

# Featured expert of the month...

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## **Mycotoxin history**

It shouldn't be a surprise that mycotoxins have become such a concern since the 1960s and the discovery of aflatoxins, but examples of their impact throughout human history have shown that they have been a threat since the very beginnings of organized agricultural production [1]. For example, some references to ergotism in the Old Testament [2] suggest that fusarium toxins, such as T-2 toxin and zearalenone, could be responsible for the decline of the Etruscan civilization [3] and the Athenian crisis, which occurred in the Fifth Century B.C. [4]. Mycotoxin control

For the purpose of raising awareness and based on the increased amount of recent work focusing on strategies to provide mycotoxin control at different levels of the food and feed chains, it's important to realize that mycotoxins represent an unavoidable risk. What is needed are analytical tools that can work as a selective "surveillance radar" and determine the need for the material that is at risk to be removed. Determining mycotoxin presence is the first step to create adequate protective methods and this requires the improvement of sampling methods and mycotoxin measuring.

Because of the detrimental effects produced by mycotoxins in food and feed grains, the level of some mycotoxins have been strictly regulated in food and feed samples, ranging from  $\mu\text{g}/\text{kg}$  to  $\text{mg}/\text{kg}$  for the majority of them. The main challenge in developing analytical procedures is the diversity of the mycotoxin molecules – around 500 currently identified – with each one exhibiting different toxicity and economic impacts. In addition, mycotoxin contamination depends on environmental conditions that favor the growth of mold and trigger mycotoxin synthesis. For these reasons, suitable techniques need to be implemented to allow the mycotoxin determination from multiple matrices, which can in turn also impact toxin extraction and may result in masked mycotoxins [5,6].

The high chemical diversity of the fungal metabolites can result in a multitude of chemical structures that can be expected from a sample extract thus rendering their search difficult and prone to errors.

## **Testing for mycotoxins**

The first food and feed evaluation tool would be the observation of signs of mold contamination either visually or via a mold count analysis. However, these evaluations poorly correlate to mycotoxin presence leaving us with mycotoxin analysis (chemotaxonomy) as a tool for high throughput screenings based on mold identification. Still, the complexity of this approach presents a challenge due to the variation of the pattern of mycotoxin synthesis in the environment. There are also more complex techniques that can be used to indirectly recognize toxigenic vs. atoxigenic strains of fungi by detecting volatile metabolites. This is generally done through the use of gas-chromatography combined with mass spectrometry detection [7] or most recently with the development of an electronic nose [8].



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The correct evaluation of mycotoxin levels is strictly linked to the right selection of representative samples, which can only be accomplished by collecting more and larger sample sizes. It has been estimated that the correct sample size could be between 1.25 to 10.0 kg according to the feed matrix, and 50 to 100 samples would need to be subsequently homogenized into sub-samples of 62.5 to 500 g.

The sample size should be homogeneous between samples with variation in weight of not more than  $\pm 5\%$  [9]. No matter what, a portion of the good lots will be eventually rejected by the sampling plan and, conversely, a portion of bad lots will be accepted by the sampling plan (seller's/buyer's risk or false negatives). The magnitude of these risks is directly related to the magnitude of the variability associated with the mycotoxin test procedure. This approach is even more complicated because of the differences in regulation among trading countries. The errors associated with sampling can be as high as 80% with 10% for sub-sampling and error during sample preparation analysis at less than 10% [10].

To be efficient and to prevent errors in the detection of mycotoxins, one should refer to quality assurance principles. The analysis should then be performed with a degree of uncertainty. Certain sampling techniques, if carried out improperly, will affect the sampling and will further induce dramatic errors in the final analysis. Training of personnel seems, therefore, mandatory to prevent side-effects related to the available sampling and sub-sampling techniques depending on the commodity. Then, the pre-treatment step of the samples should prevent inaccuracies through the use of proper instruments and tools. Finally, the analytical procedure must be performed by a sufficiently trained technician. It's important that tests use the appropriate and certified reference materials together with a validated method of analysis to guarantee final results of good quality [11].

On one hand, rapid and sensitive detection using screening tests that are cost-effective and easy to use are available to anyone in the field. The drawback is the lack of selectivity, the cross-reactivity [12] and fluctuation of the response according to variation of environmental conditions when applied in field situations (pH, temperature, matrix effects and competition, etc) [13]. They further require confirmation with a more sophisticated method that is able to show the precise speciation and quantification of the mycotoxins.

## Sensors and Biosensors

As screening tools, the immunoassay-based method uses antibodies specifically raised to detect mycotoxins. This high specificity is related to the homology between the cavity or active-site offered by the antibodies (the epitope) for a substrate (the mycotoxin). This approach suffers from the cross-reactivity evoked earlier because of the presence of analogs in the matrix. Those semi-quantitative or quantitative tests can be of different natures: radioactive in the case of radioactive immunoassays (RIA); fluorogenic in fluorescent immunoassays (FIA); or chromogenic in the case of enzyme immunoassays (EIA, ELISA).

ELISA is commonly chosen as part of mycotoxin control plans due to its speed and the significant number of samples that can be analyzed enabling semi-quantitation or a yes/no response to certain concentration levels and ranges. Other strategies are using lateral flow devices such as immunostrips, immunodipsticks, and immunofiltration with immobilized antibodies on their surface. Sensors and biosensors are also available using antibodies, enzymes, bacteria, receptors, DNA, or transducing a signal optically or electrochemically detectable (using surface plasmon resonance, infrared spectroscopy). These techniques are able to rapidly test grains for the main groups of regulated mycotoxins within reasonable detection limits in the range of 0.5 to 20  $\mu\text{g}/\text{kg}$ , but need to be used with caution again due to cross-reactivity and false-positives.



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As a screening tool, the thin-layer chromatography (TLC) can be applied in the same way ELISA is, with better repeatability and less cross-reactivity, but that requires further sample clean-up and a consequent increase in the amount of time needed to obtain a precise ratio. New emerging approaches are focusing on the use of genomics and transcriptogenomics as tools for mycotoxin analysis. These technologies are using the ability of living cells to respond to the presence of chemicals by leaving specific fingerprints in the form of gene expression, according to their type. They could turn into new screening tools with the development of DNA microchips that will evaluate the gene expression profile of specific cells when in the presence of mycotoxins extracted from feed/food material [14].

## HPLC

Secondarily, and to deal with the broad range of chemical structures, most other methods are based on liquid chromatography (HPLC). High-performance liquid chromatography (or high pressure liquid chromatography) is used to separate, identify, and quantify compounds according to their chemical properties. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. HPLC analyses still represent the “gold standard” in the detection of mycotoxins because of its reliability and the low limits of detection and quantitation achievable. However, although definitive, such methods involve complicated, time-consuming extraction and cleanup settings and a consequent rise in costs.

Even if prior clean-up and concentration procedures allow for decreased limits of detection, their limitation lies in the specific extraction, detection procedures, and conditions of analysis that are generally related to one particular mycotoxin (e.g., DON, T-2 toxin) or, to a family of mycotoxins (e.g., aflatoxins). The ability to detect multiple mycotoxins is thus quite limited and imposes a biased decision on which mycotoxin to analyze for based on assumptions. Depending on the number of toxins investigated and the limit of detection desired, obtaining fast results could be unrealistic. One could also notice that a non-detectable (ND) result does not necessarily mean non-measurable, but is generally based on the specific limit of the regulation in the region where the non-pathological incidence was observed (No-Observable Effect Level/ Lowest-Observable Effect Level - NOEL/LOEL), if available.

## What is in the pipeline?

The analytical detection of mycotoxins can be approached from different angles using different separation methods such as gas or liquid chromatography: electrophoresis. New technologies are now proposing ultra-fast elution of the analytes through a capillary based system, diminishing the length of the HPLC analytical run. Then, a multitude of connective detectors can be chosen for the determination of mycotoxins. Fluorometric and Diode array detectors are the most popular and considered methods of reference according to the volume of literature available in the official AOAC database. Some of the things to consider about these methods are: accessibility to the instrument; high throughput for quantitative analysis; and low interaction with the matrix. The main limitation of this method is in the need to use specific chromatographic separation conditions in relation to the analyte being investigated, provided the molecule fluoresces, otherwise it further requires derivatization of the sample. Other challenges lie in the recovery levels, the stability of the derivatives, and the overlapping of eluted compounds. Thus, a multi-componential approach for rapid detection of multiple analytes suited for various matrices is required.



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The 2-dimensional mass spectrometry (MS), hyphenated to separative chromatography, is becoming the new method of references for mycotoxins analysis. This approach allows the speciation through atomic mass of all the elements present in a sample, and a clear mycotoxin assignment after specific fragmentation pattern experiments. While many components have the same intact mass value, adding another dimension through fragment (tandem MS) enables the specific fingerprinting of each analyte sought. Quantification is enabled by specifically monitoring the multiple fragment ion(s) over the chromatographic elution time. Analyses are ultimately increasing in sensitivity and precision [17]. The addition of a chromatographic step enables the time-dependant elution of all the species, further proposing multi-component detection of mycotoxins within a sample.

Limiting factors are found in the suppression of ionization efficacy due to the matrix and the huge diversity of mycotoxins that can be found, in addition to the lack of any calibrant. Recent work enabled the detection and de-replication of near 500 different fungal metabolites, albeit providing only qualitative results [18]. Another interesting aspect of the LC-MS approach is the possibility to work directly with raw extracts usually obtained after solvent extraction of the sample and basic filtering, which enable a significant speed-up of the analysis.

Other technologies are also available, but probably less popular than the previously exposed techniques. Capillary electrophoresis coupled to fluorescent and UV-detectors can be used in the same way as HPLC. Ion mobility spectrometry and waveform ion mobility spectrometry – mass spectrometry are other methods available for trace analysis of mycotoxins [15].

The mass spectrometry approach, due to newly developed interfaces and the miniaturization of their components, is becoming available as bench-top device in almost every analytical laboratory. Due to its multi-detection capabilities, quality, speed, reliability, and cost, this tool is also becoming available in the field as part of the analytical tools directly available to feed and food manufacturers. Finally, the rapid application of this technology to an infinite number of other contaminants (e.g., pesticides, melamine...) or essential component analyses makes it extremely versatile.

## Conclusion

The complexity of mycotoxin detection can be easily noticed by the numerous approaches that are used more or less effectively to analyze their presence, acknowledged by the number of techniques available. The pitfalls in their use and application are also broad due to the inherent properties of mycotoxins, their chemical diversity, and their metabolization into other molecular species. The complexity of their interaction with matrices represents another limitation. In this respect, mycotoxins can be found as conjugated metabolite to one or several glycosides or glucuronides residues, masking the mycotoxin from being detected when even using an analytical procedure. Constant evolution of the analytical devices and the extraction procedures will help mitigate the errors, as well as to better understand, predict and spot the mycotoxin occurrence in the field.



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