KILLER MOULDS - A PERSPECTIVE

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Ever since his first apppearance on earth, man has been engaged in a never ending struggle with the environment to gather or produce food and, more important, to preserve it from destruction. Next to air which is freely available, food and water invariably have been the two basic requisites of paramount importance for sustenance of mankind. From time immemorial, contamination of his food, water and sometimes air too by chemical pollutants was a serious threat to man. Even today one speaks of man-made pollution of human habitat unavoidably connected with industrial development. Yet, there is another form of naturally occurring chemical pollution of food, beverages and even air that is of biological origin, irrespective of whether a country is industrialised or developing. Man was baffled for centuries by such occurrences the origin of which was not known to him but they almost invariably manifested in the form of mysterious diseases in man himself and also in his livestock causing loss of life.

At a time when science and technology were not developed as it is today, man was compelled to believe that divine involvement was the reason for any mysterious disease outbreak he could not fathom. Micro-organisms or the metabolites they secrete into the environment are the causative agents of many a disease as we know today. But without appropriate technological developments, early man had no means of either discerning most micro-organisms or detecting the presence of their metabolites in particular, for instance in his food. He was aware of the presence of moulds because he could see them growing in his food commodities and he even used some 'mushrooms' as food.

Man's association with micro-organisms extends to antiquity and he was using some of them, for example yeasts (a class of fungi as we classify them today), for leavening of dough before bread making and for brewing of fruit juices or plantcell saps to produce alcoholic beverages like grape wine and cider. He also winessed the growth of a host of harmless looking moulds on his food commodities and the damage they are doing by destroying the food itself. When food was scarce he was even forced to eat mouldy grain, nuts or food preparations made out of them. The relationship between such seemingly harmless moulds and the serious diseases they caused in man and his farm animals was beyond human comprehension for centuries. Mysterious diseases in man and his farm animals were known to have existed since medieval times but the significance of moulds as their causative agents was not brought to light until after the second half of twentieth century. With the advancement of science and technology in the fields of microbiology, biochemistry and pathology in particular man attempted to investigate and understand various natural interactions he and his livestock exhibited with other forms of life around them.

An East Anglican turkey farm in Britain lost over 100,000 poults nurtured in readiness for Christmas festivities as a consequence of mass death due to disease in 1959. This incident triggered off a series of investigations in several countries around the world that would change man's attitude towards moulds in food and feedstuff. The turkey disease in Britain was traced back to the pelleted ration fed to the birds and the incriminated peanut meal imported from Brazil to produce it proved that a mould *Aspergillus flavus* was responsible for the calamity. Further analysis of the peanut meal using Thin Layer Chromatography (TLC) revealed the presence of several new chemical compounds secreted by the mould. The first letters of the name *Aspergillus flavus* of the toxigenic mould involved were adopted in coining a name and these chemicals became known as "Aflatoxins". They exhibited fluorescence when exposed to long wavelength Ultra-Violet radiation and were designated Aflatoxins B and G because of the respective bluish and greenish-yellow colour they emitted when irradiated.

Subsequently, several other *Aspergillus* species and a number of common mould genera were found to pro-

duce similar chemical metabolites which were equally toxic to human beings and farm animals. Some were potential carcinogens. This wide assemblage of toxic chemicals was referred to as "Mycotoxins" purported to mean toxins produced by moulds, and the terms aflatoxin and mycotoxin became synonymous in early 1960. However, the term mycotoxin is a misnomer in its absolute sense because in analogy, phytotoxins and zootoxins mean compounds toxic to plants and animals respectively and one would expect mycotoxins to mean compounds toxic to moulds and not compounds secreted by moulds that are toxic to man and farm animals.

The illnesses caused in man and farm animals by consumption of food infested with toxigenic moulds are known as mycotoxicoses. Mycotoxocoses are of two kinds. Illnesses that occur in subjects due to direct ingestion of mouldy food containing toxins are termed 'primary mycotoxicoses'. It has been observed that farm animals feeding on mycotoxin contaminated feed secrete these toxins in animal products, for instance, cows milk and red meat, and they are detected even in processed dairy products like cheese. Aflatoxin B_1 and B_2 contaminated fodder when eaten by the cow, is transformed and secreted in milk as Aflatoxin M_1 and M_2 respectively in lesser concentrations than the original uptake dosage of B Aflatoxins. Illnesses caused in subjects, specially man, by consumption of animal products contaminated with mycotoxins derived in this manner are referred to as 'secondary mycotoxicoses'. The type of mycotoxicosis developed in a subject could be diverse and is dependant on the toxigenic moulds involved and also on the mycotoxins secreted.

There are only three human mycotoxicoses that are sufficiently substantiated by reasonable evidence to link the disease with the respective suspect mycotoxin. Ergotism, the proximity wasting disease of 9th and 10th centuries, then known as St. Anthony's Fire occured due to consumption of cereals or bread made from flours of rye and other grains contaminated with resting fruit bodies called sclerotia or ergots of the toxigenic strains of *Claviceps perpurea*. The toxins produced by this fungus are known as ergot alkaloids. They bring about contraction of blood vessels of limbs reducing their blood supply, and if not corrected, the affected tissues die resulting in gangrene and falling-off of limbs. Accelerated pulse, increase of glandular secretions, smooth muscle contractions manifested in uncontrollable spasms and staggering are the other symptoms of ergotism. Ergot alkaloids also induce vomiting and further, they have contratile effects on uterine blood vessels and musculature of pregnant cows resulting in reduced blood supply to the foetus causing atrophy and subsequent abortion. This latter effect has now been put to good use in giving relief to humans in that some ergot alkaloids are used as labour inducing drugs to ease the labour pains of pregnant mothers at child birth.

The second human mycotoxicosis mostly reported from Russia as early as the 19th century is Alimentary Toxic Aleukia (ATA) or absence of white blood corpuscles (leucocytes) from blood and poisoning of alimentary tract. ATA was associated with the ingestion of overwintered grain, for example wheat, rye, barley, prosomillet etc., infected with toxigenic *Fusarium* species *F. poae* and *F. sporotrichoides*. Several steroidal toxins and numerous trichothecene- type toxins have been isolated from infected materials and the current belief is that ATA is caused more likely by the latter. After ingestion of toxin contaminated food, a burning sensation in the mucous membranes of the mouth, throat, stomach occurs followed by inflammation of these regions, with vomiting, diarrhoea and abdominal pain. At the final stages, bleeding of nose, throat, gums and genital tract occurs together with the appearance of bleeding skin rashes. A decrease of white blood cell count and granulocytes occurs, the bone marrow gets exhausted, lowering body resistance to bacterial invasion and if not corrected immediately the patient dies quickly due to a multitude of complications.

The third and widely studied and documented mycotoxicosis is Aflatoxicosis due to Aflatoxins. Contamination of human food and foodstuff by Aflatoxin producing moulds is widespread and the intense carcinogenicity of Aflatoxin B_1, in particular, drew a lot of attention to this single mycotoxin group. It is strongly believed that Aflatoxin may be the causative agent of human liver cancer, as a highly significant positive correlation between the Aflatoxin levels in the diet and the incidence of liver cancer has been obtained from epidemological studies done in Africa and South-East Asia over the last three decades. In fact, thousands of people all over the world are eating lethal levels of Aflatoxins with their daily diet consisting of naturally contaminated foodstuff, peanut, maize in particular and other grains.

No food or feed could be considered safe from mould infestation because they are prone to natural contamination at one stage or another from origin to consumption. Examination of suspect material would not always show the presence of toxigenic moulds. They may die off leaving behind their deadly secretions. In many cases, one or several toxigenic moulds would grow together or in succession producing the same or widely different mycotoxins into the material and sometimes they are known to produce mixed effects and at other times synergistic effects. Certain toxigenic moulds infect the commodity while in the field viz. maize, wheat and millet. Other commodities are contaminated after harvest or during storage, transportation or processing. If adequate protection is not given, contamination of food could also occur at consumer's home itself, irrespective of whether the food is in storage or (left-overs of) prepared food set aside for later consumption.

Many food commodities have been implicated with mycotoxins. Though not exhaustive they range from cereals (rice, maize, sorghum, wheat, barley, millet, rye, oats); oil seeds (peanut, cotton seed, copra); oil seed meals; crude vegetable oils (peanut oil, coconut oil, olive oil, sunflower seed oil); fruits (apples, figs); apple juice, wine and beer; green coffee beans, cocoa beans; root crops (manioc or cassava, sweet potato); yams and tubers (carrots, radish) onions etc. Milk and cheese at times are found to be contaminated with Aflatoxin M_1. Pork, poultry, beef, dried fish, smoked fish and fish sauce are other products that have been shown to contain mycotoxins.

Since the detection of mycotoxins in commodities, techniques were developed to prevent their incidence and severity of contamination by successful application of improved practices delimiting growth of toxigenic moulds. Man was not destined with control over the climate. Therefore, natural contamination of food commodities by moulds in the field is inevitable. But man has developed techniques to decontaminate and detoxify commodities that were subjected to field and post- harvest mould contamination. The principal aim behind these techniques is to destroy or inactivate the toxins, kill the fungal spores and mycelia - which would otherwise proliferate and secrete new toxins under favourable conditions - leaving behind no toxic or carcinogenic residues in the treated commodity while preserving the nutritive value and acceptability of the product as food.

Mycotoxin decontamination is achieved firstly by physical separation of contaminated material by careful inspection of individual items and manual picking-out of immature, broken or discoloured items and insect damaged items. This is a very efficient method widely applied to food grains, coffee berries, cocoa beans and peanut. Grading by size employing sifters in mechanical separators, and sorting-out of items discoloured by mould infestation using electronic colour sensitive devices are concurrently practised with hand-picking methods. Air classification and floatation techniques also have wide applications. Mould infested and insect damaged crops are virtually lighter than sound material and as such they would either be blown over during air classification or float on the surface of Sodium Chloride solution, the separating fluid generally used in the floatation technique. Ergot sclerotia and aflatoxin contaminated peanuts are efficiently separated by these techniques.

Maize for example can be decontaminated by wet-milling, where starch, oil and other products obtained would be virtually toxin free as 80-90% of aflatoxin originally present is concentrated in the gluten fraction. When oil is expressed from peanuts, over 85% of the aflatoxin present remains in the press cake. This oil can be further decontaminated by passing through a suitable filter and an adsorbant designed for the purpose. The gluten fraction of maize and the peanut press cake with very high aflatoxin levels could be either discarded or diverted to suitable use largely by diluting with toxin free material, in bringing down toxins in the final product to virtually harmless levels.

Inactivation or total degradation of mycotoxins can be achieved by exposure to bright sunlight. If exposed in small bottles or in shallow trays for about one hour during the brightest part of the day, where about 50,000 lux units would be delivered, all remnant aflatoxins in contaminated crude coconut oil, peanut oil etc., can be economically destroyed. This is a very relevant and appropriate decontamination method suitable for developing countries including Sri Lanka.

Mycotoxin contaminated material can also be detoxified chemically. A range of inorganic and organic chemical compounds employed in detoxification trials have not proved to be successful except strong alkaline solutions. Ammonia for instance is the most useful and effective

compound used today in aflatoxin decontamination. Several processes using ammonia for detoxification of aflatoxin have been developed and patented in Europe. In U.S.A. and France, for example, small-scale pilot plants have been built and are operational. Successful transferability of the ammonia process to developing countries remains yet to be studied. Useful detoxification can also be done using chlorine gas which does not need high temperature and high moisture conditions. Though 90% of aflatoxin detoxification occurs here, its usefulness as a practicable process is doubtful because it destroys the organoleptic quality of the detoxified material.

Chemical extraction techniques are available to remove mycotoxins, aflatoxin, in particular, completely from contaminated materials. Ethanol, for example, in an aqueous solvent extraction system has been shown to remove over 95% of the total aflatoxin in peanut meal and cotton seed meal. Acetone, Chloroform and petroleum hydrocarbons in aqueous extration systems are equally effective. Though efficient, the extraction techniques are uneconomical, firstly because of high costs involved in designing special equipment to remove extraction solvents used, and secondly the resultant detoxified material loses nutritive value appreciably and would only be fit for animal feed at most.

Mycotoxin contaminated materials can also be detoxified by irradiation or heating. Aflatoxins are sensitive to Ultra-violet (U.V.) radiation. Factors like toxin concentration, duration of exposure and the solvent used govern the degradation of toxin in a contaminated product. However U.V. irradiation is not a practicable and worthwhile detoxification technique. Mycotoxins are heat-stable, but if roasted by dry heat at relatively high temperatures close to their melting points- viz. 250^0C for aflatoxin-complete degradation occurs. Such an intense heat treatment is disadvantageous because it renders the commodity unaccceptable as food due to loss of nutritive value and appearance. However, if heated in the presence of moisture, appreciable degradation of toxin levels occurs at much lower temperatures. Pressure cooking reduces as much as 70% of initial toxin levels. It is a very simple, effective and convenient method to minimise aflatoxin hazards specially in households. Perhaps microwave cooking should prove more promising and effective in this regard.

Pasteurisation, sterilisation, vacuum- packing, canning, cold storage -both refrigeration and freezing- and drying are some other useful techniques in prevention or delaying of mould growth. They give mixed results in that taste and appearance of foods suffer greatly in some cases while other methods are technically and economically not feasible.

Moulds are universally present in or on all food commodities be it grain, nut, fruit or vegetative parts, but toxin production is essentially restricted to a handful of them. At present the three mould genera *Aspergillus, Penicillium*, and *Fusarium* are universally considered most significant in toxin production. Among themselves some twenty five toxigenic species/cultivars exist which are known to produce over forty different mycotoxins.

Over a dozen forms of "Aflatoxins" are known today, of which five major forms, B_1, B_2, G_1, G_2 and M_1 are of wide interest. Aflatoxin B_1 and G_2 are detected in food in large amounts world-wide and B_1 is the mostly studied aflatoxin because of its potential carcinogenicity. Moulds of the *Aspergillus flavus* group are responsible for secretion of "Aflatoxins". Several species of *Fusarium* produce the mycotoxin called "Zearalenone". Mycotoxins known as "Ochratoxins A and B", "Penicillic Acid" and "Patulin" are produced by certain *Aspergillus* and *Penicillium* species while certain toxigenic Aspergilli also produce the toxin "Sterigmatoxystin". The group of chemical compounds known as "Trichothecenes" constituting the largest collection of mycotoxins known amounting to about thirty seven is produced by toxigenic *Fusarium* and *Stachybotrys* species. However, only six trichothecenes have been detected in field infected crops or associated with human and animal mycotoxicoses. Then there are the "Ergot Alkaloids" numbering over twelve produced by *Claviceps perpurea* and *C. paspali*.

Mycotoxin contamination of a commodity could either be a field problem or a storage problem and often a combination of both. Mould growth is a prerequisite of mycotoxin production. However, ability to isolate toxigenic moulds from a commodity does not render it mycotoxin free, because they could remain on the commodity long after the death of the moulds.

Fusarium species and *Claviceps perpurea* of St.Anthony's fire fame are good examples of moulds associated with

mycotoxin production in the field, more abundantly with unusual weather conditions. *Fusarium* invades corn and other cereal grains heavily, prior to harvest. Except for occasional heavy infections of wheat and rye in the field, contamination by *Claviceps* rarely occurs today. *Aspergillus flavus* that held the "storage fungus" tag contaminates food grains, maize for instance, in the field long before harvest. This kind of mycotoxin problems are controllable, by identifying the causative agents and avoiding contaminated material to start with, and then resorting to thorough and appropriate agricultural practices; use of proper and suitable fungicides and insecticides; and above all by developing specific plant varieties resistant to mould attack. Going along these lines of technology, fairly resistant small grain varieties of wheat and rye are being used today in greatly reducing recurrence of Ergotism in Europe. Adopting advanced agricultural practices and making every effort to get the crops out of the field before the onset of European winter and prope preparation for storage, the frequency of Alimentary Toxic Aleukia calamities has been greatly reduced.

However, contamination of commodities due to "storage fungi" is the most significant singular problem prevalent now. In addition to the damage caused by Fusaria, Aspergilli and Penicillia principally, several other mould species *Stachybotrys altra*, *Trichothecium roseum*, *Alternaria alternata* etc., for instance, are commonly encountered toxin producers in food commodities in storage.

Though it is desirable to have a mycotoxin free food rather than a mould free one, it would be more practicable and economical to have a food free of moulds. Mould proliferation and subsequent mycotoxin production can be prevented by controlling invasion and growth of moulds in stored commodities. Mould growth and toxin production in a commodity are promoted by several factors. Moisture content of the commodity, relative humidity of the storage environment, mould spore density, leakage of water or condensation, storage temperature and the influence of rodents, insects and other vermin in causing damage and generation of moisture and biological heat contribute much to enhance mould growth. But moisture, temperature and time of exposure to mould contamination are the most critical environmental factors that influence mould growth and also what could be optimised in controlling invasion.

Mould growth can be effectively controlled by cold storage, but use of low temperatures for large scale storage programmes of agricultural crops is not practicable. Control of available moisture for mould growth is the most economical and practicable approach. Water Activity (a_w) is the entity that denotes available water content for microbial growth. Moulds are capable of growing at very low available water levels and their growth can be successfully arrested below a_w levels of 0.65 ; $a_w = 1$ meaning the material being saturated with water. Food grains are dried by various techniques, and appropriate drying equipment have been evolved to suit the differnet needs. Supplemented by relevant and applicable fumigation techniques, the dried commodities are successfully stored under safe moisture levels thereby preventing invasion by moulds. Although removal of excess moisture and storage at optimum temperatures is the general practice, the combination of acceptable minimum moisture levels and the applicable safe storage temperature are considerably different for different commodities, making it a case by case situation. Even under such controlled storage conditions localised moisture and temperature increments can occur due to extraneous factors specially due to insect, rodent and vermin infestations. As such, these should be separately and appropriately dealt with if and when encountered.

Identification and quantification of mycotoxins in contaminated materials are achieved by two basic types of tests namely Chemical Analysis and Biological Assay which supplement each other in complete evaluation of mycotoxins where the identity, concentration, toxicity effects, the dosage response etc., are elucidated. Generally, the analytical procedures for mycotoxin detection follow a common flow pattern involving six or seven steps. The incriminated material is thoroughly mixed to achieve homogeniety and representative sampling is done preferably according to a good sampling plan using an appropriate sampling tool. For solids a sample of 4-5 Kg would suffice, and it is further prepared by repeatedly grinding and sifting through appropriate sieves -No. 14 and No. 20- and then it is thoroughly blended and properly sub divided to give analytical samples (50 g) which are conveniently refrigerated if not tested immediately. Mycotoxins present in very low levels (ppm-ppb) are removed from the analytical samples by an extraction process using a suitable solvent. Many interfering compounds that accumulate are removed at a clean-up operation next. Any oil component present is removed by

a defatting process either at this stage or it could be done prior to the extraction step. The clean-up operation is either a simple liquid-liquid partitioning with a separatory funnel or it could involve column chromatography and divalent metal clean-up procedures. To separate toxins from any remaining interfering compounds, the extract from clean-up is subjected to Thin Layer Chromatography (TLC). Gas-Liquid Chromatography (GLC), Liquid-Liquid Chromatograpy (LC) and mini cloumn chromatography also can be adopted for this purpose wherever applicable. A detection and quantification step is followed next, to detect and measure the response of the toxins by their fluorescence on TLC plates or in solution; or by U.V. absorption in solution or by a GLC flame detector. At this stage the toxins get separated into their various fractions which need confirmation by a TLC separation in comparison against standard mycotoxin solutions. Mass spectroscopy may be also employed in the confirmation of mycotoxins.

Biological tests are employed for confirmation of mycotoxins and also in the event of chemical analytical diffculties. The standard method employs concentrated extracts of suspect material on day-old ducklings and damage caused to the liver, for instance by aflatoxins, is estimated after 7 days or on death. In other biological confirmatory tests, toxicological effects of the suspect material on marine borer eggs, zebra fish larvae, brine shrimp eggs or fertile hens eggs are assessed. Rabbits, monkeys, mice, chicken and a host of animal models are also used in biological assays.

Mycotoxin analysis is obviously a very expensive and elaborate procedure, which introduces an exceedingly high risk for the analyst too. Next to operators who handle mycotoxin contaminated produce in trade, mycotoxin analysts are prone to gradual mycotoxin poisoning due to inhalation of toxins present in the aerosols produced during analytical procedures.

For the last three decades more than 50 countries worldwide, including Sri Lanka, have been carrying out extensive or limited studies on mycotoxins in food. It is a common yet a universal problem, imparting heavy financial and material losses to primary producers, middlemen making trade transactions, consumers -human or animal- and the national exchequers of every country. Due to international trade such losses overflow into global levels impairing international food production programmmes; commodity and food security arrangements. Though man is powerless to rid his environment of toxigenic moulds, his farsighted objective is to create a mycotoxin-free food production programme universally.