Protocols for Surveys, Sampling, Post-Collection Handling, and Analysis of Grain Samples Involved in Mycotoxin Problems

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This report examines and summarizes current knowledge regarding mycotoxin surveys, sampling techniques, conditions conducive to post-collection production of mycotoxins in grain samples, and analytical methods for mycotoxin analysis. Priority attention is given to samples of corn suspected of containing aflatoxin. The report includes recommendations where deemed appropriate by the Ad Hoc Work Group.

Surveys for Evaluating Mycotoxin Problems

General Comments

Surveys as used in this report means sampling efforts designed so that statistically sound inferences may be made concerning mycotoxins in a defined population of grain. The population may be grain in any stage of production: harvesting, transport, processing, or utilization; in any area: state, region, or nation; and for any particular time period.

Surveys to evaluate mycotoxin problems in corn have been conducted (1). It has become apparent that since many agencies (public, private, national, international, etc.) may conduct these surveys, it would be advantageous to have a set of guidelines.

Recommendations

Before a survey is made, the purpose of the survey should be carefully determined. If a survey is justified, only surveys based on probability sampling methodology should be used so that reliable inferences can be made. If possible, standard protocol should be followed as outlined elsewhere in this report. The population to be surveyed must be agreed upon at the outset. Certain questions must be considered based on inferences to be made from the survey. For example, in a survey of corn, should samples be taken of mature corn standing in the field or should samples be taken at some later stage, perhaps at time of marketing or before feeding? It depends on the results needed. If sampling is done in the field just before harvest, how many days before harvest can samples be gathered and still meet survey objectives? One must know at which stage aflatoxin forms in the field. These and other considerations must be addressed in defining the population of interest.

Once the population is specified, a suitable sampling frame to assess fields, farms, or bins must be identified or developed. The sampling frame must be able to assess sampling units with known probabilities.
A randomization process has to be part of any field sampling procedure so selectivity in sampling and resulting biases can be avoided. A bin can be adequately sampled only while it is being loaded or unloaded. Some recent research has been carried out to study the components of variance associated with sampling and subsampling from a lot of shelled corn (2). Such research is needed in order to determine optimum field sampling procedure.

Handling and testing procedures should be standardized. Efforts to plan and carry out well designed surveys will be of little value if inconsistent procedures which produce non-comparable results are used. We hope that the best procedures can be agreed upon and used uniformly. Surveys not based on sound statistical design should not be authorized or conducted.

Sampling and Subsampling Techniques

General Comments

Surveys for mycotoxins in agricultural products sometimes are based on the misconception that the distribution of the survey samples according to mycotoxin concentration closely approximates the distribution of the lots which were sampled. One reason for lack of agreement between sample distributions and lot distributions is sampling error caused by inadequate sample size. Other reasons for error in surveys are biased sampling procedures, inadequate sample comminution, and improper subsampling for analysis. Because more is known about sampling for aflatoxin, causes of sampling error will be discussed in relation to aflatoxin, but the general concepts probably apply to other mycotoxins.

Sample Size

High concentrations of aflatoxin have been found in individual kernels of corn, peanuts, and cottonseed (3, 4, 5). These high concentrations probably occur in individual seeds, nuts, or fruits of other commodities which are susceptible to aflatoxin contamination. Because aflatoxin is often highly concentrated in but a small percentage of the seed within a lot, there is a large variation in the aflatoxin concentration of samples from the lot, and determination of the true aflatoxin concentration in the lot is difficult (2, 6–8). The variance of estimated concentrations is inversely proportional to sample size.

The distribution of sample concentrations about the true aflatoxin concentration in a lot is skewed so that more than half of the sample concentrations are less than the lot concentration (9). Skewness decreases as sample size increases, and the distribution will approach a normal distribution for large samples. As a result of this skewness, the distribution of sample concentrations determined in a survey will be different from the distribution of the true concentration in the lots sampled. More than half of the individual sample concentrations will be lower than the true aflatoxin concentration in the lot from which the sample was drawn, but some of the time the sample concentration will be much higher than the lot concentration. However, when the number and size of the samples are adequate, the average aflatoxin concentration in the samples will provide a useful estimate of the average aflatoxin concentration in the lots which were sampled.

It is difficult to make a general recommendation about the size samples (amount of grain per sample) that should be used in surveys. Larger samples will increase accuracy, but the cost of the product transportation, etc., may be limiting. Cost factors must be reconciled with acceptable limits of accuracy for the anticipated survey. The effect of sample size on the agreement between the sample distribution and the lot distribution may be determined according to a procedure developed by Whitaker and Dickens (10). This same procedure may be used to estimate the lot distribution based on the sample distribution.

If the purpose of the survey is limited to a determination of the average aflatoxin concentration in all lots sampled, relatively small samples are required. When an accurate estimate of the distribution of lots according to aflatoxin concentration is desired, larger samples are required. A 10 lb sample of shelled corn is probably sufficient for most survey purposes. For the same accuracy, much larger samples of cottonseed, shelled peanuts, and possibly other products are needed.

Sampling Procedures

Samples may be taken from crops growing in the field, during handling, during storage, and at other points in the production, marketing, and processing system. Each type of sampling presents a different situation regarding the distribution of the aflatoxin-contaminated seed.
within the lot sampled and accessibility of the entire lot. When feasible, samples should be taken after the lot has been reduced to a smaller particulate size. For example, it is better to sample shelled corn rather than ear corn, and it is better to sample ground corn rather than shelled corn.

When the lot has been recently blended by harvesting, loading and unloading, turning, grinding, or other operations, a representative sample is more easily drawn than when the lot has not been blended because growth may have occurred in spots. For example, moisture condensation or leaks during storage may cause a portion of the lot to mold and contain high concentrations of aflatoxin. It is impossible to predict where to probe the contents of the storage bin in order to obtain a sample with the same aflatoxin concentration as the concentration in the entire lot.

Stream Sampling.—The most effective sampling method is to take small portions from a moving stream at periodic intervals and combine the portions into a sample. Cross-cut samplers are commercially available which automatically cut through the stream at predetermined intervals (11). When an automatic cross-cut sampler is not available, a person may be assigned to pass a cup through the stream at periodic intervals and thus collect a sample. The stream should be sampled frequently but the amount taken at each interval may be small to avoid accumulating too large a sample. The samples must be taken from the stream throughout the time the lot is moved. This is the only proper way to sample a bin.

Probe Sampling.—Probe sampling is probably adequate for lots which have recently been blended by harvesting or handling operations. Recommended methods for taking probe samples are published by the American Oil Chemists’ Society (12). As previously stated, probe sampling of lots which have not been blended recently probably will not provide representative samples because mold growth may have occurred during storage if moist conditions are present.

Field Sampling.—When samples are taken before harvest, reliable sampling representative of the field is difficult. When Aspergillus flavus grows on corn before harvest, the geographic distribution of infected ears within the field may be erratic. Also, none of the kernels, a single kernel, a group of kernels, or nearly all of the kernels on an ear may be aflatoxin-contaminated. The problem becomes one of collecting a large number of widely distributed ears in order to obtain a representative sample of ears which must then be shelled so a representative sample of the shelled corn can be subdivided. Therefore, sampling should be coordinated with harvesting so corn from a very large number of ears is represented in the sample of shelled corn. The same concept applies to other commodities where the contaminated product may be clustered before harvest.

When it is absolutely necessary to collect samples of ears from the field, one must recognize the possible errors associated with such a procedure in estimating for the field. The coefficients of variation associated with aflatoxin test data when various numbers of ears are harvested for a sample were determined. The estimated coefficients of variation (%) among aflatoxin tests when the indicated number of ears of corn are randomly selected from a field with an average of 100 ppb aflatoxin are 133 for 36 ears harvested and shelled, 83 for 100 ears, 62 for 200 ears, 48 for 400 ears, 39 for 800 ears, and 29 for the entire field (unpublished data from J. W. Dickens). (After the ears are shelled, a 4.54 kg sample of kernels is comminuted and analyzed according to the AOAC procedure (13).) The estimated coefficient of variation associated with a 36-ear sample from a field with an average aflatoxin concentration of 100 ppb is 133% compared to 29% when the entire field is harvested and a 4.54 kg sample of kernels is ground to obtain the analytical sample. The estimate of 133% is based on the assumption that the ears are selected from the field in a completely random manner.

Subsampling

Since it is not feasible to extract the entire sample for aflatoxin analysis, the large sample must be comminuted so a subsample of the comminuted material can be extracted (13, 14). A larger subsample is required for coarsely ground material than for finely ground material. Recommended methods for comminution and subsampling are given by the AOAC (13).

Post-Collection Production of Mycotoxins

General Comments

In general, grain (corn) with moisture contents of 13–13.5% is safe for long term storage. There are many examples to demonstrate the
rapidity with which fungi develop but 2 should suffice. A recent study (unpublished data from G. C. Kingsland) on the effect of grain moisture and 2 storage temperatures on growth of *Aspergillus flavus* as measured by an increase in the numbers of propagules/g corn substrate indicated dramatically the effect of these factors. For example, when corn was stored at 0°C for 8 days at 21.3% moisture, there was virtually no growth of the fungus. However, when a duplicate sample was stored at 24°C, propagules increased 1500-fold (from 278 propagules/g to 420,000 p/g). When another corn sample was stored at a moisture content of 17.2%, propagule number increased approximately 8-fold (from 40,000 to 320,000 p/g). At 12.6% moisture, there was virtually no growth (from 21,500 to 29,500 p/g) in another sample. Another study (unpublished data from D. M. Wilson) indicated an increase in aflatoxin content of approximately 10-fold (from 200 to 2300 ppb aflatoxin) in a 3-day period when a field-harvested corn sample was stored at high moisture.

Although most “storage” and “field” fungi, especially members of the genus *Aspergillus*, have rather precise minimum requirements of water activity (A_w), once these limits are breached rampant growth may occur. Also, the longer the time, the lower the A_w and temperature required to prevent fungus growth. Although mold presence is not evidence per se of aflatoxin or any other mycotoxin, mycotoxin production is not likely in the absence of mold. Average moisture contents may be misleading because they do not reflect the range among individual kernels. If the moisture and temperature at a spot in a grain sample are adequate, mold growth will occur and respiration of the mold and grain may increase the moisture content and temperature of that portion of the sample. Insect infestations that damage kernels may also contribute to fungal growth.

Corn harvested wet must be dried rapidly to prevent mold growth, i.e., to less than 13-13.5% moisture. Samples harvested dry must be kept dry. When moisture and temperature are favorable for mold growth, even for a very short period, significant production of mycotoxins can occur. Preferably, samples should be stored at 0°C, although economics generally preclude this practice in large surveys. Proper drying, as discussed above, is the most certain way of preventing formation of aflatoxin in samples of corn.

**Recommendations for Prevention and Control**

Controlling the conditions of storage to prevent mold growth and mycotoxin elaboration from harvest to chemical analysis for mycotoxins is a very difficult problem for surveys involving a large number of samples from widely dispersed locations. Current information strongly indicates that in order to prevent mold growth and/or mycotoxin elaboration it is necessary to keep the sample dry or at 0°C or below. Analysis of the problem is simple, but a solution is not. The following procedures could minimize opportunities for mycotoxin production:

1. Preferably, samples should be placed in transit in a dry condition and every effort should be made to maintain dryness.

2. Plastic bags should not be used for unrefrigerated storage unless seeds are dead and dry.

3. Closely packed piles of samples may not cool rapidly enough to 0°C under refrigeration. Therefore, the samples should be spread until they have been cooled. Since even the mass of 10 lb samples may retard cooling, sample bags should be large enough so the sample can be spread within the bag to facilitate rapid cooling.

4. Once cooled to 0°C (Dry Ice, liquid nitrogen, or refrigeration) removal therefrom can quickly result in condensation and elevate the moisture availability to unacceptable levels. Therefore, samples under refrigeration either should be analyzed immediately upon removal or other special efforts should be taken to prevent condensation on the sample, e.g., keeping samples in moistureproof containers on removal from refrigeration until samples reach ambient temperatures.

5. It may be possible to add toxicants such as acetic acid, propionic acid, etc., to a shipment before transit. There are several shortcomings to this procedure: the volume of the sample markedly influences the feasibility of this procedure; corrosive and toxic nature of such materials may represent a health hazard to personnel; this method would be lethal to all members of the microflora and thus identification of the contaminants would be impossible; and effects of such treatment on subsequent analytical procedures have not been determined. Thus, more

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research is needed before such procedures can be recommended.

(6) Time in transit should be minimized.

Analytical Problems

Current Knowledge

The official first action method adopted by the AOAC (13) and the American Association of Cereal Chemists (AACC) (15) for determining aflatoxin in corn is known as the CB method. It involves extraction of the ground corn sample with chloroform in the presence of water. The extract is purified on a silica gel column for measurement of aflatoxins by thin layer chromatography.

The coefficient of variation for one analysis by the CB method is 33% among laboratories and 17% within a laboratory. The BF method developed for peanuts has not been tested collaboratively on corn, but the coefficient of variation is 27% within a laboratory and recoveries are 30–35%, too low to be quantitative (16). The Pons method developed for cottonseed and used in some laboratories for corn has a coefficient of variation of 31% on corn and recoveries greater than 90% for samples containing less than 100 ppb (16). One proposed method includes a purification of extracts by liquid-liquid partition (17), but recoveries are low. In another proposed method, the amount of aflatoxin in an extract is determined by measuring the enhancement of fluorescence in solution after the reaction of aflatoxin with iodine (18).

Surveys can be performed using rapid screening techniques to determine the presence or absence of aflatoxin (13), and quantitating positive samples by the CB method. Minicolumn techniques have been used in surveys to determine the incidence of aflatoxin in corn samples and the approximate levels of contamination in samples. A minicolumn method for aflatoxin in corn that includes the Holaday extraction and cleanup (19) and the Velasco column (9, 20) was approved in 1978 for adoption as official first action by the AOAC.

Aflatoxin identity can be confirmed in extracts by forming the water adduct on a TLC plate with trifluoroacetic acid (13). Virtually all substances in corn determined to be aflatoxin by the CB method have been confirmed to be aflatoxin by formation of the water adduct.

A method for determining zearalenone in corn has been adopted by both the AOAC and AACC (21, 22). The extraction solvent is chloroform in the presence of water, and extracts are purified for TLC on silica gel columns. A second method designed for zearalenone in grains and feeds includes a liquid-liquid partition into basic solution. The mycotoxin is measured in solution by gas-liquid chromatography (GLC) (23). The second method has been studied collaboratively, but the results have not been published. One can confirm results obtained for zearalenone by TLC with GLC and those obtained by GLC with TLC. Final confirmation of identity for zearalenone in a feed or grain is accomplished by GLC-mass spectrometry.

A method for determining ochratoxin in barley (13) has been adopted as well as one for sterigmatocystin in several grains (13).

Recommendations

Currently validated and officially adopted methods should be used in all surveys. Research should be encouraged to improve analytical methods for mycotoxins. Lower coefficients of variation and better recoveries are needed. New methods should be considered and tested collaboratively as they are developed. The disadvantages of adopted methods are that the quantities and types of solvents required make the methods expensive, and the solvents are hazardous and toxic. Mycotoxin analytical methods are needed that require less time, less solvent, and less toxic solvents without sacrificing reliability. Such methods must be tested collaboratively before recommendation and official adoption.

Mycotoxins for which reliable, validated, sensitive methods are not available are ochratoxin (corn), penicillic acid, citrinin, and the trichotheccenes. Research is needed to develop adequate quantitative methods for these mycotoxins.

Needs and Recommendations

Research Needs

(a) Sampling of a Corn Field.—There is insufficient information on the distribution of ears according to aflatoxin concentration within a field of corn. The protocol recommended in this report should be tested and analyzed, and improvements should be made as necessary.

(b) Sampling of Containers (storage bins, truck loads, etc).—The only practical way to sample a bin is during loading and unloading.
The geometry of a bin and limited access to the bin and its contents make it difficult to obtain samples that reflect both the distribution and the mean concentration of aflatoxin in a bin. New sampling designs and equipment for obtaining samples would be of great value.

(c) Post-Collection Aflatoxin Production.— Amplification of aflatoxin concentration in samples after collection and before analysis needs to be better understood, and additional ways to prevent it should be developed.

The use of volatile fatty acids (e.g., propionic acid) as a preservative should be evaluated both for their ability to stop fungal activity and for the effect of these acids on aflatoxin. Other antifungal compounds should be tested. However, it must be recognized that some such compounds, e.g., formaldehyde and SO₂ gas, also partially degrade aflatoxin and would not be suitable for treatment of samples before analysis. Also, compounds such as propionic and acetic acid are only effective 3-5 months following treatment. The effect of these materials on quantitative analyses for aflatoxin should be investigated. Of paramount importance is the effect of such agents on the containers used to hold the grain.

(d) Analytical Problems.—There is an increasing need for an inexpensive, simple, and sensitive multitoxin assay which can be performed on a single extract from a sample.

There is an increasing need for collaborative studies and evaluations on analytical methods for mycotoxins. In particular, the FL-I and FL-IRS methods of Davis and Diener (18, 24) should be evaluated because of their reported sensitivity, reliability, low cost, and use of non-toxic solvents. It would be of great value to have additional presumptive tests for aflatoxin in corn and other grains to supplement the widely used BGYF method, which is the only truly rapid method.

Other Needs

We need to be more careful with the use of the term “survey.” Just as random sampling does not mean haphazard sampling, a survey does not mean a collection of assay results from materials of unknown origin and handling. The word survey implies a prior experimental or sampling plan and a post facto assemblage of data prescribed by the plan. Proper restriction of the term would contribute much to the development of more meaningful surveys, and provide for better planning.

RECOMMENDED PROTOCOLS FOR SURVEYS, SAMPLING, POST-COLLECTION HANDLING, AND ANALYSIS OF GRAIN SAMPLES RELATIVE TO MYCOTOXIN PROBLEMS

General Considerations

No single protocol can be recommended that will be suitable for every situation involving collection and analysis of grains for mycotoxins. Therefore, the Ad Hoc Work Group recommends protocol guidelines believed to represent best possible practices, in terms of present knowledge. The guidelines must be adapted to suit individual situations as required. These recommendations are intended primarily for use by trained personnel in connection with officially sanctioned surveys, research projects, etc.

It is recognized that the recommended protocol guidelines are somewhat inconvenient and expensive, and require certain equipment and procedures not readily available to non-technical personnel. It is hoped that research recommended elsewhere in this report can eventually provide more convenient and inexpensive procedures. It is important that all personnel receive information regarding necessary safety precautions (not covered in this report) in the handling of solvents, standards for toxins, and preparation of samples.

Protocol Guidelines for Surveys

These guidelines are statistically designed to draw inferences about the frequencies and levels of mycotoxins in grains, particularly aflatoxin in corn, of a defined population:

1. Preliminary Considerations:
   (a) The population to be surveyed must be defined as precisely as possible.
   (b) Surveys should be uniformly conducted in all areas where information is needed.
   (c) Sampling methodology should be uniform so results can be compared.
   (d) A suitable sampling frame (to assess farms, fields, bins, etc.) must be identified or developed.
   (e) Sampling procedures must be established with proper attention to randomization which is essential so that selectivity and resulting biases can be avoided.
   (f) Adequate sample size is required to provide reliable and useful results.
   (g) Post-collection mold growth and production of mycotoxins must be prevented.
(h) Adequate comminution of samples is required to uniformly blend any mycotoxin present throughout the sample.

(i) A uniform method of analysis must be selected. Preferably, only methods currently validated and approved for the commodity being surveyeded should be used.

(j) A standard format for reporting data should be adopted.

(k) Appropriate procedures for statistical analysis of survey data should be designated.

2. Procedures:

(a) The population must be defined, and the sampling frame to be used must be selected. Selection procedures must be random. In case of field sampling of corn, it would be preferable to harvest an entire field of corn and take representative stream samples of the shelled corn as previously discussed. Alternatively, probe samples can be taken (immediately after combining but before storage). Samples can be collected from randomly selected 1-acre plots, using a table of random numbers to designate plots within the field (25). Field sampling requirements are far more critical if reliable information for the individual field is needed. However, field reliability may not be an objective in some surveys.

(b) Sampling must yield a minimum of one 10 lb sample per unit sampled. Stream sampling is preferable to probe sampling which in turn is preferable to field sampling of ears of corn. However, when the latter is required it is important for ear collection to be carried out without selectivity; if sampling must serve to estimate for an individual field, it must be widely distributed throughout the field. The errors given previously should be considered when choosing the number of ears to be collected for each sample.

The following procedures for randomly selecting the desired number of ears (N) for the sample are suggested for guideline purposes: Diagram each field and divide the field into N approximately equal sections. If the field is rectangular, a count of rows for width and a count of paces for length can be helpful in dividing and identifying N equal field sections. Devise a method to randomly select one ear from each section. Composite the N ears in a mesh bag as a single sample representing the chosen field.

(c) Each sample should be handled in a manner that minimizes the post-collection production of mycotoxins. Moist samples should be held in cloth or paper bags, cooled if feasible, and transported to drying facilities as soon as possible. Samples should not be placed in bags, containers, car trunks, large piles, or other confined situations where humidity and temperature can increase around them. Significant mold growth and toxin production can occur in just a few hours and this must be avoided. As soon as possible after collection, the samples should be dried at approximately 80-90°C for 3 hours or more to reduce the grain moisture to about 12-13% (where molds are to be studied, 60°C for a longer time is recommended). If the samples were refrigerated before drying, they should be dried immediately and kept dry until analyzed.

(d) The entire 10 lb sample should be ground to pass a No. 14 sieve, thoroughly blended, and properly subdivided to a 1 kg sample. The entire 1 kg sample should be ground to pass a No. 20 sieve, thoroughly blended, and properly subdivided to 50 g analytical samples (13). When feasible, the samples may be ground and subsampled immediately after collection. The subsamples can then be analyzed immediately, stored under refrigeration, or dried for storage.

(e) Analysis of all samples for mycotoxins should be done by appropriate methods approved by AOAC (13), AAC (15), AOCS (12), or similar organizations. The method for analysis of the samples must be selected in advance and used uniformly throughout the survey. In the case of aflatoxin in corn, a revised minicolumn method that is currently recommended by AOAC for screening purposes specifies the Holaday extraction and cleanup procedure (19) in conjunction with the Velasco minicolumn (9, 20). Where more precise quantitative data are needed, the AOAC CB method is recommended (13) for determination of aflatoxin in corn.

REFERENCES


(12) *Official and Tentative Methods of Analysis of the American Oil Chemists' Society* (1973). AOCS, 508 S Sixth St, Champaign, IL.


