumigation not to kill insects and may be harmful to any person. Stack fumigation under tarpaulin sheet should be performed on polished concrete floor. The tarp must be in good condition and thick enought to hold the fumigant. Sand snakes for anchoring the sheet to concrete floor should meet the standard of proper size which is usually 4 inches in diameter. The sand snakes must be over-lapping position on the tarp around the stack. For cargo container fumigation, the rubber sealing of its door must not be torned at any part and the rubber is not expired too. Barge fumigation, if the barge is all top opened, the barge is usually covered with tarp. The tarp should be sealed with very good masking tape, otherwise it longer not fixed to the barge hold edge. So far, other temporary enclosuires should have very good seal at all possible leaks or vents as mentioned before.

Dosage

Dosage is a given quantity of a fumigant to be applied. Methyl bromide may be applied at different dosages shuch as 2 Ibs (pounds) per one thousand cubic feet for common stored insects while optimum dosage for Kahpra beetle, Trogoderma granarium is 5 Ibs per one thousand cubic

feet. For phosphine gas, the amount of fumigant given by number of pellets, tablets, sarchets, plates and strips which each can release a definite quantity of phosphine. In general, one pellet can release PH3 0.2 gram while one tablet releases 1 gram. However, good fumigater should follow recommended dosage schedule so as to apply the proper amount of fumigant at a time.

Dosage Schedules

To be effective, the fumigant must be applied in sufficient quantity. The correct amount depends on published fumigation schedules that are applicable to the infested commodity, pest, temperature, and certain other considerations. Failure to follow the specifications outlined in the schedules will result in faulty fumigation treatments.

Concentration

Concentration is the amount of a fumigant under fumigation period. The concentration of the fumigant can be measured by the thermal conductivity or Fumiscope, Kitagawa precision concentration detector tube and Toka concentration meter. The concentration under fumigation may be-varied according to leak and sorption. Even lethal concentration throughout the enclosure is needed for good fumigation. If not, insects are not kill completely and may cause insect resistant to the fumigant in not very long future.

Fumigation period

Fumigation period means the exposure period plus degas or aeration period. When the exposure

period is ended the treated commodity should be left aerated for a certain time. Then the concentration inside the commodity will be detected to ensure that the fumigant left is not harmful to any person. If so, the fumigation period is over.

Sorption

Before a constant concentration of the fumigant can be maintained the sorptive capacity of the article under fumigation must be satisfied. It varied considerably with the article being treated. Sorption includes the terms adsorption, absorption and chemisorption.

Adsorption is concerned with the loss of fumigant due to adhesion of impingement of the gas molecules to the surface of the materials under treatment.

Absorption is concerned with loss of gas within the commodity due to capilary forces.

Chemisorption is a chemical reaction that takes place with a gas and commodity. The process is irreversible. When chemisorption occurs, residues are formed. These residues are considered harmful in edible products.

Desorption

Desorption is the result of absorbed and adsorbed gas molecules. Desorption time varies with the commodities and conditions, but it can be complete.

NAP FUMIGATION

Stack fumigation

Most of grains are stored short and long term by two methods They are bagged grain and bulk grain storage. In case of bagged grain, warehouse keepers are usually piling up them as stacks in the warehouse. Each stack may be as big as 10,000 tons. When insects are found infesting grain in the stack, fumigation must be performed to eliminate all of them.

Procedure and steps of stack fumigation are as follows:

1. **Measure the dimension of the stack and calculate the volume of it. The volume is width x length x height.**
2. **Cover the stack with tarpaulin sheet. If the stack is very big, more than one sheet are needed. Sheets are connected by special clamps by rolling each edge together and cellamping.**
3. **Anchor the tarpaulin sheet to the floor by sand snakes.**
4. **Applied a fumigant according to dosage schedule. For phosphine gas tablets or any form of aluminium phosphide or magnesium phoside are applied before cornering the stack with tarpaulin sheet.**
5. **If Methyl bromide, detect leak at any suspected point by halide detecter lamp or an**

electronic detecter device. Leak should be prevented or mended.

6. **Monitor the concentration of the fumigant.**
7. **When exposure period is ending, remove all sand snakes and then remove the tarpaulin sheet against wind direction.**
8. **May be one hour later, check the concentration of the fumigant inside the stack. To allow any person working, the concentration of the fumigant must be under harmful level or less than 5 ppm.**

Barge fumigation

Barges are commercially benefit transportation for all commodities because they are able to carry much cargo at a time. When necessary to conduct a fumigation in a barge, it can be done more or less the same as stack fumigation as follows:

1. **Load all bags in a barge up to maximum loading amount.**
2. **Form free space over the cargo by tarpaulin covering over metal arches. Seal tarpaulin sheet to the edge of barge opening with good quality masking tape.**
3. **Measure the dimension of the barge and calculate its volume.**
4. **Apply a fumigant according to dosage schedule.**
5. **For Methyl bromide, detect leak at every suspected point. If any, fix or mend it.**
6. **Monitor the concentration during fumigation period.**
7. **At the end of the fumigation, just remove masking tape and tarpaulin sheet.**
8. **Aerate the fumigant for one hour or more. For Methyl bromide, carefully detect the gas**

concentration at the bottom of the barge because Methyl bromide is 2.7 heavier than air.

Fumigation of bulk commodity in barge is quite different than the bagged one. A fumigant used must be proper because of its penetration power. Only phosphine gas can be used to eliminate all insects infecting. The method of application is simply conducted like this.

1. **Apply all pellets or tablets of Aluminium phosphine into bulk cargo by probing equipments.**
2. **Cover the cargo with tarpaulin sheat. Then seal the sheet to the edge of the bage opening with good quality masking tape.**
3. **At the end of fumigation, remove masking tape and tarpaulin sheet. Then let the cargo aerated for 1-2 furs.**

Silo bin Fumigation

Silo bin is a big bulk storage of grains. Because of the complications of the silo bin system, fumigaters must learn about valves and vents. All valves and vents must be completely sealed during fumigation. To use Phosphine gas as a fumigant, pellets or tablets of Aluminium phosphide must be applied during loading cargo into a bin or during transfering from one bin to another. Application of pellets or tablets can be done by both an automatic dispenser or manual dropping.

When Methyl bromide is required for fumigation in silo bin, it must be applied in a proper manner. It's very much necessary to run recirculation system of the bin to force Methyl bromide go through the bulk cargo and form even concentration. The recirculation machine should be

continuously operated for 1-3 hours.

FUMIGANTS

There are more than ten fumigants considered as modern and effective gases. In Thailand, two popular fumigants are Methyl bromide and phosphine because of their effectiveness and easy application. However, we should know the criteria for acceptable fumigants. Because the mortality of the pest is the purpose of fumigant, so there are other factors for researchers to consider when developing a fumigant, including:

1. **Degree of volatility to produce a toxic concentration of gas.**
2. **The fire and explosive hazard.**
3. **Residue.**
4. **Ease of detection.**
5. **Ease of desorption.**
6. **Compatibility with commodity.**

However, good fumigants should have the following qualities:

1. **Must by a substance or compound that is easily converted to gaseous state and maintained in that condition for the whole exposure period.**

2. **Must have a sufficiently low boiling point so it can easily be converted to gaseous state and remain in that state during fumigation.**
3. **Be insoluble in water, since those that are highly soluble in water are highly sorbed by treated commodities, and high sorption rates of a commodity are positively correlated with an excess amount of residue.**
4. **Must not leave an excess amount of toxic residue.**
5. **Must not cause external injury as burns, pitting or blackening.**
6. **Must not cause internal injury as discoloration of fruit or malfunction of plant systems.**
7. **Must not prevent uniform maturation and softening of fruits and vegetables or retard or excite growth of plant or affect seed germination.**
8. **Must not cause off flavor in fruits, vegetables or foodstuff.**
9. **Must not shorten shelf life of commodities.**

Methyl Bromide-CH3 Br

Methyl bromide has become very popular because many plant species, seed, vegetable and some fruit are tolerant to concentrations needed to kill pests. It has become adaptable for use in treatments in temporary enclosures after the development of thermal conductivity units which can be used in the field for remote sensing of gas concentrations. Advantages and disadvantages of Methyl bromide are as follows.

Advantages:

Quick and deep penetration
Effective against all stages of insects and mites
High plant tolerance
Effective over wide range of temperature
Non-flamable and non-explosive
Readily metected

Disadvantages:

Diffuses laterally and downward rapidly, but slowly upward
Odorless
Requires volatilizing
Unfavorable response to low humidity
High sulfur content articles develop off odors Seed germination effected
Fruits and vegetable effected
High mammalian toxicity
Delayed poison symtomology
Lacks antidote

Properties-CH3 Br:
Specific gravity 1.732 (liquid) 3.27 (gas)
Boiling point 3.6C
Vapor pressure 1420 mm. at 20C

1 mg/liter (1 oz/1000 ft) 252 ppm.
1 lb. = 262 cc.
Colorless

Phosphine-PH3

Another fumigant that has become promising for stored insects in Thailand is Phosphine gas which is the result of Aluminium phosphide or Magnesium phosphide and humidity in the atmostphere. Aluminium phosphide for pest control in stored products was first used by Dr. Werner Freyburg in the 30's. The Freyburg products are marketed under the trade name Detia and include Detia Gas-Ex-b, a preparation in paper bags consisting of 57 % aluminium phosphide and 43 % inert ingredients. After that Detia began producing a pellet and tablet preparation. Phosphine gas has become very popular among tobacco leaf pests control.

Chemical and Physical Properties:
Colorless gas
Does not enter into irreversible chemical reactions with commodities.
Reacts chemically with gold, silver and copper Is flammable and explosive in high concentration cat
26 g./cu.m.
Ordor is carbide or garlic

Boiling point 87.4C
Molecular weight 34.04
One ppm. = 14 mg/liter

NECESSARY EQUIPMENT

When fumigation will be performed, fumigater must prepare necessary materials and equipment such as fumigants, sheet, masking tape, leak detection device and safety devices.

Halide Detecter Lamp.

The halide detector lamp is a device that has been widely used over many years in the refrigeration industry for detection of freon leaks from refrigeration equipment. The halide detector can be used for rough determination of concentrations of any of the halogen gases, thus its use with freon and more recently with methyl bromide and ethylene dibromide. The halide detector came into prominence with the use of temporary enclosures for on-the job determination of methyl bromide leaks. By detection of very small leaks with this instrument, temporary enclosure can be made more secure, and obtaining better effect of the available gas. Thus its use is operational as well as safety device. Its safety use is in the detection of leaks that could be harmful to humans and animals, particularly in rooms or other enclosures where there are offices and living or working areas.

The halide detector works and the principle that a flame in contact with a clean piece of copper will impart a green to blue color to the flame, if any of the organic halide vapors are present in the surrounding air. Heavy concentration will extinguish the flame.

Determining Methyl Bromide Concentrations with the Halide Detector:

ppm.	lb/1000 ft (g/m)	Flame Color
0	0	No color
25	0.00625	Faint fringe of green
50	0.0125	Moderate green
125	0.031	Green
250	0.0625	Strong green
500	0.125	Strong green-blue fringe
800	0.2	Strong blue-green
1000	0.25	Blue

Gas Mask

Gas mask consists of mask and canister that the two parts are connected by flexible rubber duct.

The canister is a compact air purifying unit which is able to protect against certain harmful gases. During inhalation, the air-gas admixture is drawn through the canister to the facepiece or mask. It is purified in the canister and expelled from the facepiece through the exhalation valve. The valve is constructed to provide near normal voice communication.

Fumiscope

Fumiscope is a thermo-conductivity analysis-device used for remote sensing of gas concentrations which are under fumigation. The fumiscope consists of an electrical pump, a glass flow rate meter, a thermal conductivity cell and a scale. By activating the pump, air sample may be drawn into the unit through the conductivity cell and expelled from the unit. The flow rate can be adjusted to provide a constant flow of air. The conductivity cell compares the thermal conductivity of the mixture of air and gas to dry air. The difference is converted to electrical energy and displayed on the scale. So by using the fumiscope we can know the concentration of methyl bromide at various parts of the temporary enclosure.

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Control of aflatoxin in maize

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Prawat Tanboon-ek(2)

ABSTRACT

Only Aspergillus flavus and A. Parasiticus have been found to be Aflatoxin producing fungi, but many other toxin producing fungi have been found in stored maize in Thailand. It has been established that Aflatoxin content is low at harvest and increases during storage. Field drying and mechanical drying were found most effective for controlling Aflatoxin contamination in maize. A Rapid BGYF test was developed to estimate Aflatoxin levels and is being used by many maize dealers. Chemicals were tested for controlling the causal fungi. Only a solution of ammonium bis proprionate and propionic acid will give temporary control and prevention of the growth of fungi in high moisture maize but will not destroy Aflatoxin - present before treatment of the grain. The grain still must be dried. Maize, highly contaminated with Aflatoxin, can be detoxified efficiently with ammonia. The resulting grain is safe and can be used for feeding cattle and swine but is not suitable for human consumption and does not move in international trade.

Maize production in Thailand

Maize is presently the fourth most important crop produced in Thailand. Maize production is particularly important in the north, north-east and central regions. Since maize cultivation is a

low cost input system, farmers tend to increase planting area rather then intensify production. Though increasing, production is still low when compared to other maize growing countries. Only some farmers accept new production technology such as fertilizing, pest control and seed of new high yielding varieties. The Fifth National Development plan (1982-1986) set a target to increase production 4.5% per year. The increased production was intended to come from extension of the cultivated area. Several trials show that more than 2.5% of yield increased when recommended fertilizing and cultural practice were used. The Six National Development Plan (1987-1991) has set a 5% production increase as the goal. The promotion by extension of maize quality improvement will be a priority. Maize is cultivated both in single and double cropping system. Generally, varieties resistant to downy mildews are grown throughout the country. There are other hybrids produced by private seed companies available in the market, but cost is the major limiting factor on use of hybrid seed. The total maize produced by Thailand is equal to only 1% of the world production, and Thailand has only a small part of total international trade. The maize importing countries of Asia and other countries prefer yellow color maize.

Postharvest handling of maize

It is estimated that Thai maize production has increased by about 11% during the period of 1960-1975. After this period, the extension to the cultivated area was limited and effected the total period is from midMay to early June, depending on average was obtained each year verying by farm price and drought conditions. A normal planting period is from mid-May to early June, depending on the arrival of early rains. Planting may start as early as mid-March to mid-April. For single cropping the growing season starts from mid-May, but for double cropping planting

must be earlier. The second crop can be planted in August/September and harvested in October/November/December. Regional variations in rainfall affect planting dates and time of harvest. Farmers try to plant early because an early crop gives a higher price. To gain this advantage, farmers may have to replant serveral times because of non-reliable rain in the early raining season. For example, the crop in 1987/1988, in some planting area, had to be replanted four times. In a maize shortage year, farmers sometimes harvest an immature crop because of the high market demand. Normal maturity for the recommended varieties is 110 days after planting, though some early maturing varieties of 90 days are available from the government seed centers or local experiment stations but give lower yields. The single crop or the first crop of double cropping are harvested in the rainy season. Only hand picking and stripping cobs are used in harvesting; no mechanical harvesting is found in Thailand. Stripped cobs are removed from the plants and packed in bags in the field. Harvested maize is transported by a range of hand, animals and engine powered carts to storage cribs or local dealers. Some farmers move the cobs immediately after harvest to a prepared area for shelling and sale. Most local dealers provide shellers for farmer's maize by contract. Shelling cost is paid by farmers. One who is not satisfied with the offered price will dry the cobs and keep them in a crib until the price is more acceptable.

Since harvesting is mainly in the rainy season, maize drying is difficult. Farmers will keep maize on the cob either wet or dry while local dealers, who always face a shortage of storage facilities, will keep maize only as shelled grain. A survey showed that 79 % of farmers sell their maize as soon as it is harvested mostly by contact with local dealers. Only 21% of the farmers store the crop to wait for a better price or until forced to sell. On-farm postharvest drying and storage are

seldom done by farmers. These practices are not considered worthwhile because farmers keep the maize only for a short period. For longer storage, typically, farmers dry the harvested maize on the cob using a straw mat on the ground. Then the dried ears will be kept in a storage crib. In some areas the ears are left on the plant before harvesting until the moisture content of the grain becames 18%. Topping, which removes the upper part of the mature plant has been recommended for the double-cropping system. This method provides the availability of the land to grow a second crop without the necessity of harvest the first crop. Ears can be left on the plant in the field for 1 month without aflatoxin formation.

During the harvesting season, local dealers are busy on travelling to and fro to buy maize to be stored in godowns, bins and silos. All the purchase is wet. The dealers must dry the grain before storing it because of the ensuing quality and value. Sun drying on cement floors is the most common practice but the amount of rain limits the utility of the floors. Aflatoxin can became a serious problem, when the wet grain cannot be dried quickly. Research by DOA indicates that if the wet grain cannot be dried within 72 hours, Aflatoxin can be a problem. As shown in Fig. 1, local dealers will move the maize to Bangkok wholesalers for export or domestic use. Some local dealers also are involved in moving directly from producing area to overseas importers. Cooperatives also take a small part in the marketing channel. It was found that Aflatoxin contamination in pre-harvest maize is relatively low but increases rapidly during the period the grain is handled by local dealers. As the problem became serious, local dealers now pay more attention to solutions. Several types of mechanical dryers, storage facilities and siloes with dryers have been placed in many parts of the country for drying and handling the grain. Some mould inhibitors also are used in domestic feeds.

Control of Aflatoxin in Maize

Aflatoxin content has become a major factor affecting the export of maize and most importers have set aflatoxin limits, usually in the range of 20 to 100 ppb. Aflatoxin restrictions and the world surplus of maize have made markets increasingly difficult to find and prices have tended to fall until the recent US drought. Since Thailand exports more than 70 % of the production, a discount of 5% on FOB price because of Aflatoxin would cost more than US$ 25 million per annum. Thailand, therefore, assigned top priority to research on aflatoxin control in maize. This work, coordinated by a national committee, has made rapid progress and many of the aflatoxin control measures that have been devised are being implemented and/or evaluated on a commercial scale. Research in Aflatoxin involves several different fields.

Chemical treatment

The DOA Division of Plant Pathology and Microbiology has recently completed screening seven reagents in the laboratory for effectiveness in preventing or reducing aflatoxin contamination of maize. Only three of the reagents were found to be effective, sodium bisulphite, ammonia, and propionic acid:ammonium bis propionate at a ratio of 9:1. Sodium bisulphite and ammonia treatments both result in grain with a strong residual odor; the ammonia treatment also produces darker grain. The most promising regent is the propionic acid-based fungicide formulation, which has been shown to effectively control both mould growth (A. flavus) and aflatoxin formation, while not adversely affecting the physical quality of the grain. The cost of the fungicide treatment may be offset by higher prices for better quality grain. Future work aims to reduce

costs by minimizing the inclusion rate and improving the application method.

Mechanical drying

The UK-Thai Aflatoxin in Maize Project (1) has identified a set of criteria, called the UK-Thai Project (UTP) System, which have been shown to reliably produce low aflatoxin-content maize during the rainy season. With the UTP system, maize is first field dried on the stalk for one to two weeks before harvesting to reduce moisture content to 18 to 22%. It is next shelled within 24 to 48 hours of harvest, and loaded into a drier within 12 hours of shelling. Thus, within 48 hours, it is dried to 14% moisture content, with no part exceeding 15%. Aflatoxin content is monitored rapidly by a special adaptation of the bright greenish-yellow fluorescence (BGYF) test. Maize dried to 14% moisture content by the UTP system can be safely stored for a minimum of two months with no increase in aflatoxin content. Using this system, 25 three-ton batches of maize were successfully processed with a mean total aflatoxin content of 2.5 ppb and a range of 0 to 16 ppb at drying sites in two provinces. The system is now being used commercially for about 50,000 tons of maize.

Improved farm storage

A USAID-funded project conducted by DOA was begun in 1985. The project aims to develop and evaluate improved farm storage and drying methods. In 1985, cribs of three sizes (0.5,1.0 and 2.5 meters) were tested, as was a solar drier developed by the Asian Institute of Technology (AIT), Bangkok.

Mycological studies

A collaborative study, involving the Division of Plant Pathology and Microbiology, the Department of Agriculture and Tropical Agricultural Research Centre (TARC) of Japan, is being conducted on the incidence and occurrence of Aspergillus flavus. A very high incidence of A. flavus has been found in soil samples, especially in soil around drying facilities and warehouses. No A. flavus spores were detected in the atmosphere in maize fields, but high levels of spores were found in the air in warehouses used for maize storage

A mycological study of maize was also done by the UNDP/FAO in collaboration with DOA January and February, 1985. The work confirms low concentrations of A. flavus spores in the air in maize fields during the dry season, as well as the high concentration of spores in warehouses. Aspergillus flavus contamination in stored maize was found to be closely associated with weevil infestation (Sitophilus zeamais); the insects carried extremely high concentrations of A. flavus spores. Virtually no A. flavus was found before harvest in the second, dry-season crop of maize, but concentrations increased slowly during temporary storage of ears and grain. It was also found that only A. flavus and A. parasitica produce aflatoxin in maize.

Quality control methods for aflatoxin

The UK-Thai project has amassed data which strongly indicate that an adaptation of the bright greenish-yellow fluorescence (BGYF) test (2) can be used in Thailand to identify the level of aflatoxin presence in maize. Batches were. classified according to the number of observed BGYF

particles (e.g. 0, 3,5,10 counts). Correlation between these BGYF counts and the mean total aflatoxin of all batches within each classification was found to be excellent (correlation coefficient, r = 0.92). Sampling was found to be a critical factor when working toward a 20 or 30 ppb aflatoxin limit. The good correlations were only found when a 10-kg representative sample was coarsely ground with a hammer mill fitted with a 6-mm screen and the sample subdivided into four 125-9 subsamples. Aflatoxin quality-control procedures based on the BGYF test have been devised for monitoring the production of low-aflatoxin content maize, and for assisting grain management at regional and export storage facilities. Monitoring is best done in conjunction with mini-column testing to minimize consumer risk.

Analytical Services

Aflatoxin analysis is routinely done at a number of laboratories in Thailand. Unfortunately, sampling methods, sample preparation and analytical methods vary widely, although efforts are being made to standardize them. Inspection companies offer an aflatoxin analysis service that is predominantly semiquantitative, based on mini-column determination which is sometimes linked to a fluorotoxin meter. Fully quantitative aflatoxin determination is mainly performed in government laboratories, using quantitation by thin-layer chromatography (TLC). Sophisticated techniques, such as high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC), are gradually being introduced, and should soon enable a faster and more accurate analysis of samples.

Future Research

Future research has been approved by the national committee in the areas of:

- **Continued work on inhibition of aflatoxin-producing fungi by chemical treatment;**
- **Aflatoxin detoxification;**
- **Evaluation of the UTP system for producing lowaflatoxin maize on a commercial scale;**
- **Determination of the feasibility of increasing the proporting of second-crop, dry-season maize, which is known to have a low aflatoxin content, and to determine where such changes might be most appropriate;**
- **Study of aflatoxin distribution in low-aflatoxin content batches in order to devise appropriate sampling plans for use throughout the maize marketing chain;**
- **Development and evaluation of analytical techniques, both fully quantitative and semi-quantitative, for use in quality control;**
- **Reduction of the risk of aflatoxin contamination in unshelled maize, e.g., in crib storage and extended field drying; and**
- **Study of the risk of aflatoxin contamination associated with maize shipping, and the development of suitable control measures.**

Cooperative Research

Much of the aflatoxin research in Thailand can now be considered to be coordinated and cooperative, due to the influence of the national committee. Assistance from other countries to provide funding, training and staff is still needed; such support has played a significant role in aflatoxin research in the past. Various foreign agencies have given support to the Department of

Agriculture through bilateral or multilateral assistance.

The United Kingdom has provided training, equipment, staff and volunteers to join in collaborative projects with Thai researchers, at a value of approximately 15 million baht (US$ 600,000). The United States Agency for International Development in Phase 1 of its contract, has approved a soft loan of approximately US$ 200,000 and a grant for research staff and overseas training and study tours for Thai scientists for 1985 and 1986. The United Nations Development Programme (UNDP) has approved funds of US$ 38,500 for 1985 and 1986. In addition, the Tropical Agricultural Research Centre (Japan) has approved a cooperative project with the Division of Plant Pathology and Microbiology of the Department of Agriculture on quality and preservation of maize by preventing aflatoxin contamination. The Tropical Agricultural Research Centre supplies senior researchers, training, analytical equipment and software.

Acknowledgements

Credit for the rapid progress in the battle against aflatoxin must go to the National Committee on Mycotoxin Control in Agricultural Commodities and its constituent organizations. Particular mention should be made of the research carried out by the Department of Agriculture, which through its own research and in collaboration with the United Nations, Japanese teams (TARC and JICA), USAID and British teams.

REFERENCES

1. **Boonma, C., Rodvinit P., Reasanon, S., Bumrungtai, N. and Artchinda, S. (1980). Thailand Corn Commodity System, (in Thai), Kasestart University.**
2. **MOAC (1983). Agricultural Statistics of Thailand, Crop Year 1982/1983, Report 202, Centre for Agric. States. and Office of Agric. Econ., Bangkok.**
3. **Dept. of Agri. (1985). Report on AFLATOXIN IN MAIZE IN THAILAND Volume Two, DOA, Bangkok.**

Fig 1 Marketing Channel for Maize In Thailand

	1985/86			
	Planted Area (rai)	Production (rai)	Yield (ton)	Average Yield (kg./rai)
Northern	7,566,655	7,268,451	3,042,553	5,925
North Eastern	3,119,853	3,078,534	1,202,532	2,447
Central	1,210,814	1,172,679	537,234	2,513
	1986/87			
Northern	7,431,431	6,949,942	2,687,464	5,937
North Eastern	3,176,232	2,866,322	1,012,226	2,253

Central	1,197,851	1,161,164	493,457	2,207
	1987/88			
Northern	7,039,457	5,385,255	1,697,075	4,871
North Eastern	2,708,235	2,352,159	763,477	2,205
Central	1,128,555	969,237	383,098	1,935

MAIZE PRODUCING PROVINCES IN THAILAND

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