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Application Bulletin

AFLATOXIN DETECTION USING BLAK-RAY® ULTRAVIOLET LAMPS

In late 1975 there was increased concern about the possibility of the presence of carcinogenic mold metabolites, particularly aflatoxins in food and animal feed products. This has been an annually recurrent problem in recent years at about harvest time but has received increasingly more attention because of the involvement of the U.S. Department of Agriculture, FDA and public awareness of potentially dangerous food contaminants generated by newspaper articles and the Consumers' Union.(1)

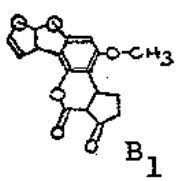
WHY ARE AFLATOXINS IMPORTANT?

It has been found that they can be carcinogenic to some lab animals in very small concentrations, e.g., rats, ferrets, ducks, trout, dogs, turkeys, cattle and pigs. The toxins prevent protein synthesis within the cell and the ability to mobilize fats. Poor growth and changes in the liver consequently take place, at levels of 1 ppm and up.

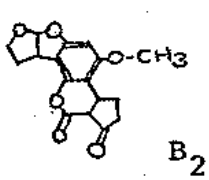
Most animals affected become depressed, have poor hair coats, are anaemic, suffer chronic lesions from long feeding and frequently have convulsions. Thus the feed efficiency and rate of gain of weight are affected. The FDA has established an "actionable" level of 15-20 ppb.

WHAT IS AN AFLATOXIN?

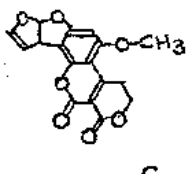
The word AFLATOXIN is an acronym formed from Aspergillus FLAvus TOXIN and is used as a generic name for several related metabolites (mycotoxins) produced by the mold Aspergillus Flavus on various grains and nuts, e.g., corn sorghum, cottonseed, peanuts, pistachio nuts and copra. These toxins can be identified as and are separable into Aflatoxins B_1, B_2, G_1, G_2, M_1, B_{2a} and G_{2a}. They are a class of heterocyclic compounds, the structures of the main aflatoxins being:



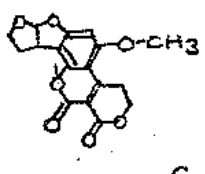
B_1



B_2



G_1



G_2

WHY DOES AFLATOXIN OCCUR?

Aspergillus flavus can occur on virtually all grains, fruits and vegetables if they are stored in damp places at temperatures that permit the growth of the mold and subsequent aflatoxin formation. It is not found in the field very much but is essentially formed in storage.[2] In the case of corn, toxin-free kernels can be found adjacent to highly contaminated kernels and presumably infected by A. flavus so that factors additional to humidity and temperature are probably involved such as physical damage to the kernels or the grain maturity.[2] Attempts[3] & [4] have been made to correlate insect activity (corn earworm) and subsequent infection of the damaged corn by A. flavus.

These were unsuccessful and no relationship was established. However, more recently[5] insect damage has been related to the presence of aflatoxin in preharvest corn. No aflatoxin-contaminated samples came from fields where preharvest ears escaped insect activity. In all probability[5] A. flavus invades preharvest corn damaged by insects.

HOW CAN IT BE DETECTED?

Since most samples do not contain a detectable amount of aflatoxin, there is a need for a method which correctly identifies the many negative samples with minimum expenditure of time and money. Such a method is known as the BLACK LIGHT TEST and UVP BLAK-RAY® lamps are recommended for this application. Refer to UVP Brochure 818.

The aflatoxins all have absorption maxima around 360nm with a molar absorptivity of about 20,000. The B toxins are named for their blue fluorescence (425nm) and the G toxins for their green-blue fluorescence (450nm). The B_1 toxin is the most common, followed by B_2 toxin, while the G toxins are fairly rare. The fluorescence sensitivity of the G toxins is more than 10 times greater than that for the B toxins. The minimum detectable level is about 100 pgrams for the G toxins and 1 ngram for the B toxins.

Corn is inspected under the BLAK-RAY lamp for a characteristic bright greenish-yellow (BGY) fluorescence in broken and damaged kernels. The test takes 5 minutes or less. If the fluorescence is observed, aflatoxin may be present but not necessarily in appreciable or detectable levels. There are substances in corn and other food that fluoresce under long wave ultraviolet irradiation, but are not associated with aflatoxin. Many other fungi[6] such as aspergillus niger, various penicillium species, aspergillus repens and other species which do not produce aflatoxin may produce fluorescent harmless metabolites so that the fluorescence is not a specific indication of the presence of toxicogenic molds, although it may indicate that conditions have been favorable for growth of the toxicogenic molds.

Additionally, A. flavus, isolated from corn, has displayed a broad spectrum of aflatoxin production, ranging from no detectable yields

to high levels [7] (> 100 ppm). A probably contributory factor to this is that the fluorescence in naturally contaminated corn is not stable and disappears in 4 to 6 weeks on continuous exposure to visible or ultraviolet radiation although the toxin does not disappear. Fresh samples must therefore be taken.

The reliability of the method depends on the size of the sample taken for analysis and how it is taken. A sample must be large enough to be representative of the entire lot of corn and must be taken from all parts of the lot--whether bin, truck or railroad car. One highly contaminated corn kernel may account for objectionable levels of aflatoxin in a 3000-kernel (2-lb.) sample. If only a 1 to 2 lb. sample is collected, the one highly contaminated kernel could be missed. Usually from 5 to 10-lb. samples are collected, but even larger ones would be better. If the sampling is not performed properly, the results obtained by this screening method could be of little value.

The black light test is an excellent screening test for possible presence of aflatoxin, but it does not give quantitative indication. Even with good technique, brightly fluorescing samples may contain less aflatoxin than weakly fluorescing samples. Because of this, additional confirmatory and quantitative measurements are needed to be applied to those samples that reacted positively to the black light test. Non-fluorescing samples need not be subjected to this.

FURTHER SCREENING

Since the black light test is only a preliminary confirmatory test, another screening test is used in feed mills immediately following the black light test. This test involves the use of a small chromatographic column (mini-column) in the final separation and detection step of the method.

If the black light test gives a positive answer, the 10 lb. sample of cracked corn is ground to pass a twenty mesh screen and then mixed thoroughly. A 50 g representative subsample of the 10 lb. of finely ground corn is used for the mini-column test. The final separation step of the mini-column method involves transferring 3 ml of chloroform extract to the top of a mini-column. After the chloroform drains into the column, 3 ml of an elution solvent are passed through the mini-column.

The mini-column is then inspected in the dark by shining a long wave black light on it. This, again, should be one of the BLAK-RAY Lamps. If a bluish-green fluorescent band is detected at the proper height in the mini-column, the sample is judged to be positive to aflatoxin. In this instance, the property detected by the test is the bluish-green fluorescence.

This test, like the black light test, is a good screening test because it gives few false negative answers. However, this test also has the characteristic of giving only a few false positive answers. This makes

it a better screening test, from a scientific viewpoint, than the black light test; however, it is better costwise to run the two tests one after the other.

After the mini-column test is completed, a judgment can be made as to whether or not a shipment of corn will be accepted by a feed mill. The chances of accepting a truckload of corn that contains more than 20 ppb aflatoxin is slight, as is the chance of rejecting a truckload of corn that does not contain detectable aflatoxin.

If further confirmation is required, a subsample of the ground sample of corn can be sent to a chemical laboratory, or the extract remaining from the mini-column test can be used for a simple confirmatory test.

In this test, a portion of the chloroform extract not used for the mini-column test is evaporated to a small volume and some of this concentrated extract is spotted on a thin layer plate. A small amount of trifluoroacetic acid (TFA) is placed on top of the spot where the sample extract was placed. If aflatoxin is present in the sample spot on the thin layer plate, it will be derivatized to a water adduct of the parent aflatoxin compound. After thin layer chromatography is performed, this plate is inspected in the dark under a long wave black light. If a bluish-green fluorescent spot is detected on the thin layer plate at the same height as a reference spot known to be the derivative of aflatoxin formed by the TFA reaction, this confirmatory test (the TFA test) is positive and the concentration probably greater than 5 to 10 ppb.

QUALITATIVE TESTS (7)

Although the TFA test is a confirmatory test, it is not essentially different from the mini-column test. Both are qualitative tests, that is, both test for qualities or properties of aflatoxin. The final property of aflatoxin involved in the mini-column test is the property fluorescing bluish-green under a long wave black light.

The TFA test involves three additional properties of aflatoxin. One of the properties involved is that aflatoxin will from a derivative in the presence of trifluoroacetic acid while on a thin layer plate at room temperature. Another property is that this derivative will move to a certain height on the thin layer plate using a certain developing solvent. A third property is that this derivative will fluoresce bluish-green under a long wave black light.

However, even if this test also is positive, we are still not 100% certain that aflatoxin has been detected. At this point, the degree of certainty is probably over 99%.

If we desire a further confirmation that aflatoxin is in the sample, a small amount of the underivatized compound can be isolated on a thin layer plate and extracted. This small amount of sample can then be inserted into the entrance of a mass spectrometer. The mass spectrometer is an instrument which will break the aflatoxin into many fragments. The original mass of the aflatoxin molecule, plus that of each of the fragments produced, will be indicated by the output of the instrument.

The pattern of the various masses is characteristic of the particular aflatoxin involved. If the pattern of the chemical suspected to be aflatoxin is identical with the pattern given by a known aflatoxin, we are about 99.99% certain that the suspect chemical is aflatoxin.

HOW IS THE CONCENTRATION OF AFLATOXIN MEASURED?

Quantitative Methods -

A list of quantitative methods available for aflatoxin analyses is given in Table 1. Most of these methods have been validated by Association of Official Analytical Chemists (AOAC) collaborative studies. The main differences between the methods listed in Table 1 is the types of samples to which each has been applied.

The last two methods listed apply to most of the major ingredients and mixed feeds. The primary difference between these two methods is that the former is much more time-consuming and costly.

Neither of the last two methods, however, applies to ammoniated cottonseed meal or roasted corn. New analytical interferences arise during the aflatoxin decontamination processes used on these types of samples.

Thus, each step taken in attempting to solve the mycotoxin problem seems to generate a need for a new type of analytical method. Oftentimes, however, the new analytical method developed is a modification of a previous method.

There is a relationship between the major clean-up step and the disadvantages of each of the methods used.

TABLE 1 - Quantitative Methods for Aflatoxin [7]

Applicable to	Major Clean-up Step	Final Sep.	Detection	Adv.	Disadvantage
Peanut meal	Column chromatography	TLC	Fl.	Good clean-up	Solvent costs Elapsed time
Peanut meal	Liquid-liquid partitioning	"	"	Low-cost	Fair clean-up Fair precision
Cottonseed meal	Column	"	"	Good clean-up	Solvent costs Elapsed time
Cottonseed meal and ammoniated cottonseed meal	Divalent metal clean-up	"	"	Good clean-up	Elapsed time for ammoniated cottonseed meal
Corn	Column chromatography	"	"	Good clean-up	Elapsed time
Roasted corn	Column chromatography	"	"	Rigorous clean-up	Solvent costs Elapsed time
Ingredients and mixed feeds	Column chromatography	"	"	One method for many products	Solvent costs Elapsed time
Ingredients and mixed feeds	Divalent metal clean-up	"	"	Good clean-up Low-cost One method for many products	Requires much glassware

DETOXIFICATION AND PREVENTION

Once corn and other products have been contaminated, there really is no practical way of removing the toxin completely without altering the product. Heat, for example, will not destroy the toxin; however, a certain degree of detoxification has been achieved by treating aflatoxin B_1 contaminated corn with an ammonia solution (8)(9), but this is not viable commercially. It is thought that B_1 binds irreversibly to the protein fractions through the dihydrofurofuran ring system and that the hemiacetal part of the molecule can lead to a phenolic dialdehyde derivative capable of reacting with amino groups on proteins and amino acids of the product by electrostatic or hydrogen bonding.

The problem is essentially that of determining how and when A. flavus invades the commodity, what factors are involved in aflatoxin formation by the mold and how the growth of the mold can be prevented. This can be achieved by at least three methods: (1) control of the environment; (2) use of chemical antifungal agents and (3) utilization of natural resistance factors.

It is known that A. flavus will not grow at a relative humidity level less than 16% but control of the environment is not practical. The use of chemical antifungal agents to control aflatoxin production has been extensively investigated, but the technique must be viewed with reservations since there is a possibility of ecological problems developing later.

The present distribution of A. flavus infection, fluorescence and toxin contamination on a varietal basis, has shown no significant difference between several common commercial corn hybrids. However, a variety of corn has been discovered that had a noticeably lower aflatoxin production, compared with other varieties.(10) Such discoveries help plant geneticists to breed more resistant strains.

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