AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

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Aflatoxicosis

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Contents

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1. Introduction

Aflatoxicosis is the syndrome caused by the ingestion of a group of substances called aflatoxins, which are produced by the fungi *Aspergillus flavus* and *A. parasiticus*. In addition to a high acute toxicity, aflatoxins are among the most potent carcinogens known to humans (Newberne, 1973).

Attention was first directed to this condition in 1960 by the loss of many thousands of turkey poults and ducklings on farms in Great Britain after they were fed imported groundnut (peanut) meal. Since 1960, aflatoxin has been incriminated in the death or debilitation of many other animal species, including humans (Table 1). Early studies of the toxic peanut meal demonstrated that the toxic factor could be separated chromatographically into four fractions which were fluorescent under ultraviolet (UV) light.

Table 1. Cases of aflatoxicosis in domestic animals reported in Australia

Species	*Source of aflatoxin*	*Reference*
Chickens	Peanut meal	Gardiner and Oldroyd (1965)
Turkeys	Peanut meal	Hart (1965)
Dogs	Mouldy bread	Ketterer *et al.* (1975)
Cattle	Peanut hay	McKenzie *et al.* (1981)
Pigs	Sorghum	Connole and Hill (1970)
Pigs	Mouldy bread	Ketterer *et al.* (1982)
Pigs	Peanut waste	Ketterer *et al.* (1982)
Pigs	Sorghum	Ketterer *et al.* (1982)
Pigs	Sorghum	Ketterer *et al.* (1982)

All four components were given the name 'aflatoxin'. The letters B (blue) and G (green) were added to refer to their fluorescent colour and the subscripts 1 and 2 to refer to their relative chromatographic mobility. Later, two other compounds were isolated from the milk of cows fed the groundnut meal and these were called aflatoxins M1 and M2 (milk). Although a large number of closely related compounds have been isolated or synthesised, only these six aflatoxins (Fig. 1) are important contaminants of foods and feeding stuffs. Aflatoxin B1 is the most common and most toxic. Aflatoxins B2, G1 and G2 are rarely found in the absence of B1. Traces of aflatoxin Ml may be detected in milk or liver extracts if large quantities of aflatoxin Bl have been ingested Aflatoxins B2a, G2a and M2a shown in Fig. 1 are acid-catalysed hydrolysis products of aflatoxins B1, G1 and M1, respectively, and have been reported in stomach contents (Ketterer *et al.*, 1982).

Crops most frequently contaminated with aflatoxins before harvest are peanuts and maize. During storage, many commodities can support growth of *A. flavus*, providing conditions of moisture and temperature are suitable. High aflatoxin concentrations are commonly formed in sorghum and maize if these are stored with high moisture contents (16–20%) and in warm conditions (25–40°C). Aflatoxin contamination of wheat, barley and oats also occurs, but grass and fodder crops are unsuitable substrates for the growth of *A. flavus*.

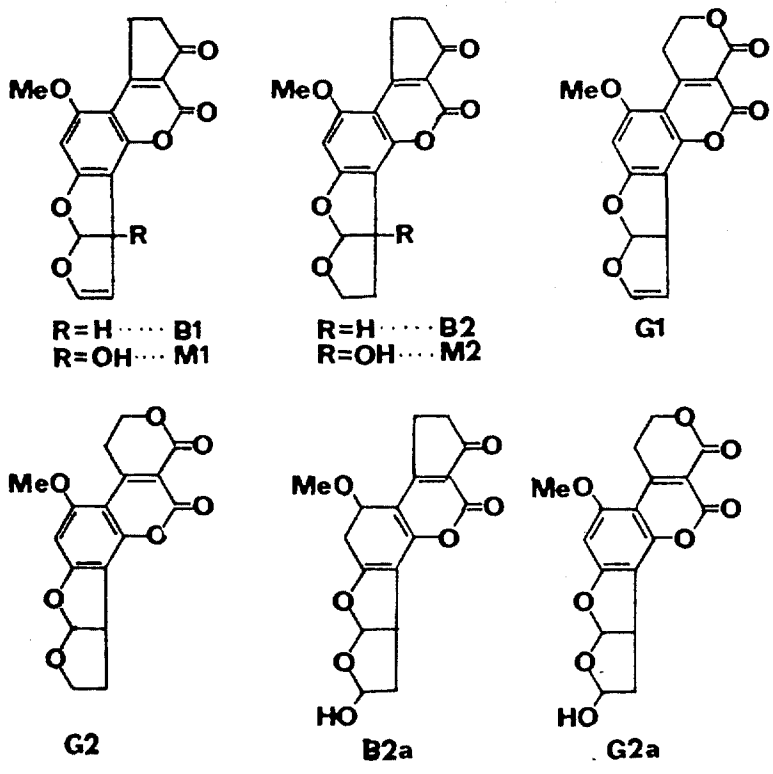

Figure 1. Structures of aflatoxins.

Diagnosis of aflatoxicosis depends on some or all of the following factors.

(a) History: the problem is associated with a specific feed; there are either signs of mould activity (visible mould, heating) in the feed or a possibility of poor feed storage and wetting of feed; treatment has little effect.
(b) Clinical signs and pathology consistent with aflatoxicosis.
(c) Detection of the *A. flavus* group in the feed.
(d) Detection of significant concentrations of aflatoxins in either the feed, stomach contents, vomitus, excreta, milk or liver.

2. Clinical Signs and Pathology

2.1. Clinical Signs

The domestic species vary in their susceptibility to aflatoxin. Listed in approximate decreasing order of susceptibility, they are: duckling, turkey, pig, dog, chicken and cattle. The disease has been rarely reported in cats, goats, horses and sheep.

Clinical signs of aflatoxicosis are referable to hepatic damage and consist of anorexia, depression, jaundice and sometimes blood in the faeces and haematuria. Cattle are likely to be more chronically affected, presumably because of their increased resistance to the toxin; they have a tendency to show nervous signs particularly grinding of the teeth and possible blindness. Tenesmus is another common sign in cattle, often leading to eversion of the rectum. Scouring is usually mild, when present.

Serum bilirubin levels are usually elevated and alkaline phosphatase may be elevated. In acute cases serum glutamic oxaloacetic transaminase and ornithine carbamyl transferase are also raised.

Prolonged feeding of aflatoxin concentrations not quite high enough to produce the above signs will cause reduced weight gain and decreased resistance to infectious disease. Long-term exposure also results in an increased incidence of neoplasia.

2.2. Gross Pathology

Pathological changes in the acute disease are generalised icterus and a yellow swollen liver with an oedematous gall bladder. Frequently there are widespread haemorrhages on serosal surfaces and in the gastrointestinal tract.

In chronic cases the liver is pale and firm due to fibrosis and the gall bladder may be greatly distended. There is usually ascites and oedema of the mesentery. In cattle the abomasal wall can be very oedematous.

2.3. Microscopic Pathology

The livers of acute cases have contrilobular or diffuse necrosis, marked accumulation of fat in hepatocytes and early biliary ductule proliferation. Variable degrees of haemorrhage and hepatocyte karyomegaly also occur. In subacute cases the main findings are fatty change. biliary ductule proliferation and fibrosis. With increasing chronicity there is progressive biliary ductule proliferation and dissecting fibrosis resulting in distortion of the hepatic architecture. Nodular hyperplasia also occurs.

An additional microscopic finding reported in bovine livers is an intimal proliferation affecting centrilobular and hepatic veins and resulting in obliteration of the lumen. Therefore, the hepatic changes seen in aflatoxicosis, namely biliary ductule proliferation, fibrosis and intimal proliferation are similar to the lesion of pyrrolizidine alkaloid toxicity. The presence of megalocytosis would favour a diagnosis of the latter but there is need for caution because megalocytosis has also occasionally been reported in aflatoxicosis.

For more detailed information see Allcroft and Lewis (1963), Asplin and Carnaghan (1961), Newberne (1973), Newberne *et al.* (1966) and Sisk *et al.* (1968).

3. Cultural Methods for the Study of the *Aspergillus flavus* Group

3.1. Isolation

An optimum culture medium for comparative study of the Aspergilli should contain the necessary chemical elements in pure and assimilable form, supplemented by utilisable carbohydrates.

Aspergilli, as they are isolated from nature, usually do not require vitamins or other growth factors. A recommended medium is Czapek's solution agar which is a solid neutral medium of known chemical composition with nitrate as the only source of nitrogen (see 3.4.1. for formula). Descriptions of growth characteristics on this medium are used in standard textbooks of the Aspergilli, e.g. Raper and Fennell (1965).

Another suitable medium is malt extract agar (see 3.4.3. for formula). This medium is less diagnostic than Czapek's agar but many species sporulate more freely when grown on it.

Feedstuffs such as grains usually possess a variety of fungal species, most of which are contaminants from the soil or air. To eliminate such contaminants, surface sterilisation can be used. The material should be immersed in a 10% solution of a commercial chlorine bleaching agent for two minutes then washed in sterile water. Grains treated may be dried on filter paper before culturing.

If the test sample is contaminated heavily a dilution method may be necessary. A satisfactory suspension may be prepared by agitating about 1 g of the material in a 9 mL sterile water blank containing a wetting agent such as sodium lauryl sulfonate at a concentration of 1:10 000 or 1:100 000. The suspension may be used directly for streaking on agar plates. Hay infusion agar (see 3.4.2. for formula) is the recommended

medium for isolation. If needed, further dilution of the original material may be done by diluting in successive water blanks.

Isolation of *Aspergillus* spp. colonies is usually made by directly transferring small portions of the test material (prepared feedstuffs, mashes, grains, grasses, etc.) by means of an inoculating wire on to several points on the surface of the agar medium in Petri plates. Up to 10 grains or pieces, or 0.1–0.5 g of powdered food can be used per plate. Liquids (0.1 mL) can be spread on the agar surface. Usually three agar plates are sufficient for culturing. Plate cultures are incubated at 20–25°C (room temperature). Growth is examined daily by eye or with a low power microscope (x40–x100) for one to two weeks.

Microscopic examination of an *Aspergillus* colony under low power shows masses of conidia in discrete clumps or heads, circular masses or in columns. Suspected *Aspergillus* spp. colonies are subcultured on fresh agar slopes to obtain pure cultures. It is usual to find several types of colonies growing from a feedstuff sample. *A. flavus* type cultures grow rapidly and the original cultures can be discarded after 10–14 days.

3.2. Microscopic Examination and Identification of Aspergillus flavus

Mounts may be prepared by using a dissecting needle or nichrome wire to pick off a sporing portion of a young colony, placing it on a slide and adding a drop of 70% alcohol to wet the spores. It may be necessary to tease out the growth with a needle then stain with a drop of lactophenol blue (Conant *et al.*, 1971) (see 3.4.4.). Add a coverslip carefully to avoid air bubbles. Examine under bright field under low power (x40–x100), then high power or even oil immersion (up to x400) if necessary. One sees conidia borne on stalks (or conidiophores), ending in a swollen body (or vesicle) bearing one or two sets of specialised cells (phialides or metulae). This sporing apparatus is diagnostic for the genus *Aspergillus*.

As well, other characteristics such as the colour of the massed spores and the structure of the spore-bearing apparatus are used to determine the species present.

Keys based either on morphology or on colour are used to identify strains of Aspergilli down to groups (Raper and Fennell, 1965). The *A. flavus* group has these characters.

(a) Conidial heads globose to radiate or columnar; very light yellow–green, deep yellow–green, olive brown or brown.
(b) Conidiophores colourless — usually roughened but varying from either smooth or nearly so to coarsely roughened.
(c) Vesicles globose to subglobose at maturity in species with large heads, remaining clavate or flask shaped in species with small heads; fertile over most of their surface.
(d) Sterigmata uniseriate or biseriate with both conditions commonly seen in the same strain or in a single vesicle.
(e) Conidia in most species globose or subglobose when mature with roughening either conspicuous or almost absent. Often showing considerable intrastrain variability in size; in other species, elliptical and smooth or delicately roughened;
(f) Sclerotia dark red, brown to purple-brown or black at maturity; globose, subglobose or vertically elongate.

Colonies of suspect *A. flavus* should be examined for:

(a) colony diameter;
(b) colony colour;
(c) whether liquid droplets form on colony surface; and
(d) shape of sporing heads.

A wet mount will be necessary for examination under low power and high power to determine vesicle shape and either presence or absence of metulae.

Having decided that the strain fits into the *A. flavus* group, a further key is used.

(a) Conidial heads in pale to intense yellow or yellow–green shades when young;
(b) Colonies not shifting to brown on Czapek's agar;
(c) Conidia definitely echinulate;
(d) (i) Sterigmata either single or double with the latter predominant; heads radiate or very loosely columnar .... *A. flavus* Link.
(ii) Sterigmata typically in a single series.
(A) heads columnar; sterigmata usually uniserlate ..*A. flavus* var. *columnaris.*
(B) heats radiate; sterigmata uniseriate.... .....*A. parasiticus*

Results of the detailed examination are compared with the description in a standard text book, e.g. Raper and Fennell (1965). As there are some 150 *Aspergillus* species recognised and the *A. flavus* group contains 11 species, some experience is necessary to identify strains to species level.

3.3. Screening for Aflatoxin Production

A modified Czapek Dox groundnut agar medium (de Vogel *et al.*, 1965) can be used successfully as a screening medium to examine strains of the *A. flavus* group for their ability to synthesise aflatoxin (Connole *et al.*, 1981). The blue fluorescence in UV light is specific for aflatoxin B1. Three consecutive subcultures on the medium appear necessary in the case of an unknown strain, which is negative in the first test. It enables many isolates to be examined in a short time.

The agar is prepared and inoculated as in 3.4.5. After incubation for 48–72 hours, luxuriant growth has occurred and sporulation has begun, and the plates are inverted and examined under a UV lamp which should radiate strongly in the

350–370 nm range. A bright blue fluorescence is observed in the medium on which aflatoxin B1-producing strains are growing. The plates should be re-incubated for another 24 hours if fluorescence is doubtful.

It has been calculated that 5 µg aflatoxin B1/mL of medium is the lowest concentration at which the typically blue fluorescence just occurs. The fluorescence is stable and after a few days is still perceptible. At very low concentrations the colour tends to become violet due to reflection of UV light. Table 2 shows the intensity of fluorescence at various concentrations of aflatoxin B1 in the screening medium.

3.4. Media Formulation

3.4.1. Czapek's Solution Agar (Adapted by Dox, 1910)

Sodium nitrate, $NaNO_3$	3.0 g
Potassium dihydrogen phosphate K_2HPO_4	1.0 g
Magnesium sulfate, $MgSO_4.7H_2O$	0.5 g
Potassium chloride, KCl	0.5 g
Iron sulfate, $FeSO_4.7H_2O$	0.01 g
Sucrose	30.0 g
Agar	15.0 g
Distilled water	1 L

To reduce caramelisation, the sucrose is added just prior to final sterilisation. Sterilise at 120°C for 20 min.

3.4.2. Hay Infusion Agar (Raper, 1937)

Distilled water	1 L
Decomposing hay	50 g

Autoclave for 30 min at 120°C. Filter.

Infusion filtrate	1 L
Dipotassium hydrogen phosphate K_2HPO_4	2 g
Agar	15.0 g

Adjust pH to 6.2; sterilise at 120°C for 20 min.

3.4.3. Malt Extract Agar (Blakeslee, 1915)

Distilled water	1 L
Malt extract	20.0 g
Peptone	1.0 g
Dextrose	20.0 g
Agar	20.0 g

Add dextrose just prior to final sterilisation at 120°C for 20 min.

3.4.4. Lactophenol Cotton Blue Stain (Conant *et al.*, 1971)

Phenol crystals, C_6H_5OH	20 g
Lactic acid, $C_3H_6O_3$	20 mL
Glycerol	40 mL
Distilled water	20 mL
Cotton blue stain	0.05 g

Dissolve by heating gently under a hot water tap. Add 0.05 g cotton blue stain.

3.4.5. Aflatoxin Screening Medium (de Vogel *et al.*, 1965)

The following are dissolved in 900 mL distilled water.

Sucrose	30 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Potassium chloride, KCl	0.5 g
Ammonium chloride, NH_4Cl	1.6 g
Iron sulfate, $FeSO_4.7H_2O$	0.01 g

Mill toxin-free groundnuts (100 g) in a mixer at 12 000 rpm and then add after which 100 mL distilled water. The slurry is heated for 10 min at 100°C in a water bath and then mixed with the salt solution. The mixture is boiled for a five minutes and then filtered hot through a double layer of cheese cloth. Then 20 g of Bacto agar (Difco) is added and the pH adjusted to 7.2. The medium is sterilised in steam at 115°C for 20 min.

A thin layer of Hyflo Supercel (Johns-Manville, New York, NY, USA) is spread on the bottom of the Petri plate before pouring. The Hyflo Supercel layer prevents the appearance of green, grey or yellow fluorescence produced by the mycelium itself.

Hyflo Supercel (6 g), sterilised by dry heat for one hour at 180°C, is mixed with 10 mL sterile water in a 12 cm diameter Petri plate and the paste is distributed over the bottom of the plate by gentle shaking. The plates are partly dried by warming them carefully on an asbestos gauze to prevent the formation of bubbles or cracks in the Hyflo Supercel layer. A thin layer (0.5–1 cm) of the agar medium, cooled to 50°C, is now slowly poured on to the Hyflo Supercel. After solidification, another 15 mL medium is added. A loopful of a spore suspension of the *A. flavus* strain is streaked evenly over half the surface of the agar, the other half being used as a blank. The plates are incubated at 30°C.

Table 2. Intensity of fluorescence at various concentrations of aflatoxin B1 in screening medium

Aflatoxin B1 concentration (µg/mL)	*Relative intensity of fluorescence*
5	±
10	+
20	++
40	++
80	+++
160	+++
320	+++

± Very weakly positive; + weakly positive; ++ strongly positive; +++ very strongly positive.
Although this method gives only an approximate assay of aflatoxin B1 concentration it is a very useful and rapid screening test for the recognition of aflatoxin B1-producing strains, especially in laboratories where chemical assays are not readily available.

4. Aflatoxin Assays

4.1. General

Two types of tests are discussed here for the detection and determination of aflatoxins: biological and chemical. The bioassay techniques are only semiquantitative and generally non-specific. Those currently available are unsuitable for routine screening purposes and do not respond to the low levels required for food control. The chemical assay techniques, although more reliable and faster, can produce false identifications, particularly on commodities with which the analyst has had little experience. Chemical assay involves extraction of aflatoxins with various organic solvents followed by a number of steps to clean the extract. The final step usually involves visual examination of aflatoxin fluorescence after separation by thin-layer chromatography (TLC). Spots on a TLC plate can be verified as aflatoxins with increasing certainty by additional chromatography with different solvent systems, by derivative formation and finally by mass spectrometry. The toxicity of the compound can be verified by bioassay. Aflatoxin concentrations can be determined by visual comparison of fluorescence intensity with that of standards. Densitometers have been used to increase accuracy.

High pressure liquid chromatography (HPLC) can provide the best accuracy of determination, but while HPLC methods are available for a few commodities, problems of sample cleanliness have to be overcome for some mixed feeds. The methods also suffer from erratic recoveries of aflatoxins that occur during the additional clean-up and derivatisation steps necessary for HPLC.

A review of aflatoxin methodology was presented by Schuller *et al.* (1976).

The requirements of a diagnostic laboratory differ from those of a food quality control laboratory where large numbers of a limited range of foods are analysed. The diagnostic laboratory must cope with small numbers of samples from many different sources and with a wide range of compositions. The selected method should be capable of handling all substances presented with only minor modifications. The procedure recommended here, adapted from a range of published methods, has been found satisfactory in this laboratory for all commodities so far examined. With practice, detection limits of 1 µg/kg aflatoxins can be attained.

4.2. Sampling

Sampling is undoubtedly the most important contributor to errors in the analysis of feeds. This arises from the discontinuous nature of mould growth and aflatoxin production in raw agricultural commodities. Since the most heavily contaminated portion of feed may have been consumed by the animal, it can be impossible to relate the levels actually detected in the feedstuff to that eaten. Nevertheless, every attempt should be made to ensure that the analytical sample is as representative as possible of the bulk. It may also be of value to analyse mouldy lumps separately.

Prepare samples by one of the following procedures:

(a) CAUTION: Hammer-milling of dry samples is not recommended since hazards exist to the operator from air borne dust and mould spores. If these operations are unavoidable, protective masks and exhaust hoods *must be used.*

(b) With homogenous, finely divided material such as formulated rations and oilseed meals, mix well and draw the analytical sample by a random dividing procedure.

(c) With samples of high fat or oil content. mixing/grinding is best accomplished using a large meat mincer. Small samples can be ground by blender with addition of grinding aid such as oyster shells.

(d) If large samples containing visible mould are received, a large soxhlet extractor may be used and a portion of extract used for determination.

4.3. Reagents and Apparatus

(a) Diatomaceous earth: e.g. Hyflo Supercel

(b) Distilled acetone (CH_3COCH_3)

(c) Extraction solvent: Dilute 850 mL acetone to 1 L with distilled water

(d) Distilled hexane [$CH_3(CH_2)_4CH_3$]

(e) Distilled dichloromethane (CH_2Cl)

(f) Distilled, dried chloroform ($CHCl_3$) and additional solvents for TLC (see 5.6.2.)

(g) Diethyl ether [$(C_2H_5)_2O$]: distilled, dried, low in ethanol (C_2H_5OH) content

(h) Lead acetate solution — 20 g analytical reagent (AR) grade lead acetate [$(CH_3CO_2)_2Pb$]/100 mL distilled water

(i) Citric acid monohydrate ($C_6H_8O_7.H_2O$)AR

(j) Ammonium sulfate [$(NH_4)_2SO_4$]AR

(k) Sodium sulphate (Na_2SO_4)AR anhydrous granular

(l) Acidic alumina activity II: Add 3% distilled water to acidic alumina activity I, mix well and stand overnight to equilibrate

(m) Explosion-proof high-speed blender

(n) Separatory funnels 500 mL capacity

(o) Erlenmeyer flasks 250 mL capacity

(p) Filter funnels 120 mm diameter with fluted filter papers No. 4

(q) Heated water bath with air blower or rotary vacuum evaporator

(r) Chromatography columns: 1.5 cm internal diameter — add 2 cm acidic alumina activity II. Tamp down. Top with 10 cm anhydrous sodium sulfate (see 4.4. *N.B.2)*

(s) Phase separating filter paper (Whatman PS is suitable)

(t) Tapered bottom 16 mL test tubes. Capped centrifuge tubes are most suitable
(u) Thin-layer chromatography plates (see 4.6.3.)
(v) TLC Development Tank: suitable for 20 x 20 cm plates
(w) UV Lamp or Chromato-Vue Lamp: Longwave 15 watt UV lamp (use with UV absorbing eyeglasses) or chromato-Vue cabinet equipped with 15 watt lamp (Ultra-Violet Products, Inc. or equivalent)
(x) Microsyringes: 10 µL and 100 µL capacities Grind tips off needles to avoid damage to surface of TLC plate.

4.4. Extraction Procedure: Grains and Feedstuffs

(a) Weigh 50 g into the blender vessel.
(b) Add 250 mL extraction solvent plus 10 g Hyflo Supercel.
(c) Blend five minutes at high speed, releasing any pressure build-up at regular intervals.
(d) Filter into an Erlenmeyer flask, collecting 100 mL filtrate.
(e) Add 20 mL lead acetate solution, 80 mL distilled water and 10 g diatomaceous earth. Mix and stand for five minutes.
(f) Filter. Take 100 mL filtrate into a separatory funnel.
(g) Extract fats with 100 mL hexane. Discard hexane.
(h) Extract with two portions each of 50 mL dichloromethane and pass the extracts in turn through the prepared chromatography column. Add the second extract as the first reaches the top of the sodium sulfate. Collect in a 250 mL flask, allowing column to run to dryness.

N.B. 1: Low recoveries of aflatoxins Gl and G2 may be experienced with some batches of alumina. Perform recovery experiments with each new batch.

N.B. 2: Although the alumina produces a cleaner extract, it is not essential to use alumina with most grain-based samples.

(a) Reduce eluate to near dryness, using a rotary evaporator, or an air stream with mild heating in a water bath.
(b) Transfer to a tapered test tube. Blow to dryness under either an air or (preferably) nitrogen stream. Cap and store at 0°C for TLC assay.

4.5. Extraction Procedure: Liver, Kidney, Milk, Vomitus, Stomach Contents — Samples Containing Aflatoxins M1 and M2

(a) Liver, kidney — take 100 g into blender vessel. Add 50 mL distilled water, 20 g sodium chloride and 3 g citric acid. Blend for one minute.
Milk, vomitus, stomach contents — Take 100 mL. Add 20 g sodium chloride and 3 g citric acid. Blend for one minute.
(b) Add 300 mL acetone plus 10 g Hyflo Supercel. Blend for five minutes at high speed, releasing any pressure built up at regular intervals.
(c) Filter and collect 250 mL filtrate.
(d) Add 20 mL lead acetate solution, 130 mL distilled water, 7 g ammonium sulfate and 10 g Hyflo Supercel. Mix and stand for five minutes.
(e) Filter. Take 300 mL filtrate into a separatory funnel.
(f) Extract fat with 100 mL hexane. Discard hexane extract.
(g) Extract aflatoxins with 100 mL dichloromethane, followed by 50 mL dichloromethane: acetone (1:1). Emulsions at this point are best broken by warming, and passing through phase separating paper. The dichloromethane extracts need not be completely clear.
(h) Dry extracts with anhydrous sodium sulfate and continue as in 4.4.(h).

4.6. Determination: Thin Layer Chromatography

4.6.1. Introduction

Final separation and estimation by thin layer chromatography is the most critical step in the estimation of aflatoxin. Differences in separations achieved between laboratories are the rule rather than the exception. These differences arise from variations in absorbents, solvents and environmental conditions such as temperature and humidity. Because of this, thin layer absorbents and development solvents must be adjusted to optimum in each individual laboratory. (Perform TLC only in a laboratory free of volatile reagents, since these can affect adsorbents and adsorbed spots).

4.6.2. TLC Techniques

Many techniques are available for the resolution of aflatoxins from interfering spots on a chromatogram. The particular commodity will determine which method is used. The simplest procedure is a single solvent - single development. Given below is a list of solvent systems that may be useful in different situations. With difficult separations, a double development with the same or different solvent systems may achieve the desired results.

(a) Chloroform–acetone (9:1) unequilibrated.
(b) Benzene (C_6H_6)–methanol (CH_3OH)–acetic acid (CH_3CO_2H)(90:5:5) unequilibrated.
(c) Diethyl ether –methanol–water (96:3:1) unequilibrated.
(d) Dichloromethane (CH_2Cl_2)–trichoroethene (CH_3CCl_3)–amyl alcohol–formic acid (80:15:4:1) unequilibrated. (Order of R_f changed to Bl, Gl, B2, G2).
(e) Chloroform–trichloroethene–n-amyl alcohol–formic acid (80:15:4:1) equilibrated.

(f) Chloroform–acetone–water (88:12:1.5) unequilibrated.
(g) Chloroform–acetone–isopropanol–water (88:12:1.5:1) unequilibrated.
(h) Chloroform–isopropanol (99:1).
(i) Chloroform–methanol (95:5) equilibrated.
(j) Benzene–ethanol–water (40:6:3). Vapour phase in equilibrium with vapour phase of benzene–ethanol water (40:27:20).
(k) Diethylether–ethanol (97:3).
(l) Chloroform–isopropanol–acetone (85:5:10). (Aflatoxin Ml).

The two-dimensional development given as standard procedure below has been found to separate aflatoxins from interferences in all commodities so far examined. It is most useful in a diagnostic situation where a limited number of various substances have to be analysed. It suffers from the disadvantages that only one extract is examined per plate, and the double development is more time consuming. Once the presence of aflatoxin has been shown by this method, quantitation can usually be performed by single development TLC.

4.6.3. Thin-Layer Chromatography Plates

If the analyst has little experience with the preparation of TLC plates, it is recommended that precoated aluminium or glass silica gel plates be used. TLC aluminium sheets silica gel 60 precoated, layer thickness 0.2 mm (Merck Corporation) have been found to be satisfactory (Store in box until just before use).

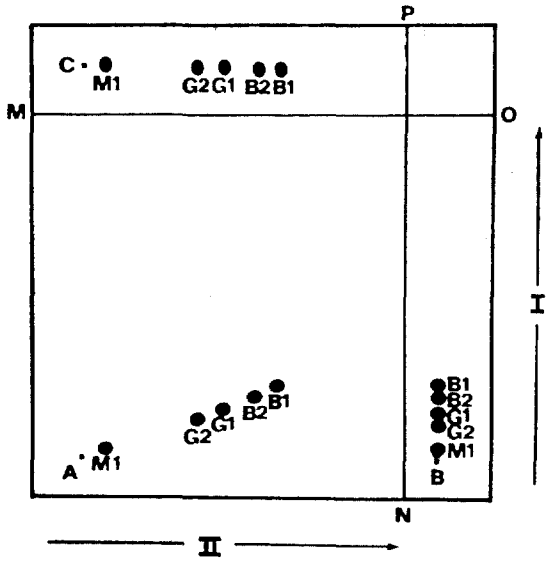

Figure 2. Diagram showing the location of aflatoxins on a two-dimensional TLC plate after developing the plate with diethyl ether in dimension I to the line shown (M–O) followed by chloroform–acetone (9:1) in dimension II to line N–P. The sample, applied at point A, is run in both dimensions while the standards, applied at points B and C, are subjected to single dimension development. Spots are located using long wavelength UV light.

Prepare plates as follows: weigh 30 g silica gel into 200 mL beaker and add amount of water recommended by manufacturer. Mix well and quickly pour into applicator. Immediately coat ten 20 x 10 cm glass plates with 0.25 mm thickness of silica gel suspension and allow to rest undisturbed until gelled. Dry 1 hour at 110°C and store in desiccating cabinet until just before use.

The silica gel plates used must pass the following test. Spot 10 ng each of aflatoxins B1 G1 and M1 plus 2 ng each of aflatoxins B2 and G2 on the same origin spot. On another origin spot these aflatoxin standards at a dilution that is just visible under the UV illumination employed. Develop plate in development solvent to be used for a distance of 12 cm. This must take <1.5 hours. Dry the plate and store in absence of light overnight. Examine under UV light. Spots should be well separated. Disappearance of any spot is evidence of excessive fading and silica gel is unsuitable.

4.6.4. Procedure — Two Dimensional Thin-Layer Chromatography

Using a 100 µL syringe, add 200 µL chloroform to the sample extracts, and cap the tube. Mix well to dissolve. As quickly as possible, withdraw 20 µL and spot on point A (shown in Fig. 2).

On point B, spot 4 µL aflatoxin standard mixture (4.8) containing 4 ng B1, 1 ng B2, 4 ng G1, 1 ng G2, and 4 ng M1. On point C, spot 6 µL aflatoxin standard mixture. Allow the spots to dry and develop in Dimension I with diethyl ether (dry, low in ethanol) in an equilibrated tank for a distance of 8 cm (line MO). Remove the plate from the tank and air-dry in the dark. Turn the plate and develop in Dimension II with chloroform-acetone (9:1) for 8 cm (line PN). Allow to dry in the dark.

Irradiate with longwave UV light by placing the plate 10 cm from the lamp. Aflatoxin spots should be well separated and roughly at positions shown in diagram (actual location of spots may be ascertained by repeating above two dimensional development with standard mixture).

Absence of spots in aflatoxin region indicates a negative result. If spots are seen corresponding to aflatoxins, confirm identity as in 4.7. Quantify results as in 4.6.5.

4.6.5. Quantification

Determine the quantity of aflatoxin in the extract by matching the fluorescence intensity of the extract spots with that of spots B and C of the standard solution. Interpolate if necessary. If the fluorescence intensity given by 20 µL of the extract is greater than that of the standard spots, dilute the extract with chloroform and reduce the volume spotted before repeating TLC. Each aflatoxin must be quantified individually by comparing with the respective standard aflatoxin solution.

The content in micrograms of aflatoxin per kg of sample is given by the formula:

$$(SRV)/(XW)$$

where S and X are the respective volumes in microlitres of the standard solutions of aflatoxin and of the extract, which have the same fluorescence intensity. R is concentration in micrograms of aflatoxin per mL of standard solution. V is final volume of extract in microlitres, allowing for any dilution that was necessary. W is weight in grams of the sample in the final extract.

4.7. Chemical Confirmation

Conform the identity of aflatoxins in the sample extract by the following processes.

4.7.1. Treatment with Sulphuric Acid

Spray the developed TLC plate with 25% sulfuric acid (4.5 mol/L). The fluorescence of the aflatoxins under UV illumination must show the following colour changes:

(a) Aflatoxins B1 and B2. Bright blue – greenish yellow.
(b) Aflatoxins G1 and G2. Greenish blue – yellowish blue.
(c) Aflatoxins M1 and M2. Bright blue – yellow.

N.B. Compounds other than aflatoxins may show similar changes.

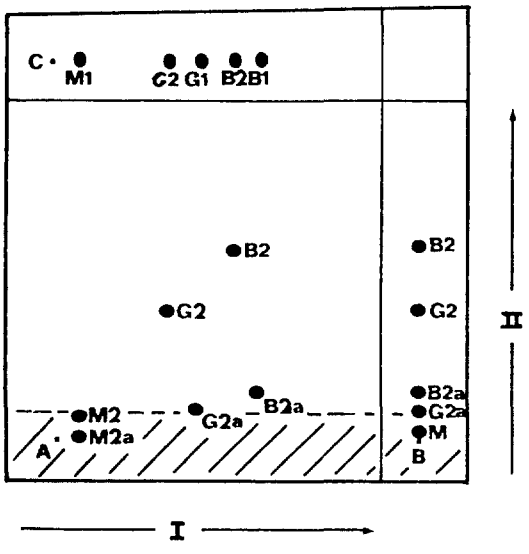

Figure 3. Diagram showing the location of aflatoxin standards, applied at points A, B and C, on a two-dimensional TLC plate after developing the plate to the line indicated with chloroform:acetone (9:1) in dimension I, spraying the hatched area with 1 mol/L hydrochloric acid and developing the plate in the same solvent system in dimension II. Spots are located by viewing the plate under UV light.

4.7.2. Different Thin Layer Chromatography Solvent Systems

Coincidence of migration with standard aflatoxins in several different solvents will provide supportive evidence of identity (see 4.6.2). The technique of adding standards to samples before TLC (spiking) must be used to show exact position of spots because of variations in R_f brought about by other components of the sample extracts.

4.7.3. Derivative Formation

Apply to points A, B and C (Fig. 3), of a 20 x 20 cm TLC plate, 6 µL of standard mixture (see 4.8.).

Develop in dimension I with chloroform–acetone (9:1) for a distance of 12 cm. Dry in dark. Carefully shielding remainder of the plate, spray the hatched area on Fig. 3 with 1 mol/L hydrochloric acid (HCl). Allow to react in the dark for 15 min, and then dry plate thoroughly in the dark under an air stream. Develop in Dimension II using the same solvent system. Dry in the dark.

Examine under UV illumination and check for the following features:-

(a) Appearance of well-separated aflatoxin spots originating from standard solution applied at point
(b) Appearance of spots originating from standard solution applied at point B. Aflatoxins B2, G2 and M2 do not react and will appear at unchanged R_f values. Traces of unreacted B1, G1 and M1 may remain. The reaction products of B1, G1 and M1 (hemiacetals) will appear as dominant intensity fluorescent spots at about 10% of the R_f of B1, G1 and M1.
(c) Check that spots originating from point A show correspondence with spots from B and C.

To confirm identity of aflatoxins in a sample, repeat the procedure, spotting sample solution at point A, and standard mixture as before at points B and C.

N.B. For confirmation of aflatoxin M1 only, increase Rf of standard and derivative by using the solvent system chloroform - isopropanol - acetone (85:5:10). Increase time of reaction with I mol/L hydrochloric acid to 30 min.

4.8. Aflatoxin Standards

CAUTION: DO NOT TRANSFER DRY AFLATOXIN FOR WEIGHING OR OTHER PURPOSE UNLESS FACILITIES ARE AVAILABLE TO PREVENT DISSEMINATION OF AFLATOXINS TO SURROUNDINGS, DUE TO ELECTROSTATIC CHARGE ON PARTICLES.

Primary standards are available from a number of chemical suppliers. If standards are received in solution, adjust concentration to 8–10 µg/mL. For standards received as dry films or crystals. proceed as follows: using label statement of aflatoxin content as a guide, introduce

Table 3. Molecular weight and e values for aflatoxins

Aflatoxin	*MW*	ε
B1	312	19 800
B2	314	20 900
G1	328	17 100
G2	330	18 200
M1	328	19 950

into the vial a volume of solvent calculated to give a concentration of 8–10 µg/mL. For aflatoxins B1, B2, G1 and G2, use benzene–acetonitrile (CH_3CN)(98:2) and for aflatoxin M1, M2 use chloroform. Agitate vigorously to dissolve. Transfer without rinsing into a ground glass stoppered flask.

Using a calibrated spectrophotometer and matched quartzface cells, record the UV spectrum of the aflatoxin solutions from 330 to 370 nm against the pure solvent used. Determine concentration of aflatoxin solutions by measuring A at wavelength of maximum absorption close to 350 nm and using the following equation:

$$\mu g \text{ aflatoxin/mL} = (A \times MW \times 1000)/\varepsilon$$

where MW and ε are as follows (Table 3). Solutions of aflatoxin in benzene–acetonitrile (98:2) are stable in deep freeze for more than one year.

Prepare working TLC standards by diluting the primary standard solutions to give concentrations as follows (Table 4).

Standard mixture — include the concentrations of aflatoxins in Table 4 in a single solution.

5. Biological Assay

5.1. Duckling assay

The use of day-old ducklings (Report, 1962) combined with chemical identification of the aflatoxins is probably the most widely used and accepted procedure for aflatoxin identification in various commodities. The sample of meal is extracted with methanol, the methanol residue dispersed in water and then extracted with chloroform. An aqueous suspension of the chloroform residue is dosed to day-old White Pekin ducklings.

Table 4. Concentration of working standard of aflatoxins for thin layer chromatography

Aflatoxin	*Concentration* ng/µL (1 µg/mL)
B1	1
B2	0.2
G1	1
G2	0.2
M1	1

The birds are housed in electric brooders and fed on a chick mash known to contain no aflatoxin. To a group of three ducklings administer the extract by means of a thin polythene tube attached to a hypodermic syringe. Insert the tube via the oesophagus into the gizzard and deliver the required amount. On day one give 0.5 mL of the well-shaken extract. Gradually increase this daily dose so that during a period of four days the equivalent of 80 g of original material is given to each duckling. Kill any survivors on the seventh day.

Remove the liver from any that die and from those killed at the end of the period; fix in 10% formol saline, section and stain with haematoxylin and eosin. In ducklings that die within the first two or three days of dosing, the histological changes consist of massive necrosis of the parenchymal cells with diffuse haemorrhages. In these cases it may be necessary to repeat the dosing at lower levels. Those that die three days or later after dosing, and survivors given toxic extracts, develop proliferative changes of the bile duct epithelium. These proliferating cords Or cells are easily recognised as they are markedly basophilic. The extent to which proliferation occurs has been found to be a reliable indication of the relative toxicity of the extracts. Lesions are assessed on an empirical basis using symbols, +, ++, +++, ++++ to indicate severity.

Results obtained by this test may be conveniently classified into the following categories:

5.1.1. Strong

The ducklings die with the characteristic histological liver lesions. It is usually possible in this case to indicate the approximate weight of the original sample which contains sufficient aflatoxin to kill ducklings.

5.1.2. Moderate

The ducklings survive and are killed seven days after the start of dosing. Characteristic histological liver lesions are present to a marked degree (+++ or ++++).

5.1.3. Slight

The ducklings survive and are killed seven days after the start of dosing. Histological liver lesions are present but are less severe than in the preceding category.

5.1.4. Nil

The ducklings survive and are killed seven days after the start of dosing. There are no histological changes in the livers.

This method should only be considered to be semiquantitative or qualitative.

5.2. Chicken Embryo

This form of assay has proved to be simple, reproducible and sensitive. The method is that of Verrett *et al.* (1964).

(a) Samples that have been chemically evaluated for presence of aflatoxin are purified by TLC.
(b) Aflatoxin Bl is eluted from the TLC plates and the solvent removed by evaporation.
(c) Residue is taken up in absolute ethanol to yield a concentration of 10 ug/mL based on chemical analysis.
(d) Fertile White Leghorn eggs are used and injected before incubation.
(e) For injections into the air cells. a hole about 5 mm diameter is drilled in the shell over the air cell and the test solution is deposited on the egg membrane. The hole is sealed with adhesive cellophane tape.
(f) The eggs are allowed to remain undisturbed in a vertical position (air cell up) for about an hour to let the material disperse.
(g) The eggs are candled daily from the fourth day of incubation on, at which time all non-viable embryos are removed. The evaluation is based on the mortality at 21 days although mortality with eggs receiving higher levels of aflatoxin is usually seen as early as the fourth day after incubation.

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