

## **The Screening of Agricultural Commodities and Physiological Samples for Fungi and Mycotoxins**

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### **Introduction**

Currently the problem of agricultural commodities, feed and foodstuffs becoming contaminated with mycotoxins is receiving increase attention due to the more stringent food quality control and legislation being practiced. Therefore, it is of importance to food producing countries to be in a position to detect where commodities are/or have been infected by fungi and possibly contaminated with their toxins know as mycotoxins. This presentation attempts to go through the problems facing such activities, possible solutions and current approaches.

The first question to be addressed is that of what is a fungus. The type we are discussing are the so called filamentous fungi that are saprophytic (sometimes plant and animal pathogens) that use organic material to promote their growth. They form mats on growth substrates called mycelium and can invade any crops such as cereals, oil seeds and legumes either in the field as plant pathogens or in storage as saprophytes. The mycelium is made up of hyphae which are microscopic strands of cells, which may also contain so-called fruiting structure that produce spores. They propagate themselves by means of spores that may be sexually or asexually formed and are found throughout the environment, including soil, air and water.

The next question to address is what are mycotoxins? These are produced as secondary metabolites that are only formed during the sporulation phase of the organism growth cycle. They are generally fairly specific to a species of fungus or related species and, therefore, tend to have unique chemical structures. They are defined as being toxic to animals and humans when ingested, inhaled or absorbed, causing mycotoxicoses. They may produce acute symptoms if the concentration is high enough, i.e., cause death or chronic symptoms at lower levels where ingestion may occur over a time period. The latter may be a very insidious process and can give rise to cancers, heart disease and brain lesions.

### **Problems facing mycotoxin analyst**

There are fundamental difficulties facing analysts who wish to examine commodities for mycotoxins some of which may be listed as follows:

- There are over 300 known each with different chemical structure & activity.
- They are present in commodities at low concentration in ppb (parts per billion, µg/kg) at most ppm (parts per million, mg/kg) levels.
- They are not uniformly distributed through non-processed commodities.
- They usually cause chronic effects and are slow acting and insidious.
- Although most are solvent soluble allowing an extract free of carbohydrates etc. some are not, e.g., the fumonisin which means that extra clean up is required.

To decide which toxins to look for in a sample, several pointers may be used, i.e.,:

- List any animal symptoms – certain toxins give certain symptoms, e.g., vulvo vaginitis in pigs is caused by zearalenone (Aucock *et al.* 1980).
- Take note of the matrix (commodity) – Certain crops are associated with certain toxins (Blunden *et al.* 1991) e.g., aflatoxin in peanut.
- Screen for fungi – as mentioned mycotoxins are only produced by certain fungi, e.g., *Aspergillus parasiticus* produces all four aflatoxins.
- Look for the commonly occurring ones – some mycotoxins are more commonly found than others, e.g. aflatoxins, fumonisin, ochratoxin, deoxynivalenol, & zearalenone.
- Run a cytotoxicity screen – this gives some idea if a toxin is present in an extract, and indicates which positives to analyze.
- Run a multi-mycotoxin screen – this can identify several mycotoxins present in an extract – usually depends upon thin layer chromatography (TLC).
- Then do a definitive analysis – once some idea of what may be present is formulated specific methods of analysis may be obtained from the literature, e.g., high performance liquid chromatography (HPLC) for aflatoxins.

#### **Approaches to mycotoxin analysis**

Two main approaches to screening for fungi in commodities are applied:

1. A portion of the material is surface sterilised (if suitable) and placed (or sprinkled) on the surface of a suitable nutrient agar such as potato dextrose agar and incubated. Fungi present in the material will grow out, produce mycelium and are identified.
2. The material is surface sterilised and aseptically milled, a known quantity is suspended in a known volume of sterile Ringer's and serially diluted. The dilutions are mixed with a restriction agar and incubated. Fungal colonies are identified and counted (Kaufman *et al.* 1963).

Cytotoxicity screen can be a useful tool where the presence of toxic materials is unknown. It is unspecific as it will detect any toxins ranging from mycotoxins to pesticides and inorganic poisons. The method depends on growing a living culture of animals or human cells in nutrient broth. These are dispensed into the well of a microtitre plate where they can be exposed to known levels of mycotoxins, the extract under consideration and any other controls required, e.g., carrier solvent (ethanol or dimethylsulphoxide are commonly used). The mixtures are incubated for at least 24 hours and then the viability of cells are measured. This can be done by a live cell count (dye exclusion) or by the reducing activity of the cells by adding methyl thiazole tetrazolium salt which is reduced to a purple dye. In theory the more viable the cells the more reducing activity and the more purple dye released. This can be quantitated against a control which had no toxin added (Hanelt *et al.* 1994).

Another approach to finding out toxins are present is the use of a mycotoxin multi-screen. The method used by our group was one developed by the Ministry of Agriculture, Food and Fisheries in the United Kingdom. It depends upon extraction of the commodity with aqueous acetonitrile, followed by a dialysis clean up (Patterson & Roberts 1979). The extract is examined by two dimensional TLC, the plates being

evaluated by viewing under ultra violet light (UV) and by spraying with selective staining reagents. The extract is also useful for investigation by the cytotoxicity assay (Robb & Norval 1983).

Disadvantages are:

- Experienced operators are needed
- Only semi quantitative
- Variable sensitivity depending up matrix and toxin

Advantages are:

- Rapid –result obtained within 24hours
- Cheap – does not require sophisticated equipment

Once an idea of what mycotoxins are present a method for a specific mycotoxins has to be applied. Whatever methodology is used several criteria have to be met, i.e., it has to be:

- Specific
- Accurate
- Sensitive
- Repeatable and reproducible
- Meet international standards, e.g., iso-accredited laboratories, commensurate with legislation.

### **Suggest methodologies**

Like the analysis of any component in a commodity there are recognised steps. The most important is the extraction and clean up of the analyte from the matrix. For mycotoxins this is particularly important as they are general present at low concentration so an extraction procedure must aim at removing as much of the toxin from matrix which minimising the extraction of other components which would become contaminants. Fortunately most mycotoxins are not very polar and may be extracted with lower polarity solvents such as dichloromethane or ethyl acetate. Usually some “clean-up” step is required and this may range from solvent-solvent extraction, to the use of C18 and ion exchange cartridges to immunoaffinity columns such as those supplied by Vicam Inc, Waterton, USA.

Once a cleaned up extract is obtained these can be analysed using modern instrumental methods, such as HPLC (Thiel *et al.* 1986; Reinhart & Zimmerli 1996; Zollner *et al.* 1999) and GC/MS. It may be in certain cases that the analyte does not have a chromophore that can be used in its detection so derivatisation is a solution, e.g., amine reagents OPA) for fumonisins and silylating agents for trichothecenes (Ternhune *et al.* 1984).

One fairly recent advancement is the use of Elisa methods using anti-bodies raised to specific mycotoxins. Several companies, e.g., Neogen provide such kits for most of the more common mycotoxins (Trucksess & Stack 1984).

Currently HPLC is the method of choice for most mycotoxins, apart from the trichothecenes. Usually reverse phase is used with fairly polar eluting solvents based on ones such as methanol and water. Most mycotoxins have a UV absorbance spectrum

and can be detected using UV absorbance including diode array. Where this is not sensitive enough then fluorescence detection is used. This is particularly useful for aflatoxins (Thiel *et al.* 1991). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) the principle member of the group has somewhat quenched fluorescence as compared to its congener aflatoxin B<sub>2</sub> which is minus an isolated double bond. The fluorescence of AFB<sub>1</sub> can be enhanced (hence also sensitivity) by adding groups across the double bond such as halogens. This can be conveniently done by post column derivatisation using a Cobra cell (now called a Coris Cell, Dr. Weber Consulting Kft) which generates atomic bromine.

Mycotoxins such as fumonisin B<sub>1</sub> (FB<sub>1</sub>) that do not contain convenient chromophore groups can be derivatised with reagents which add these. O-Phthaldialdehyde is a commonly used reagent for FB<sub>1</sub> but suffers from the disadvantage that the derivative is somewhat unstable and each sample has to be derivatised in the same time scale and run immediately on the machine (Sydenham *et al.* 1992).

Gas liquid chromatography is not used as frequently as HPLC for analysing mycotoxins and its main use lies in the analysis of trichothecenes (such as deoxynivalenol) which does not have a suitable chromophore. Silylating agents are commonly used yield a more volatile derivative that may be chromatographed and detected using flame ionisation or more recently mass spectrometry (Schollenberger *et al.* 1998).

Mass spectrometry (MS) has been developed more recently as a detector connected to output of both liquid and gas chromatographs. As the detector provides detection and identification of analyses it is an ideal and powerful method of analysis. A more recent methodology is to use MS/MS whereby specific ions can be selected and further analysed. In principle using this approach it is possible to directly analyse crude extracts for a range of analytes provided a suitable library of spectra is available, even by direct insertion into the machine without prior chromatography (Spanjer *et al.* 2008). Although this sounds an ideal method it requires high investment in instrumentation and skilled staff.

### **Physiological samples**

Time does not permit any in depth discussion of the analysis of physiological samples from animals and humans but this is of some importance in the research into the effect and targets of mycotoxins in living systems. Most fluids and tissues have at some stage been screened and analysed for exposure to mycotoxins. Such fluids examined have been urine, blood, faeces, CSF, breast milk and saliva; tissues: the major organs, brain, hair and placenta (Garner *et al.* 1985; Fernandez *et al.* 1997; Coulter *et al.* 1986). The instrumental methods already discussed can be used for the final measurement of toxins but the important part is the application of suitable extraction and clean-up methods. Other methods in these analyses that can be applied are flow cytometry and immuno cytochemistry (Santella *et al.* 1993).

### **Conclusion**

- Mycotoxins are ubiquitous dangerous contaminants of food commodities
- It is therefore important that appropriate rapid and sensitive methods of analysis are developed and applied.

- This requires a deliberate and focussed investment in the infra structure, instrumentation and skilled personnel required for this purpose.

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