Romer Labs® Guide to Mycotoxins

Vol. 2: Sampling and Sample Preparation for Mycotoxin Analysis

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Romer® test kits enable rapid, easy to use and reliable testing for a range of mycotoxins.

**AgraQuant® ELISA Test Kits**

All below ELISA test kits are available in both 48- and 96-well format. Quantitative results are determined using an ELISA reader.

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**AgraStrip™ Total Aflatoxin**

The newly launched AgraStrip™ Total Aflatoxin is a USDA/GIPSA approved kit that allows rapid on-site screening for total aflatoxin. To meet the needs of different markets, the AgraStrip™ is available at three cut-off levels; 4 ppb, 10 ppb and 20 ppb. The kit is noted for its speed, user-friendliness, performance, compactness and ruggedness. Distinctive results are ready within 5 minutes!

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Sampling and Sample Preparation for Mycotoxin Analysis

www.romerlabs.com
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1. Introduction

Mycotoxins are a chemically diverse group of compounds, which are secondary metabolites of fungi. They are produced by the toxigenic molds that contaminate grains either while still in the field or during their storage following harvest. Mycotoxin contamination and the severity of the mycotoxin problem varies from year to year and from one geographic region to another. However, in the last 20 years, there has been only one or two years where there was not a significant problem with mycotoxins in the United States.

Mycotoxins have been linked to problems in animal husbandry ranging from poor feed conversion, low weight gain, digestive distress, reproductive deficiency, tumor development, immunity deficiency, stagggers or tremorgenic problems and death. In certain areas of the world human diseases are attributed to mycotoxins and continue to occur. Therefore most countries have regulatory levels for the occurrence of mycotoxins in certain commodities and require all grains to be tested for those specific regulated mycotoxins.

One might suspect mold contamination if certain signs or characteristics appear in or on the grain. Such signs may include:

- Low bushel weight (density below 48 pounds per bushel)
- Discolored kernels (pink or black tipped on wheat or corn)
- Tombstone/scabby kernels on wheat
- “Stained” discoloration on barley
- Musty odor
- Excessive amount of broken kernels (above 5% broken corn and foreign material)
- Moisture content above 14%. Although moisture is not an indication of mold contamination, mold growth is possible.

However, it is important to keep in mind that the presence or absence of mold does not correspond to the presence or absence of mycotoxins. Molds do not always produce toxins and molds can be easily destroyed, whereas mycotoxins are hard to destroy. Therefore, the only proven way to determine if grain or foods contain mycotoxins is to test for them.

Testing for mycotoxins consists of three steps:

1. Sampling: several small samples are taken at random from the lot and are composed into one larger “lot sample”.
2. Sample preparation, which consists of:
   - Grinding: the entire lot sample is ground to a fine particle size
   - Subsampling: a representative subsample (the “analytical sample”) is removed for analysis
   - Extraction: the analytical sample is extracted with a solvent1.
3. Analysis: the analytical sample is analyzed and the amount of mycotoxins contained is determined.

As time and money are being spent for the analyses of mycotoxins, the extra time for proper sampling and sample preparation is crucial: the test results are meaningless if the analyzed sample is not representative of the lot. Therefore a well-enforced sampling program is crucial for accurate mycotoxin results.

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1 Depending on the author, extraction is considered part of the sample preparation or part of the analysis. This topic is not included in the present booklet but will be discussed in a later volume of the Romer Labs’ Guides to Mycotoxins.
Sampling must be monitored and proper techniques implemented. Sampling procedures must be written, reviewed and followed by everyone involved. Shortcuts cannot be taken, even when several trucks or railcars are waiting to be sampled.

2. Mycotoxin-specific Challenges

2.1. Overview

A crucial aspect of mycotoxin analysis is its replicability, which means that the same result should be reached again and again when a specific lot is repeatedly analyzed. Only this ensures that the result is accurate and correct. A high replicability means that the variability among results is low. In other words, variability is the amount your test value varies from the true value. Variability is due to the combination of the many steps required to produce a mycotoxin test result.

Although there are sources of variability at each of the 3 steps of mycotoxin testing (sampling, sample preparation and analysis), sampling variability is the largest source of error in determining mycotoxin levels. For example nearly 90% of the error associated with aflatoxin testing can be attributed to sampling.

The high sampling error when testing for mycotoxins is due to two main factors: low concentration of mycotoxins in a given commodity and the unequal distribution in the lot. For example, in a lot of corn, the vast majority of kernels will be free of mycotoxins, with less than 0.1% of kernels, which may be contaminated. However, individual kernels of corn have been found to contain as high as 400,000,000 ppb aflatoxin.

An accurate sample therefore tries to capture an adequate proportion of the kernels with a very high contamination level. If that is not the case, the test results will be “false negatives” or “false positives”; either way resulting in high potential costs.

2.2. Low concentration

Despite extremely high levels of mycotoxins in some kernels, the overall concentration of mycotoxins in a lot of grain is usually very low. The unit of measurement is commonly either:

• “parts per million” (ppm) for such mycotoxins as DON (also called vomitoxin), fumonisin and citrinin; or
• “parts per billion” (ppb) for such mycotoxins as aflatoxin, ochratoxin A and patulin.

To illustrate the meaning of these low levels, some examples of how small these units are:

One part per million (ppm):
- There are about 13,960 kernels of wheat in one pound. One kernel in 71 pounds (32 kg) is equivalent to 1 ppm.
- There are approximately 3,500,000 to 4,000,000 grains of sand per pound. If you take four grains out of the pound, you have removed 1 ppm.

One part per billion (ppb):
- One kernel of corn in 3.5 railcars.
- One ppb of the area of Texas is the size of a basketball court.
- One corn plant in 40,000 acres of corn (A typical farmer would plant some 25,000 corn plants per acre. 40,000 acres of corn would therefore equal 1 billion plants).
2.3. Uneven distribution

Unlike protein or moisture content in corn or wheat, where every kernel tested has a similar level of content (i.e. protein is uniformly distributed), mycotoxin content does not occur in every kernel. In the extreme it may only occur in a few ears or heads in an entire field. This means that some kernels may contain high levels of toxins while others contain no toxin at all, resulting in an uneven distribution of the mycotoxin.

This is due to the fact that molds do not grow evenly throughout a field or a bin of grain, and the toxins produced are deposited in some kernels of grain and not in others. Therefore mycotoxins tend to be concentrated in one spot, a so called “hot spot” or “nugget”, whereas the remainder of the lot is free of toxins (Fig. 1).

The greater the extent of contamination, the more likely that the distribution will be uniform and test results accurate. Conversely, when the overall concentration of a toxin in a “lot” of grain is low, uneven distribution is accentuated.

As stated before, correct analysis means determining the average contamination of the whole lot. If the proper sampling procedures are not followed, it is likely that the analysis results will either:
- under-estimate the true mycotoxin concentration, if only the non-contaminated areas are sampled; or
- over-estimate the true mycotoxin concentration, if the samples are taken from the contaminated nuggets.

2.4. False negatives and false positives

A “false negative” is a mycotoxin test result, which reflects an answer lower than the correct answer. This type of answer is very common in mycotoxin testing, largely due to improper sampling and sample preparation. When too few incremental samples are taken or the total lot sample is too small, it is much more common to “miss” one of the contaminated kernels than to “hit” it. This type of result is also common when the entire sample probed is divided or split prior to grinding because contaminated kernels are “divided out” of the analytical sample.

The number of false negative results obtained depends on:
- Number of probes taken and total sample size: the smaller the total sample taken from a truck or railcar, the less chance there is of obtaining a representative number of contaminated kernels.
- The particle size: if the sample is not ground finely enough before the analytical sample is taken, the true contamination level may be underestimated.

The number of false negatives obtained can range from 5%, which is normal, to about 90% which can result from one pound sample being coarsely ground (e.g. with a blender) and an analytical sample taken from the top of the ground corn.
False negative test results are detrimental as they can cause substantial financial losses, including:

- Expenses for mycotoxin testing are wasted if unreliable results are generated due to poor sampling procedures.
- Fines can be incurred if grain contaminated with more than 20 ppb of aflatoxin is sold across state lines. Additional transportation costs are also incurred when the grain processor rejects the load and returns it to the sellers’ facility. The seller loses credibility and confidence, leading to reduced grain sales.
- A processing plant can be subject to down-time until suitable grain can be purchased, if a false negative grain is retested and the correct value analyzed, making the grain unusable.
- Costly lawsuits may follow if a commodity is processed and detrimental health effects result from the consumption of the food or feed.

On the other hand, a “false positive” is the result of an analysis for mycotoxin which reflects a higher than correct answer. This type of answer is not as common as false negatives because when sampling is done incorrectly it is much easier to “miss” the contaminated kernel than it is to “hit” too many of them.

Still, false positive test results also cause financial losses:

- Good grain is sold at lower prices, since contaminated commodities command a lower price than if uncontaminated.
- Blending or treating good grain incurs unnecessary costs.
- Inaccurate results reflect poorly on the overall testing program and deters potential grain sellers to offer grain for purchase.

3. Sampling Procedures

3.1. Overview

Sampling is defined as the process of removing an appropriate quantity for testing from a larger bulk, in such a way that the proportion and distribution of the factors being tested are the same in both the whole (lot) and the part removed (sample). The sampling process consists of taking a number of small samples (incremental samples) from a lot of feed or ingredient and pooling them into a large aggregate sample.

The importance of proper sampling becomes clear when we realize, for example, that most railcars contain 2,000 to 3,000 bushels (or about 120,000 to 180,000 pounds) and trucks contain approx. 825 bushels (or about 49,500 pounds) of corn, and we ultimately analyze 50 g of ground sample that must represent the entire railcar or truck.

To ensure that the tested sample is representative, proper sampling techniques must be used. A “boot” sample from the exposed layer of grain in a hopper car or truck, or a “bucket” sample as a truck or railcar is unloaded is not representative of the lot as a whole and therefore should never be used. Also, people collecting grain samples can influence how well the sample represents the lot of grain by sampling only a portion of the grain stream. For this reason, the Federal Grain Inspection Service (FGIS) does not allow scoop sampling and hand-grab sampling for official inspections.
Romer Labs® has a durable mill that has been specifically developed for products that are difficult to grind due to high moisture and high oil content such as pet foods, cottonseed, tree nuts or spices.

The Romer Analytical Sampling (RAS®) Mill features interchangeable grinding burrs and power drive screws to allow for the grinding of practically any product. The moving parts are made of stainless steel and flashed chrome for food safety analysis. The mill is very versatile and will easily grind samples for moisture, protein, fat, fiber, pesticide and mycotoxin analyses.

Benefits
- **Easy-to-Clean** – Prevents sample cross-contamination
- **Powerful** – Designed for moist & high oil products
- **Safe** – Generates little heat or dust
- **Durable** – Rugged steel construction for years of use
- **Approved** – CE Approved
The distribution of constituents, such as broken kernels or foreign material, is generally not uniform throughout the load. As grain is loaded into a container (truck, wagon, railcar or storage), constituents of the grain mass stratify and segregate depending on size, density, and shape. During loading fine particles tend to concentrate in the area near the center and larger-sized materials migrate to the outside of the storage container. When unloading, a reverse segregation occurs. This explains why the number of incremental samples and the proper sampling pattern is crucial to ensure that the sample is truly representative of the whole grain mass.

For a sample to be considered representative, it must be:

1. Obtained with appropriate equipment, such as a probe (trier) for stationary grain, a diverter-type mechanical sampler or pelican sampler for moving grain.
2. Obtained using a sampling pattern and procedure designed to collect samples from all areas of the lot.
3. Of appropriate size, which depends on the lot size and the commodity; e.g. a 5 to 10 pound sample of corn and a 3 to 5 pound sample of wheat or barley should be taken from a truck or railcar of grain.
4. Adequately identified and labeled on the bag.
5. Handled in such a way as to maintain representativeness. This means that samples should be stored in a cool and dry place, and submitted in double or triple lined paper bags or breathable cloth bags. Samples should never be shipped in plastic bags as these may promote mold growth if the sample moisture level is above 14%.

3.2. Sampling methods for flowing grain

3.2.1. Manual sampling

The Extension Service of the North Dakota State University of Agriculture and Applied Science (NDSU) issued the following recommendations to obtain a representative sample from the grain stream falling from the endgate of a truck or wagon:

1. Use an appropriate sampling device that will collect grain from the entire grain stream without overflowing. The grain stream may need to be controlled to allow the sampling device to pass through the stream without overflowing.
2. Make sure the sampling container is empty before sampling.
3. Samples should not be taken from the first or the last portions of a load since this grain contains excessive amounts of chaff and other larger material, which accumulates next to the endgate.
4. Samples should be collected from the grain stream where the stream is established, about 12 inches (30 cm) below the endgate.
5. The entire stream must be cut (sampled) with a side to side sweep of the sampling device, cutting the full thickness of the stream – front to back. Hold the sampling device in a horizontal position while passing it through the grain stream to facilitate even filling of the sampling device.
6. Take at least two, preferably more, samples at random intervals. Sampling intervals should be selected so the entire lot of grain is equally represented. For example, three samples taken in the middle of each third of the load; at \( \frac{1}{6} \), \( \frac{1}{2} \) and \( \frac{5}{6} \) unloaded. For official inspection the FGIS requires a minimum of two separate passes of the sampling tool for quantities of less than 500 bushels. Each additional 500 bushels of grain are to be sampled at least once.
A pelican sampler is most commonly used for sampling grain in a falling stream. It is named for its resemblance to the beak of a pelican, and is a leather pouch, approximately 7 inches deep, 2 inches wide and 18 inches long, that is attached to a long pole. A metal band along the edge of the pouch holds it open. The back edge of the frame is higher than the front to help catch more grain and direct it into the pouch, even when the pelican is not perfectly vertical in the grain stream. To obtain a representative sample, the following minimum number of cuts are required for each type of carrier:

- Hopper car: min. 2 cuts per compartment
- Boxcar: min. 4 cuts
- Truck: min. 2 cuts
- Barge/ship: min. 1 cut per 500 bushels

The samples should be taken in the following manner:
1. Make sure the loading spout is positioned so that the pelican will swing easily through the entire falling stream.
2. Hold the pelican so that its pouch is next to the stream.
3. Grasping the pelican firmly, swing it completely through the stream in a continuous motion. Don’t allow it to overflow.
4. Pour the contents of the pelican into a sample container.

If the grain lot is moving on a conveyor belt, an Ellis cup must be used. The Ellis cup is constructed of lightweight aluminum. To draw a representative sample, three cupfuls (one set) must be drawn from the moving grain approximately once each 500 bushels. The minimum number of sets for each type of carrier is the same as the minimum number of cuts when using a pelican sampler (see above).

When using an Ellis cup, the sample must be drawn at a location that will ensure its representativeness: for outbound grain, the samples should be drawn as close as physically possible to the end of the loading spout. For inbound grain, the samples should be drawn before or immediately after the initial elevation of the grain. The three cupfuls making up one set, should be drawn from the center of the grain stream, halfway between the center and the right edge of the stream and halfway between the center and the left edge of the stream.

### 3.2.2. Mechanical sampling

Increasingly, elevators are equipped with mechanical sampling systems. Due to their continuous sampling they are able to draw the most representative sample from lots of grain.

![Fig. 2: Diverter-type sampler installed in spout](image)

The diverter-type (D/T) mechanical system is used for commodities with large particle size such as whole grain. Even though D/T’s vary in design, all operate on the same principle. Installed at the end of a conveyor belt or within a spout, they draw their sample by periodically moving a diverter (often called the “pelican”) through the entire grain stream. The frequency of these “cuts” is regulated by timer controls. After grain enters the primary sample, it flows through a tube into a secondary sampler. The secondary sampler reduces the size of the sample. From the secondary sampler, the sample flows to a sample collection box.

Before using a D/T, it is important to ensure that it’s working properly and not clogged with old grain or dust and that the electric timer is correctly set. If the flow-rate is 4,000 bushels or fewer per hour, the timer should be set so that the diverter crosses the sampling area at least once every 3 minutes. If the flow-rate is faster, there should be one diverter cut for every 200 bushels loaded.
Point-type (P/T) mechanical sampling systems are commonly used for powdered commodities. These commodities are more homogeneous than whole grain and have less particle segregation. They do not use a pelican to completely cut across the stream of commodity through a spout. Instead they use a tube with a hole or slot and an auger delivery system.

3.3. Sampling patterns for stationary grain

A large percentage of grain, as it travels from the farm to the final consumer, is sampled with a probe, also called a trier.

Probes are used for lift top and roll top barges, hopper cars, boxcars, trucks, hopper-bottom containers as well as sacked grains and it is the only sampling method approved by the Grain Inspection, Packers & Stockyards Administration (GIPSA) for stationary lots. Because mold growth usually occurs in spots in the grain lot, the most accurate sampling is done on recently blended lots of grain.

Probes are constructed of brass or aluminum and come in various sizes with standard lengths of 5, 6, 8, 10 and 12 feet. The type of carrier dictates which probe length is used. As a general rule, a probe should be used that will reach the bottom of the container. Probes consist of two tubes, one inside the other. GIPSA approved grain probes are 1 3/8 inches in diameter (outer tube). Depending on the model, the inner tube can be divided into compartments. The outer tube has slots which match the compartment openings of the inner tube. When the tubes are aligned, grain may enter into or be emptied from the compartments of the probe. The latter is used only when the stratification in a lot needs to be examined. As this is not relevant for mycotoxin analysis, emptying usually occurs from the opening in the handle end if a non-compartmentalized probe is used. To combine the individual samples taken with a probe, a sampling canvas is often used. These are usually made of flat duck cloth or similar material.

The lengths of the double-tube compartmentalized probes approved by GIPSA for sampling stationary lots of grain are, depending on the carrier:

- Flat-bed truck/trailer: 5 or 6 feet (11 or 12 compartments)
- Hopper bottom trucks: 6, 8 or 10 feet (12, 16 or 20 compartments)
- Box car: 6 feet (12 compartments)
- Hopper car: 10 or 12 feet (20 compartments)
- Barges and bay boats: 12 feet (20 compartments)
The general rules to obtain a representative sample with a probe are:

1. Lay out the sampling canvas.
2. Insert the probe at a 10-degree angle from the vertical, with the slots facing upwards and completely closed. Keep the slots closed until the probe is inserted as deeply as possible into the grain. If the slots are not kept closed, a disproportionate amount of grain from the top of the lot will fall into the probe’s compartments as it is being inserted. If the grain contains sand or grit, it is permissible to insert the probe with the slots facing downward to avoid “freezing” the probe. After the probe is inserted, turn the slots upward before opening.
3. After the probe is fully inserted (with the slots facing upward), open the slots and move the probe up-and-down in two quick, short motions. When sampling grains, such as oats and barley, additional up-and-down movements may be necessary to fill the probe.
4. Close the slots completely. Then, grasp the probe by the outer tube and withdraw it from the grain.
5. Empty the probe on the canvas.
6. Take the remaining number of probe samples and empty them on the canvas.
7. When transferring the sample from the canvas to the sample bag, take care not to spill any portion of the sample or allow fine material to be blown away.
8. Close the sample bag securely and label the sample. Preferably use a heavy paper bag, never use a plastic bag.

3.3.1. Sampling patterns for large grain carriers

To ensure that all parts of a container are adequately sampled, GIPSA has established a sampling pattern for each type of carrier. Each lot should be probed in as many additional locations as necessary to assure that the sample is the required size and representative of the lot. Additional probes should be drawn in a balanced manner. For example, one compartment of a hopper car should not be probed twice unless the other compartments are also probed twice, regardless of the amount of grain in any one compartment or the amount of additional sample needed.

The following diagrams, reproduced by courtesy of FGIS, indicate the recommended standard sampling patterns. Insert the probe at the points marked, with the tip of the probe angled ten degrees in the direction of the arrow. When two arrows are shown, the tip of the probe may be pointed in either of the indicated directions at the sampler’s discretion.

<table>
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<th>Diagram</th>
<th>Description</th>
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<tr>
<td>Fig. 5: Seven probe pattern</td>
<td>For flat-bottom trucks or trailers containing grain more than four feet (1.2 m) deep (Fig. 5), a seven probe pattern should be implemented. On tandem trucks, each trailer should be treated as a separate load.</td>
</tr>
<tr>
<td>Fig. 6: Nine probe pattern</td>
<td>For flat-bottom trucks or trailers containing grain less than four feet deep, a nine probe pattern should be followed (Fig. 6).</td>
</tr>
<tr>
<td>Fig. 7: Pattern for hopper bottom</td>
<td>For hopper bottom trailers or containers, the sampling pattern is shown in figure 7.</td>
</tr>
<tr>
<td>Fig. 8: Pattern for box car</td>
<td>For box cars, insert the probe at an approximately 10 degree angle in either direction shown in the diagram. The sample from the center of the car may be taken with the slots facing toward either end of the car. The other samples should be drawn approx. 2-4 feet from the side of the car and 3-5 feet from the end of the car.</td>
</tr>
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For 3-compartment, through or door type hopper cars, insert probe in the direction of the arrow at an approximately 10-degree angle. The probe may be inserted either in the center of each hopper or slightly off center in order to miss the cross beam (Fig. 9).

For lift-top and roll-top barges, draw the first probe approx. 4 feet in from the stern end of the barge and approximately 7 feet from the side. Take the next probe approx. 15 feet from the first probe. Proceed to take probes at 15-foot intervals until the bow end of the barge is reached (Fig. 10). The last probe should be taken approx. 4 feet from the opposite end and approximately 7 feet from the side. Sample both sides of the barge in this manner until the entire barge is sampled.

For flat-top barges and fiberglass hatch top barges, draw one probe from each opening in the direction of the arrowhead. Insert the probe in the center of the opening, approx. 7 feet from the side edge (Fig. 11).

### 3.3.2. Sampling sacked grain

When grain is sampled from sacks, a double-tubed, grain probe (minimum 4 feet long) should be used (Fig. 4). If the lot contains more than 10,000 sacks, divide the lot into two or more approximately equal-sized sublots. For each lot or subplot, randomly select 36 sacks for sampling.

Stand each selected sack on end and insert the probe into the top corner of the sack. Move the probe diagonally through the sack until the probe touches the opposite bottom corner (Fig. 12).
3.3.3. Sampling storage bins

The only practical way to obtain a representative sample from a storage bin is during the loading or unloading process. Either automatic sampling equipment or an adequate probe can be used (see sampling for flowing grain). If this is not possible, probed samples need to be taken as described below:

Collect 5 probes of feed or meal, or 9 probes of whole grain according to the sampling pattern shown in figure 13.

Collect about 0.5 pounds from the bottom of the bin using an auger and combine this with the probed sample (if an augured sample is collected, omit one of the probes from the top of the stored material).

If moisture accumulation is suspected, use the probe pattern shown in figure 14 to collect potentially moist material from the edges of the bin separately from potentially less moist material from the center of the bin. Transfer the edges-sample and the center-sample to two separate sample bags, close securely and label appropriately.

Fig. 13: Bin sampling

Fig. 14: Bin sampling if moisture is suspected

3.3.4. Sampling feed troughs

To sample pans in a poultry house, collect twelve 75 g grab samples in a sample bag, four at the first station the feed reaches, four at the middle station and four at the last station the feed reaches.

For feed troughs, collect twelve 75 g grab samples randomly from the trough. Take some samples from each section of the trough.

3.4. Sample size

3.4.1. Influence of sample size

Studies have confirmed that the variability of mycotoxin test results in samples of whole kernel grain is proportionate to the number of kernels being tested. Therefore, with all other things being equal, 1000 kernels of contaminated corn will show the same variability as 1000 kernels of contaminated wheat or barley. This means that the number of kernels sampled is more important than the weight of the sample. Because the kernels of wheat and barley are much smaller than corn, the number of kernels tested can easily be increased and the variability of sample results quickly decreased because more kernels are being tested.

This also means that there is a smaller sampling error associated with processed commodities, such as flour, than is associated with whole seeds. This is due to the smaller particle size, which increases the number of possibly contaminated particles, and the greater degree of mixing associated with the production process.

The importance of adequate sample size for the accuracy of the mycotoxin analysis result is confirmed in the following study. The study, made by Romer® Labs, shows the variability of test results in relation...
Probes and Sampler

16-OHT 72" Brass Probe

22-OH 63" Brass Probe

Pelican Sampler

Ellis Sampler

Photos provided courtesy of Seedburo Equipment Company
to the sample size. The samples were taken from a truck containing corn contaminated with aflatoxin at the 20 ppb level.

<table>
<thead>
<tr>
<th>Sample size probed from truck</th>
<th>Approx. number of kernels</th>
<th>Range(^1) of analysis results</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 pounds (4.5 kg)</td>
<td>30,000</td>
<td>11.6 – 28.4 ppb</td>
</tr>
<tr>
<td>5 pounds (2.2 kg)</td>
<td>15,000</td>
<td>8.1 – 31.9 ppb</td>
</tr>
<tr>
<td>2.5 pounds (1.1 kg)</td>
<td>7,500</td>
<td>3.2 – 38.8 ppb</td>
</tr>
<tr>
<td>1 pound (0.4 kg)</td>
<td>3,000</td>
<td>0 – 46.9 ppb</td>
</tr>
</tbody>
</table>

\(^1\) at the 95% confidence range

Thus if 10 pounds were probed and analyzed repeatedly, the answers would range from 11.6 ppb to 28.4 ppb. This variability increases substantially, when only 2.5 or 1 pound samples are taken. If only 2.5 pounds of whole kernel corn is taken for an aflatoxin analysis it is very possible to detect only 3.2 ppb out of a lot that actually contains 20 ppb. This has nothing to do with the test method, it is simply because the sample probed “missed” the aflatoxin.

By taking a sample that is too small, the toxins are either missed completely, or found at much lower levels than truly present. This is referred to as a “false negative”: e.g. when a one-pound sample with a true contamination level of 20 ppb aflatoxin gives a “non-detect” test result.

### 3.4.2. Sample size for aflatoxin analysis

GIPSA has determined that the optimum sample size for aflatoxin analysis in corn is 10 pounds. GIPSA has not established an optimum aflatoxin sample size for wheat, barley, sorghum and other grains. Currently the 10 pound sample size is used by default for all grains when testing for aflatoxin.

GIPSA established reduced sample sizes for domestic shipments of corn by truck and rail. The smaller sample sizes were adopted in response to industry concerns that the 10 pound sample size would significantly increase the cost of inspection. But it should be noted that using the reduced sample sizes significantly increases the sampling variability. The GIPSA minimum sample sizes are:

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Minimum sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trucks</td>
<td>2 pounds 0.908 kg</td>
</tr>
<tr>
<td>Railcars</td>
<td>3 pounds 1.363 kg</td>
</tr>
<tr>
<td>Sublots / Barges</td>
<td>10 pounds 4.540 kg</td>
</tr>
</tbody>
</table>

Because aflatoxin is usually analyzed at lower concentrations than the other mycotoxins, any sampling and sample preparation scheme that applies to aflatoxin can be applied to other mycotoxins.

### 3.4.3. Sample size for DON (vomitoxin) analysis

The minimum sample size required by GIPSA for DON analysis of barley and wheat is 200 g. Larger samples are preferred. Grains with larger kernel size, such as corn, require larger sample sizes to maintain the same level or accuracy.
3.4.4. **General guidelines**

The following sample sizes can be used as a rough guideline when sampling for mycotoxin analysis:

- 5 pounds (2.3 kg) for whole corn.
- 3 pounds (1.4 kg) for whole wheat, wheat midds, barley, pearled barley, milo and grain of similar size.
- 2 pounds (0.9 kg) for finished feed or meal (soybean, peanut, cottonseed, corn meal, etc.).
- 1 pound (0.5 kg) for pelleted or mashed feed.
- 300 ml for liquids.

3.5. **Sampling of mixed feeds**

When mixed feeds are sampled for mycotoxin analysis, two situations are possible:

1. A mycotoxin was present in one or more of the feed ingredients when the feed was mixed, or
2. A mycotoxin was produced in the feed after it was mixed due to poor storage conditions (14% moisture or more).

In the first case, the mycotoxin is more evenly distributed in the feed than it was in the contaminated ingredient, because the ingredient has been coarsely ground and mixed into the feed. In cases like this, a two-pound sample of feed is sufficient to provide a representative sample of a truck or rail car. In the second case, the mycotoxin is usually less evenly distributed. The feed will first become moldy in the moist areas of the storage bin and the mold will slowly migrate to less moist areas as it grows. A good way to sample feed in this case is to take at least a 2 pound sample from the moist areas of the bin (usually the outer edges and corners) and a 2 pound sample from the center (Fig. 14).

By analyzing the outer samples separately from the inner samples, it is possible to tell (1) if there are any toxins present and (2) if these toxins come from a contaminated ingredient or from poor storage conditions. If toxins are found in the outer samples and not in the center, poor storage conditions are probably responsible for the contamination. If toxins are found in both the composite center and outer samples at similar concentrations, the contamination most likely came from an ingredient.

4. **Sample Preparation**

4.1. **Overview**

The objective of sample preparation is to obtain a small sample of grain or feed to be used in the analysis. It should contain the same concentration of toxin as the large sample from which it is taken.

Sample preparation consists of two steps: grinding the lot sample and taking an analytical sample. For example, a 10 pound corn sample for aflatoxin analysis is ground and a subsample of 500 g obtained. This subsample is then mixed and a 50 g analytical sample obtained.

As with sampling, sample preparation demands considerable time and attention. The importance of proper sample preparation can be exemplified by considering 10 kernels of corn, one of which is contaminated with aflatoxin. If each kernel weighs 1 g and the contaminated kernel contains 200 ppb of aflatoxin, the 10 kernels together therefore contain 20 ppb aflatoxin. A representative sample of these 10 kernels will, by definition, contain 20 ppb aflatoxin. To obtain a 1 g representative sample of these 10 kernels of corn, all 10 of the kernels need to be ground and mixed together so that a 1 g subsample will contain 20 ppb of aflatoxin. Thus, 10% of the corn and 10% of the aflatoxin are present in the 1 g sample.
Therefore the best way to ensure that the analytical sample contains the same concentration of toxin as the large sample from which it was taken is for the analytical sample to contain particles from each kernel of grain in the large sample. This can be done by the methods described below or by using specialized pieces of equipment, combining the grinding and subsampling steps (e.g. the Romer Series II® Mill).

Due to the uneven distribution of the contaminated kernels in a sample, and the fact that it is impossible to evenly divide a sample of corn that has only 1 kernel of contaminated corn out of 200 kernels, it is imperative to grind the entire sample before subsampling. Never split or riffle the sample before grinding. If only a part of the 5-10 pound sample is ground, or a sample is smaller than the recommended size, the analysis will tend to produce false negative results.

Below are two standard methods for sample preparation that illustrate the typical steps involved:

The Association of Official Analytical Chemists (AOAC) method:
1. Grind the total sample collected using a hammer, Whiley or disk mill to pass a #14 mesh sieve.
2. Split the sample using a sample splitter such as a riffle unit until 1-2 kg is obtained.
3. Regrind 1-2 kg to completely pass a #20 sieve.
4. Mix reground portion thoroughly in tumble blender or planetary mixer.
5. Take analytical sample from this mix using sample splits.

Whitaker method:
1. Coarse grind entire sample
2. Mix the entire sample thoroughly
3. Using a riffle divider, collect a 2.5 pound portion
4. Fine grind the 2.5 pound portion
5. Mix the entire sample thoroughly
6. Using a riffle divider, collect a 500 g sample
7. Mix thoroughly and remove 50 g sample for analysis

4.2. Grinding

The complete lot sample needs to be ground to a fine particle size (20 mesh size or 1 mm opening size). The purpose of grinding is threefold:
- To open up contaminated kernels and distribute the particles throughout the sample, thereby increasing the chance of detecting contaminated particles.
- To increase the uniformity of the commodity and therefore allow subsampling with greater precision and accuracy.
- To accelerate the process of chemical reaction or extraction procedure due to the smaller particle size.

Various types of grinders exist to handle different products and the varying degrees of humidity they are subjected to in storage. Some grinders allow the screen size to be changed, others provide removable disc heads and various hammer speeds. Grinders such as the Romer Series II® Mill, Bunn Model G3, Viking Hammermill, Falling Number Mill and UDY grinder are a few examples. It is important to use an adequate piece of equipment. A blender does not grind corn fine enough to obtain a representative analytical sample.

Few mills are available that will grind corn to 20 mesh particle size. With most mills, it is necessary to first grind the total sample coarsely (about 14 mesh) before grinding to 20 mesh. If this is the case, only 2.5 pounds of the coarsely ground corn need to be further ground to 20 mesh.
Care should be taken to always use a clean grinding system. More care must be taken after grinding a “hot” sample than after a “clean” one. Some labs run a “clean” sample between lots for the purpose of cleaning the mill. Other labs run at least 50 g of the next sample and discard it to “purge” the mill. These practices do not always eliminate cross contamination. The best method to avoid cross contamination between successive samples is to completely open up and clean the grinder, e.g. using a vacuum cleaner or suitable chemical solvents.

When grinding a sample for mycotoxin analysis, following guidelines should be followed:

- 75% of particles should pass through a 20 mesh screen
- Grind to the consistency of corn meal or whole wheat flour
- Do not grind to a fine flour or powder (it does not significantly increase extraction efficiency, but might pack).
- The consistency of corn grits, bulgur or semolina is too coarse

### 4.3. Mixing

The Association of American Feed Control Officials (AAFCO) warns that the mixing often included in sample preparation methods can lead to higher analytical result variability if not performed properly. The AAFCO also states that if the subsampling is performed with the appropriate equipment, mixing is not an issue and does not have to be done.

Mixing is often performed in an attempt to “homogenize” the sample. Once the sample is “homogenized”, any increment or grab from the sample is deemed representative without further consideration. But this is not always appropriate for particulate matter, especially for material with different particle sizes and densities, which can segregate with mixing. This is why stirring with a spatula or shaking a container is not recommended for dry, ground material, especially when the jar is full or almost full. The material at the bottom is often not adequately mixed in and the stirring may actually promote segregation.

One type of mixing technique that will work for many materials is the Paul Schatz motion which is a three dimensional motion combining a figure eight movement with rotation (see web site at http://www.wab.ch/e/produkte/turbula/turbula.html).

### 4.4. Subsampling

Once the complete lot sample has been ground, a smaller sample needs to be taken for the actual chemical analysis. It is not advisable to simply “dip” a portion from the sample as this can cause significant sampling variability. Instead, one of two basic methods, splitting or subsampling, should be applied.

The most common techniques for sample splitting is riffle splitting. Riffling is the separation of a free-flowing material into (usually) equal parts by means of a mechanical device composed of diverter chutes. There are two basic types of stationary rifflers: gated and nongated.

- Gated rifflers have a trap door between the hopper and the chutes. After the sample is poured into the hopper, the trap door must be opened to allow the sample to flow through the chutes into the receiving pans.
- A nongated riffler does not have a trap door. The sample is poured over the chutes and immediately falls through the chutes into the receiving pans.

Of the two types of stationary riffling, it is generally accepted that gated riffling provides more accurate splits as it is more forgiving of operator error.
The other splitting technique is called rotary riffling. This is a motorized mechanical device in which the sample is split into hundreds of increments. Because of the large number of increments, this device provides the best splits possible.

Samples can also be split using a divider. The purpose of a divider is to reduce the size of a grain sample while maintaining the representativeness of the original sample. Particularly, if the grain sample contains foreign substances of different specific gravity or size than of the grain with which they are mixed, a mechanical device, such as a Boerner divider, is necessary. There are several approved types of dividers that maintain the uniformity of the sample while reducing the sample size:

- Cargo model (originally a modified Boerner): used to reduce large (larger than 2.5 kg) samples down to proper size for Boerner Divider
- Boerner Laboratory Model: used to reduce 2.5 kg grain samples to “file” and “work” samples
- Gamet Laboratory 3- and 4-way models

With subsampling, anywhere from a single increment to as many as several hundred increments are selected, usually at random, from the primary sample to form the subsample. Unfortunately, it is a common practice to take only very few increments (in some cases only one). If only a small number of increments are selected, there can be a very large subsampling error due to segregation. This is why rotary splitters achieve very low sample splitting errors (hundreds of increments) and why coning and quartering achieves such large sample splitting errors (only two increments). When determining how many increments to collect, remember that more is always better, e.g. three increments are better than one.

4.5. Romer Series II® Mill

The sample preparation process as recommended by the AOAC takes about 25-30 minutes per sample and requires 3 pieces of equipment (mill, riffler and mixer) each of which needs to be cleaned after each sample to prevent cross contamination. The method is also cumbersome since few mills are available that will grind corn to 20 mesh particle size. With most mills, it is necessary to first grind coarsely before grinding to 20 mesh.

The Romer Series II® Mill simultaneously grinds and splits a sample of grain, food or feed, and provides a much simpler and more efficient alternative. Its key features are:

- Corn is ground to 20 mesh particle size in one step.
- 5 pounds of corn are ground and subsampled in 2.5 minutes.
- A representative analytical sample is split off at the same time as the sample is ground, combining the two sample preparation steps and reducing the equipment needed.
- Each individual kernel of grain or coarse particle of feed or food is ground and split among the 3 chutes ensuring a homogenous and representative sample.
- Three chutes are available for sample collection (one for the analytical testing, one for file sample, and one for an extra sample, e.g. for confirmation testing).
- The collection chute contains a restrictor door, which can be adjusted to vary the amount of ground sample allowed into the chute. By adjusting the restrictor door, 0.1 to 50% of the ground commodity can be segregated.
- The time required to clean the mill between samples is only 30 seconds.

Even with this simplicity, the Romer Series II® Mill provides a rigorously prepared analytical sample – one as “representative” as that obtained with the longer AOAC method.
Romer Labs® has a durable mill that subsamples each kernel or coarse feed particle as it is ground. This is the only mill available that combines these two steps into one patented labor saving piece of equipment. The simultaneous process ensures proper sample preparation which is essential to obtain accurate test results.

The Romer Series II® Mill is a long-lasting powerful piece of equipment that was developed specifically for mycotoxin analysis. It can also be used for preparing samples prior to analysis of moisture, proteins, fat, fiber and pesticides.

Benefits

- Consistent – Representative sub-sampling
- Easy-to-Clean – Prevents sample cross-contamination
- Efficient – Grinds 1-2 lbs. per minute
- Flexible – Suitable for grains & small pellet feeds
- Safe – Generates little heat or dust
- Durable – Rugged steel construction for years of use
- Referenced – USDA/GIPSA
With the Romer Series II® Mill, the sample preparation steps can be reduced to:
1. The complete lot sample is ground and a 100-300 g subsample is collected from the front chute of the Romer Series II® Mill.
2. The subsample is mixed thoroughly for approx. 15 seconds.
3. A 50 g analytical sample is taken for mycotoxin analysis.

A detailed description of sample preparation using the Romer Series II® Mill is included in Chapter 4.5 of the “Aflatoxin Handbook” published by the FGIS.

5. Regulations

5.1. In the United States
In the United States both the Food and Drug Administration (FDA) as well as the U.S. Department of Agriculture (USDA) are involved in mycotoxin-related regulations.

The FDA monitors the mycotoxin content in various commodities, but action levels have only been defined for aflatoxin. Guidelines for sampling and sample preparation refer to this mycotoxin. But since aflatoxin is found at a much lower level than other toxins, procedures defined for aflatoxin will also apply to other mycotoxins.

The FDA has published an Investigations Operations Manual (IOM). Its fourth chapter covers sampling, with subchapter 450 dedicated to “Sampling: preparation, handling, shipping”. As part of its Sample Schedule, the IOM also includes a chart on “Mycotoxin Sample Sizes” (chart 6), which defines the number of incremental sample units to be collected from as many random sites in the lot as possible, their size and the resulting total sample size. These sample sizes for mycotoxin analysis are defined for specific products. The IOM is available at the following website: www.fda.gov/ora/inspect_ref/iom

The USDA’s Federal Grain Inspection Service (FGIS) provides the U.S. grain market with federal quality standards and a uniform system for applying them. Accordingly, it has published the “Aflatoxin Handbook”, in which it describes sampling and testing procedures for aflatoxin. The sample sizes are laid out in chapter 4.2. of the handbook. The samples should be obtained according to the guidelines in the Grain Inspection Handbook, Book I “Grain Sampling”. A minimum 10-pound (approx. 4.5 kg) sample is required for testing of official samples and is recommended for submitted samples. As discussed in chapter 3.4.2. of the present Romer Guide, reduced sample sizes can also be used.

The sample preparation procedures have been published in the FGIS Program Bulletin 93.1 “Aflatoxin Sampling and Reconditioning Procedures”:
1. Grind the entire corn sample obtained for aflatoxin testing
2. Prepare three 500 g subportions from the ground sample:
   - Test portion: used by the original inspection service
   - File portion: used by the review inspection service
   - FDA portion: needs to be retained for FDA analysis if the results exceed 20 ppb (for human food).

5.2. In the European Union
The European Union has set maximum levels for aflatoxin B₁, B₂, G₁, G₂ and M₁ in foodstuffs (Commission Regulation 1525/98) and has published the sampling methods in its Directive 98/53/EC
dated July 16, 1998. This directive has been published in the Official Journal of the European Communities L 201/93. In 2004 this regulation was amended by the Directive 2004/43/EC dated April 2004 adding methods of analysis for the official control of the levels of aflatoxin and ochratoxin A in food for infants and young children.

Annex I of Directive 98/53/EC defines the methods of sampling for certain foodstuffs. Specific provisions are defined when sampling groundnuts, nuts, dried fruit, cereals, milk, derived products and compound foods.

For example, for cereal lots under 50 tonnes, depending on the lot weight, 10 to 100 incremental samples of 100 g each must be taken, resulting in an aggregate sample of 1 to 10 kg.

For cereal lots between 50 and 300 tonnes, the lot must be divided into sublots of 100 tonnes and 100 incremental samples, of about 300 g each, must be taken resulting in an aggregate sample of 30 kg. This aggregate sample must be mixed and divided into three equal subsamples of 10 kg before grinding.

Annex II of the directive defines the sample preparation and criteria for methods of analysis used in official checking of the levels of aflatoxins. As far as sample preparation is concerned, it only states to “finely grind and mix thoroughly using a process that has been demonstrated to achieve complete homogenization”. The replicate samples for enforcement, trade and referee purposes are to be taken from the homogenized material.

The full text of the directive can be ordered from the European Union’s Publisher at the EU web site: http://publications.eu.int/index_en.html
6. Further Reading

More detailed information on the issues addressed in this guide can be found in the following publications:

AAFCO (2000). Guidelines for Preparing Laboratory Samples. Prepared by: Laboratory Methods and Services Committee, Sample Preparation Working Group of the Association of American Feed Control Officials Inc. It covers sample preparation, quality control procedures and guidelines (SOPs) for the preparation of a wide variety of feed samples.


USDA (1999) Grain Fungal Diseases & Mycotoxin Reference. The publication gives an overview of fungal diseases of plants, the most common mycotoxins and their effects, sampling methods and patterns, as well as the GIPSA approved test kits for mycotoxin analysis. It is available in PDF format from the information/publications section of the website: www.usda.gov/gipsa

USDA / GIPSA / FGIS (1992) Aflatoxin Handbook. This handbook gives information on laboratory practices, procedures for sampling and sample processing, as well as procedures to extract aflatoxin, and describes various tests for aflatoxin. Although the handbook was published in 1992, later updates are available.

USDA / GIPSA / FGIS (1995) Grain Inspection Handbook - Book I: Grain Sampling. This handbook sets forth the policies and procedures for sampling grain in accordance with the regulations under the United States Grain Standards Act. It is available in PDF format from the information/publications section of the website: www.usda.gov/gipsa

USDA / GIPSA / FGIS (1995) Mechanical Sampling Systems Handbook. This handbook sets forth the policies and procedures regarding the equipment requirements, installation, authorization, examination and testing of mechanical sampling systems. It is available in PDF format from the information/publications section of the website: www.usda.gov/gipsa
Reference Standards

Biopure, an affiliate of Romer Labs®, offers a wide variety of reference standards for mycotoxin analysis. Biopure assures the high quality and purity of mycotoxin standards offered through in-house production, synthesis, purification and analysis with ‘state-of-the-art’ equipment. The reference standards are available in both liquid, for ease and readiness of use, as well as in crystalline forms. Biopure offers also Matrix Reference Materials (MRM) for DON in maize and will continue to extend this product line.

Mycotoxin Analytical Service

For over 20 years, Romer Labs® has proven to be a fast, accurate, and reliable partner in mycotoxin analysis. We provide analytical service for mycotoxins globally through our offices in the USA, Austria and Singapore. Our analytical program covers more than 20 different mycotoxins and employs the latest technologies available (HPLC, LC-MS, GC-MS).
Proper sample preparation is key to assure quality in mycotoxin testing to obtain accurate, consistent and reliable results. Romer® Mills are specially designed to meet this criterion.

**Romer® Analytical Sampling (RAS®) Mill** is specifically developed for products that are difficult to grind due to high moisture and/or high oil content. This patented design grinds and splits samples using one chute with a divider, thus providing two representative, homogenous sub-samples for further analysis.

**Series II® Mill** is a patented, USDA-GIPSA approved mill that grinds and splits each kernel among three chutes, providing a representative, homogenous sub-sample for mycotoxin testing. Like the RAS® mill, it grinds and sub-samples at the same time in a single-step.

### Clean-up Columns

A good sample clean-up is another important criterion for accurate and reliable results in mycotoxin analysis. Romer offers a selection of clean-up columns in different formats:

**MycoSep®/MultiSep®**
- 1-step, rapid clean up
- Available in 2 formats
- Stability – 18 months
- Recoveries >90%
- Part of AOAC and CEN methods
- Unique Principle – Interferences are retained, analyte passes through the column

**StarLine™ Immuno-Affinity Column (IAC)**
- 3-step clean up
- For a wide variety of commodities and foodstuffs
- Compliant with EC commission directive 2002/26/EC
- Stability – 18 months
- Recoveries >95%
- Repeatability <5%
- Different extraction solutions can be used
**History of Romer Labs®**

1982  **Tom & Marie Romer** founded Romer Labs® in Washington, MO, as a Testing Service for mycotoxins.

1992  **Romer Labs®** moved to its current location in Union, MO Industrial Park.

1995  **The first test kit** was developed.

1999  **Erber AG from Austria** acquired Romer Labs and Tom Romer retired.

2002  **Romer Labs® Group** was conceptualized resulting in the establishment of Regional Business Units in the USA, Austria and Singapore.

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**Worldwide expertise**

**in**

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