



Mycotoxin prevention and control in foodgrains

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A collaborative publication of the UNDP/FAO Regional Network Inter-Country Cooperation on Preharvest Technology and Quality Control of Foodgrains (REGNET)

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Foreword

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This publication is an offshoot of the Regional Training Course on Mycotoxin Prevention and Control in Foodgrains sponsored by the Bangkok-based UNDP/FAO Regional Network for Inter-Country Cooperation on Postharvest Technology and Quality Control of FoodGrains (REGNET) and the ASEAN Grain Postharvest Programme (AGPP). The course was held in Thailand 31 July-12 August 1989 and was conducted by the Plant Protection and Microbiology Division of the Department of Agriculture of Thailand.

As part of their objectives, REGNET and AGPP are mandated to organise short-term training courses on grain postharvest technology. These courses are designed for

postharvest research and development technologists in the Asian and ASEAN regions.

This book therefore serves as a training reference manual. Our intended users included the following: a) those who would be planning and conducting similar training courses; b) research and development technologists, and policy makers, who may use the information for research, extension and policy-generating activities; and c) students, who can use the manual as a text reference.

The manual contains the text of lectures and laboratory exercises presented by the resource speakers in the training course. In addition, country reports from the course participants and other selected relevant materials are included as references.

R.L. Semple, A.S. Frio, P.A. Hicks and J.V. Lozare

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An introduction to mycotoxins

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by J.I. Pitt

Introduction

Mycotoxins are toxic metabolites produced by fungi, especially by saprophytic moulds growing on foodstuffs or animal feeds. They must always have been a hazard to man and domestic animals, but until the past 30 years their effects have been largely overlooked. Although poisonous mushrooms are carefully avoided, moulds growing on foods have generally been considered to cause unaesthetic spoilage,

without being dangerous to health. Between 1960 and 1970 it was established that some fungal metabolites, now called mycotoxins, were responsible for animal disease and death. In the decade following 1970 it became clear that mycotoxins have been the cause of human illness and death as well, and are still causing it.

It is now well established that mycotoxicoses (the diseases caused by mycotoxins) have been responsible for major epidemics in man and animals at least during recent historic times. The most important have been ergotism, which killed thousands of people in Europe in the last thousand years, alimentary toxic aleukia (ATA) which was responsible for the death of many thousands of people in the USSR in the 1940s; stachybotryotoxicosis, which killed tens of thousands of horses and cattle in the USSR in the 1930s; and aflatoxicosis, which killed 100,000 young turkeys in England in 1960 and has caused death and disease in many other animals, and perhaps man as well. Each of these diseases is now known to have been caused by growth of specific moulds which produced one or more potent toxins, usually in one specific kind of commodity or feed.

It is important to distinguish between the effects of bacterial toxins and mycotoxins. The classic bacterial toxins are proteins, which produce characteristic symptoms in only a few hours, as the human body recognises them, and produces antibody

mediated reactions to them. Fungal toxins on the other hand, are almost all low molecular weight chemical compounds which are not detected by antigens, and hence produce no obvious symptoms. Mycotoxins are insidious poisons.

Mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin and the dose. In animals, acute diseases include liver and kidney damage, attack on the central nervous system, skin disorders and hormonal effects. Nerve toxins may cause trembling or even death. Skin disorders may be necrotic lesions or photosensitivity, while hormonal effects include abortions in cattle, swollen genitals in pigs and a variety of poorly defined disorders including vomiting in pigs, feed refusal and failure to thrive. Toxins which act on the liver and kidney are especially difficult to detect and levels much lower than those producing acute effects are often carcinogenic. When eaten in minute quantities in the daily diet, they can cause cancers in experimental animals long after the time of eating. It is probable that humans can be affected the same way.

Acute mycotoxicoses

Table 1 lists a number of mycotoxins, some of the moulds which are known to produce them, and known or possible acute diseases with which they may be

involved. In some cases, the connection between mycotoxin and disease is fairly well documented. In other, cause and effect are less certain. The most important disease which may have been due to mycotoxins, are reviewed briefly below.

Ergotism

Ergotism occurred throughout the past thousand years in central Europe, and has certainly killed many thousands of people. The fact that it was caused by a fungus has been known for a long time, since at least 1750. The fungus, *Claviceps purpurea*, grows in the ovaries of grains, especially rye, and the resulting sclerotia, called ergots, are difficult to separate from normal grain at milling, and become dispersed in flour made from the grain.

Ergotism causes constrictions in blood vessels leading to the hands and feet. In extreme cases death of cells (necrosis), bacterial infections (gangrene) and effects on the mind (hallucinations) may occur, and in some cases death results. The toxins in ergots are now known to be alkaloids, some of which find use in pharmaceuticals. The last outbreak of ergotism in Europe occurred in 1954. So far as I am aware, ergotism has not occurred in Asia, but it has occurred in Ethiopia quite recently (King, 1979).

Alimentary toxic aleukia

ATA caused the deaths of many thousands of people in the USSR, especially in the Orenburg District around the Caspian Sea, from 1942 to 1948. In some localities, mortalities were as high as 60% of those afflicted, and 10% of the entire population. Records show that ATA was also prevalent in earlier years.

ATA is an exceptionally nasty disease, causing fever, bleeding from the skin, nose, throat and gums, necrosis, and suppression of the immune system. These features are similar to radiation poisoning, and quite different from those caused by most other mycotoxins, or bacterial toxins.

During World War II, labour was very scarce in Russia, resulting in delays in harvest, and also food was very scarce, causing consumption of poor quality grain. Early studies on ATA were inconclusive. During the 1970s it became clear that ATA was a mycotoxicosis, and that the toxin responsible for ATA was the trichothecene toxin known as T-2. It was produced by the growth of *Fusarium* species, *F. sporotrichioides* and *F. poae*, in grain allowed to remain in the fields unharvested during winter.

Trichothecenes are now regarded as probably the most important mycotoxins, believed to be responsible for a variety of diseases of both man and domestic animals. Most have occurred in Europe, the USSR, Japan and the United States (Ueno, 1980). Toxicoses are usually acute, but Marasas et al. (1979) have suggested that trichothecenes may be involved in the high incidence of oesophageal cancer in the Republic of Transkei (South Africa).

Trichothecenes are also at the centre of the "Yellow Rain" controversy which occurred earlier this decade. According to some sources, trichothecenes were used as a chemical warfare agent in South East Asia, causing the deaths of thousands of villagers in Laos and along the ThaiKampuchean border (Watson et al., 1984). The facts in the Yellow rain controversy have become obscured by politics, but two things are clear: people have died from chemical poisoning in those areas, and trichothecenes are sufficiently toxic to have been the cause.

There are no records of ATA in Asia, and it is not clear if trichothecene production is likely in this region. We are currently carrying out studies aimed at providing information about this point.

Acute cardiac beriberi

Another human mycotoxicosis of significance, acute cardiac beriberi was a common disease in Japan, especially in the second half of last century. This disease is characterised by difficulties with breathing, nausea and vomiting, and after 2 to 3 days, severe pain and distress. Progressive paralysis may lead to respiratory failure and death.

Beriberi is the general name for vitamin deficiencies resulting from the consumption of polished rice. Careful work by Uraguchi (1971) showed that acute cardiac beriberi may not be a vitamin deficiency, but a toxicosis. In 1910 the incidence of acute cardiac beriberi suddenly decreased in Japan: Uraguchi points out that this coincided with implementation of a government inspection scheme which dramatically reduced the sale of mouldy rice. The incidence of true beriberi, resulting from the consumption of polished rice, was unaffected. It is notable that victims of this acute cardiac beriberi were often young healthy adults.

Acute cardiac beriberi is caused by citreoviridin, a mycotoxin produced by the comparatively rare species, *Penicillium citreonigrum*. Although it no longer occurs in Japan, there is no proof that acute cardiac beriberi does not still exist in some other part of Asia.

Onyalai

Onyalai is an acute disease characterised by haemorrhaging lesions in the mouth. It has been endemic in Africa, especially in Southern Sahara regions for at least 80 years (Rabie et al., 1975). It is much more common in rural than urban populations, Since many of the people affected by onaylai subsist on millet, Rabie et al. (1975) suggested the possible role of a mycotoxin in this disease. Toxigenic isolates of *Phoma sorghina* were found to be common in millet consumed by affected populations, and Rabie et al. (1975) were able to reproduce many of the symptoms of onyalai in rats fed maize and wheat on which *P. sorghina* had been grown.

Chronic mycotoxicoses

Some of the toxins discussed in this section (Table 2) may produce acute effects, but they are more significant because of their ability to cause long term disease. The best known and most studied of chronic mycotoxicoses are produced by aflatoxins.

Aflatoxins

Aflatoxins were discovered in 1960 following the deaths of 100,000 young turkeys

in England, and high incidences of liver disease in ducklings in Kenya and hatchery reared trout in the United States, English scientists soon established the cause of all these problems to be toxins produced by the common moulds *Aspergillus flavus* and *A. parasiticus*. Assay techniques were devised and preliminary toxicological studies carried out by 1963 (Sargent et al., 1963).

Aflatoxins are named by letters and subscripts. Aflatoxin B₁, the most toxic compound, is usually associated with aflatoxin B₂: these compounds are usually formed by both *A. flavus* and *A. parasiticus*. Aflatoxins G₁ and G₂ are formed only by *A. parasiticus* (Klich and Pitt, 1988). Aflatoxins M₁ and M₂ are formed in milk when aflatoxin B₁ and G₁ are ingested in feed.

Aflatoxins have both acute and chronic toxicity in animals, and produce four quite different effects: acute liver damage, liver cirrhosis, induction of tumours and teratogenic and other genetic effects.

Acute toxicity of aflatoxins to humans has been encountered only rarely (Shank, 1978). In 1967, 26 Taiwanese in two farming communities became ill with apparent food poisoning. Nineteen of those affected were children of whom three died. Rice from affected households was blackish green and mouldy, and appeared to be of

poorer quality than rice from households which were unaffected. Samples of the mouldy rice contained about 200 g/kg of aflatoxin B1, which was probably responsible for the outbreak. Post mortem examinations were not carried out.

In 1974, an outbreak of hepatitis that affected 400 Indian people, of whom 100 died, was almost certainly due to aflatoxins. The outbreak was traced to corn heavily contaminated with *Aspergillus flavus* and containing up to 15 mg/kg aflatoxins. Consumption by some of the affected adults was estimated to be 2-6 mg in a single day.

It has been suspected for some time that aflatoxin may be a factor in Reye's syndrome, a common cause of death in South East Asian children. Shank et al. (1971) found significant levels of aflatoxins (1-4 g/kg) in livers of 23 Thai children who had died of Reye's syndrome. Children who have died from Reye's syndrome in Czechoslovakia and in New Zealand have also been found to have had aflatoxins in their livers at autopsy.

Kwashiorkor, a disease of children in Northern Africa and elsewhere in undernourished populations, which is usually attributed to nutritional deficiencies, may also be related to aflatoxin intake (Hendrickse et al. 1982). Aflatoxin-induced

liver damage may make these children less able to cope with the high protein diets usually recommended as the cure for kwashiorkor (Newell, 1983).

Aflatoxins and primary liver cancer

Scarcely two years after the discovery of aflatoxins came the first warnings that they may cause human liver cancer. This disease has a high incidence in central Africa and South East Asia. When epidemiological evidence suggested a possible correlation with mycotoxins in the food supply, field studies were initiated on an international basis. Epidemiological data were coupled with analyses of those foods that form the staple diets of stable indigenous populations. Stability in both diet and population is essential in studies of this kind because of the long induction period (10-20 years) for human liver cancer.

Studies of this kind were carried out in Kenya, Swaziland, Uganda, Thailand, Mozambique and rural areas in the Southeastern United States. By 1976, sufficient data existed to allow plotting and statistical analysis (van Rensburg, 1977). These indicated a positive correlation between the logarithm of aflatoxin ingestion and the occurrence of human primary liver cancer, at least in Africa and South East Asia.

Epidemiological studies in the USA have produced results differing from those of van Rensburg (1977). Stoloff and Friedman (1976) estimated that children in rural communities in the southern states of the USA may ingest as much as 40 g aflatoxin per kilogram body weight per day, mostly from maize. From van Rensburg's figures, such a level should produce four to ten deaths from primary liver cancer per 100,000 population per year.

The actual level, however, is about one, less than in some other regions of the USA where aflatoxin is unlikely to be ingested in significant amounts.

Some doubt has recently been cast on the role of aflatoxin in human liver cancer. Earlier or simultaneous exposure to hepatitis B virus appears to be a prerequisite. Equally or more important may be the course of events after the cancerous lesion is initiated by aflatoxin. Campbell (1983) has shown that in rats a high protein diet after exposure to aflatoxin caused a higher rate of liver cancer than a low protein diet. Thus it would seem that nutrition can play a vital role in the induction of liver cancer by aflatoxins.

Ochratoxins

In the early 1970s, observers in Denmark noted a high incidence of nephritis (kidney inflammation) in pigs at slaughter. A search for possible causes eventually showed the presence of ochratoxin A, a mycotoxin originally reported from *Aspergillus ochraceus*. Analysis of pig feeds showed that 50% of samples contained ochratoxin A at levels up to 27 mg/kg. The mould responsible was reported to be *Penicillium viridicatum*, but has more recently been shown to be *P. verrucosum* (Pitt, 1987). This species occurs commonly in Danish barley (Frisvad and Viuf, 1985).

The discovery of ochratoxin led to analyses of pork and bacon. It was found that a significant proportion of ingested ochratoxin lodged unchanged in depot fat. The risk to humans is difficult to assess, but as pig meats are an important part of the Danish diet and rural populations usually eat their own uninspected pigs, a risk certainly exists. Death rates from kidney failure are high in some Danish rural areas and it is reasonable to suppose the cause is ochratoxin.

Penicillium verrucosum has not been reported to occur in Asia. However, *Aspergillus ochraceus* and related species which also produce ochratoxin do. The significance of ochratoxin A in tropical climates has not yet been assessed however.

Conclusions

The potential role of aflatoxins and other mycotoxins in cancer should be sufficient incentive for further investigations, especially in Asia and other tropical areas where the occurrence and significance of mycotoxins has not yet been fully assessed.

While it may not be possible to produce a food supply completely free of mycotoxins, improvements in storage and handling of grains, nuts and other commodities can minimise mould growth, and so reduce the risk of mycotoxin contamination in food supplies.

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Mycotoxins and human health risks an overview

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Subhkij Angsubhakorn

INTRODUCTION

This is a review and brief historical report of mycotoxins as risks to human health. Mycotoxins are recorded in history as far back as 5,000 years ago in China. Besides ergot and mushroom poisonings, reports as early 1861, indicate that a suspected mycotoxin affecting humans was reported in Russia, and 1891, there was a report of mouldy rice in Japan to be toxic to man. This review reports on the early literature on mycotoxins affecting human up to 1960, which is the time of the discovery of aflatoxin, and to our present knowledge where this problem still persists.

ABSTRACT

All reviews described here deal with fungi that are toxic in one way or another, either the fungi are themselves toxic when consumed, as either poisonous mushrooms

or ergot, or the fungi, as they grow, secrete toxic compounds that diffuse into the substrate on which they are growing and so make it toxic when eaten. Antibiotics which are produced by fungi are mycotoxin too, but in general usage, this term is applied to compounds that are toxic to microbes.

AMANITA TOXIN

History

Poisonous mushrooms (toadstool), which are called mycetismus, have been known, written and talked about for well over two thousand years. The *Amanita phalloides* group is estimated to be responsible for 95 percent of the fatal cases of mushroom poisoning throughout the world. An outbreak of mass poisoning occurred near Poznan, Poland, in 1918, in which 31 school children died from eating a dish of mushrooms containing this fungus-presumably in their school lunch (Simons, 1971). *A. phalloides* evidently is more common in Europe, and *A. Verna* and its varieties are more common in the United States.

Mycology

Higher fungi such as the *Amanita phalloides* group is estimated to be responsible for 95 percent of fatal cases of mushroom poisoning throughout the world.

Higher fungi such as *Amanita phalloides* group is estimated to be responsible for 95 percent of fatal case of mushroom poisoning through-out the world.

Chemistry

All the substances are cyclic polypeptides with molecular weights varying from about 800 to 1100.

The lethal dose of amanithins is about 0.1 g/kg body weight and 1-2 mg/kg body weight for phalloidin. *A. phalloides* Cactophores contain around 10, 8, 7 and 1.5 mg of phalloidin, alpha-amanitin, beta-amanitin and gamma-amanitin, respectively per 100 g of fresh tissue.

Clinical Symptoms

There are two groups of toxins that are present in these mushrooms—the phallotoxins, composed of phallin and phalloidin, and the amanitins, made up of alpha amanitin and beta amanitin.

The gastro-intestinal symptoms, which appear first and some hours after the mushrooms are consumed, are produced by the phallotoxins. The amanitins are 10 to 20 times as toxic as the phallotoxins, and cause the hepatorenal damage that results in the symptoms appearing later and that in many cases ends in death.

There is a characteristic latency of 6 to 15 or even 20 hours. The illness begins as a severe gastroenteritis, sometimes with associated cardiovascular collapse, prostration, delirium and coma, but after a day or two the patient often seems to improve. The most characteristic manifestations do not occur until the third or fourth day after the ingestion, when jaundice and other signs of hepatic cell necrosis appear. Severe damage may also arise in the renal tubular epithelium, myocardium and brain. Death may occur with 5 to 10 days; it is due to liver and renal failure, cerebral damage or with attending coma or cardiac arrest.

ERGOT ALKALOIDS

History

Infection of grasses and cereals with fungal species of genus *Claviceps* produces sclerotia (compact hyphal structure). The pharmacological properties of ergot were recognized in the latter part of the 16th century, and about 100 years later, cereal grains containing ergot were shown to be the cause of these epidemics in Europe.

In 1951 there was an outbreak of "bread poisoning" in a small town in France. Apparently moldy rye was sold illegally (to avoid a grain tax) in central France to a miller, who ground the rye and mixed it with flour which he then sold to a baker.

On August 15, 1951, many people ate small amounts of bread which may have been made from this contaminated flour. About 200 people became ill, as did many domestic animals. dogs, cats, poultry, fish, fed the same bread. A total of 25 people suffered severe delirium, and four people, one previously healthy young man and three old people suffered severe delirium. A recent outbreak occurred in Ethiopia, 97 of 42,000 people developed gangrenous ergotism. (King, 1979).

Mycology

Claviceps purpurea is the common ergot on rye and wheat. The fungus lives over winter in the form of sclerotium, a dense mass of fungus cells. Usually the sclerotia are somewhat larger and also less dense than the seeds of the host plant on which they are borne.

Chemistry

The ergot alkaloids are derivatives of lysergic acid (ergotamine), isolysergic acid (ergocristine) or dimethyl ergoline (agoclacine). Pharmacologically, these compounds are rapid acting, powerful oxytoxics, i.e., they stimulate the smooth muscle of the uterus. They are also weak vaso-constrictors.

Clinical Symptoms

Descriptions of ergot poisoning written in the Middle Ages were quite vivid. There were severe internal feeling of heat and intense thirst, multiple ulcerations of skin, a burning sensation of the limbs, the feeling of ants and mice crawling underneath the skin, the drying and turning black of hands, arms, feet and legs, blindness, dementia

and mental degeneration.

Acute ergot poisoning today is essentially a problem only in chemotherapy and rarely has it occurred recently as a result of eating. The symptoms include vomiting, diarrhea, intense thirst, a tingling, itchy and cold skin, a rapid, weak pulse, confusion, and unconsciousness.

Chronic ergot poisoning today seems limited to accidents in treating patients for migraine headaches. The extremities, especially the feet and legs, become cold, pale, and numb because of the constriction of the local blood vessels and result in diminished blood flow. Walking becomes painful and eventually gangrene develops.

PINK ROT DISEASE

History

In 1961, Birmingham et al/ reported a phototoxic dermatitis which had been shown to be endemic among white harvesters of celery. It was believed that exposure to

celery oil and sunlight was responsible for the dermatitis. Also, it was noted that the dermatitis was more severe when rotted celery was handled. Experimentally, only extracts of rotted celery produced the characteristic blistering lesions of celery dermatitis.

Mycology

Celery rot ("pink rot") is a fungus disease produced by *Sclerotinia sclerotiorum*.

Chemistry

Two psoralens were isolated and identified from "pink rot" celery which were not delectable in healthy extracts. The psoralens and a group of linear furocoumarins containing a furan ring fuse at the 2, 3 position to the c-6 and c-7 of the coumarin structure.

Clinical Symptoms

The affected areas were the hands and forearms, but the lower legs, chest, abdomen, and back also affected if the worker wore shorts and no shirt. The lesions healed,

with depigmentation and lasting many months. The incidence was said to be greater after a rain and before the use of fungicides to control fungal growth on the celery.

Psoralens, especially 8 - methoxypsoralen and trimethypsoralen have been used successfully to treat vitiligo, which is a progressive, localized achromasia of the skin resulting from a functional abnormality of the melanocytes, apparently due to the loss of function of the tyrosinase system.

In apparent careless use of psoralens as suntanning agents to augment the tanning capabilities of the skin, excessive amounts of the agents were used, and after exposure to the sun, a severe dermatitis developed.

Nausea, vomiting, vertigo, and mental excitation resulted from ingestion of 20 mg or more of 8methoxypsoralen; whether liver damage resulted is still an unsolved question (Becker, 1960).

ALIMENTARY TOXIC ALEUKIA

History

The bulk of the literature on blood-forming disease is written in Russian, for it is in the Soviet Union that the disease has been a problem. Perhaps as early as 1913, a food - borne disease, possibly from eating fusarium - contaminated bread, occurred in Siberia. During the war years of 1941-1945 larger outbreaks occurred more frequently than in the 1930, is involving several districts in Western Siberia and European Soviet Russia.

Soon the toxicity of rye was related to the growth of toxigenic strains of Fusarium on the cereal seed which absorbed much moisture and provided a suitable medium for the growth of cryophilic fungi.

It has been suggested that the epoxytrichothecenes, T-2 toxin, played a role in Russian alimentary toxic aleukia (Bamburg, et al. 1969). It is shown that T-2 poisoning in chickens resembles the mycotoxin poisoning associated with Russian overwintered grain.

Mycology

The trichothecenes are produced by various species of *Fusarium* especially *Fusarium graminearum* (*F. roseum*), *F. moniliforme*, etc.

Chemistry

The trichothecenes are a complex group of sesquiterpenoids containing the trichothecane nucleus, characterized by an olefinic bond at the 9, 10 position and an epoxy group at the 12, 13 position.

Clinical Symptoms

The Russian descriptions of the disease, dividing the clinical feature into four stages, indicate that the disease seems to result from toxic injury to the hematopoietic, autonomic nervous, and endocrine systems.

First Stage

This is a rapid onset of irritation to the upper gastrointestinal tract, beginning a few hours after ingestion of the toxic cereal product, often bread. The contaminated food would have a peppery taste and produce a burning sensation from the mouth to the

stomach. Within a few days, the patient develops acute gastro-enteritis, with nausea, vomiting and diarrhea, This local effect persists for 3 to 9 days and then spontaneously ends, even when the victim continues to eat the poisoned grain.

Second Stage

A slow degeneration of the bone marrow occurs within 9 weeks. Visible skin hemorrhage appears and hematologic examination reveals a marked decrease in the total number of leukocytes. Before hemorrhages appear, some patients display nervous system problems: irritation, weakness, fatigue, vertigo, headache, palpitation and slight asthma.

Third Stage

The most serious stage comes about suddenly and has four essential features:

1. Hemorrhagic syndromes begin with petechial hemorrhage on the skin, axillary and inguinal areas, arms, thighs, face and head.
2. Necrosis begins in the throat and spreads throughout the mouth and into the larynx, vocal cords, lungs stomach and bowels.

3. Impaired hematopoietic and reticuloendothelial systems permit widespread bacterial infection in the necrotic areas. Lymph nodes become enlarged.
4. Laryngeal edema and stenosis of the glottis caused by esophageal lesion, resulting in death because of asphyxiation.

Fourth Stage

Recovery period if clinical help is provided in time.

YELLOWED RICE SYNDROME

History

Epidemics of an acute heart disease broke out in rural Japan a hundred years ago, the etiology of which was never determined. Uraguchi (1971) analyzed the records of cases of the disease called acute cardiac beriberi (Shoshinkakke) and concluded that the ailment was probably a human mycotoxicosis.

Acute cardiac beriberi was associated with the consumption of polished rice and was initially thought to be an avitaminosis. In 1910, however, the Japanese government took action to exclude mouldy rice from the markets and the incidence of acute cardiac beriberi dropped dramatically.

Uraguchi (1971) suggested that acute cardiac beriberi may have resulted from eating "yellowed rice" Such a foodstuff became pigmented and toxic to rats, and produced symptoms similar to those observed in humans affected with beriberi.

Penicillium sp.

phialide conidia conidiophore

Mycology

Toxin producing fungi: *Penicillium islandicum* (Luteoskyrin and cyclochlorotine), *P. citreoviride* (citreoviridin), *P. rugulosum* (Rugutosin) and *citrinum* (citrinin).

Chemistry

The empirical formula of citreoviridin is C₂₃ H₃₀ O₆. It contains one methoxy

group and double bonds.

Clinical Symptoms

The clinical manifestations of acute cardiac beriberi, begin with palpitation, precordial distress, and tachypnea; followed by nausea and vomiting, and difficult breathing.

Within a few days, the patient suffers severe anguish, pain, severe restlessness, or sometimes violent mania. The right heart is dilated, heart sounds are abnormal, blood pressure is low, and pulse is rapid, sometimes exceeding 120 beats/min, and the patient faints.

The dyspnea increases, the skin of the extremities becomes cold, dry, and cyanotic, and the voice becomes husky, Finally the pulse becomes feeble, the pupils dilated, consciousness is lost, and respiration fails.

BALKAN NEPHROPATHY

History

In 1957 to 1958, an unusual chronic disease of the kidney occurred endemically in Yugoslavia, Rumania, and Bulgaria with a prevalence of 3-8 percent, mainly in rural areas where food is home grown. It was common in 30-50 year- old - females. In Yugoslavia, 6.5 percent of blood samples contained ochratoxin A at concentrations between 3 and 5 mg/g serum (Hurt et a/, 1982).

Barnes (1967) suggested that plant toxins or mycotoxins may be an environmental factor causing this human disease. Krogh et al, (1974) presented preliminary evidence to associate the human disease with ingestion of ochratoxin A (OTA).

This nephrotoxic compound occurs in feeds and foodstuffs and is considered a major determinant of porcine nephropathy, a form of kidney damage strikingly similar to that seen in Balkan nephropathy cases.

Renal porcine nephropathy has been reported regularly from Denmark since 1928. The law in Denmark requires that all abnormal gross appearance of kidneys must be analysed for OTA and the toxin concentration exceeds 10 g/g which corresponds to 50 g/ml in the blood, the entire carcass is condemned. Nephropathy has also been

reported in chickens.

Mycology

Toxin-producing fungi: *Aspergillus ochraceus* and *Penicillium viridicatum*

Chemistry

Ochratoxin is a dihydroisocoumarin derivative produced by seven species of *Penicillium* and six species of *Aspergillus* including *A. ochraceus*.

Clinical Symptoms

The disease is of indefinite onset without acute manifestations. Among the earliest and most frequent complaints are headache, lassitude, easy fatigue, and anorexia.

The typical syndrome includes a shallow, coppercolored skin, yellowing of the palms and soles, anemia in the preazotemic stage, and perhaps occasional profuse intermitent hematuria, due to tumors of the urinary passages; there is no hypertension or edema.

It becomes evident that in the endemic villages, when a high incidence of urinary tract tumors parallels the EN occurrence and both diseases follow a similar pattern of geographic clustering, age and a sex distribution (Chernozemsky et al, 1977).

AFLATOXINS

History

Historically, the aflatoxins were discovered as a consequence to the death of 100,000 of turkey poults ("Turkey X disease"), ducklings and chicks in England in 1960 with a loss of at least several hundred thousand dollars. The problem was eventually traced to feed contamination, specifically a shipment of Brazilian peanut meal used as poultry feed produced by Old Cake Mills, Ltd. in London. This meal, termed Rosetti meal (from the name of the ship in which it was imported), proved to be both toxic and carcinogenic and was found to be contaminated with the common fungus, *Aspergillus flavus*.

The active principles were extracted and isolated from *A. flavus* cultures by a group

in England and the Netherlands (Van der Zijden. et al, 1962; Nesbitt et al, 1962), chemically identified by a research group in the U.S. (Asao et al, 1963), and named aflatoxin the "a" from *Aspergillus* and the "fla" from flavus.

The aflatoxins are a group of secondary fungal metabolites which have been epidemiologically implicated as environmental toxin and carcinogens in man. They are substituted coumarins containing a fused dihydrofurofuran moiety. There are four primary aflatoxins, named B1, B2, G1 and G2, from their blue and green fluorescence, respectively, on thin-layer chromatographic plates. As was generally known to be the case with aflatoxin toxicity and carcinogenicity, a similar potency series, namely $AFB1 > AFB2 > AFG1 > AFG2 >$ has been established for aflatoxin - induced mutagenic activity and DNA damage.

Aflatoxin metabolites

AFM1 is a 4-hydroxylation of AFB1 at the 4 position. This metabolite was first detected in the milk of cows ingesting AFB1. It has also been detected in the urine of humans consuming AFB1 contaminated peanut butter. AFM1 could induce typical bile duct hyperplasia in day - old ducklings characteristic of AFB1.

This hemiacetal AFB₂a metabolite is produced by hepatic microsomes from AFB₁ by hydration of the 2, 3 vinyl ether double bond resulting in hydroxylation at the 2 position.

It is possible that AFB₂a plays an important role in aflatoxin acute toxicity by binding and inhibiting key enzymes of intermediary metabolism and resulting in liver cell necrosis.

AFP₁ is produced by the O-demethylation of AFB₁ and was the major excretory product in the urine of AFB₁-treated rhesus monkeys, where it was present as glucuronide and sulfate conjugates. It is formed in vitro by human microsomes. AFP₁ was nontoxic to chicken embryos and nonmutagenic.

AFQ₁ is formed from AFB₁ by ring hydroxylation of the carbon atom to the carbonyl function of the cyclopentenone ring. It represents one-third to one-half of the metabolites produced from AFB₁ by monkey and human liver microsomes. This metabolite is nontoxic and only 1-2% is mutagenic as AFB₁ in Ames assay.

Reduction of the cyclopentenone carbonyl function of AFB₁ to hydroxy group by an NADPH dependent cytoplasmic enzyme produced AFL. The toxicity of AFL is only

1/18 that of AFB1 in one day - old duckling bile duct hyperplasia assay.

AFLH was formed from AFB1, using both the microsomes and soluble enzyme preparation from human liver. (Salhab and Hisch, 1975). The compound is a dihydroxyl derivative of AFB1, with substitutions at a cyclopentenone carbonyl function and the β carbon. It was not toxic to the chicken embryo test.

Aflatoxin and Acute Poisoning

1. Taiwan Outbreak

In 1967, there was an outbreak of apparent poisoning of 26 persons in two Taiwan rural villages (Ling et al, 1967). The victims had consumed moldy rice for up to 3 weeks; they developed edema of the legs and feet, abdominal pain, vomiting, and palpable livers, but no fever. The three fatal cases were children between 4 and 8 years. Autopsies were not done, and the caused of death could not be established. In a retrospective analysis of the outbreak, a few rice samples from affected households were assayed for aflatoxins. Two of the samples contained up to 200 ppb

aflatoxin B1.

2. Kenya Case

In 1982, an acute hepatitis was reported in Kenya. There were 12 of 20 cases who died with malaise, abdominal discomfort, with subsequent appearance of dark urine and jaundice. Local dogs who shared the food were affected, with many deaths. Stored grain appeared to be the cause of the outbreak. Aflatoxin was detected in two liver samples (39 and 89 ppb). Histologically, there was centrolobular necrosis.

3. Uganda Case

Aflatoxin B1 was circumstantially associated with the death of a 15-year-old African boy in Uganda (Serch - Hanssen, 1970). The youth, his younger brother, and his sister became ill at the same time; the young sibling survived, but the older boy died 6 days later with symptoms resembling the victims in the Taiwan outbreak.

An autopsy revealed pulmonary edema, flabby heart, and diffuse necrosis of the liver. Histology demonstrated centrolobular necrosis with a mild fatty liver, in addition to the edema and congestion in the lungs.

A sample of the cassava eaten by these children contained 1.7 ppm aflatoxin which Alpert and Serck Hanssen (1970) suggest may be lethal if such a diet is consumed over a few weeks. This estimate is based on the acute toxicity of aflatoxin B1 in monkeys.

4. Reye's Syndrome

Reye's syndrome is an acute and often fatal childhood illness which is characterized by encephalopathy and fatty degeneration of viscera (EFDV). This syndrome was first described between 1951 and 1962 in Austraria by Reye et al.

Clinically, the main features of this syndrome are vomiting, convulsions and coma. Hypoglycemia, hypogly, corrhachia and elevated serum transaminases are the most constant biochemical abnormalities. Fatty degeneration in the liver and kidneys, and cerebral edema are the major autopsy findings.

Thailand case

As reported by Bourgeois et al (1971), a 3-year-old Thai boy was brought to a Northeast provincial hospital after a 12-hr illness of fever, vomiting, coma and

convulsions. The child died 6 hours later, and an autopsy revealed marked cerebral with neuronal degeneration, severe fatty metamorphosis of the liver, kidneys, and heart, and lymphocytolysis in the spleen, thymus, and lymph nodes.

Upon admission of the child to the hospital, a medical team travelled to the boy's home and obtained a small sample of steamed glutinous rice which had been cooked 2 days before the onset of the child's illness and reportedly has been the only food the family had for the past 2 days. The small size of the sample precluded an accurate measurement of the amount of aflatoxins present but clinical assay indicated the amount was in the parts per million range. The rice examined also contained toxigenic strains of *A. flavus*, *A. clavatus*, *A. ochraceus*, and *A. niger*, (as shown in Table 1, Angsubhakorn et al, 1978).

Table 1: Toxins which may have caused death in a boy with Reye's syndrome in the village of Baan Kota, Khonkaen Province, Thailand.

Fungi isolated	Toxins produced	LD50(mg.kg ⁻¹) in rat	Organs involved
A. clavatus	Kotantin	—	—

	Desmethyl Kotantin	—	—
	Cytochalasin E	0.98 (1 day-Old)	Brain-edema
	Tryptoquivaline	—	Tremorgen
	Tryptoquivalone	—	Tremorgen
A. niger	Malciformin G.	0.9 (newborn)	—
		0.87	—
		(28 day-old)	—
A. ochraceous	Ochratoxin A.	22	Renal tubular necrosis
A. flavus	Aflatoxin B1	7.2 (weanling)	Hepatic periportal necrosis

The B. form was found in one or more autopsy specimens from 22 of the 23 Reye's syndrome cases (Shank et al, 1971) and in several instances, these

aflatoxin concentrations were as high as those seen in specimens from monkeys poisoned with the aflatoxins (Bourgeois, et al, 1971) (Table 2).

Table 2: Comparison of aflatoxin B1 concentration in autopsy specimens from Reye's syndromecases and experimetal monkeys poisoned with aflatoxin B1.

Specimen	Aflatoxin B1 concentrations (g/kg specimen or ml fulid)	
	Human*	Monkey (AFB1 mg.kg-1)
Brain	1-4	30 (40 5)
Liver	93	163(40.5)
Kidney	1-4	87(40.5)
Bile	8	163(40.5)
Stool	123	—
Stomach content	127	—

The trace amounts of aflatoxins in tissue specimens from control cases is thought to reflect chronic low-level ingestion of the mycotoxin in that area of Thailand.

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New Zealand Case

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Others have also reported aflatoxin residues in autopsy specimens of children dying of Reye's syndrome. Becroft suggested that contamination of foods by aflatoxin may have a role in the etiology of Reye's syndrome, in an analysis of

liver specimens from two children who died of Reye's syndrome (Becroft and Webster, 1972). The amount of aflatoxin B. present was estimated to be in the range of 5 to 50 g/kg liver in each specimen (5-50 ppb).

Czechoslovakia Case

Dvorachova et al (1972), reported finding aflatoxin B. in liver specimens of two infants, who died with liver damage and encephalopathy, and later added four more cases of infants, who died similarly and also had detectable concentrations of afitoxin B1 in their livers.

The United States Case

Two reports in the U.S. have suggested an association between Reye's syndrome and aflatoxin exposure. Chaves-Carballo et al., (1976) found fluorescing material chromatographically similar to aflatoxin G2 in the formaldehyde fixed - liver of a 15 year old Reye's syndrome patient. Similar material could not be found in seven other cases or in 12 control.

German Case

Rosenberg (1972) described the case of a 45-year-old man, who died a short time after an apparent gastric illness. He had eaten an unusually large amount of nuts, which were apparently quite mouldy. The illness was diagnosed as acute yellow atrophy of the liver, but analysis of the liver revealed the presence of a blue fluorescing material which cochromatographed with aflatoxin B. on a thin layer chromatographic (TLC) plate. The author suggests the case may be one of acute aflatoxin poisoning.

Aflatoxin and Sub-acute Poisoning

There are reports which suggest that some outbreaks of sub-acute poisonings contain resulted from ingestion of large amounts of aflatoxins over a period of time; most of those outbreaks involve children.

1. Possible association with Indian Hepatitis

In October 1974, unseasonal rains in 150 villages in Gujerat and Rajasthan western India resulted in extensive mould damage to standing corn crops. The people in these rural areas were poor and were forced to eat the contaminated

grain for lack of alternate foodstuffs. After a few weeks of consuming the mouldy corn, many people became ill with symptoms of liver injury (Krishnamachari, et al, 1975). One hundred and six of 397 patients died. The disease mainly affected male adults and spared infants and children (ages of 6 and 30 years). Patients suffered a sub-acute poisoning with anorexia, vomiting, jaundice and ascites.

Dogs that shared food of affected households also developed ascites and jaundice and died a few weeks after onset. Other domestic animals which did not share the family food were not affected.

Five specimens of mouldy corn were collected from affected households and chemical analysis revealed aflatoxin contents ranging from 6.25 to 15.6 mg/kg corn which is extremely heavy contamination. Aflatoxin B1 was detected in 2 of 7 serum samples collected from patients. Histopathologically, liver specimens revealed extensive bile duct proliferation, periportal fibrosis, and occasional multinucleated giant cells. The authors estimated that the patients had ingested 2 to 6 mg of aflatoxin each day for several weeks.

2. Possible association with Indian Childhood Cirrhosis

In India, liver cirrhosis is the third most common cause of death in hospital among children under the age of 5 years. With its characteristically insidious onset, involving low grade fever, mild abdominal distension followed by enlarged liver with a characteristic leafy border, the disease may progress to jaundice, ascites, fibrosis, cirrhosis, and hepatic coma (Yadgiri et al, 1970, Amla et al, 1971). In one episode (Amla, et al, 1967), children suffering from kwashiorkor were given peanut flour supplement for several weeks until it was discovered that the peanut flour contained 300 ppb aflatoxins. Liver biopsies taken 1-2 months after consumption of the toxic meal showed fatty liver while after some 4 months fibrosis and cirrhosis were apparent.

Aflatoxin and Liver Cancer

Geographic distribution of liver cancer

Primary liver cancer is not a common disease in most areas of the world. There

are particular geographic areas, however, where the annual liver cancer rate is reported to be well above the level (2 cases per 100,000 people). Certain populations in Africa, southern India, Japan, and Southeast Asia have unusually high incidences of liver cancer.

The hazards from chronic exposures to mycotoxins are potential rather than documented.' The evidence for the association of aflatoxins in the cause of liver cancer has been considered strong enough to justify intervention in the food contamination cycle. However, other factors such as the part played by hepatitis, must be assessed.

Several field studies which have associated consumption of aflatoxins with human liver cancer have been documented. The studies took place from 1966 to 1973 in Uganda, the Philippines, Thailand, Kenya, and Swaziland, in approximately that order.

Uganda

The pioneering effort in the field associations was undertaken by Alpert et al/,

(1971) at Harvard Medical School Massachusetts Institute of Technology,

Food samples were collected during the ninemonth period from September 1966 to June 1967 from village markets and home granaries throughout Uganda by staffs and medical students on vacation leave from Kampala. All food specimens were sealed upon collection and kept in cold storage until shipped by air freight to Boston, for chemical assay for aflatoxins.

Analysis of a total of 480 samples of food (Table 3), revealed 29% containing more than 1 ppb aflatoxin and 4% containing more than 1 ppm. Aflatoxins occurred most frequently in bean (72%), whereas maize (45%) peanut (18%) and cassava (12%) were contaminated less frequently. The aflatoxin concentration sometimes exceeded 1000 ppb.

Table 3: Aflatoxin concentration in some Uganda foodstuffs

Foodstuff	Not contaminated	Total aflatoxin cone. (ppb)		
	Not assayed	1-100	100-	1000

	(%)		1000	
Bean	46/64 (72)	30	11	5
Maize	22/49 (45)	13	9	0
Sorghum	26/69 (38)	19	5	5
Groundnut	27/152 (18)	11	8	8
Cassava	4/34 (12)	0	2	2
Others	21/112 (18)	14	3	3
Total (%)	142/480 (29)	87 (29)	37 (8)	18 (4)

At the time the aflatoxin survey was being made, local cancer registry records covering 1964 to 1966 were being studied to estimate the geographical distribution of liver cancer in Uganda.

Table 4 gives the relation between the incidence of liver cancer and the aflatoxin contamination in foodstuffs in Uganda. Hepatoma occurred at an annual rate of 1.0 to 2.7 cases per 100,000 people.

Table 4: Hepatoma incidence and frequency of aflatoxin contamination of

foodstuffs in Uganda.

Area	Hepatoma incidence	% Contamination	Aflatoxin contamination of foodstuffs (%)		
			Total aflatoxin cone. (ppb)		
			1-100	100-1000	1000
Toro	No data	79	10	31	38
Karamoja	6.8	44	24	15	5
Buganda	2-3	29	23	4	1
West Nole	2.7	23	19	4	0
Acholi	2.7	15	15	0	0
Busoga	2.4	10	5	5	0
Ankole	1.4	11	11	0	0

For the Karamojan tribe, hepatoma incidence was 6.8 cases per 100,000 per

year, and the frequency of aflatoxin contamination was 44 %.

Thailand

Over a 23 - month period from September 1967 through July 1969 (Shank et al, 1972) mycological studies on cereal, ok seeds, beans, cassava, dried fish, dried and fresh vegetables and prepared foods showed *Aspergillus flavus* to be the most common contamination fungus. *Penicillium*, *Fusarium*, and *Rhizopus* fungi were also prevalent.

The consumption of aflatoxin was determined by three separate surveys, each of 2 - day duration, over a period of 1 year. Within the three survey areas of Thailand (Singburi, Ratchaburi and Songkhla), samples of food served were collected, and the amounts of each food eaten by the family were measured. Daily aflatoxin ingestion, expressed as nanograms of total aflatoxins consumed per kilogram body weight on family rather than individual basis (Table 5), was highest in Singburi (73 to 81), intermediate in Ratchaburi (45 to 77), and lowest in Songkhia. (5 to 8).

Table 5: Liver cancer incidence and aflatoxin consumption in Thailand

Province	Liver cancer incidence (cases/100,000/year)	Average daily aflatoxin intake (ng/kg bo.wt.)	
		B1	Total
Singburi	—	51 - 55	73 - 81
Ratchaburi	6.0	31-48	45-77
Songkhla	2.0	(5-6)	(5-8)

Incidence of liver cancer, as measured in this survey, was two new cases per year in Songkhla and 6 new cases /100,0001 year in Ratchaburi. National health records indicated that the incidence of primary liver cancer in Singburi area was 14 deaths /100,00/ year, but this rate could not be measured directly as part of the aflatoxin study due to the unavailability of a key figure in the study.

Kenya

Another investigation was conducted in Kenya at the time of the Thailand study (Peers and Linseli, 1973). The main evening meal was sampled over 24 times in sample clusters of individuals distributed in 132 sublocations in the district. The collection period was 21 months. Estimation of the incidence of primary liver cancer in the district was based on data from the Kenya Cancer Registry (Table 6).

Table 6: Hepatoma Incidence and aflatoxin consumption in Kenya.

Altitude area	Liver cancer incidence cases/100,000/year (1967-1970)		Average daily aflatoxin B1 intake (ng/kg bo.wt.)	
	male	female	male	female
Low	12.9	5.4	14.81	10.03
Middle	10.8	3.3	17.84	5.86
High	3.1	1/10,000/4	4.88	3.46

year

In the high altitude area, 39 of 808 samples (5%) contained aflatoxins, mean concentration of which was 0.121 ppb. In the middle area, 7% (54/909) of the samples were contaminated with mean concentration of 0.205 ppb; the low altitude area had a highest frequency, 78 of 816 (10%) of the samples, with 0.351 g/kg, of aflatoxin contamination.

Mozambique

The 1974 Van Rensburg et al study reported results in measuring aflatoxin consumption in Mozambique, in particular the Inhambans district, which showed a liver cancer rate of 35.5 and 25.4/ 105/year for the periods 1964-68 and 1969-71, respectively, with more than twice as many cases in males as in females.

Aflatoxin contamination of prepared foods consumed by the study population was measured by chemical assay of 880 meals. The mean daily per capita consumption of aflatoxins was calculated to be 222.4 mg/kg body weight. Thus, the highest primary liver cancer rate correlates with the highest known aflatoxin intake in the world.

Swaziland

Two studies on aflatoxin and human liver cancer have been performed in Swaziland. In 1971, Keen and Martin found a geographical distribution for aflatoxins in peanut samples from low, middle, and high velds with the distribution of liver cancer cases.

A retrospective survey of primary liver cancer among a group of workers showed that Shangaans had a higher incidence than Swazis, although both tribes were provided with the same lot of groundnut. Interviews from tribal groups indicated that Shangaans ate more peanuts in powder form more often and for longer periods than did the Swazis. The Shangaans powdered the nuts in wooden, fungus - infected mortar, and supplemented their rations with locally purchased groundnut, whereas Swazis did neither of these things.

In 1972, the International Agency for Research on Cancer (IARC) and Tropical Products Institute (TPI) of London initiated a study in Swaziland which was modeled on their earlier study in Murang's district of Kenya.

Table 7: Relation between aflatoxin consumption and liver cancer in Swaziland.

Area	Average daily AFB1 mtake (ng/kg bo.wt.)		Liver cancer incidence adults/100,000/year	
	male	female	male	female
Lowveld	53.34	43.14	26.65	5.62
Lebombo	19.89	15.40	18.65	1/10,000
Middleveld	14.43	8.89	14.79	2.21
Highveld	8.34	5.11	7.02	1.42

Aflatoxin determinations were made from 1056 samples of the main meal and 45.5 sample of beer, etc. The result showed clear correlation between estimated aflatoxin consumption and liver cancer rates.

The Philippines

Peanut butter and maize have been shown to be contributors of aflatoxins to the Philippines food products (Campbell and Salamat, 1971). Aflatoxins were found in almost all of the 149 samples of peanut butter, with an average concentration of AFB1 of 213 ppb. The most heavily contaminated sample of peanut butter contained 8.6 ppm AFB1 whereas 95 of 98 maize samples analysed contained an average of 110 ppb AFB1.

EPILOGUE

Epidemiological studies have associated ingestion of aflatoxins with human liver disease and others in certain populations, but this does not necessarily indicate that the aflatoxins are the sole causative agents.

1. Relation between chemicals and aflatoxins

Diets that can contain appreciable quantities of aflatoxins would appear capable of containing other mycotoxins, i.e. sterigmatocystin, ochratoxin, penicillic acid, etc; organochlorine insecticides ie. DDT, Lindane; nitrosamines and certain

plant toxins, ie. pyrrolizidine alkaloids, etc.

The synergistic effect of dimethylnitrosamine on liver tumor induced by sterigmatocystin, a fungal product of *Aspergillus versicolor* which is about ten times less carcinogenic than AFB1 was reported (Terao, et al. 1978). Moreover in our previous study, we also observed the synergistic effect of dimethylnitrosamine on liver tumor induced by the most potent hepatocarcinogenic mycotoxin, aflatoxin B. (Angsubhakorn, et al., 1981).

Table 8: Summary of histopathological changes during DMN and AFB1 induced liver cancer in rats at 12 month.

Diet	Rats with Lesions			
	No. of rats	Foci of cell alteration	Neoplastic nodules	Hepatocellular carcinomas(%)
Control	14	0	0	0
AFB1	20	27	7	9(45)

DMN	21	13	0	1(5)
AFB1 + DMN	14	12	11	11(79)

The alpha isomer of 1, 2, 3, 4, 5, 6 hexachlorocyclohexane of lindane, inhibits the development of liver tumors induced by AFB1 (Table 9) (Angsub hakorn et al, 1978,1981).

Table 9: Liver tumor in long term experiment.

Experimental group.	Strain of rats	
	Fisher	Buffalo
Control	0/3	0/7
AFB1	5/5(100%)	6/6(100%)
??- BHC	0/5	0/10
??- BHC + AFB1	0/8	0/8

Our previous work using gamma isomer of lindane supports the previous results

demonstrating protection against AFB1 - induced carcinoma in rodent liver after long term administration of high doses of delta - HCH (Angsubhakorn, et al, 1989) (Table 10).

Table 10: Liver tumor in long term experiment.

Experimental group	No. animals examined	No. liver-tumor-bearing animals (%)
1. control	23	0
2. LD + AF, 1 wk	20	1(5)
3. LD + AF, 3 wk	23	0
4. LD + AF, 5 wk	17	0
5. LD + AF, 10 wk	22	0
6. LD + AF, 15 wk	13	0
7. LD, 15 wk	18	0
8. AF, 15 wk	19	6(315)

It has indicated that aflatoxin and alcohol when consumed concurrently, can act synergistically in the development of primary liver cancer in man (BulatoJayme et al. 1982) and hepatotoxicity in rats (Toskulkao et. al. 1982).

2. Relation between virus and aflatoxins

Epidemiological evidence strongly implicates both hepatitis B virus and aflatoxin as important agents in the production of hepatocellular carcinoma. Many have speculated that the two factors are co-carcinogens. However, data derived by many workers suggest that the risk of hepatoma -is no greater in chronic H. BV carriers from Mozambique than in those from the U.S.A. The difference between hepatoma-rates in the two countries, therefore, relates significantly to the H.B.V. carrier rate in the population. Aflatoxin may act primarily as an immuno-suppressive agent causing an increase in H.B.V. carriers.

Experimentally, the newly described woodchuck hepatitis virus (Summers, et at, 1978) which morphologically and clinico-pathologically seems strikingly similar to H.RV., could provide an ideal system to test this hypothesis. If this hypothesis proves to be correct, a vaccine to prevent hepatitis B could be used to eradicate

hepatoma without the major economic problem of changing farming and crop storage and food consumption by populations likely to be exposed to aflatoxin.

3. Relation between parasites and aflatoxins

Parasitic infection may influence carcinogens. The northeastern part of Thailand which has a higher death rate due to liver cancer in adult who suffered to some extent from liver fluke disease, is also an area of high exposure to aflatoxins.

The interaction between human liver flukes nitrosamine and bile-duct tumor was first demonstrated in hamsters (Thamavit et al, 1978). Moreover, interaction between rodent malarial parasite, aflatoxin and hepatic cell tumor were also reported (Angsubhakorn, et al, 1986, 1988). The mechanism by which malarial infection enhances or reduces aflatoxin-induced liver tumor has not been determined but may be related to effects of immunologic or metabolic activation of malarial parasite antigen.

4. Relation between other cancer and aflatoxin

Human health risk associated with occupational exposure to aflatoxin-contaminated food stuffs, an issue that has received little attention, should be considered in the present time. Aflatoxincontaminated dust may be a causative factor in the cause of nasal cancer but not cancer of liver.

Czechoslovakia

In Czechoslovakia, two chemical engineers who had worked on a method for sterilizing peanut meal infected with *A. flavus* were reported to have died from lung cancer. One of them, who died at 68 year of age, had done this work only 3 months, and developed symptoms 3 months later. No details were provided for the second engineer (Dvorachava, 1976).

England

Two British biochemists developed cancer of the colon after exposure to purified aflatoxins. One of them had worked with this material from 1962 to 1964 and developed symptoms in 1971 at 42 year of age. The second had done this work for 12 months between 1969 and 1970 and developed symptoms in

1972, at 28 years of age (Deger, 1979).

5. Other Possible Cases

It has generally been assumed that exposures to large amounts of aflatoxin and presumably most other mycotoxins occur only in areas of the world with a tropical climate and with agricultural practices not common to the technologically modern countries in the western hemisphere. With greater experience and at closer scrutiny, however, it appears this assumption may not be valid. However, most foodstuff produced and sold in the advanced temperate countries appear to contain at most only trace amounts of aflatoxins.

It is conceivable that silo operators when handling mouldy grains could be exposed to high concentrations of airborne mycotoxins; similar conditions may also occur in a food - processing factory where vegetable oils are obtained from nut and seeds mainly peanut and linseed (Van Nienwenhize, et al, 1973). During all phases of the process great quantities of dust are created in the work areas, especially during the dumping, transportation and bagging of the residues. The authors estimated a range of exposures for a workweek (45 hr) of

0.039-2.5 g aflatoxin which is approximately 10 % of the exposure seen in the dietary studies of Asia and Africa. From an epidemiological study, 11 out of 55 workers developed various forms of cancer (4 cases of bronchial carcinomas and carcinoma of the bladder (1), gastrointestinal tract (1), liver (1), maxillary sinus (1), prostate (1), anaplastic carcinoma (1) and pleura mesothelioma).

6. Mycotoxin Residues in Animal Tissues

The greatest demonstrated potentials for encountering mycotoxin residue in edible animal tissue are aflatoxin M in cow's milk and ochratoxin A in swine kidney. Aflatoxin B. is the mycotoxin that has been most extensively studied for possible residues in tissues. The various controlled studies of aflatoxin transmission from feed to edible tissue have been reduced to a table of feed to tissue ratios for cow's milk, chicken eggs, and the livers of cattle, swine and chickens. (Table 11)

Table 11 Ratios of AFB1 levels in feed to AFB1 or M1 levels in edible tissue.

Animal	Tissue	Aflatoxin in	Feed to
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		tissue	tissue ratio
Layers	Eggs	B1	2,200
Dairy cattle	Milk	M1	300
Beef cattle	Liver	B1	14,000
Swine	Liver	B1	800
Boiler	Liver	M1	1,200

Most of the eggs and milk data are based on a continuous feeding regimen; the liver data are based on slaughter 18-24 hours after the last exposure of aflatoxin. It is shown that milk is the tissue most vulnerable to aflatoxin contamination, particularly swine feed consumption and lactation are concurrent events, i.e. there is no withdrawal period.

Occurrence of ochratoxin A residues in poultry and swine kidneys was established in Denmark, Sweden and Yugoslavia. The obvious explanation was that both diseases had common cause in ochratoxincontaminated grains used for feed

In the United States, an action level of 0.5 g/ml has been set for AFM1 in milk

and 20 ppb in food. In Denmark, If ochratoxin is detected in the kidneys, the whole carcass will be condemned.

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Common mycotoxigenic species of fusarium

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by Ailsa D. Hocking

Fusarium

Fusarium is one of the most important genera of plant pathogenic fungi on earth, with a record of devastating infections in many kinds of economically important plants. Fusarium species are responsible for wilts, blights, root rots and cankers in legumes, coffee, pine trees, wheat, corn, carnations and grasses. The importance of Fusarium species in the current context is that infection may sometimes occur in developing seeds, especially in cereals, and also in maturing fruits and vegetables. An immediate potential for toxin production in foods is apparent.

The very important role of *Fusaria* as mycotoxin producers appears to have remained largely unsuspected until the 1970s. Research has now unequivocally established the role of *Fusaria* as the cause of alimentary toxic aleukia (ATA). This was the previously mentioned human mycotoxicosis epidemic in the USSR which killed an estimated 100,000 people between 1942 and 1948 (Joffe, 1978). ATA is also known to have occurred in Russia in 1932 and 1913, and there is little doubt that outbreaks occurred in earlier years as well. Matossian (1981) has argued persuasively that ATA occurred in other countries, including England, in the 16th to 18th centuries at least.

Research since 1970 has shown that *Fusarium* species are capable of producing a bewildering array of mycotoxins. Foremost among these are the trichothecenes, of which at least 50 are known: the majority are produced by *Fusaria*. The most notorious is T-2 toxin, which was responsible for ATA (Joffe, 1978). Other *Fusarium* mycotoxins are known to be highly toxic to animals, and are suspected to be responsible for acute and chronic human diseases also.

Taxonomy

The taxonomy of *Fusarium* was in disarray until recently, with several competing taxonomic schemes, recognising from 9 to 60 species in the genus. A determined attack on this problem by an international collaborative group has resolved most of the conflict, and the taxonomy of Nelson et al. (1983) has met with widespread approval. Nelson et al. (1983) accepted 30 species.

A direct consequence of confusion in taxonomy is confusion over species mycotoxin associations. *Fusarium* isolates producing a particular toxin have been given different names as a result of the different taxonomic systems used, or simply as a result of misidentification. Marasas et al. (1984) intensively studied more than 200 toxigenic *Fusarium* isolates, and provided accurate information on species identifications and the corresponding toxins produced. They listed 24 *Fusarium* species with confirmed toxigenicity. The four species judged to be most important from the viewpoint of human health, *F. sporotrichioides*, *F. equiseti*, *F. graminearum* and *F. moniliforme*, are discussed below.

Enumeration

Growth of Fusarium species is favoured by dilute, high aw (water activity) media. Enumeration of fusaria can be effectively carried out on PDA (Booth, 1971) provided chloramphenicol or other broad spectrum antibiotics are added to suppress bacteria. However, acidified PDA, a frequently used antibacterial medium, is not recommended because it may inhibit sensitive cells (King et al., 1986). DCPA (Andrews and Pitt, 1986) is an effective enumeration and isolation medium for most food-borne Fusarium species. Recognition of Fusarium colonies on any of these media requires careful observation and experience. Presumptive identification to genus level can usually be made from colony appearance: low to floccose colonies, coloured white, pink or purple, with pale to red or purple reverses, are indicative of Fusarium. Confirmation requires microscopical examination, where the crescent-shaped macroconidia characteristic of the genus should be observed. These are not always produced on enumeration media, especially PDA, however. Differentiation of some species on enumeration media is possible, but also requires experience.

Identification

Identification of Fusarium species is ideally carried from growth on carnation

leaf agar, an effective medium for macroconidium production (Nelson et al., 1983), but this medium is not readily available to the nonspecialist. Pitt and Hocking (1985a) provided keys and descriptions enabling identification of the species of interest in foods after growth on the readily available media CYA, MEA and PDA. Hocking and Andrews (1987) reported that DCPA, a medium which encourages production of macroconidia, is a practical alternative medium to carnation leaves for identifying most food-borne species.

As noted above, the current definitive taxonomy is that of Nelson et al. (1983). Burgess et al. (1988) have published a very useful and up to date guide to most species. Pitt and Hocking (1985a) give keys and descriptions of common food-borne species.

Toxins, toxicity and symptoms: General

Trichothecenes, the principal toxins produced by Fusaria, are sesquiterpenes with a basic 12,13-epoxytrichothec-9-ene ring system. Trichothecenes are often produced in mixtures even under pure culture conditions, and are very difficult to separate, so the toxicity of many of these compounds remains uncertain.

Some are known to be highly toxic: none appear to be benign (Cole and Cox, 1981).

Because of the variable quality of the available data, trichothecene toxicity will not be considered in detail. However, as an example, the acute LD50 values for T-2 toxin are of the order of 8-4 mg/kg in rats, pigs and mice. LD50 values for the much less toxic deoxynivalenol have been reported as at least 70 mg/kg in mice; however, only 5 mg/kg in feed causes vomiting in pigs.

The biochemical basis of trichothecene toxicity is noncompetitive inhibition of protein synthesis (Cole and Cox, 1981; Ueno, 1983). Points of attack at the molecular level differ: some trichothecenes attack initiation of protein synthesis, others inhibit elongation or termination. Differences in toxicity and symptoms result.

It is difficult indeed to catalogue the symptoms of trichothecene poisoning. Vomiting, diarrhoea, anorexia and gastro-intestinal inflammation are rapid responses which sometimes occur, but less immediate effects such as skin necrosis, leukopenia, ataxia, haemorrhaging of muscular tissue, and

degeneration of nerve cells are all known (Cole and Cox, 1981; Ueno, 1983). Mortalities may result not only from injection or oral ingestion, but sometimes also from topical application. With the possible exception of the hepatocarcinogenicity of the aflatoxins, trichothecenes constitute the greatest known mycotoxin threat to human and animal health, the more insidious because the symptoms are so variable.

Some other mycotoxins are also produced by Fusarium species: zearalenone, in fact an oestrogen rather than a true mycotoxin; moniliformin, which has a unique four carbon ring structure; fusaric acid, better known as a phytotoxin involved in plant pathogenicity; and, from obviously toxic isolates, toxic principles which have defied isolation or characterisation (Maracas et al., 1984). The most notable of these, produced by *F. moniliforme*, eluded investigators for 15 years (Maracas et al., 1984). It has recently been isolated, characterised, and named fumoinisin B (Bezuidenhout et al., 1988).

Most Fusarium toxins have been shown to possess only acute toxicity. However, strong circumstantial evidence suggests that some may be involved in human cancer. Fumonisin B, which is a bizarre molecule believed to cause

leucoencephalomalasia in horses, is also reported to induce liver cancer in rats (Gelderblom et al., 1988). Involvement of this compound in oesophageal cancer in southern Africa appears likely.

Finally, the possible implication of trichothecenes in the controversial "yellow rain" episode in Laos must be mentioned (Mirocha et al., 1983; Nowicke and Meselson, 1984). The discussions of this controversy show strong political bias and the facts about the alleged use of mycotoxins as a biological warfare agent remain obscure. However, the fact that trichothecene toxins could have caused many of the reported symptoms is beyond dispute (Maracas et al., 1984).

Distribution in nature and in foods

Fusarium species are primarily plant pathogens, and occur mostly in association with plants and cultivated soils. In many cases, particular plant species associations are known or can be predicted. Such associations will be described under the individual species which follow.

Unlike most *Aspergillus* and *Penicillium* species, *Fusaria* grow in crops before

harvest, and grow only at high aw levels. Mycotoxins are therefore usually only produced before or immediately after harvest.

F. sporotrichloides

In the years 1942-1948, at least 100,000 Russian people died from a mysterious epidemic. Illness and death occurred mostly but not exclusively in the Orenburg district near the Caspian Sea. In some localities, up to 60% of the population were affected, and up to 10% died (Joffe, 1978). The disease, now called Alimentary Toxic Aleukia (ATA), has since been shown to have occurred in Russia twice previously in this century, in 1932 and 1913, and perhaps elsewhere in Europe, including England, in earlier centuries (Matossian, 1981).

ATA is an exceptionally unpleasant disease. Symptoms include fever, haemorrhagic rash, bleeding from nose, throat and gums, necrotic angina, extreme leucopenia, agranulocytosis, sepsis and exhaustion of the bone marrow (Joffe, 1978). These symptoms more closely resemble those of radiation sickness than bacterial or other fungal toxicoses.

The direct cause of ATA in Russia in the 1940s was consumption of bread and other cereal products made from grains which were left in fields over winter due to wartime labour shortages. This became clear about 1950. However the fact that ATA was a mycotoxicosis was not finally established until the mid1970s, when it was proved that T-2 toxin was produced by *Fusarium sporotrichioides* and the closely related species *F. poae* during growth in freezing and thawing cycles (Yagen et al., 1977; Joffe, 1978).

***F. sporotrichioides* has also been implicated in a variety of very serious animal diseases, including scabby grain intoxication and bean hull poisoning in Japan, mouldy corn toxicosis in the USA, and fescue foot in the USA, Australia and New Zealand (Maracas et al., 1984).**

Taxonomy

***Fusarium sporotrichioides*, classified in *Fusarium* Section *Sporotrichiella*, is now a well circumscribed species (Nelson et al., 1983). However, many literature reports have misidentified this species as *F. tricinctum* sensu Snyder and no boldface currently accepted species. *F. tricinctum* sensu stricto is in fact a**

species of low toxicity. Much of this confusion has been rectified by Marasas et al. (1984).

Identification

Colonies of *F. sporotrichioides* grow rapidly on CYA, MEA, PDA or DCPA, are deep and floccose, with mycelium coloured pale pink or salmon, and reverse on PDA greyish rose to burgundy. Macroconidia and microconidia are abundant on DCPA: microconidia are both fusiform and pear-shaped, and are borne from phialides with more than one fertile pore.

Toxins and toxicity

As well as T-2 toxin, some isolates of *F. sporotrichioides* are known to produce butenolide, fusarenon-X, neosolaniol and nivalenol. Zearalenone, deoxynivalenol and some less well characterised trichothecenes occur less frequently (Maracas et al. 1984).

The patterns of toxicity shown by isolates of this species depend on the relative production of these various toxins. All animal species studied are affected by

them, in various ways.

Symptoms

T-2 toxin, produced by *F. sporotrichioides*, is the most important human food poison which can result from ingestion of mouldy grain. The symptoms of this intoxication have been described above.

Distribution in nature and food

Fortunately, *Fusarium sporotrichioides* is not a commonly occurring species. It is found mainly in temperate regions on cereal crops, although it has also been isolated from peanuts and soy beans (Pitt and Hocking, 1985a). Indications are that T-2 production is favoured by growth at low temperatures, but experimental evidence remains incomplete.

F. equiseti

A broad spectrum plant pathogen and soil saprophyte of widespread distribution, *F. equiseti* has a long history of association with animal disease, and

a possible implication in human leukaemia. Conclusive evidence of the latter is lacking. However, given its widespread distribution and the long list of mycotoxins produced (Maracas et al., 1984), the potential for this species to cause human and animal disease cannot be ignored. Confusion over the name of this species in earlier years precludes any detailed discussion of its history.

Taxonomy

F. equiseti is classified by Nelson et al. (1983) in Fusarium Section Gibbosum. It has a teleomorph (sexual state) called Gibberella intricans, which has been recorded from nature quite rarely (Booth, 1971). Consequently, use of the Fusarium name for this species is to be preferred.

Much of the earlier literature on F. equiseti is located under the names F. roseum sensu Snyder and Hansen (1940), F. roseum 'Gibbosum', F. roseum 'Avenaceum', F. roseum 'Culmorum', or F. roseum 'equiseti' and several other distinct species as well. Hence it is now frequently impossible to determine which of the names used by earlier authors actually refers to F. equiseti (Maracas et al., 1984).

Identification

Colonies of *F. equiseti* on CYA, MEA and PDA usually cover the whole Petri dish, while those on DCPA are smaller. Mycelium and colony reverses are not strongly coloured, but white or pale salmon to brown. Macroconidia are distinctly curved ("hunch backed"); microconidia are not produced.

Toxins and toxicity

Mycotoxins produced by *F. equiseti* include nivalenol, fusarenon X, T-2, diacetoxyscirpenol, butenolide, zearalenone and several others less well characterised: an impressive list (Maracas et al., 1984). Several of these may be produced simultaneously. Suzuki et al. (1980) reported production of nivalenol, diacetoxyscirpenol and fusarenon-X by 16 of 25 isolates of *F. equiseti* in Japan. T-2, zearalenone and butenolide occur less commonly.

Association of *F. equiseti* with human leukaemia has been reported, and diacetoxyscirpenol is suggested as a possible cause. *F. equiseti* was isolated from dust in a house in the United States where two people had developed

leukaemia, and the isolate was capable of depressing the immune response of guinea pigs (Wray et al., 1979). Previous work by the same authors had suggested a *Fusarium* species was the cause of leukaemia in a U.S. house where four cases had occurred (Wray and O'Steen, 1975).

Associations have been reported linking *F. equiseti* with animal diseases such as degnala, a disease of buffalo eating rice straw in Pakistan and India, bean hull poisoning of horses in Japan, and tibial dyschondroplasia, a bone disease of poultry (Maracas et al., 1984). Clear evidence of causation remains elusive in each case

Symptoms

As with other intoxications caused by *Fusaria*, symptoms of poisoning caused by *F. equiseti* are very varied, reflecting the insidious nature of trichothecene toxicoses, the range of toxins produced by a single species and the proportion of each toxin formed under the influence of substrate, temperature and water activity.

F. equiseti has been reported from several types of grains, and no doubt toxins produced by this species are consumed in human food in many places from time to time. The wide range of diseases produced in animals, as indicated above, provide little clue to the recognition of symptoms in humans.

Distribution in nature and foods

A cosmopolitan soil fungus, **F. equiseti** has a distribution extending from Alaska to the tropics (Domsch et al., 1980). It has also been isolated from a wide variety of plants, where it causes stem and root rots in particular (Booth, 1971; Nelson et al., 1983). **F. equiseti** has been reported from a variety of cereal grains, especially maize and barley (Maracas et al., 1979) but relatively from other foods (Pitt and Hocking, 1985a).

F. graminearum

As with most **Fusarium** species, the history and toxicological importance of **F. graminearum** is obscured by the confusion over its identity. Now that the taxonomic problems have been clarified, it is recognised that **F. graminearum**

causes oestrogenic syndromes, feed refusal and emetic syndromes in pigs and sometimes other animals, and is very likely to be the cause of human Akakabi-byo (scabby grain intoxication) in Japan (Yoshizawa, 1983).

Taxonomy

In the classification of Nelson et al. (1983), *F. graminearum* is placed in the Section *Discolor*. This species has a well recognised teleomorph (sexual state), *Gibberella zeae*. Literature references to *G. zeae* are frequent, and usually correct. Choice of the *Fusarium* or *Gibberella* name for this species ultimately depends on the individual author's preference, but should also reflect the predominant state being isolated or studied.

Two quite distinct biotypes of this species have been encountered in Australia. *F. graminearum* Group I, the cause of crown rot of wheat, produces the *Gibberella* state only when isolates are mated in compatible pairs. However, *F. graminearum* Group II, which causes diseases in aerial parts and grains of cereals, readily forms the *Gibberella* state in single isolate (and single spore) culture (Burgess et al., 1988).

As with *F. equiseti*, the name *F. roseum sensu Snyder and Hansen (1940)* has caused great confusion in the literature related to *F. graminearum*.

Identification

Colonies of *F. graminearum* fill the Petri dish when grown on CYA, MEA or PDA for 7 days; colonies on DCPA are somewhat smaller. On CYA and MEA, colonies are floccose, in muted or pastel shades of greyish red or yellow. On PDA, colonies are usually highly coloured, with dense to floccose greyish rose to golden brown mycelium and a dark ruby reverse, while on DCPA, colony appearance is dominated by salmon to orange clusters of macroconidia, often in concentric rings. Macroconidia are relatively straight and thick walled, with a foot-shaped basal cell. Microconidia are not produced.

Toxins, toxicity and symptoms

The principal toxins produced by *F. graminearum* are well defined: deoxynivalenol (DON; also known as vomitoxin), nivalenol and zearalenone (Maracas et al., 1984). Reports of the occurrence of diacetoxyscirpenol,

fusarenon-X and butenolide are accepted by Marasas et al. (1984), but the frequency of production appears to be much lower. Production of T-2 remains equivocal. Some minor toxins are also produced (Maracas et al., 1984).

The toxicity of the major F: graminearum toxins is undoubted, but until recently the picture has been clouded by the fact that some isolates identified as this species can produce T-2. Even low levels of this very toxic compound, difficult to separate out or often even to detect, can cause symptoms which have been wrongly attributed to the other toxins. The recent production of gram quantities of pure DON (Miller et al., 1984) will shortly result in much more accurate toxicity studies.

One point is clear: DON causes vomiting and feed refusal in pigs at levels near 5 mg/kg of feed. Although very low limits have been set for DON in human foods in the USA, Canada and Japan, its toxicity to species other than pigs remains to be defined, and appears unlikely to be high.

The oestrogenic effect of zearalenone in animals is a well defined syndrome. Corn, barley and wheat grains infected with F. graminearum and producing

zearalenone cause genital problems in domestic animals, especially pigs. Symptoms include hyperemia and edematous swelling of the vulva in prepubertal gilts, or in more severe cases prolapse of the vagina and rectum. Reproductive disorders in sows include infertility, foetal resorption or mummification, abortions, reduced litter size and small piglets. Male pigs are also affected: atrophy of testes, decreased libido and hypertrophy of the mammary glands are all well documented (Maracas et a/., 1984).

At present, *F. graminearum* isolates which produce nivalenol are known only from Japan, and the significance of this mycotoxin in the environment is not clear. However, in Japan, sporadic epiphytotics of "akakabi-byo" (red mould disease) occur, most probably due to the common occurrence of *F. graminearum* on wheat, barley, oats, rye and rice in Japan. Symptoms include anorexia, nausea, vomiting, headache, abdominal pain, diarrhoea, chills, giddiness and convulsions (Yoshizaw, 1983). Deoxynivalenol and zearalenone appear unlikely to be the prime causes of this range of symptoms: nivalenol is a more likely candidate (Yoshizawa, 1983; Marasas et al., 1984).

Metabolites of *F. graminearum* enter the human diet through cereal

consumption in other parts of the world also. Deoxynivalenol, nivalenol and zearalenone have all been reported from corn, corn meal and other corn products, wheat and breakfast cereals in the USA, Canada and Africa. Possible effects in humans remain undefined.

Distribution in nature and food

F. graminearum is primarily a pathogen of gramineous plants, particularly wheat, causing crown rot at the base of the stem, and head scab in developing grain. It also causes cob rot of corn in many countries, including the wetter areas of Europe, North America, Africa and Australia (Maracas et al., 1984) It is uncommon in other situations, or other foods (Pitt and Hocking 1985a).

F. moniliforme

Fusarium moniliforme Sheldon, also known as verticillioides (Sacc.) Nirenberg, was described more than a century ago as a species occurring on corn. Reports of its possible involvement in human or animal disease date back almost as far, coming from Italy, Russia and the United States by 1904 (Maracas et al., 1984).

The only animal disease for which the causal role of *F. moniliforme* has been established beyond doubt is the disease of horses and related animals known as equine leukoencephalomalacia (LEM). This disease was known as early as 1850 in the corn belts in the United States, with epidemics involving hundreds or thousands of horses in 1900, the 1930s and as recently as 197879. It also occurs in other parts of the world, including Argentina, China, Egypt, New Caledonia and South Africa (Maracas et a./., 1984). However, *F. moniliforme* was not positively identified as the cause of LEM until 1971 (Wilson, 1971).

***F. moniliforme* has been suggested to be the cause of a variety of other animal diseases, including bean hull poisoning of horses in Japan, abnormal bone development or rickets in chickens and pigs in the U.S.A., France and Germany, and a toxicosis due to mouldy sweet potatoes in the U.S.A. (Maracas et al., 1984).**

The high rate of human oesophageal cancer which occurs in some parts of Transkei in southern Africa appears to be associated with corn consumption, and perhaps, therefore, with *F. moniliforme* (Maracas et al., 1984).

Taxonomy

Unlike other species considered here, *F. moniliforme* has been recognised as a distinct entity for many years. Disagreement still exists over the correct name: the name *F. verticillioides* (Sacc.) Nirenberg undoubtedly has nomenclatural priority, but has not been accepted by Nelson et al. (1983), their grounds being that the provisions of the International Code of Botanical Nomenclature are difficult to apply to *Fusarium* species in the absence of type material. Eventual conservation of *F. moniliforme* seems likely.

Identification

Colonies of *F. moniliforme* on PDA are white, sometimes tinged with purple. Macroconidia vary from slightly sickle shaped to almost straight. Microconidia are abundant, and are usually single celled, ellipsoidal to clavate with a flattened base, and formed in long chains.

Toxins and toxicity

Despite early warnings that *F. moniliforme* was a highly toxic fungus, the road

to understanding of the toxins responsible has been long and difficult. The most important toxin produced by this species is undoubtedly fumonisin B, a mycotoxin only characterised very recently (Bezuidenhout et al., 1988). Fumonisin B is a bizarre molecule, consisting of a 20 carbon aliphatic chain with two ester-linked hydrophilic side chains.

F. moniliforme growing in corn is known to be responsible for LEM, a brain disease of horses. LEM has been as a serious problem in the United States corn belt for more than 100 years, and has caused the deaths of thousands of horses. Recent work indicates that fumonisins are the toxins most likely to be responsible for LEM.

The effect of fumonisins on humans is not known, but the fact that fumonisin B can induce cancer in rats suggests that this toxin may have a role in human oesophageal cancer. Corn is the major staple food in areas of the Transkei where oesophageal cancer is endemic, and the most striking difference between areas of low and high incidence was the much greater infection of corn by F. moniliforme in the high incidence areas (Maracas et al., 1981). Kriek et al (1981) showed that several isolates of F. moniliforme from high incidence areas

were acutely toxic to ducklings, but did not produce other known toxins such as moniliformin. The discovery of the fumonisins should help in the elucidation of the role of moniliforme in human oesophageal cancer.

Because of the intense interest over the past two decades in the toxicity of *F. moniliforme* which has recently culminated in the discovery of the fumonisins, other toxins produced by this species have been carefully studied. A few isolates of moniliforme produces moniliformin, a compound which is known to be toxic, but which lacks a known disease role. Other known compounds of greater or less toxicity include fusaric acid, fusarins and fusariocins (Maracas et al., 1984). The production of T-2 toxin, diacetoxyscirpenol and zearalenone by *F. moniliforme* have been reported, but are regarded as unlikely by Marasas et al. (1984).

The known toxicity of *F. moniliforme* to a wide variety of animals, and its probable role in human oesophageal cancer, may well result from the production of the newly discovered fumonisins. However, the diversity of the demonstrated toxicity of authentic isolates of *F. moniliforme* to a wide variety of animals (Maracas et al., 1984) is such that the possibility of other potent toxins cannot

be ruled out.

Symptoms

Symptoms of *F. moniliforme* poisoning vary widely with animal type, dosage and toxigenic fungal isolate. The best defined disease produced by *F. moniliforme*, LEM, is characterised by liquefactive necrotic lesions in the white matter of the cerebral hemispheres of horses and other equine species. Marked neurotoxicity is evident, with aimless walking and loss of muscle control followed by death, which usually occurs about 2 weeks after toxin ingestion. In baboons, *F. moniliforme* toxicity has been shown to lead to heart failure. Chickens and ducklings are sensitive to feed containing *F. moniliforme*, and there is some evidence that the toxin responsible is moniliformin (Cole et al., 1973).

The toxicity of one *F. moniliforme* isolate to rats was characterised by cirrhosis and hyperplasia in the liver, and thrombosis in the heart and other organs (Kriek et al., 1981). Of 20 other isolates, 15 showed mortalities and some symptoms, while 5 were nontoxic.

F. moniliforme may also be involved in abnormal bone development and diseases similar to rickets in chickens, sometimes with high mortalities.

The principal human disease with which F. moniliforme may be associated is oesophageal cancer, which has an abnormally high prevalence in the Transkei and in Henan Province, China. However, direct evidence that fumonisins or other known F. moniliforme toxins are causally related to this disease remain lacking.

Distribution in nature and foods

Like other Fusarium species, F. moniliforme is primarily a plant pathogen, causing both stalk and cob rot of corn, and diseases in rice, sorghum, sugar cane and other Graminae. It is much more common in the tropics than temperate zones (Domsch et al., 1980; Pitt and Hocking, 1985a). By far its most common source in foods is corn, both under field and storage conditions, but it has also been isolated from nuts, yams and occasionally other commodities (Pitt and Hocking, 1985a).

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Toxigenic aspergillus and penicillium species

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by J.I. Pitt

Aspergillus

Aspergillus is a large genus, with 100 or more well on common synthetic or semi-synthetic media. The most widely used taxonomy is by Raper and Fennell (1965). Keys and descriptions of common species may be found in Pitt and Hocking (1985a) and Klich and Pitt (1988a). Aspergillus species are of very common occurrence in the environment, principally in soils and decaying vegetation, but a number of species are also closely associated with human foods, particularly cereals and nuts (Pitt and Hocking, 1985a). Many species are xerophilic, and capable of spoiling foods only just above safe moisture limits. The most significant mycotoxigenic species are *A. flavus* and *A. parasiticus*, which make aflatoxins, *A. ochraceus*, which makes ochratoxin, and *A. versicolor*, which produces sterigmatocystin. These species are treated below: *A. flavus* and *A. parasiticus* are very closely related, and are treated together.

Aspergillus flavus and *A. parasiticus*

Aspergillus flavus and A. parasiticus were classified by Raper and Fennell (1965) in what was termed the "Aspergillus flavus group", an incorrect terminology now replaced by the correct term "Aspergillus Section Flavi" (Gems et al., 1985). A. flavus and A. parasiticus are closely related to A. Oryzee and A. sojae, species which are important in the manufacture of fermented foods in Asia, but which do not produce aflatoxins. For obvious reasons, accurate differentiation of these four species is important.

Identification

Differentiation of A. flavus and A. parasiticus from nearly all other species is not difficult. Both grow rapidly on standard identification media such as Czapek agar or Malt Extract agar (MEA; Raper and Fennell, 1965). or Czapek Yeast Extract agar (CYA; Pitt and Hocking, 1985a), and produce yellow green conidia on colonies which are otherwise uncoloured (Raper and Fennell, 1965; Pitt and Hocking, 1985a; Klich and Pitt, 1988a).

Distinguishing A. flavus from A. parasiticus is more difficult, with a lack of agreement among the students of these species (Klich and Pitt, 1985). A recent

study has concluded that the texture of conidial walls is the most reliable differentiating feature: walls of *A. flavus* conidia are usually smooth to finely roughened, while those of *A. parasiticus* are definitely rough when viewed under a 100 X objective. A variety of other characters are also of taxonomic value (Klich and Pitt, 1988b).

Differentiating these species is of some importance: *A. flavus* isolates usually make only B aflatoxins (see below), and less than 50% of isolates are toxigenic, while *A. parasiticus* isolates make G as well as B aflatoxins, and are invariably toxigenic (Klich and Pitt, 1988b).

Toxins and toxicity: aflatoxins

Aflatoxins are produced in nature only by *A. flavus* and *A. parasiticus*. The four major naturally produced aflatoxins are known as aflatoxins B1, B2, G1 and G2. 'B' and 'G' refer to the blue and green fluorescent colours produced by these compounds under ultraviolet light illumination on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds respectively (Fig. 4). When aflatoxin B1 and B2 are ingested by lactating cows,

a proportion (ca 1.5%; Frobish et al.,1986) is hydroxylated and excreted in the milk as aflatoxins M1 and M2, compounds of lower toxicity than the parent molecules, but significant because of the widespread consumption of cows' milk by infants.

Aflatoxins are both acutely and chronically toxic to animals, including man. They produce four distinct effects: acute liver damage; liver cirrhosis; induction of tumours; and teratogenic effects (Stoloff, 1977).

Acute toxicity of aflatoxins to humans has been observed only rarely (Shank, 1978). In 1967, 26 Taiwanese in two farming communities became ill with apparent food poisoning. Nineteen of them were children, three of whom died. Rice from affected households was blackish green and mouldy, and appeared to be of poorer quality than that from neighbouring households which were unaffected. Samples of the mouldy rice contained about 200 g/kg of aflatoxin B1, and this was probably responsible for the outbreak. Post mortem examinations were not carried out.

In 1974 an outbreak of hepatitis that affected 400 Indian people of whom 100

died, almost certainly resulted from aflatoxins (Krishnamachari et al., 1975). The outbreak was traced to corn heavily contaminated with *A. flavus*, and containing up to 15 mg/kg of aflatoxins. Consumption of toxin by some of the affected adults was calculated to be 2-6 mg in a single day. It can be concluded that the acute lethal dose for adult humans is of the order of 10 mg.

Kwashiorkor, a disease of children in Northern Africa and elsewhere, is usually attributed to nutritional deficiencies, but may also be related to aflatoxin intake (Hendrickse et al., 1982). Aflatoxin-induced liver damage may render children with kwashiorkor less able to assimilate the high protein diets usually recommended as the cure for this disease (Newwell, 1983).

Aflatoxin B1 has been demonstrated, in a variety of animal species, to be the most potent liver carcinogen known. Human liver cancer has a high incidence in central Africa and parts of Southeast Asia. Epidemiological data suggested a link between human liver cancer and aflatoxins. Studies in several African countries and Thailand showed a correlation between the logarithm of aflatoxin intake and the occurrence of primary human liver cancer (van Rensburg, 1977).

Studies in the United States produced different results. On the basis of van Rensburg's figures, Stoloff and Friedman (1976) estimated that children in rural areas of the USA consumed enough aflatoxins from corn to induce 4-10 deaths from liver cancer per 100,000 adult population. However, the actual death rate is less than 1 per 100,000. Refinements to those calculations by Stoloff (1983) reinforced his conclusion that aflatoxins are unlikely to contribute significantly to the incidence of liver cancer in the USA.

The resolution of the conflict in the conclusions drawn by van Rensburg (1977) and Stoloff (1983) lies in recent information which suggests that prior or simultaneous exposure to hepatitis B virus may be a prerequisite for the induction of this form of liver cancer in humans. It appears that aflatoxins and hepatitis B are cocarcinogens, and the probability of cancer of the liver in humans is high only in areas where both aflatoxins and hepatitis B are prevalent (Campbell, 1983).

Recent evidence suggests that primates are able to detoxify aflatoxins by pathways which do not exist in lower animals (Masri, 1984). The toxicity of aflatoxins to humans is certainly lower than was assumed in earlier years;

nevertheless the known carcinogenicity in lower animals is so high that every effort must be made to monitor aflatoxin levels in human foods and to reduce them as far as possible.

Symptoms

The symptoms of acute aflatoxin poisoning were studied following the hepatitis outbreak in India mentioned above (Krishnamachari et al., 1975). This disease outbreak was characterised by jaundice, rapidly developing ascites and portal hypertension, with the implication of a food-borne toxin involving the liver. However, the ingestion of aflatoxins at the low levels necessary to induce liver cancer is totally asymptomatic and has a very long induction period as well.

Distribution in nature and in foods

Aspergillus flavus is of ubiquitous occurrence in nature. Since the discovery of aflatoxins, it has become the most widely reported food-borne fungus, reflecting its economic and medical importance, and ease of recognition, as well as its universal occurrence. A. parasiticus is less common, but the extent of its

occurrence is obscured by the tendency for *A. flavus* and *A. parasiticus* to be reported only as *A. flavus*.

***A. flavus* (and *A. parasiticus*) have a particular affinity for nuts and oilseeds, though the reason is not clear. Peanuts, corn and cottonseed are the three most important crops invaded by *A. flavus*. Earlier work assumed that invasion was primarily a function of inadequate drying or improper storage, and these factors are certainly important in the occurrence of aflatoxins in the humid tropics. However, in temperate zones, recent work has stressed the importance of *A. flavus* invasion of these crops before harvest. Invasion of peanuts occurs as a result of drought stress and related factors (Cole et al., 1982).**

Pre-harvest invasion in corn is partly dependent on insect damage to the developing cobs, but the fungus can also invade down the silks. cobs, but the fungus can also invade down the silks of the developing ears (Lillehoj et al., 1980). In cottonseed, invasion is now believed to occur by entry through the nectaries (Klich et al., 1984). Most other nuts are also susceptible to invasion at some time (Pitt and Hocking, 1985a).

Cereals are also a common substrate for growth of *A. flavus* but, unlike the case of nuts and oilseeds, small grain cereal spoilage by *A. flavus* is almost always the result of poor handling. Aflatoxin levels in small grains are rarely significant (Stoloff, 1977). Spices sometimes contain *A. flavus* (Pitt and Hocking, 1985a) and viable counts may be very high. However, the quantities of spices consumed are so small that aflatoxin in spices does not appear to be a real hazard.

In developed countries, stringent sorting and clean up procedures are used to reduce aflatoxins to low levels in foods with a perceived risk. For peanuts, where fungal growth is usually accompanied by discolouration of the kernel, this includes the use of sophisticated colour sorting equipment. Statistically based sampling, the drawing of large samples, homogenising before sub-sampling and standardised aflatoxin assays are used to ensure that susceptible crops and foods meet the stringent requirements of health laws in both exporting and importing countries. Developing countries are often less fortunate. Established patterns of local consumption, where substandard nuts and corn may be consumed without any form of sorting or inspection, mean that aflatoxin ingestion remains far too high in many countries, especially in rural areas.

A. ochraceus and closely related species

A. ochraceus is the most commonly occurring species in the "Aspergillus ochraceus group" of Raper and Fennell (1965), now correctly referred to as Aspergillus Section Circumdati (Gems et al., 1985).

Identification

A. ochraceus and other closely related ochratoxin producing species - A. alliaceus, A. melleus, A. sclerotiorum and A. sulphureus - all grow moderately slowly on standard identification media such as CYA and MEA (Pitt and Hocking, 1985a). Colonies are deep but not very dense, and coloured pale brown to yellow brown; stipes are long, heads are large, vesicles are spherical and metulae and phialides are densely packed. Conidia are small, pale brown and smooth walled (Raper and Fennell, 1965; Pitt and Hocking, 1985a; Klich and Pitt, 1988a). Differentiation of these species is not always easy (Raper and Fennell, 1965; Klich and Pitt, 1988a). However, A. ochraceus is by far the most commonly occurring of these species, and differentiation of the others from A. ochraceus is rarely necessary.

Toxins and toxicity: ochratoxins

The major toxin produced by *A. ochraceus* and the other closely related species mentioned above is ochratoxin A. This toxin is discussed under *Penicillium verrucosum*, the most important species producing ochratoxins. *A. ochraceus* also produces penicillin acid, a mycotoxin of lower toxicity and uncertain importance in human health.

Symptoms

For a discussion of the symptoms of ochratoxin A poisoning, see under *Penicillium verrucosum*. Penicillic acid is a hepatocarcinogen in some animal species, and has also been reported to affect the heart (Reiss, 1988).

Distribution in nature and foods

The natural habitat for *A. ochraceus* and the other closely related species is drying or decaying vegetation, seeds, nuts and fruits. *A. ochraceus* is widely distributed in foods, especially dried foods, with records from such diverse sources as various kinds of beans, dried fruit, biltong and salt fish (Pitt and

Hocking, 1985a). Nuts, including peanuts, pecans and betel nuts, are also a major source. Although *A. ochraceus* has been isolated from a wide range of cereals, including barley, wheat, flour and rice, records are rather infrequent (Pitt and Hocking, 1985a).

A. versicolor

The most important species in the "Aspergillus versicolor group" of Raper and Fennell (1965), *A. versicolor* is now classified correctly in Aspergillus Section Versicolores (Gems et al. 1985).

Identification

Slowly growing, low, dense, green colonies on CYA or MEA are characteristic of *A. versicolor*, and a variety of other Aspergillus and penicillium species. Reddish drops of exudate and orange or reddish brown reverse colours on CYA are valuable additional characters. The microscopic appearance of *A. versicolor* is more distinctive. Stipes are relatively short, with heavy yellow walls, vesicles are usually ellipsoidal rather than spherical, and bear metulae and phialides only

over the upper two thirds. Conidia are very small (22.5 μ m in diameter), smooth walled, and coloured pale green (Raper and Fennell, 1965; Pitt and Hocking, 1985a; Klich and Pitt, 1988a).

The closely related species *A. sydowii* has many characteristics in common with *A. versicolor*, but colonies are coloured blue rather than grey green. *A. sydowii* produces no mycotoxins of significance.

Toxins and toxicity: sterigmatocystin

Sterigmatocystin is produced by several species of fungi (Cole and Cox, 1981), but *A. versicolor* is by far the most important. This toxin is a precursor of the aflatoxins, but acute oral toxicity is low because of very low solubility in water or gastric juices. Hence sterigmatocystin is unlikely to be responsible for acute poisoning outbreaks in man or animals (Terao, 1983). Due to this insolubility, experimental doses given to animals are only adsorbed to a small extent. As absorption is dependent on the method of administration, LD50 figures lack accuracy: literature values vary from 60 to 800mg/kg body weight. Even low doses cause pathological changes to the livers of rats, however(Terao, 1983).

As a liver carcinogen, sterigmatocystin appears to be only about 1/150th as potent as aflatoxin B1, but this is still much more potent than most other known liver carcinogens. Levels as low as 15 g/day fed continuously, or a single 10 mg dose, caused liver cancer in 30% or more of male Wistar rats (Terao, 1983). Sterigmatocystin has the potential to cause human liver cancer.

Symptoms

Again due to its very low solubility, oral ingestion of sterigmatocystin is undoubtedly asymptomatic. Detection of a disease syndrome due to this mycotoxin appears to be a very difficult epidemiological problem indeed.

Distribution in nature and in food

Distribution of *A. versicolor* in nature appears to be widespread though sparse. Its slow growth and exophilic nature undoubtedly contribute to poor recovery in surveys of fungal populations.

***A. versicolor* has been reported from a very wide variety of foods. It occurs at harvest in some crops such as wheat, but is much more commonly isolated from**

stored commodities, particularly wheat, corn, flour and rice. It has also been found in a variety of nuts, fermented and cured meats and biltong, spices and cheese (Pitt and Hocking, 1985a).

Penicillium

Penicillium is a large genus, with 150 recognised species (Pitt, 1979a), of which 50 or more are of common occurrence (Pitt, 1988a). All common species grow and sporulate well on synthetic or semi-synthetic media, and usually can be readily recognised at genus level.

Classification of Penicillium is based primarily on microscopic morphology: the genus is divided into subgenera based on the number and arrangement of phialides (elements producing conidia) and metulae and rami (elements supporting phialides) on the main stalk cells (stipes). The classification of Pitt (1979a) includes four subgenera: Aspergilloides, where phialides are borne directly on the stipes without intervening supporting elements; Furcatum and

Biverticillium, where phialides are supported by metulae; and **Penicillium**, where both metulae and rami are usually present. The majority of important toxigenic and food spoilage species are found in subgenus **Penicillium**.

Identification

Identification of Penicillium isolates to species level is not easy, preferably being carried out under carefully standardised conditions of media, incubation time, and temperature. As well as microscopic morphology, gross physiological features, including colony diameters, colours of conidia and colony pigments, etc. are used to distinguish species. A complete taxonomy of all species may be found in Pitt (1979a) or Raper and Thom (1949), though the latter is out of date now. Pitt and Hocking (1985a) and Pitt (1988a) provide keys and descriptions to many common species. A computer assisted key to the common species will be available shortly (Pitt, 1988b).

The most important toxigenic Penicillium species in foods are *P. citreonigrum* (which makes citreoviridin), *P. citrinum* (citrinin), *P. crustosum* (penitrem A), *P. islandicum* (cyclochlorotine, islanditoxin, luteoskyrin and erythrokyrin), and *P.*

verrucosum (ochratoxin A). Each of these species and toxins is discussed below.

Penicillium citreonigrum

The Oriental disease known as "beriberi" has traditionally been regarded as a nutritional disease, an avitaminosis. However, beriberi is more than a single disease, and one form of it, known in Japan as acute cardiac beriberi, has been established to be a mycotoxicosis. The work of Sakaki in the 1890s (Ueno and Ueno, 1972) implicated mouldy "yellow rice" as a probable cause of acute cardiac beriberi, and led to a ban on the sale of yellow rice in Japan in 1910. The disease subsequently disappeared from Japan.

Uraguchi (1969) and Ueno and Ueno (1972) showed that acute cardiac beriberi was due to the growth in rice of *P. citreonigrum* (synonyms */? citreoviride*, *P. toxicarium*), and that the mycotoxin responsible was citreoviridin.

Acute cardiac beriberi in Japan is now only of historical interest. However, *P. citreonigrum* and Citreoviridin may still occur in other parts of Asia.

Citreoviridin is also produced by *P. ochrosalmoneum* (see below).

***P. citreonigrum* is a member of subgenus *Aspergilloides*. *P. citreonigrum* was described in 1901, but ignored in many more recent taxonomies because the description was meagre. However, on the basis of neotypification by Biourge in 1923, Pitt (1979a) revived the name, which has priority over the more commonly used name *P. citreoviride*.**

Identification

When grown on standard identification media (Pitt, 1979a; 1988a), *P. citreonigrum* is a distinctive species. Colonies grow quite slowly (after 7 days: 2080 mm diameter on CYA, 22-26 mm diameter on MEA, 0-10 mm at 37Q) sparsely produce pale grey green conidia, and exhibit yellow mycelium, soluble pigment and reverse colours. Penicilli consist of small clusters of phialides only, stipes are slender and not apically enlarged, and conidia are spherical, smooth walled and tiny (1.8-2.8 μ m in diameter).

Toxins and toxicity

As noted above, *P. citreonigrum* produces citreoviridin, the cause of acute cardiac beriberi. Citreoviridin is also produced by *Penicillium ochrosalmoneum*, which has an ascomycete state, *Eupenicillium ochrosalmoneum*. It is not closely related to *P. citreonigrum*.

Citreoviridin is an unusual molecule consisting of a lactone ring conjugated to a furan ring, with a molecular weight of 402 (Cole and Cox, 1981). It is a neurotoxin, acutely toxic to mice, with intraperitoneal and oral LD50s of 7.5 mg/kg and 20 mg/kg respectively (Ueno and Ueno, 1972).

Symptoms

In several animal species, Citreoviridin caused vomiting, convulsions, ascending paralysis and respiratory arrest. Less frequent signs were reported to be ataxia, enforced movements or stiffness in the extremities, and later cardiovascular disturbance, flaccid paralysis and hypothermia (Uraguchi, 1969). In higher mammals, neurological symptoms or depressed sensory responses are also evident.

In man, the disease called acute cardiac beriberi has been recognised for the past three centuries (Ueno and Ueno, 1972). The disease frequently occurred in young healthy adults, and death could occur within a few days. In many respects, the acute symptoms in man paralleled those in animals (Uraguchi, 1969).

Distribution in nature and in foods

***P. citreonigrum* is not a commonly isolated species, but it is widely distributed (Pitt and Hocking, 1985a). According to Miyake et al. (1940), as reported by Uraguchi (1969), *P. citreonigrum* grows in rice after harvest, when the moisture content reaches 14.6%. At 1% higher moisture, other fungi will overgrow it, so the moisture band for invasion is narrow. The fungus is reported to be favoured by the lower temperatures and shorter hours of daylight occurring in the more temperate rice growing areas.**

***P. ochrosalmoneum* is also an uncommon species in most environments. However, it has been found colonising unharvested corn in the USA, where it may produce citreoviridin under natural conditions (Wicklowsky and Cole, 1984).**

P. citrinum

Described in 1910, *P. citrinum* has been a well recognised species for most of this century. Its importance in the present context lies not so much in the production of a mycotoxin of particular human significance, but in its ubiquity, so that any toxins produced can be expected to be very widely distributed in food and feed supplies. *P. citrinum* is the major producer of citrinin, a compound discovered during the 1940s, and considered then to be a potentially valuable antibiotic. Like several other *Penicillium* metabolises, it proved to be too toxic for therapeutic use, and became known in time as a potentially hazardous mycotoxin.

Classified in subgenus *Furcatum* section *Furcatum* (Pitt, 1979a), *P. citrinum* is a very well circumscribed species, accepted without controversy for many years.

Identification

The most distinctive feature of *P. citrinum* is its benicillus, which consists of a

cluster of three to five divergent metulae, usually epically swollen. Under the stereomicroscope, the phialides from each metula usually bear conidia as a long column, producing a distinctive pattern which can be of diagnostic value. Colonies of this species on CYA and MEA are of moderate size (25-30 mm and 14-18 mm, respectively), with the smaller size on MEA also a distinctive feature. Growth normally occurs at 37C but colonies seldom exceed 10 mm after 7 days.

Toxins and toxicity

Citrinin is the only mycotoxin produced by *P. citrinum*. *P. citrinum* is the major producer of this toxin, but production by *P. expansum* and *P. verrucosum* has also been reliably reported. Literature citations indicate that at least 22 *Penicillium* species have been reported to produce citrinin, but the great majority of these are either regarded as synonyms, or require confirmation (Pitt and Leistner, 1988).

Citrinin is a significant renal toxin to monogastric domestic animals, including pigs (Frlis et al., 1969) and dogs (Cariton et al., 1974). Domestic birds are also

susceptible: citrinin causes watery diarrhoea, increased food consumption and reduced weight gain due to kidney degeneration in chickens (Mehdi et al., 1981), ducklings and turkeys (Mehdi et al., 1984). LD50 figures have been reported to be imprecise due to delayed deaths. The oral LD50 in mice is 110 mg/kg (Scott, 1977).

Chronic kidney degeneration in Danish pigs was at first considered to be due to citrinin (Frlis et al., 1969), but it was later shown that ochratoxin A was more significant (Krogh et al., 1973).

The effect of citrinin on humans remains undocumented. However, kidney damage appears to be a likely result of prolonged ingestion.

Symptoms

Like many other mycotoxins, citrinin is essentially asymptomatic in animals (as distinct from birds), causing a nonspecific deterioration in kidney function.

Distribution in nature and foods

P. citrinum is a ubiquitous fungus, and has been isolated from nearly every kind of food surveyed for fungi. The most common sources are milled grains and flour, and whole cereals, especially rice, wheat and corn (Pitt and Hocking, 1985a). Instances of spoilage are rare, but growth, and toxin production, are likely to be a common occurrence.

P. crustosum

Described in 1930, P. crustosum was regarded by Raper and Thom (1949) as uncommon, with its major source has been shown to be P. crustosum in consequence, this species remained more or less unrecognised until recently. The occurrence of serious outbreaks of tremorgenic and other neurotoxicity in domestic animals was linked to other species, notably P. cyclopium (now P. aurantiogriseum; Pitt, 1979a), P. palitans (now P. commune; Pitt, 1988a) and P. viridicatum. The toxin responsible is now known as penitrem A, and its major source has been shown to be P. crustosum (Pitt, 1979b). P. crustosum is now recognised as a very common species in foods and feeds (Pitt and Hocking,

1985a).

Identification

A member of *Penicillium* subgenus *Penicillium*, *P. crustosum* produces the large penicilli with rami, metulae and phialides characteristic of this subgenus. It is one of the faster growing species in section *Penicillium*, within that subgenus, producing dull green colonies with a granular texture on both CYA and MEA. Microscopically, *P. crustosum* is characterised by large rough walled stipes and smooth walled, usually spherical conidia. However, the most distinctive feature of typical isolates is the production of enormous numbers of conidia on MEA, which become detached from the colony when the Petri dish is jarred.

Toxins and toxicity

Naturally occurring compounds which can cause sustained trembling are rare, and most of those known are produced by fungi. One of the most potent is penitrem A, which is produced by *P. canescens*, *P. crustosum*, *P. glandicola* (= *P. granulatum*; Seifert and Samson, 1985) and *P. janozowskii* (= *P. nigricans*;

Pitt, 1979a). *P. crustosum* is by far the most important source, as virtually all known isolates are producers, and it is by far the most commonly occurring of these species in foods. *P. crustosum* has also been reliably reported to produce cyclopiazonic acid and roquefortine, though rarely (Pitt and Leistner, 1988).

Penitrem A is a potent neurotoxin, with an intraperitoneal LD50 of 1 mg/kg in mice. Oral LD50 data do not appear to be available, but death or severe brain damage have been reported in field outbreaks involving sheep, cows, horses and dogs (Wilson et al., 1968; Hocking et al., 1988).

Symptoms

In laboratory animals, the main symptom of poisoning by penitrem A is the onset of sustained trembling, which may continue for long periods without appearing to interfere with the normal functions of the animal. Trembling has been sustained in experiments without apparent ill effects or residual effects for as long as 18 days (Jortner et al., 1986). However, relatively small increases in does (5 to 20 fold) can be rapidly lethal (Hou et al., 1971 a). Post mortem diagnosis of tremorigenic toxins such as penitrem A is virtually impossible, as no

pathological effects are evident.

The symptoms of penitrem A are essentially the same as those of a range of other fungal tremorgens, including those from *Claviceps paspali* growing in *Paspalum* grass. *Acremonium lolii* growing in *Lolium perenne* (ryegrass), or any of several *Penicillium* and *Aspergillus* species growing in foods or feeds (Pitt and Leistner, 1988).

The potential hazard of penitrem A to man remains unknown, and puzzling. Its known toxicity to large domestic animals and dogs is such that it is unlikely to be non-toxic to humans. However, the only symptoms in man which can be attributed to *P. crustosum* have been unlikely, though quite well documented, instances of dizziness and vomiting after consuming beverages which contained mould growth . Recovery of patients was complete in all cases. Available evidence is fragmentary, and direct experimentation impossible. The role of penitrem A and perhaps other fungal neurotoxins in human illness or neurological disorders still awaits elucidation.

Distribution in nature and foods

P. crustosum is an ubiquitous spoilage fungus. Pitt and Hocking (1985a) reported isolating it from the majority of cereal and animal feed samples examined by them over more than a decade. P crustosum can cause spoilage of corn, processed meats, nuts, cheese and fruit, as well as being a weak pathogen on pomaceous fruits and cucurbits (Pitt and Hocking, 1985a). The occurrence of penitrem A in animal feeds is well documented (Wilson et al., 1968; Hou et al., 1971b). Its occurrence in human foods appears equally certain.

P. islandicum

Described by Sopp in 1912, P. islandicum attracted little attention from taxonomists until Raper and Thom (1949) recognised it. It has been accepted unchanged in concept since that time.

When tested against experimental animals, P. islandicum has been shown to produce several highly toxic compounds. However, the significance of P. islandicum as a toxigenic fungus and of the toxins themselves remains in doubt.

The species is included here as a potential problem, rather than because of known outbreaks of disease.

Neither the name of this species nor its circumscription has materially altered during the 80 years since it was described. *P. islandicum* is a representative of subgenus *Biverticillium*, which is characterised by the production of penicilli with more than five appressed metulae, of metulae and phialides of approximately equal length, and of phialides which are acerose (shaped like a pine needle) (Pitt, 1979a; 1988).

Among species which produce the penicillus type characteristic of subgenus *Biverticillium*, *P. islandicum* can be readily distinguished by its slowly growing, dense colonies with brilliant orange to brown colours in both mycelium and reverse. Colonies at 37C are usually similar to those at 25C Conidia are usually blue.

Toxins and toxicity

***P. islandicum* produces at least four mycotoxins, unique to the species.**

Cyclochlorotine and islanditoxin are chlorine-containing cyclic peptides which have the same toxic moiety, a pyrrolidine ring with two attached chlorine atoms, and share a number of other physical and chemical properties (Scott, 1977). Both compounds are very toxic: cyclochlorotine has an oral LD50 in mice of 6.5 mg/kg, while that of islanditoxin by subcutaneous injection was 3 mg/kg. Fed to mice at the rate of 40 g per day, cyclochlorotine caused liver cirrhosis, fibrosis and tumors (Uraguchi et al., 1972).

Luteoskyrin is a dimeric anthroquinone and erythrokyrin a heterocyclic red pigment. Both are liver and kidney toxins, though less acutely toxic than cyclochlorotine. Luteoskyrin is also carcinogenic.

Because of the toxic "yellow rice" syndrome, described above under *P. citreonigrum*, Japanese scientists have taken a particular interest in *P. islandicum*, which also can cause yellowing of rice (Saito et al., 1971). However, the significance of the toxins produced by *P. islandicum* remains unclear.

Symptoms

Little information exists about the symptoms caused by the *P. islandicum* toxins.

Distribution in nature and foods

Reports of *P. islandicum* in nature have been infrequent (Pitt and Hocking, 1985a). Considering the striking appearance of colonies and the ease with which this species can be identified, the indications are that it is uncommon, at least in the temperate zones where most studies of *Penicillia* in foods have been undertaken.

P. varrucosum

For half a century or more, nephropathy has been an important disease in Danish pigs. Etiological studies first showed it to be associated with mouldy grain, and then with a fungus identified as *P. viridicatum* (Krogh and Hasselager, 1968). A representative isolate was first shown to produce oxalic acid and citrinin, and then ochraoxin A (Krogh et al., 1973). The major source of fungus and toxin was barley (Krogh, 1978).

Pitt (1987) showed that the major *Penicillium* species producing ochratoxin A was *P. verrucosum*, not *P. viridicatum*. *P. verrucosum* is the main source of ochratoxin A in temperate regions. In the tropics, *Aspergillus ochraceus* may be more significant.

Ochratoxin A is fat-soluble, and not readily excreted, so it accumulates in fatty tissues. In consequence, it poses a serious health risk to humans, especially in rural areas where pigs are not subject to rigorous inspection, and pork and bacon may contain high levels of ochratoxin.

***P. verrucosum* is classified in subgenus *Penicillium*, a large subgenus, and in section *Penicillium*, which includes many mycotoxigenic species of common occurrence in foods.**

Identification

***P. verrucosum* is distinguished by slow growth on CYA and MEA at 25C (17-24 mm and 10-20 mm after 7 days, respectively; Pitt, 1987), bright green conidia, clear to pale yellow exudate, and rough stipes (Pitt, 1988a). It is similar in**

general appearance to *P. viridicatum*, differing most obviously by slower growth, and to *P. solitum*, from which it differs by having green rather than blue conidia.

Toxins and toxicity

***P. verrucosum* is the principal producer of the nephrotoxin ochratoxin A. Pitt (1987) reported that 47 of 84 *P. verrucosum* isolates (56%) from West German and Australian sources produced this toxin. A few isolates also produce citrinin (6 of 84, 7%; Pitt, 1987).**

Ochratoxin A is also produced by some isolates of *Aspergillus ochraceus* (5 of 17, 30%; Ciegler, 1972). Ochratoxin A is an acute nephrotoxin, with oral LD50 values of 20 mg/kg in young rats and 3.6 mg/kg in day old chicks. It is also lethal to mice, trout, dogs and pigs (Scott, 1977). Necroses of the renal tubules and periportal liver cells were the main pathological changes observed after fatal doses.

In humans, ochratoxin A appears to be responsible for kidney degeneration,

which in extreme cases can lead to death. Kidney failure rates in rural Scandinavian populations are high, and a possible cause is the ingestion of pig tissues containing excessive levels of ochratoxin A (Krogh et al., 1974).

Distribution in nature and foods

P. verrucosum has been reported almost exclusively from temperate zones. It is associated with Scandinavian barley: in one survey of farms where pigs were suffering from nephritis, 67 of 70 barley samples contained high levels of P. verrucosum, and 66 contained ochratoxin A (Frisvad and Viuf, 1986). This species has also been isolated quite frequently from meat products in Germany and other European countries. It appears to be uncommon elsewhere (Pitt and Hocking, 1985a).

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Table 1. Acute mycolaxicoses of human significance.

Date Disease	Toxin	Cause	Diagnosis
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To 1954 Ergotism	Ergot alhaloids	Claviceps purpurea in rye	1800, fungal cause suspected
To 1910 Acute cardiac beriberi	Citreoviridin	Penicillium cilreonigrum in rice	1910, yellow rice sale banned
			1969, fungal cause demonstrated
To 1948 Alimentary toxic aleukia	T-2	Fusarium poae in millet and rye	1950, fungal origin suspected
			1976, toxin established correctly
1965-66 'Cobalt-beer' cardio-myopathy	T-2	Fusarium spp in barley	1980, fungal origin poposed
1974 Hepatitis	A/laloxin	Aspergillus flavus in maize	1975, fungal cause demonstrated
Current Pellagra	T-2	Fusarium spp. in maize	1980, fungai origin proposed
Current Reye's	Aflatoxin	Aspergillus flavus in	1977, fungal origin

syndrome		nuts and maize	proposed
Current Kwashiorkor	Aflatoxin	Aspergillus flavus in nuts and cereals	1983, fungal involvement proposed

Table2. Chronic mycotoxicoses of human significance.

Date Disease	Taxin	Cause	Diagnosis
Current Liver cancer	Aflatoxin	Aspergillus flavus in foods	1963, fungal cause
			1970-75, epidemiological studies
			1977, statements of human risk
Current Oesophageal cancer in Transkei	Deoxynivaleno, zearalenone	Fusarium spp. in maize	1979, fungal cause suspected

Current Nephropathy in Denmark	Ochratoxin	Penicillium vindicatum in barley fed to pigs	1973, fungal cause known in pigs suspected in humans from residues in pork
Current Balkan endemic nephropathy	?	?	1972, fungal cause suspected

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Mycotoxin economic aspects

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by Thirapong Tangthirasunan

Thailand is an agricultural country. Agriculture has played a vital role in the Thai economy contributing around 25% of the gross domestic product (GDP) and about 55% of export by value.

Thailand's economy still depends on the successful export of its major agricultural commodities. Problems in export of agricultural products are many and varied.

Firstly, it subjects to the uncertainty of the world commodities market including the importing policy of the buying country.

Secondly, for some specific crops such as maize, it is more difficult as world production continue to rise faster than demand. The maize buyers now emphasize quality particularly the level of aflatoxin.

Formerly, Thai maize was preferred for its bright yellow colour and high protein

content. Today, however, these qualities are overlooked as a result of high level of aflatoxin. This has seriously affected the demand for Thai maize.

In recent years the quality of Thai maize has suffered through high level of aflatoxin, a mycotoxin produced by a fungus thriving in moist condition and poisonous in concentration. The effect on Thai economy has been substantial through lost export market and discounted prices. The Thai farmers, many of whom are often subject to unfair practices of the middlemen and the private local traders, have seen the price depressed accordingly.

Thailand is the world's third largest exporter of maize, and maize contributes significantly to the country's foreign exchange earnings. Thai maize is exported freely, with major customers in Singapore, Malaysia, Hongkong, Taiwan, Korea, Middle East and more recently in Africa. Over 60% is exported in the period from September to January, largely influenced by the season of harvest and seasonal price advantage.

It is generally realized throughout that Thai maize suffers from a US\$ 10-20 per tonne quality discount on the world market. These losses will continue to rise

unless real efforts are made to control aflatoxin and improve quality.

There have been significant changes in the export markets for Thai maize. In 1981/82 Japan purchased 20,400 tonnes and Taiwan purchased 235,000 tonnes. In 1983/84 these purchases were 9,500 and 1,000 tonnes respectively. Such decline purchasing always referred to aflatoxin. In recent years the price of Thai maize has also fallen in relation to other sources of supply reflecting a decline in quality associated with the incidence of aflatoxin.

Levels of aflatoxin acceptable to Japan are less than 20 ppb. Other countries required levels of less than 50 or 100 ppb. Thai maize frequently exceeds these levels, in the rainy season. Because of the high incidence of aflatoxin, Thai maize regularly trades at \$15-20 per tonne below comparable maize from other sources. The annual cost to Thailand of the aflatoxin problem is not less than \$50 million per annum according to the report on the findings of the ThaiBritish aflatoxin Project, 1984.

Aflatoxin is measured in terms of parts per billion (ppb.) Acceptable levels in importing countries are generally in the range 30-100 ppb. Levels of aflatoxin

found in the stores of regional merchants in Thailand generally exceeded these levels.

In Thailand there is little contamination before harvest and contamination occurs mainly in the period immediately after harvesting and during primary processing.

It appears that most farmers dispose of their maize as soon as possible after harvest. Usually, harvesting may occur over a 10-14 day period, during which time the maize is bulked up often in moist, if not in wet conditions. In terms of aflatoxin contamination, this bulking up period is not considered as "storage" although in critical period. This storage period may be longer if the roads are impassable due to heavy rain, or if there is delay with transport or in the arrival of the mechanical sheller.

Maize is typically a low input-low output system. About half of the maize crop is stored on farm for at least 60 days-frequently in a moist condition. Many farmers store temporarily while bulking up enough for sale. Storage bulking up delays in drying and queuing problems in the merchant sector, together with the

humid ambient conditions, create ideal conditions for aflatoxin contamination.

It appears that, in general, those involved in the marketing and utilisation of maize in Thailand are generally aware of the problem of aflatoxin. What they lack is information about the problem, practical proposal for its solution and financial incentives to implement the appropriate measure.

Following the above, the Thai-British Project for the control of aflatoxin in maize was established in 1984 to investigate the incidence of aflatoxin in Thai maize and to determine whether its level can be controlled by effective drying. This project was undertaken by the staff of the Rural Investment Overseas Company Limited in conjunction with Silsoe College and the Tropical Development and Research Institute now the Overseas

Development and Natural Resource Institute, or ODNRD from the U.K. Also the Thai team comprised of officers and technicians from the Department of Agriculture, Office of Agricultural Economics, Bank for Agriculture and Agricultural Co-operatives and some private sector in the upcountry rural areas.

After Phase III is completed it indicated that the combined effort of the Thai Government, the maize industry and the participants of this project have been successful in demonstrating an effective method of aflatoxin control. Thailand is now in a better position to reduce the aflatoxin problem in maize to acceptable levels following the application of this research on a much wider national scale (see following sections for more comprehensive review of conclusions and recommendations from the project)

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Mycotoxicosis in animals

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by Sunchai Luxsanakoses

INTRODUCTION

History

Mycotoxins may be produced on hay, cereals, pastures, or fodder, or may be present in constituents used in the manufacture of meals or pelleted animal diets. Some animal diets, especially those containing grain or nuts, may contain several toxigenic species of mould, which may produce a number of mycotoxins having different toxic or pharmacological properties. Under these situations, clinical signs and lesions found in disease outbreaks may not conform to the usual descriptions of outbreaks, or to experimental findings in animals dosed with mycotoxins isolated from pure mould cultures.

Tentative Diagnosis (Confirmation)

History

Clinical signs

Lesions

requires extraction & identification of responsible mycotoxins from suspect foodstuffs.

Treatment

Generally ineffective

But some animals recover if the source of toxin is removed

Others are stunted or die.

CLASSIFICATION OF MYCOTOXINS

Classified according to the main organ system, they affect.;

Hepatotoxins

- **Sporidesmin**
- **Aflatoxins**
- **Luteoskyrin**

- **Cyclochlorotine**
- **Rubratoxins**
- **Sterigmatocystin**

Nephrotoxins

- **Ochratoxin**
- **Citrinin**

Neurotoxins

- **Penitrema**
- **Patulin**
- **Citreoviridin**
- **Misc. neurotoxins**

Cytotoxins (Alimentary Tract Toxins)

- **Trichothecenes**

- **T-2 Toxin**
- **Diacetoxyscripenal**
- **Neosolaniol**
- **Nivalenol**
- **Diacetylnivalenol**
- **Deoxynivalenol (Don, Vomitoxin)**
- **HT2 toxin**
- **Fusarenon X**

Estrogenic Mycotoxins

- **F-2 Toxin (Zearalenone)**

Other Mycotoxins

- **Ergot**
- **Fescue**
- **Lupinosis**

Discovered by:

Stevens et al. 1960: Turkey H disease outbreak Wannop, 1961; Abrams, 1965: duckling is more susceptible

Asao et al (1963): isolated the Chemical structure & referred to it as "Aflatoxin"

Harley et al (1963): found FBI, FB2, FG1, and FG2 Aflatoxicosis increases the susceptibility of turkeys to candidiasis, pasteurellosis and salmonellosis, and of chicks to coccidiosis and Marek's disease: Experimental work has revealed a significant interaction between aflatoxin and vitamin D with respect to bone calcification in chicks, this increasing their vitamin D requirements. (Bird, 1978).

Vitamin A increases mortality rate in Aflatoxin chicks (Bryden et al., 1979)

Affected: Animals:***Poultry***

ducks; turkey poult; pheasant chicks; chickens quail

Mammals

young pigs; pregnant sows; dogs; calves; cattle; sheep; cats; monkeys; man

Fish

Lab. animals

Cause(s):

Some strains of *Aspergillus flavus* & most strains of *A. parasiticus*

Sign(s):

a high mortality

sudden onset

inappetite

dejected appearance

Farm animals:

- **malabsorption of various nutrients**

- **coagulopathy**
- **less tissue integrity**
- **poor growth**
- **less FCR**
- **greater susceptibility to infection**
- **vaccine failures**
- **drug failures**
- **reproductive problems**
- **greater sensitivity to temperature extremes**

Other animals:

Chicken:(5)

depression, inappetence, lower growth rates, poor condition, bruising, lowered egg production, fertility, and hatchability, with high mortality. Also, ataxia, convulsion and opisthotonos are common signs.

less plasma testosterone(22)

paralysis & lameness(24)

Turkey poults & ducklings are particularly susceptible. In acute outbreaks, death occurs after only a short period of inappetence. Subacute symptoms are more usual.

- **lower growth rate**
- **lower FCR**
- **depressed appetite**
- **interferes with absorption of food especially carotenoid**
- **with low level of aflatoxin:**
- **lower quantity of meat of carcass**
- **lower meat & bone ratio**
- **susceptibility to body wands**
- **lower fat in liver**
- **destroyed protein synthesis in liver**
- **lowered resistance to diseases**
- **lower level of plasma amino acids**

Fish

highly sensitive

1 ppb B1 - cancer in Rainbow trout(5)

Rat

highly sensitive p.(6)

1 ppb B1 - cancer in Fisher rat(5)

Severity ~ Biological Effect of Toxin(3)

Dosages:

Duration of Expose:

Species:

Sheep - highest resistance

Duck - lowest resistance & the most susceptible

breed:

Age:

young animals are more susceptible to acute effect(22)

diet:

dietary protein (11): The effect of aflatoxin is enhanced by a low protein diet.

A low fat diet also: increases the aflatoxin effect

animal's health

individual susceptibility

Type of Toxin

PM Lesion(s):

Target organs(5)

Liver- major

Kidney - minor (in some condition)

Colon - minor (in some condition)

Chich(23)

Testes

Liver

general edema with congested spleen & kidneys marked degenerative changes in the parenchymal liver cells

bile duct proliferation a membranous glomerulonephritis hyaline droplet necrosis some degree of ascites & visceral edema pale & mottled liver with widespread necrosis excessive bile production (common) marked catarrhal enteritis (characteristic) (especifically duodenum).

Diagnosis:

A. flavus may be isolated from feed.

Biological assays for the toxin (duckling/poults).

Chemical assays: fluorescent/chromatographic

techniques.

Suspect: if his topath. shows that hyperplasia of

bile ducts (Common)

Chickens hepatic cells enlarged with some necrotic foci. if feed aflatoxin 25-15,000 g/Kg for 8wks the highest B1 residue will be found in liver (muscles)

Swine

if feed aflatoxin 300-500 ug/Kg for 4 months B1

residue can be found ~137 ug/kg in liver but if stop

feed aflatoxin for 2 wks-no liver aflatoxin residue

can be found

FDA (3)

to condemn foods with 15 ppb B1 (6)

Mild inhibitors can control the growth of *A.flavus* in the feed, i.e.

- 8-Hydroxyquinoline (500 ppm [0.5 gm/kg]) - Gentian violet (500-1500 ppm [0.5-1.5 g/kg]) - Propionic acid (500-1500 ppm [0.5-1.5g/kg]) - Thiabendazole (100 ppm [100 mg/kg])

Fed a high energy, good quality protein diet, increased water, and fat soluble vitamins.

Luteoskyrin

Animals Affected: chicken rat

Cause(s): *Penicillium islandicum*

PM Lesion (s): liver necrosis

Cyclochlorotine

Animals Affected: chicken rat

Cause(s):

Penicillium islandicum

PM Lesion(s): liver necrosis

Rubratoxins

Sources: maize; legume; cereal; peanut kernels; pods; sunflower seeds; bran

Charcteristics: Classification: Rubratoxin A Rubratoxin B

Animals Affected: several spp.

Cause(s):

penicillium rubrum sign(s): several organs & tissues hemorrhage, especially - liver

Sterigmatocystin

Sources: Green coffee/moldy wheat/Dutch cheeses Characteristics: low acute toxicity about 1/10 as potent a carcinogen as B1 Spp. Affected: Lab. animals mice/rats Cause(s): Several spp. of Aspergillus Penicillium luteum Bipolaris sp.

NEPHROTOXINS

Ochratoxin

Sources:

Cereal grains

wheat/Barley/Oats/Corn/Dry beans/

Moldy peanuts/Cheese/Tissues of

swine

Characteristics:

Ochratoxin A-a potent nephrotoxin in

Rats/Dogs/swine

teratogenic to Mice; Rats, & Chicken embryos

involved in Porcine nephropathy?

Animals Affected: Poultry ducks/chickens Mammals swine/dogs/man(?)

**Cause(s): Aspergillus ochraceus & related spp. Penicillium uiridicatum other
Penicillium spp.**

Sign(s):

3.6 mg/Kg Ochratoxin A causes 5% mortality

rate(4)

54 mg/Kg Ochratoxin B(4)

lowered growth rate

edema of visceral organs

accumulated of uric acid in

kidneys

ureters

heart

spleen

liver

suppress as blood-forming in bone marrow

suppress as lymph formation in spleen

bursa of Fabricius

highest toxicity in broiler (4)

5 ppm Ochratoxin in feed- growth rate

4-8 ppm Ochratoxin in feed-high mortality

rate

4 ppm Ochratoxin in laying hen-ceases egg

production

Citrinin

Sources:

Cereal grains wheat/Barley/Corn/Rice

**Characteristics: a yellow colored compound involved in Porcine nephropathy?
lower toxicity than Ochratoxin**

Animals Affected:

Mammals

swine/dogs

Lab. animals

Cause(s): several Penicillium spp. several Aspergillus spp.

NEUROTOXINS

Penitrem A

Sources: silage

Characteristics: produces neurological & renal effects

Discovered by: Wilson et al. 1968

Spp. Affected: Cattle sheep horses

Causes(s): Penicillium cyclopium

Sign(s): incoordination syndrome (staggers)

Patulin

Sources: Moldy feed/Rotted apples/Apple juice/Wheat straw residue

Characteristics: carcinogenic in mice found Pexpansum in apple & apple juice

Spp. Affected: Poultry chicken embryo/chickens/quail Mammals cattle/cats

Lab. animals mice/rats/rabbits Aquatics brine shrimp/guppies/zebra fish larvae

Cause(s): numerous *Penicillium* spp. numerous *Aspergillus* spp. *Byssochlamys nivea*

Citreoviridin

Cause(s): *Penicillium citreoviride*

**Sign(s): directly affects the central nervous system paralysis convulsion
respiratory failure cardiac failure death**

Miscellaneous Neurotoxins

Cause(S): *Claviceps paspali* *aspergillus fumigatus*

**Sign(s): nervous disorder: ataxialparesis/apathyl hypersensitivity/frenzy
locomotor problems**

CYTOTOXINS (ALIMENTARY TRACT TOXINS)

Trichothecenes

T-2 Toxin (Fusariotoxin)

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

Characteristics:

quite toxic to rats/trout/calves

involved in ATA (Alimentary toxic Aleukid) in human

has been implicated in "Yellow Rain" in Southeast

Asia

is not thought to be carcinogenic

is very toxic, but low contamination in foods & feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

Cause(s): Fusarium tricinctum(4) Fusarium spp.

Sign(S):

Poultry:

affect to G1 tract of broiler

1 ppm T-2 causes stomatitis

20 mg/Kg in Laying hen

stomatitis

<<appetite

wt. loss

<< egg production

soft shell

abnormal of hair coat

large doses in chickens cause

oral lesions

large intestine hemorrhage

severe ascites

neurotoxic effects

death

tibial dyschondroplasia (26)

Skin of Rabbits/Rats/& other animals (+Man) severe dermal responses

Cattle:(16) similar to other spp. coagulopathy immunosuppression hematologic changes lymphatic necrosis or atrophy decreased feed consumption decreased weight gain

Swine:(19) anorexia lethargy posterior weakness/paresis persistent high fever decreased weight gain prominent neutrophilia decreased serum glucose/albumin/alkaline phosphatase increased serum globulin topical exposure can cause systemic effects reduced cardiac output (hypotension) (21) circulatory shock (21)

Treatment Swine superactivated charcoal with magnesium sulfate improved survival times and rates (25)

Diacetoxyscripenai

Sources:

Corn/Wheat/commercial cattle feed/Mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

Neosolaniol

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

Nivalenol

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

Diacetylnivalenol

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals**

Deoxynivalenol (DON, vomitoxin)

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

**Characteristics: moldy corn toxicosis of swine may have teratogenic effect
unlike T-2 toxin, DON contamination in feed is significant**

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

**Sign(s): vomiting in animals inappetite lack of weight gain digestive disorders
diarrhea death**

HT-2 Toxin

Sources:

Corn/Wheat/Commercial cattle feed/mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

Fusarenon H

Sources:

Corn/Wheat/Commercial cattle feed/Mixed feeds

**Spp. Affected: Poultry turkey/chickens Mammals
swine/cattle/horses/dogs/cats/man Lab. animals mice/rats**

ESTROGENIC MYCOTOXINS

F-2 Toxin (Zearalenone)

Sources:

Corn/Oats/Barley/Wheat/Sorghum(both fresh and stored)/Moldy hay/Pelleted commercial feed high moisture corn

Characteristics:

- a potent nonsteroidal estrogen**
- one of the Resorcylic acid lactones (RAL)**
- under controlled administration; "zearalanol", a closely related RAL, is widely used in cattle as an anabolic agent**
- not highly toxic**

- needs 1-5 ppm to cause responses in swine

- can be transmitted to piglets via sow's milk and causes estrogenism in young pigs

Spp. Affected: Poultry turkey/chickens Mammals swine/dairy cattle/lambs Lab. animals mice/rats/guinea pigs

Cause(s): Fusarium Spp.(4) primarily from *F. graminearium* (*roseum*)

Sign(S)

affect Reproductive system of mammal

Poultry:

***Fusarium roseum*(4)**

swollen & prolapse of poultry's cloaca

bursa of Fabricius enlargement of turkey

25 ppm F-2 causes no effect to chicken

300 ppm F-2/10 days(4)

no effect to growth rate of Leghorn chicken

but found cysts in reproductive tract

Swine:(48) no effect on libido/mating behavior slight increase in sperm abnormalities vulvovaginitis & estrogenic responses in swine

Cattle:(20) abortion

OTHER MYCOTOXINS

Ergot toxin

Sources: flowers or spikelets of cereals (rye) & of grasses perennial rye

grass/cocksfoot/timothy/crested dog's tail/oat grass/yorkshire fog

Characteristics: a worldwide disease of farm animals ergot contains a number of alkaloids ergotamine & ergonovine (ergometrine) are the most important alkaloids ergotamine stimulates the central nervous system

Discovered by:

Edwards (1953)

Spp. Affected: cattle swine sheep

Cause(s):

Claviceps purpurea

(ergot)

Sign(s):

Ergotamine: — Weak clonic convulsions followed by depression — lethargy — weakness — ataxia — stupor — paralysis of respiratory center — hypertension —> tone of uterus — death

Ergometrine:

—capillary endothelium damage

— blocking of capillary flow

—vascular stasis

— thrombosis in small arterioles

— dry gangrene

Cattle:

— lameness for 2-6 weeks or longer

— **hindlimbs are affected before forelimbs**

— **high fever**

— **high pulse rate**

— **high respiration rate**

— **swelling & tenderness of fetlock & pastern**

joints

— **in 1 week, loss of sensation of the affected**

area

— **dry gangrene at the distal part**

— **sloughing of distal part**

Swine: — lower feed intake — lower weight gain — lack of udder development of sow pregnancy — weak and undersized newborn piglets — low survival rate of the piglets

Sheep: — similar signs to cattle with additional, mouth ulceration

PM Lesion(s): marked intestinal inflammation in sheep

Control: immediate change to an ergot-free diet grazing or topping of pastures to reduce flower head production.

Fescue toxin (Fescue Foot/Tall Fescue Lameness)

Sources: perennial grass

Characteristics: cool season perennial grass in Australia, New Zealand, Eastern & Central USA, France, Italy

Discovered by: Case (1966) and Keyl et al. (1967)

SDD. Affected: cattle sheep

Cause(s): unidentified, may be related to Acremonium coenophialum, Fusarium spp., and Aspergillus terreus

Sign(s): lameness in one or both hind feet may progress to necrosis of the distal part of the affected limb tail & ears may also be affected with or without lameness.

Lupins Toxin (Mycotic Lupinosis)

sources: sweet lupines

Characteristics: differs from Lupine poisoning (a nervous syndrome caused by alkaloids present in bitter lupines)

Discovered by: van Warmelo et al. (1970)

Spp. Affected: sheep cattle

Cause(s): Phomopsis leptostromiformis

Sign(s): — inappetite — listlessness — photosensitive (sheep) — ketosis (common) — lacrimation (cattle) — salivation (cattle) — jaundice — death occur in 2-14 days in acute case

PM Lesion(s):

— liver—damage, enlarge, orange-yellow, fatty (acute)

— liver - bronze-or tan -colored, firm, contracted in size, distorted and fibrosed

— copious transudates in abdominal, thoracic and pericardial sac

control:

— frequent surveillance of sheep and of lupine fodder material for characteristic black spot fungal infestation, especially after rains

—oral doses of zinc (0.5 g or more per day) for protection from phomopsin-

induced liver in jury in sheep.

REGULATION AND CONTROL

The U.S. Food, Drug & Cosmetic Act: defines food as adulterated if it contains "any poisonous or deleterious substance which may render it injurious to health". (Labuza 1982: Rodricks & Roberts, 1977)

Safe tolerance levels have not been established for any of the mycotoxins. In the absence of tolerances, FDA has set a regulation which apply to all products known to be susceptible to aflatoxin contamination, including animal feeds. In the U.S., the FDA has an action level of 20 ppb (ug/kg) for aflatoxin in susceptible commodities. Since 1984, FDA has relaxed its action level for aflatoxin levels in feed. The new aflatoxin in feed level should not exceed 100 ppb except those for dairy cows, in which the level still should not exceed 20 ppb. The action level for aflatoxin in whole milk, skim milk and low fat milk is 0.5 ppb.

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-

Overview of analytical methods for mycotoxin contamination in maize and peanuts

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There are two main types of assay which have been developed for detection and/or determination of mycotoxins, viz biological and chemical. Bioassay techniques, are only qualitative or semi-quantitative and are often non-specific. They are useful for indicating the presence of unknown toxins, and are useful in studies to isolate new mycotoxins. Once a new toxin has been identified, it is then usually possible to develop a suitable chemical assay for its detection and quantification. The chemical assay is almost invariably quicker, cheaper, more specific, more reproducible, and more sensitive than the corresponding bioassay. Chemical assays are suitable for routine analyses, as required for quality control and survey work. Immunoassays, are a combination of a chemical and a biological assay. They are very sensitive and can be specific, making them

suitable for screening body fluids etc. for traces of toxin.

CHEMICAL ANALYSES

All methods of chemical analysis for mycotoxins incorporate a combination of procedures, as indicated in the flow-chart in Figure 1.

Sampling and Sample Preparation

This aspect of the analysis is discussed in detail in the previous section. However, it must be emphasised here that this is a most important step. If the sample taken for analysis is not representative of the bulk, then the analytical results are meaningless. Because of the very uneven distribution of mycotoxins that are usually found in naturally contaminated commodities, it is essential to follow a suitable sampling plan. Sample preparation involves grinding and sample division to obtain a representative analytical sample. The use of water slurries enables larger, and therefore more representative analytical samples to be taken. Typically, 1 kg of ground material is blended at high speed with an

appropriate amount of water to give an homogeneous slurry (paste) from which 100g aliquots are taken for analysis.

Extraction

The organic solvents most commonly used for extraction of mycotoxins are: chloroform, acetonitrile, methanol and acetone. These solvents are mixed with a given ratio of a more polar solvent (water, dilute acid, aqueous solution of salts) to aid the breaking of weak electrostatic bonds which bind some mycotoxins to other substrate molecules (eg proteins).

[Figure 1. Flow diagram for Mycotoxin Analysis](#)

The ground sample, or preferably an aqueous slurry, is either shaken with the extraction solvent for 30-45 minutes or blended at high speed for about 3 minutes. It should be noted that an explosion proof blender is recommended for use with inflammable solvents such as acetone and methanol.

Clean-Up

Mycotoxins are such a diverse group of chemical compounds that it is difficult to find a simple procedure which specifically removes non- mycotoxin "interfering" compounds whilst leaving the mycotoxins in the extract. For this reason it is difficult to find a good method for screening a wide-range of mycotoxins simultaneously. It is possible, however, to devise procedures which remove interfering non-mycotoxin compounds from the extract of a particular commodity and leave a particular mycotoxin or group of mycotoxins in the extract.

Common clean-up techniques which have been used are:

- a. Defatting-this is carried out prior to the toxin extraction step and uses petroleum ether or hexane to extract lipids from the sample using a Soxhlet extractor. This step is only required when the subsequent clean-up step is not capable of removing the lipids.**
- b. Column-chromatography-this technique has very wide application, and is used in a number of regulatory or officially approved methods. A glass column is packed with one or more adsorbent materials and the crude extract is added to the top of the column. The column is then eluted with a**

series of solvents or solvent mixtures which are designed to first wash off interfering compounds and then elute the desired mycotoxins, whilst other interfering compounds remain strongly bound on the column.

A miniature column, called a mini-column is used in many rapid aflatoxin assay methods to remove interfering compounds and to qualitatively detect aflatoxin down to a few parts per billion (ppb)

- c. Precipitation-this is an extremely useful technique whereby certain chemicals, sometimes in colloidal form, are added to the crude extract and these absorb certain pigments, proteins and other interfering compounds onto their surface. The complex so formed precipitates out of solution and can be filtered off, leaving a "cleaned-up" solution. Useful precipitating agents include: cupric carbonate, ammonium sulphate, lead acetate, and ferric gel.**
- d. Liquid-liquid partition-is commonly used, often in conjunction with one of the other clean-up procedures, to provide additional clean-up and also to transfer toxins from one solvent system to another whilst at the same time effecting a considerable increase in concentration of the toxins. The partition is carried out in a separating funnel which contains the two**

immiscible solvents. The funnel is shaken for a few minutes to allow the dissolved compounds, including the mycotoxins, to partition between the two phases. The solvents are selected so that the mycotoxins are preferentially partitioned into one of the solvents. Care must be taken in the choice of solvents in order to minimise the risk of emulsion formation.

e. Thin layer chromatography (TLC) -although this technique is used mainly for detection and quantification of toxins it is also useful for separating the mycotoxins from interfering compounds in the extract. Sometimes it is necessary to try a range of developing solvents in order to separate the toxins of interest from the interfering compounds. If this proves impossible, or too time consuming, then twodimensional TLC may be applicable.

f. Other clean-up procedures.

The clean-up procedures outline above are those employed in officially approved methods for aflatoxin analysis, and they are also used in analytical methods for many other mycotoxins. Other clean-up procedures, including reverse dialysis and base extraction are explained in the next period.

Work-Up

After the clean-up step the extract must be "worked-up" in order to prepare it for the detection and/or quantification step. Following clean-up the extract is often dissolved in a large volume of aqueous solvent, so it must be transferred into a small volume (10-50ml) of a volatile solvent, such as chloroform. The chloroform solution often requires drying and this is achieved by passing it through a bed of anhydrous sodium sulphate. The solvent is evaporated off, as described below, to near dryness. Care must be taken at this stage as some mycotoxins, eg the aflatoxins, can break-down if the dry extract is heated at 100C. To avoid this the evaporation is best carried out using a rotary evaporator at 30.C to reduce the volume to a few ml, followed by evaporation to dryness in an inert atmosphere using a sample concentrator. If such items of equipment are not available, then evaporation should be carried out using a steam-bath, preferably under a stream of nitrogen, and great care should be taken to ensure that flasks are removed from the steam-bath just before they go dry, and that vials are removed on the point of dryness. The extracts are now ready for detection and quantification.

Detection and Quantification

It is fortunate that the aflatoxins and many of the other important mycotoxins are fluorescent under ultraviolet light. This enable them to be detected at very low levels (parts per billion), and this method of detection is used in the majority of analytical methods for mycotoxins.

Detection or "qualitative assay" is usually by TLC or mini-column and only requires the use of a qualitative (not accurately determined) standard. Such a standard is also sufficient to enable a semi-quantitative assessment to be made by TLC, using a "dilution-toextinction" principle. Fully quantitative determinations, which require the use of a standard of known concentration, can be carried out by a variety of techniques including: TLC high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), and the fluorotoxinmeter (FTM). These, and other quantitative techiques are discussed in subsequent sections of this manual.

Confirmation

It is essential that confirmatory tests be carried out if a mycotoxin is thought to have been detected, especially when a new commodity, or new source of commodity is being analysed. Failure to do this could easily lead to false-positive results (ie., an interfering compound is mistaken for a known mycotoxin).

Most confirmatory tests involve the formation of a derivative which has different properties (eg., colour of fluorescence or polarity), than the presumptive mycotoxin. When detection and/or quantification is by TLC, then derivatives can be formed "on-the-plate" either by spotting a reagent on the plate before development or by spraying on a reagent after development. An example of the former approach is the use of trifluoroacetic acid (TFA) to form the hemi-acetal derivatives of aflatoxin B1, G1 and M1 which are identified, if present, as slower running fluorescent spots. A commonly used spray reagent for the aflatoxins is 50% sulphuric acid which reacts with the blue and green-blue fluorescent aflatoxins to give yellow fluorescent derivatives.

DETERMINATION OF THE PURITY AND CONCENTRATION OF MYCOTOXIN

STANDARDS

Pure mycotoxins are available from several manufacturers (listed in Appendix: 2) and these are used to prepare standard solutions for use in mycotoxin analytical procedures. It is essential that the exact concentrations of the standard solutions are known; these may be determined by UV absorbance measurements on a spectrophotometer.

THEORETICAL ASPECTS OF CONCENTRATION MEASUREMENTS

There are two laws which describe the absorption of light by matter.—

Lambert's Law: The proportion of monochromatic light absorbed by a homogeneous medium is independent of the intensity of the incident light, and each successive unit layer absorbs an equal fraction of the light incident upon it.

Beer's law: The fraction of the incident light absorbed by a solute in a transparent solvent depends on the concentration of the solution (c) and on the

path length (1).

These two laws can be combined to give the "socalled" Beer-Lambert Law which leads to the expression

$$A = \log_{10} (I_0/I) \quad cl = kcl$$

where

A = The absorbing capacity, absorbance or optical density of the solution

I = intensity of the transitted radiation

I₀ = intensity of the transmitted radiation

c = concentration of the absorbing substance

l = path length of the solution

k = extinction coefficient of the absorbing substance (a constant)

In practice, the quantities actually measured are the relative intensities of the radiation beam transmitted by a cell full of pure solvent, and by an indentical

cell full of the solution. When these intensities are taken as I_0 and I respectively (see Figure 1), the resulting absorbance, which is generally read directly from the logarithmic chart paper, is that of the dissolved solute only. In manual UV single-beam spectrophotometers the absorbance scale is first 'zeroed' with the solvent blank alone. The solvent blank is then replaced by the cell containing the solution and the absorbance read off.

FIGURE 1: Measurement of absorbance (A). The right hand diagram represents an absorption band as recorded by a double-beam spectrophotometer $A = \log_{10} (I_0/I)$

The extinction coefficient (k), at a given wave length, is a constant, characteristic of the substance under examination. When l is expressed in centimetres and the concentration as a percentage, k is called A , $\text{cm } 1\% \text{ cm}$; the optical density for a 1% w/v solution in a 1cm cell. If the molecular weight of the substance under examination is known, the concentration can be expressed in g. moles/litre and k is then called the molar absorptivity and is designated E (the optical density for a molar solution in a 1cm cell). It follows that:

$$E = \frac{A \times M. Wt}{c \times l} \text{ (c in g/litre (or mg/ml), l in cm)}$$

$$\text{ie } c = \frac{A \times M. Wt}{1 \times E} \text{ mg/ml}$$

$$\text{or } c = \frac{A \times M. Wt \times 1000}{1 \times E} \text{ ug/ml}$$

CALIBRATION OF THE SPECTROPHOTOMETER

In order to avoid errors in determining the concentration of the mycotoxin standards, the spectrophotometer which is to be used should be checked to ensure that it is functioning correctly. This is done using three serially diluted solutions of potassium bichromate to confirm that absorbance of the solutions is directly proportional to the concentration. The correction factor (CF) should be as near to 1.0 as possible (ie no correction to any calculation being necessary).

Method

Make up the solutions of potassium dichromate in 0.018N sulphuric acid as described in Appendix:1. Determine the absorbance of the three solutions at the wavelength of maximum absorption (about 350 nm) using 0.018N sulphuric acid as the reference blank.

Calculate the absorptivity (E) for each of the solutions using the equation.

$$E = \frac{\text{absorbance} \times 1,000}{\text{concentration (mM)}}$$

Take an average of the three values obtained to give E

Determine the correction factor (CF) for the particular instrument and cells used by applying the equation:

$$CF = \frac{3,160}{E}$$

If CF is less than 0.95 or greater than 1.05 check the instrument or the technique.

DETERMINATION OF CONCENTRATION OF MYCOTOXIN STANDARD SOLUTIONS

If the standard is received as a dry film in a vial or as a crystalline sample, add a small known volume of appropriate solvent by means of a syringe through the rubber septum of the vial to dissolve the standard. Quantitatively withdraw a known volume of the concentrated solution and transfer it to a volumetric flask and dilute accordingly to give the required concentration.

This method of dissolving the pure standard minimises the hazards of handling highly toxic material. Measure the absorbance of the mycotoxin standard solution at the wavelength of the maximum absorption, the majority of mycotoxins have a maximum absorption at wavelength between 200-400 nm and details of these and other spectrophotometric parameters are given in Table 1. Calculate the concentration using the equation:

$$\text{Concentration (ug/ml)} = \frac{\text{Absorbance} \times \text{Molecular Weight} \times \text{CF} \times 1,000}{E}$$

Where CF is the correction factor obtained above and E is the absorptivity of the particular toxin being examined in the Particular solvent used.

Table 1: Spectrophotometric Parameters for Various Mycotoxins

Mycotoxin	Molecular weight	Solvent	Absorbivity (E)	- Max (nm)
Aflatoxin B.	312	Benzene:acetonitrile		
		(98:2 v/v)	19,800	353
Aflatoxin B1	312	Chloroform	22,300	353
Aflatoxin B2	314	Benzene:acetonitrile		
		(98:2 v/v)	20,900	355
Aflatoxin G1	328	Benzene:acetonitrile		
		(98:2 v/v)	17,100	355
Aflatoxin G2	330	Benzene:acetonitrile		

		(98:2 v/v)		357
Aflatoxin M1	328	Chloroform	19,950	357
Ochratoxin A	403	Benzene:acetic acid		
		(99:1 v/v)	5,550	333
Ochratoxin B	369	Benzene:acetic acid		
		(99:1 v/v)	6,000	320
Ochratoxin A	431	Benzene:acetic acid		
ethyl ester		(99:1 v/v)	6,200	333
Ochratoxin B	397	Benzene:acetic acid		
ethylester		(99:1 v/v)		320
Patulin	154	Absolute ethanol	14,540	276
Patulin	154	Methanol	12,880	275
Sterigmatocystin	324	Benzene	15,200	325
Citrinin	259	Chloroform	16,100	322
Zearalenone	318	Ethanol	29,700	236

Zearalenone	318	Ethanol	13,909	274
Zearalenone	318	Ethanol	6,020	316

Nb If the standard is dissolved in benzene: acetonitrike 98:2, then use this solvent as the reference blask.

MYCOTOXIN STANDARD SOLUTION CONCENTRATIONS

Although the list in Table 2 below does not necessarily correspond with the concentrations quoted in the literature, we have found them to be most useful.

- a. Stock solutions - these are made directly from the pure crystals or dry film.**
- b. UV solutions - these are direct dilutions from the stock solution and are those which are used for determining the correct concentration of standard by UV spectrophotometric measurement.**
- c. TLC working solutions - these are made by dilution from either the stock or the UV solutions.**

TABLE 2: CONCENTRATIONS OF MYCOTOXIN STANDARD SOLUTIONS

Mycotoxin	Stock (ug/ml)	UV (ug/ml)	TLC (ug/ml)
Aflatoxin B1	100	10	1
Aflatoxin B2	100	10	1
Aflatoxin G1	100	10	1
Aflatoxin G2	100	10	1
Aflatoxin M1	10	10	1
Ochratoxin	25	25	5
Patulin	40	10	40
Sterigmatocystin	100	100	100
Citrinin	40	20	40
Zearalenone	100	10	50
T-2 Toxin	5,000	—	100
Diacetoxyscirpenol	5,000	—	100

0.05 65 Available from suppliers.

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APPARATUS AND REAGENTS

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Reagents

0.018 N Sulphuric acid - dissolve 1.0ml concentrated sulphuric acid in 2.0 litres distilled water. Potassium dichromate solutions - weight accurately approximately 80 mg of potassium dichromate and dissolve it in 1.0 litre 0.018 N sulphuric acid.

Calculate its molarity as follows:

$$\text{Molality in mM} = \frac{\text{Weight in mg}}{\text{Molecular weight}}$$

It should give a solution of approximately 0.25 mM. make two accurate, successive half dilutions of this solution with 0.018 N sulphuric acid to give solutions of approximately 0.125 mM and 0.0625 mM.

Acetic acid A.R.

Acetonitrile A.R.

Benzene A.R.

Absolute ethanol A.R.

Methanol A.R.

Chloroform A.R.

Ethanol A.R.

Apparatus

Spectrophotometer - capable of measurements from 200 - 400 nm.

Quartz - Faced cells - 1 cm.

Lens tissue.

Disposable pipettes and rubber teats.

Acetone wash bottle.

Beaker - 25 Oml, for waste solvent.

Volumetric flasks - 100, 250ml.

MYCOTOXIN STANDARDS

(a) Suppliers(a)

- 1. Aldrich Chemical Co Ltd**
The Old Brickyard
New Road
Gillingham
Dorset SP 8 4JL
UK
- 2. Sigma Chemical Co Ltd**
Fancy Road
Poole
Dorset BH17 7NH
UK
- 3. Phase Separations Ltd**
Deeside Industrial Estate
Queensferry
Clwyd CH5 2LR
UK

- 4. Uniscience Ltd**
Uniscience House
8 Jesus Lane
Cambridge CB5 8BA
UK
- 5. Makor Chemicals Ltd**
P.O.B. 6570
Jerusalem 91060
Israel
- 6. Pierce and Warriner (UK) Ltd**
44 Upper Northgate Street
Chester
Cheshire CH1 4EF
UK
- 7. C.P. Laboratories Ltd**
(Calbiochem)
PO Box 22
Bishops Stortford

Herts CM22 7RQ

UK

8. Pfaltz and Bauer, Inc.,

375 Fairfield Avenue

Stamford

Connecticut 06902

USA

9. Sigma Chemical Co

PO Box 14508

St Louis

Missouri 63178

USA

Calbiochem Agencies in Middle East and Africa

Nigeria

Nigerian Hoechst Ltd

PO Box 261

Ikeja

Lagos

Lebanon

Hosta Pharma

B.P. 5767

R L Beyrouth

Liban

Kuwait

Bader Sultan and Bros Co

PO Box Safat 867

KWT - Kuwait it

If no agency is listed for a country order from C.P. Laboratories Ltd. UK.

Calbiochem Agencies in South East Asia and the Far East

Australia

Calbiochem - Behring

Australia Pty Ltd

PO Box 37

Carlingford

NSW 2118

Philippines

Hoechst Far East Marketing corp

Hoechst House

Salcedo Street

Legaspi Village

Makati, Metro Manila

PO Box 433

Commercial Centre 3117

Malaysia

Hoechst Malaysia Sdn Bhd

468-6C Jalan Ipoh

**PO Box 540
Kuala Lumpur**

**Singapore
Hoechst Singapore Pte Ltd
Circuit Road
PO Box 89
Singapore 9137**

**Republic of Korea
Han-Dok Remedia Ind. co Ltd
344 Sangbong-dong
Dongdaemun - ku
C.P.O. Box 30
Seoul**

**Japan
Hoechst Japan Ltd
C.P.O. Box 1256**

Tokyo

New Zealand

Hoechst New Zealand Ltd

PO Box 67

Auckland

Calbiochem Agencies in Central and South America

Brazil

Casa Fachada Ltda

Setor Diagnosticos

Rua Julio Verne 55

04725 Santo Amaro

Sao Paulo

Chile

Victor Manuel Grez O

Ahumada 312

Oficina 721

Casilla 14656

Santiago

Colombia

Hoechst Colombiana S.A.

Apartado Aero 80188

Bogota

Costa Rica

Productos Hoechst Ltda

Apartado 10158

San Jose

Dominican Republic

Hoechst Dominicana S.A.

Apartado Postal 830

Santo Domingo

Ecuador

Hoechst - Eteco S.A.

Casilla 1408

Quito

Guatemala

Quimica Hoechst de Guatemala S.A.

Km. 15.5 Caratera Roosevelt

apartado 155

Guatemala, GA.

Venezuela

Hoechst Remedial S.A.

Apartado 80222

Caracas 108

(b) Source and approximate prices

1. Allatoxin B1(d)	Amount(b) (mg)	Approximate Cost(c) ()
Available from all suppliers	5	10
	10	15
	50	70
	250	320
NB It may be possible to obtain, free of charge, a solution in chloroform containing approximately 10 ug/ml aflatoxin B1 from: -		
Dr P L Schuller		
Rijks Instituut voor de Volksgezondheid		
Postbus 1, Bilthoven		
The Netherlands		
2. Aflaloxin B2(d)		
Available from all suppliers	5	18
	10	30

	50	150
3. Allatoxin G1(d)		
Available from all suppliers	5	17
	10	30
	50	125
4. Atlatoxin G2(d)		
Available from all suppliers	5	20
	10	30
	50	150
5. Aflaloxin M1(d)		
Available from suppliers	0.01	20
(2) (4) (5) (6) (7) (9)	0.05	57
vacuum dried or as a 10 ug/ml	0.25	200
solution in chloroform		
6. Allatoxin M2(d)		

Available from suppliers	0.01	20
(2) (4) (5) (6) (9) vacuum dried or as a 10 ug/ml solution in chloroform	0.05	65
7. Aflatoxin Qualitative Standard Kit		
(Contains 10 mg each of B1, B2, G1, G2)		90-140
Available from suppliers		
(1) (2) (4) (5) (6) (9)		
8. Aflatoxin Quantitative Standard Kit		
(Contains 1mg each of B1, B2, G1, G2)		30
Available from suppliers		
(1) (2) (4) (5) (6) (9)		
9. Citrinin		
Available from suppliers	5	20
(1) (2) (4) (5) (7) (9)) (9)	10	30
	25	40

	50	107
10. Cytochalasin A		
Available from suppliers	1	12
(1) (2) (4) (5) (7) (9)	5	30
	10	47
11. Cytochalasin B		
Available from suppliers	1	6
(1) (2) (4) (5) (7) (9)	5	20
	10	32
	50	135
12. Cytochalasin C		
Available from suppliers	1	14
(1) (2) (4) (5) (7) (9)	5	40
	10	65

13. Cytochalasin D		
Available from suppliers	1	13
(1) (2) (4) (5) (7) (9)	5	35
	10	65
14. Cytochalasin E		
Available from suppliers	1	10
(1) (2) (4) (5) (9)	5	30
	10	50
15. Diacetoxyscirpenol		
Available from suppliers	5	13
(2) (4) (5) (6) (7) (9)	10	25
	25	50
	50	95
16. Luleoskyrin		
Available from suppliers	5	25

(2) (4) (5) (6) (7) (9)	25	80
	100	280
17. Ochraloxin A		
Available from suppliers	5	30
(1) (2) (4) (5) (6) (7) (9)	25	120
18. Patulin		
Available from suppliers	10	26
(1) (2) (4) (5) (6) (7) (9)	25	35
	50	100
19. Penicillic Acid		
Available from suppliers	5	8
(1) (2) (4) (5) (6) (9)	10	12
	25	23
	100	75
20. Roridin A		

Available from suppliers	5	25
(2) (4) (5) (6) (9)	25	80
21. Rubratoxin B		
Available from suppliers	5	20
(1) (2) (4) (5) (6) (7) (9)	10	35
	25	64
22. Sterigmatocystin		
Available from suppliers	5	20
(1) (2) (4) (5) (6) (7) (9)	10	36
	25	100
	50	150
23. T-2 Toxin'		
Available from suppliers	5	24
(2) (4) (5) (6) (7) (9)	25	56-100
24. Verrucarin A		

Available from suppliers	1	7
(2) (4) (5) (6) (9)	5	25
	25	85
25. Verrucarol		
Available from suppliers	1	12
(2) (4) (5) (9)	5	50
26. Zearalenone		
Available from suppliers	10	5
(2) (4) (5) (9)	25	12
27. Mycoloxin Kits(f)		
Available from suppliers		
(2) (4) (5) (9)		
1. Ochratoxin 5mg, Patulin 10mg, Penicillic acid 10-25 mg, Rubratoxin 5- 10mg.		86
2. Citrinin 10mg, Luteoskyrin 5mg,		68

Sampling, sample handling and preparation in grains and cereals

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by Traisat Hongsuwong,

This paper is presented in the Training Course on Mycotoxin Prevention and Control in Field of Sampling, Sample Handling and Preparation in Grains/Cereals. It is a collection from many ideas of selected literatures and is aimed to share some knowledge to improve your work.

SAMPLING

By the result of some sort of a test of a portion of the material with its quality

criterion to judge whether each article is non-defective or defective, or with an acceptability criterion to judge whether a lot is acceptable or not, the portion of the material in a sample used to judge the whole material, improper sampling will lead to inappropriate grading even with correct testing.

In general, sampling is conducted in such a way that the sample represents the population, but in the same case a sample is taken from an especially good or bad section. Without understanding the sampling method of the test sample, one can not evaluate correctly about the quality of the material being inspected.

Uniform sampling

In this method, a sample is taken so as to represent the average of the whole population. Samples are taken in a small quantity from each section of the population. In this case, the total amount of the sampling method of the test sample, one cannot of it is used for testing. Sampling in this case has to be evenly reduced. The reduction procedure is called dividing, which is performed by quartering, dividing or the use of divider.

Selective sampling

When the products are disposed according to the lowest quality, sampling is made from sections with particularly poor quality. For example, to judge baking condition of bread through determination of moisture a sample is taken from the central part of the bread.

Random sampling

This method is applied in cases of the several samples are taken from a product to be uniform and when they do not have the same quality. In this sampling, individual samples, an amount of sampling, and in some case, sampling period are not fixed before sampling. Strictly random sampling is rather difficult, and so the subjects of sampling are chosen by the use of dice, lottery, or random table. The random sampling can prevent unfair action of inspect.

SAMPLING, SAMPLE HANDLING IN GRAIN AND CEREALS (ISO)

Correct sampling is an operation that requires most careful attention. Emphasis cannot therefore be too strongly laid on the necessity of obtaining a properly

representative sample of grain. Careless or inaccurate sampling could lead to misunderstanding and unwarranted financial adjustments.

Samples shall be fully representative of the lots from which they are taken. Therefore, as the composition of the lot is seldom uniform, a sufficient number of increments shall be taken and carefully mixed, thus giving a bulk sample from which are obtained, by successive divisions, the laboratory samples.

Apparatus

Apparatus is required as follows, and many types and variations of apparatus are available.

Method of taking samples from carried in bulk.

When sampling takes place while the product is in motion, increments shall be taken at time intervals dependent on the rate of flow.

When bulk grain is sampled in the hold during discharge, increments shall be taken from as many places as possible, excluding the run, and at intervals determined by the rate of discharge.

Method of taking samples from cereals carried in bags.

The increments shall be taken from different parts of bag example top, middle and bottom, by means of a sack-type spear from the number of bags specified in the table below.

If sampling takes place from weight hoppers, increments shall be taken by means of cylindrical samplers, shovels, or mechanical samplers in accordance with the practice of the port.

The procedure for silos or warehouses is necessarily dependent on local conditions.

If sampling takes place from laden wagons or lorries, the increments shall be taken throughout the whole depth of the layer, by means of a cylindrical sampler and at the following points.

Table 1. Number of bags to be sampled.

in consignment	Number of bags to be sampled.

Up to 10 10 to 100	Each bag 10, taken at random.
More than 100	Square root (approximately) of total number, taken according to a suitable sampling scheme.

Table 2. Sampling scheme for consignments of more than 100 bags.

N = Number of bags in consignment; n = Number of bags in group.

N	n	N	n	N	n
101 - 121	11	1,601- 1,681	41	4,901- 5,041	71
122 - 144	12	1,682- 1,764	42	5,024- 5,184	72
145 - 169	13	1,765- 1,849	43	5,185- 5,329	73
170 - 196	14	1,850- 1,936	44	5,330- 5,476	74

197 - 225	15	1,937- 2,025	45	5,477- 5,625	75
226 - 256	16	2,026- 2,116	46	5,626- 5,766	76
257 - 289	17	2,117- 2,209	47	5,777- 5,929	77
290 - 324	18	2,210- 2,304	48	5,930- 6,084	78
325 - 361	19	2,305- 2,401	49	6,085- 6,241	79
326 - 400	20	2,402- 2,500	50	6,242- 6,400	80
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
1,226- 1,296	36	4,226- 4,356	66	9,026- 9,216	96

1,297- 1,369	37	4,357- 4,489	67	9,217- 9,409	97
1,370- 1,444	38	4,490- 4,624	68	9,410- 9,604	98
1,445- 1,521	39	4,625- 4,761	69	9,605- 9,801	99
1,522- 1,600	40	4,762- 4,900	70	9,802- 10,000	100

For consignments larger than 10,000 bags, n equals the square root Of N. rounded upwards.

Samples

- Laboratory Samples

The bulk sample shall be divided to obtain the required number of laboratory samples by use of the apparatus mentioned as follow. The number of laboratory

samples to be taken for analysis and arbitration shall be specified in the contract or otherwise agreed between the buyer and the seller.

- Size of samples

Samples of the sizes given in Table 3 are usually suitable for all grains. Larger or smaller laboratory samples may be required in some cases, according to the tests to be carried out.

Table 3. Sizes of samples

LOT	Increment	Bulk sample	Laboratory sample
Up to	1 Kg.	100 Kg.	5 Kg.
500 tons.	(max.)		

MAIZE - SAMPLING. (OCS)

The Office of Commodity Standards, Department of Foreign Trade, Ministry of Commerce empowered by the Export Standards Act B.E. 2503 (1960) amended by the Export Standards Act (No.2), B.E. 2522 (1979), is responsible for the control of products to be exported as follows:

- **To specify the standardized products**
- **To prepare export commodity standards**
- **To control exporters, surveyors and inspectors**
- **To provide inspection service**
- **To issue certificates for commodity standards on quality, volume, weight, and origin of products**
- **To prevent and suppress deception of commodities to be exported, and**
- **To collect statistics concerning manufacture, market needs, price level and value of exports and publicize such information to concerned persons so that they can use the information for production and export targets.**

At the time being, twelve products have already been standardized. According to the 6th National Economic and Social Development Plan (1987/1991), seven additional products will also be standardized.

Standardized commodities:

- **Jute and Kenaf**
- **Maize (corn)**
- **Castor seed**
- **Kapok**
- **Salt**
- **Teak Conversions**
- **Sorghums**
- **Thai silverware**
- **Tapioca products**
- **Thai silk and silk products**
- **Green bean**
- **Fish meal**

(6) "Greatly spoiled seeds" mean seeds which, the whole part, are rotten, mouldy, containing no starch, sprout.

(7) "Seeds destroyed by weevils" mean seeds which are bitten or bore by

weevils or other insects.

(8) "Broken seeds" mean sound seeds which are broken into pieces and each piece less than a half of natural sound seed, but not immature seeds, spoiled seeds, or weevilled seeds.

(9) "Foreign material" means all matter other than maize.

Clause 2. The standards of maize shall be classified into two grades as follows:

(1) Grade 1 maize.

(2) Grade 2 maize.

Clause 3. The standard specifications for each grade of maize shall be as follows:

A. Grade 1 maize shall be sound seeds, but the following tolerances are allowed:

- (1) Seeds of other colours, not exceeding 1.0 per cent by weight.**
- (2) Partially spoiled seeds together with greatly spoiled seeds, not exceeding 4.0 per cent by weight but greatly spoiled seeds, not exceeding 1.5 per cent by weight.**
- (3) Seeds destroyed by weevils, not exceeding 2.0 per cent by weight.**
- (4) Broken seeds together with immature seeds, not exceeding 2.0 per cent by weight.**
- (5) Foreign material, not exceeding 1.5 per cent by weight, but have no oil seeds or poisonous matter.**
- (6) Moisture content on the average, not exceeding 14.5 per cent by weight and there shall be no part having moisture content in excess of 15.0 per cent by weight.**

B. Grade 2 maize shall be sound seeds, but the following tolerances are allowed:

Standards for Maize

Clause 1. Definitions

- (1) "Maize" means seeds of *Zea mays* which are split from the cob.**
- (2) "Sound seeds" mean seeds which are not immature, spoiled, destroyed by weevils, broken, or seeds of other colours.**
- (3) "Seeds of other colours" mean seeds which are not of the colours as agreed upon.**
- (4) "Immature seeds" mean seeds which are not fully developed.**
- (5) "Partially spoiled seeds" mean seeds which, any part, are rotten, mouldy or containing no starch.**

Clause 4. In case of disputes or contentious problems concerning to clause 3, the latest sample provided by the Office of Commodity Standards shall be taken as the basis of determination.

Clause 5. In case of selling maize by sample, which has been approved by the Office of Commodity Standards, the standard of such maize shall not be inferior to the sample or condition agreed by buyers.

Clause 6. In case of maize exported in gunny bags, those gunny bags shall be new ones, which are the same type, size and weight as the gunny bags used for packing rice (Heavy Cee); they shall be in good condition, suitable for export, not torn, not leaked and free from bad odor. The mouth of the bags shall be tightly sewn across and reverse, with double jute twine, each way not less than 8 stitches for the bags with width not exceeding 60 cm. and not less than 11 stitches for the bags with width exceeding 60 cm., but not over 86 cm.

Nevertheless, except the buyer has made an agreement with the exporter concerning the type, size and weight of the gunny bags including the sewing of the mouth of the gunny bags which differs from the preceded mention and the exporter has declared such agreement in details in the application form for standard certificate.

In case of maize exported in bulk, but due to the necessity to use gunny bags for packing some portion of maize in order to prevent the movement of maize in

the hatch of the outgoing vessel, those gunny bags may be used ones, but they shall be strong, durable and in good condition, not torn, not leaked and free from bad odor. The mouth of the bags shall be tightly sewn, in order to prevent the maize moving or leak from the gunny bags in the loading time.

(1) Seeds of other colours, not exceeding 3.0 per cent by weight.

(2) Partially spoiled seeds together with greatly spoiled seeds, not exceeding 6.0 per cent by weight but greatly spoiled seeds, not exceeding 2.0 per cent by weight.

(3) Seeds destroyed by weevils, not exceeding 3.0 per cent by weight.

(4) Broken seeds together with immature seeds, not exceeding 3.0 per cent by weight.

(5) Foreign material, not exceeding 2.0 per cent by weight, but have no oil seeds or poisonous matter.

(6) Moisture content not exceeding 15.5 per cent by weight.

A. Pre-Loading Samples

(1) In case of maize in bags, the increments shall be taken by random at least 2 sides of the pile (upper and other sides) and the number of bags no less than 5% of bags in the pile.

(2) In case of maize in bulk pile, the increment shall be taken by random and throughout no less than 0.5 meter depth of the layer, and 2 meters of neighboring points, overall the pile, each point at least 0.5 Kg. of sample by means of a cylindrical sampler.

(3) In case of maize in a storage silo, the increments shall be taken no less than 1 Kg. each of 1 meter depth from upper layer until 3/4 of maize height, by means of pneumatic probe sampler or sampling takes place by means of maize in circulate motion, increments shall by taken no less than 0.5 Kg. each 1 M.ton. maize circulation, until 2 % or more of maize which stored in the silo.

(4) When the bulk maize is sampled in the hold during storage in silos or

warehouses, increments shall be taken at least 0.5 Kg. lorries, or unit. In case of bag, it shall be taken at least 0.5 Kg. per truck or wagon or unit and no less than 5% of bag in each unit.

(5) The bulk sample of each pile, bin or silo shall be formed by combining the increments and mixing to obtain uniformity and sub-dividing to obtain about 3 Kg., and six samples to be taken for analysis and arbitration.

B. Loading samples

(1) The maize, which is to be dispatched, have to be certified as to the quality by means of preloading samples to be based on standards or agreement.

Sampling Procedure (Official Inspection) for maize.

There are two kinds of inspection samples. The first is called a "pre - loading" sample and is taken to be representative of maize in a storage bin, silo, godown (in bag or in bulk). The second is a "loading" sample and is collected as maize is loaded, onto a barge, lighter or ship for export.

(2) Physical quality inspection and packaging checks are to be performed, and samples taken from every bag of maize by means of sampling spear or 0.5 Kg. from each wagon, lorry, unit or time intervals dependent on the rate of flow.

(3) During inspection, each 100 M.tons. loaded, take at least 0.5 Kg. sample by random from pile or silo for moisture testing and each of two for discrimination testing.

When the quality of maize tested inferior to the standards or agreements, the inspection shall be temporary stopped to take away the inferior part. Then, samples are taken by random from another part for moisture testing and discrimination testing, if the quality is to be accepted, the maize shall be loaded again.

In case the inferior maize is not due to moisture content, it can be mixed with another that its quality has to be accepted by means of pre-loading samples, and it can be loaded again. If the quality is to be accepted, samples are taken and tested as in the first paragraph.

SAMPLING, SAMPLE PREPARATION, AND SAMPLING PLANS FOR MYCOTOXIN ANALYSIS IN U.S.A.

It is now well established that aflatoxin (mycotoxin) tends to be distributed very heterogeneously

The official first action method for corn specified by the Association of Official Analytical chemists (AOAC) does not designate sample size, but it requires that the entire sample of shelled corn be ground to pass a No.14 sieve, and that a 1-2 Kg. sub-sample of this material be ground to pass a No. 20 sieve. A 50 Kg. sub-sample of the finely ground material is then analysed by the CB. method.

Whitaker, Dickens and Monroe developed the following equations for variance (error) terms related to this test procedure:

$$V = S + C + F + Q$$

V = Total variance (total error)

S = Error in sampling = 3.9539 P/Ws

C = Error in sub-sampling the coarse ground material = 0.1196 P/Wc

F = Error in sub-sampling the fine ground material = 0.0125 P/Wf

within a batch of maize. Traditional means of sampling and sample preparation of agricultural crops and foodstuffs are generally not adequate for mycotoxin analyses.

Associated errors and error reduction

In a study with corn, the total error was broken down into four components: sampling error, coarse subsampling, fine sub-sampling, and analytical error.

Q = Error due to quantification = 0.0699 P/Nq

Ws = mass of sample in kg.

WC = mass of coarse sub-sample in kg.

Wf = mass of fine sub-sample in kg.

Nq = the number of times the aflatoxin in the solvent extract is quantified on a separate TLC.

P = Aflatoxin concentration (ug/kg) in the lot.

These studies draw attention to the fact that the sampling error is usually the largest contributor to the total error, so improved sampling can make the greatest contribution toward the accuracy of analytical results from which acceptance or rejection decisions are made.

Some methods to increase the precision of aflatoxin tests are to increase sample size, to increase the size of the sub-sample used for aflatoxin analysis, and to increase the number of analyses. Different costs are associated with each method and careful study is required to determine the testing program that will provide the most precision for a given cost. The optimum balance in sample size, degree of comminution, sub-sample size, and number of analyses will vary according to the cost of the sample to be comminuted, the cost of sample and subsampling, the cost of analysis, and other factors. In general, the costs of properly designed aflatoxin testing programs will increase as precision increases.

Sampling Procedure

Samples may be taken from crops growing in the field, during handling, storage,

and at other points in the production. Marketing samples can best be obtained by the use of automatic continuous samplers in situations where such equipment can be used, such as manufacturing process streams of materials. When this is not possible, e.g., when a bulk lot is in a bin, truck, box car or similar container, probe samples should be taken by means of probes which can reach to the bottom of the container. When the lot is bagged, samples are best taken from the bags while they are being gridded or emptied into containers. These samples may consist of portions taken by scoop or by hand, "grabs" and composited in a collection container. After the bags are closed the job becomes more difficult, but samples can be removed by means of small triers (probes). For lots comprising a relatively small number of bags it is best to sample each bag. As the number of bags in a lot becomes large, a good practice is to remove material from one-fourth of the bags.

Since the recognition of the aflatoxin problem, it has generally been the practice to require at least 1 kg. samples; and the U.S. Food and Drug Administration has advocated a minimum of a 15 lb. (6.8 kg.) sample. The size of the lot under investigation usually does not affect the random variability associated with sampling if the sample size is small compared to the size of the lot. A properly

drawn 48 lb. sample is as representative of a 100,000 lb. lot of raw shelled peanuts as it is for a 40,000 lb. lot. Over the years, the size of the sample for the control of aflatoxin in peanuts in the United States has risen from 12 lbs. (5.4 kg.), to 24 lbs. (10.9 kg.), to 48 lbs. (21.8 kg.), to the current 144 lb. sample (three 48 lb. samples). This increase in size evolved as more reliable test results were required by the manufacturer. Increasing sample size has the advantage of simultaneously reducing the number of good lots rejected and the number of bad lots accepted by a testing program.

Usually the amount of sample material removed from the lot is more than is required, so it is necessary to thoroughly mix this material before removing the required amount of sample. After mixing, the sample can be sub-divided to the required size by use of mechanical dividers or by applying the "quartering" technique.

Sample Preparation

Assuming that a representative lot sample can be obtained, the next step in the process is to prepare the sample for analysis. In general, this will involve mixing

and blending of the material, coarse grinding to reduce the portticle size so the material will pass a standard # 14 mesh screen, mixing to obtain a portion for further grinding to produce a flowable material which can be sub-divided to the specified size of analytical sample.

[Product sample sizes used by the United States Food and Drug Administration for mycotoxin analysis.](#)

[Schematic of the United States peanut aflatoxin testing program.](#)

INSPECTION SCHEMES FOR SHELLED PEANUTS WITH REGARD TO AFLATOXIN IN THE NETHERLANDS

In the Netherlands, a provisional Code of Practice for the peanut wholesalers and processors has been laid down. The provisional Code deals with inspection practices with regard to aflatoxin for lots of shelled peanuts prior to processing and/or selling to retailers, restaurants, etc. The Code should be used by wholesalers and processors.

Provisional Inspection Scheme

Classification of peanuts in the lot (average)	Number and weight of subsamples per lot 1	Acceptance criterion (microgram Aflatoxine B1 per kg)
60 nuts per ounce or more	4 x 5 kg	In each subsample 3 ppb or less
less than 60 nuts per ounce	4 x 10 kg	Ditto.

Before sampling, the lot should be divided into four equal parts. From each part a sub-sample is taken. The sub-sample should be made up of small equal samples which are taken out of each 250 kg of the part of the lot.

Classification of the peanuts in a	Probability of acceptance (%) when the average aflatoxine B1 content of the lot is (microgram
---	--

lot (average)	per kg):							
	Aflatoxine							
	1	2	3	5	10	15	20	30
60/oz or more	71	29	15	7	4	3	3	2.5
less than 60/oz	74	29	14	6	3	2	2	2

Probability of Acceptance

When the above described scheme is applied, the probability of acceptance calculated according to the method and on the basis of the distribution of Aflatoxine B. in peanuts as described by J. Walbel in his article "Stichprobengrosse für die Bestimmung von Aflatoxin in Erdnüssen", in Deutschen Lebensmittel-Rundschau (vol. 73, nr. 11, November 1977, page 353 t/m 357), is as follows.

In this case it is assumed that a sorted and cleaned lot has a degree of contamination of 1 peanut/15.000 peanuts. Besides, it is assumed that the average weight per peanut is respectively 0.35 g (classification 60/oz or more) and 0.65 g (classification less than 60/oz).

Desirable Inspection Scheme

The inspection scheme described above is agreed upon only for the time being.

Regarding the intake of aflatoxin, the stand of the State Supervisory Agency for Public Health in the Netherlands is as follows:

- 1. All measures which are feasible should be taken to avoid the contamination of foods with aflatoxine.**
- 2. In case of an accidental "one-off" intake of aflatoxine (for instance when products with contaminated whole or broken peanuts are consumed), the intake should in no case be more than 50 microgram aflatoxine B1.**
- 3. In case of an accidental "sub-chronic" intake of aflatoxine (for instance when products with contaminated milled peanuts like peanut butter are**

consumed) the intake should in no case be more than 0.5 microgram Aflatoxine B1 per day over a short period.

Condition 2 implies in fact that lots of peanuts which contain contaminated peanuts with 50 microgram Aflatoxine B1 or more should always be rejected when inspected. This means that an inspection scheme should be used which offers a probability of acceptance of 0 for lots with nuts containing 50 microgram Aflatoxine B1 or more. Using the same method of calculation and the same assumptions as referred to above, this implies that in case of gradings of 60/oz or more the probability of acceptance of a lot with on average Aflatoxine B. content of 10 micrograms per kg should be 0 ($\leq 0.1\%$) and in case of gradings of less than 60/oz the probability of acceptance should be 0 ($\leq 0.1\%$) when the lot contains on average 5 microgram per kg.

Condition 3 implies that since in the Netherlands a high individual consumption of peanuts from products like peanut butter is about 35 g per person per day, the probability of acceptance of a lot of peanuts which is intended for milled peanut products should be 0 ($\leq 0.1\%$) when the average Aflatoxine B. content of the lot is 15 microgram per kg (for all classifications).

The provisional inspection scheme which is now used for some months by the wholesalers and processors may after some time be adapted so that they conform more with the conditions described above.

**State Supervisory Agency for Public Health
Chief Inspectorate for Foodstuffs
PO. Box 5406
2280 HK RIJSWIJK
The Netherlands**

SAMPLING, SAMPLE HANDLING AND PREPARATION IN TAIWAN. (R.O.C.)

The Bureau of Commodity Inspection and Quarantine, Ministry of Economic Affairs is responsible for the control of products (such as corn) to be imported by means of CNS. (Chinese Standards)

Sampling Procedure

- 1. The products (corn) are to be rejected if the quantity of aflatoxin is greater than 50 ppb.**
- 2. Pneumatic-Sampler or Probe-A-Vac. are apparatus for taking sample from corn carried in bulk from hatch of the vessel.**
- 3. The increments shall be taken every meter throughout the whole depth of the layer, with 4-6 sampling points taken at random.**

Size of sample (Laboratory sample)

The bulk sample shall be formed by combining the increments at least 66 Kg., and mixing them well and sub-dividing to obtain three of 21 Kgs. samples.

Sample Preparation

The 21 kgs. sample will mixing, blending and coarse grinding (about 1 mm.), then mixing to obtain uniformity and sub-dividing to obtain about 2 Kgs. And further grinding to reduce the particle size so the material will pass a standard # 20 Mesh screen for aflatoxin analysis.

Aflatoxin analysis

A high pressure liquid chromatographic (HPLC) method is designed for determining aflatoxins in corn.

Schematic of Corn Aflatoxin Testing Program.

[Schematic of Corn Aflatoxin Testing Program.](#)

SAMPLING, SAMPLE HANDLING AND PREPARATION FOR AFLATOXIN DETERMINATION IN MAIZE (OCS unofficial)

7.1 Method of taking samples from maize stacked in bulk.

7.1.1 1,000 metric tons presume to be a lot, and 200 up to 1,000 metric tons remains to be a lot.

7.1.2 Increment shall be taken from a single position in the 200 metric tons lot,

and the remains less than 200 metric tons is an increment.

7.1.3 Increments shall be taken through at least half depth of the layer. The sub-sample shall be formed by combining five increments (no less than 5 kgs./increment) and mixing them well, and dividing to obtain 2 kgs.

7.1.4 The sub-sample shall be divided to obtain four of 2 kgs. Laboratory samples.

Eg. 2,400 metrictons maize stock in bulk.

Lot.	1,000	1,000	400	metrictons
Increments	5	5	5	
Laboratory Samples	4	4	4	

7.2 Method of taking samples from maize stocked in bags.

7.2.1 1,000 metric tons presumed to be a lot and 200 up to 1,000 metric tons remains to be a lot.

7.2.2 Increments shall be taken from bags of each lot from at least four sides of stock, and from bags at top side which is pulled off one fourth of bags height. The number of bags to be sampled shall be no less than 20% of stock, and sub-sample shall be no less than 16 kgs.

7.2.3 The sub-sample shall be formed by combining the increments and mixing then well, and divided to obtain four of 2 kgs. Laboratory samples.

	Eg. 2,400 metric tons maize stock in bags.			
Lot	1,000	1,000	400	metric tons
Increment	16	16	16	kgs.
Laboratory Samples	4	4	4	

7.2.4 100% sampling of the bags shall be takes place while the bags is in motion

to stock in go-down, a sample per pile and divided to obtain four of 2 kgs. Laboratory samples.

7.3 Method of taking samples from maize stocked in silo.

7.3.1 Sub-sample shall be taken from 200 metric tons of maize, and 100 upto 200 metric tons remains is a sub-sample. The sub-sample has to be no less than 5 kgs., mixing them well and divided to obtain 2 kgs.

7.3.2 The sub-samples shall be taken from the top and bottom of the silo by the ratio of 2:3 if sampling from the top is not available, it shall be taken from the bottom only.

Eg. 1,500 metric tons of maize stocked in silo.

$$= \frac{1,500}{200} \times \frac{2}{5} = 3$$

Number of sub-sample taken from the bottom of silo.

$$= \frac{1,500}{200} \times \frac{3}{5} = 5$$

7.3.3 How to get sampling from the top.

The increment shall be taken from every meter until about 5 - 7 meters depth of the layer or no less than 1/4 of maize height in silo, and the sub-sample shall be taken from different directions.

7.3.4 Bottom Sampling.

The increments shall be taken by recycle 10 % of silo contents.

10 % of 1,500 M.ton. = 150 tons

5 % sub-sample taken from = $\frac{150}{5} = 30$ tons lot.

Sample for 30 tons lot taken at random for 5 kgs. sample size, are mixed well and divided to obtain 2 kgs. sub-sample.

In case top sampling is not possible to take, the increments (bottom sampling) shall be taken by recycling 20% of silo contents, and similar sampling as above.

7.4 The bulk sample.

The bulk sample shall be formed by combining the increments, and mixing them well, and dividing to obtain four 2 kgs.-samples for analysis.

7.5 Schematic of Corn Aflatoxin Testing Program.

$$2 \text{ kgs. SAMPLE 1 } \begin{matrix} A & B \\ \frac{1A + AB}{2} \end{matrix}$$

(Contract, accept. >Contract. << 150 % Run sample 2 > 150 % Contract, reject.

$$2 \text{ kgs. SAMPLE 2 } \begin{matrix} A & B \\ \frac{1A + 1B + 2A + 2B}{4} \end{matrix}$$

<= Contract., accept. > Contract <= 150 % Run sample 3) 150 % contract, reject. 2 Kgs. Sample 3 A B

$$\frac{1A + 1B + 2A + 2B + 3A + 3B}{6}$$

< Contract, accept. >Contract, reject. 4th sample hold for evidence.

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