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NUTRITION: *Invited Review*

Invited Review: Use of technology to assess and monitor multimycotoxin and emerging mycotoxin challenges in feedstuffs*

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ABSTRACT

Purpose: Published literature on available methods of mycotoxin analysis was reviewed to create a reference for implementing mycotoxin management programs.

Sources: The information in this review came from scientific journals and published books.

Synthesis: Mycotoxins are nonliving, invisible, toxic secondary metabolites produced by a variety of fungal species found around the world. To determine mycotoxin type, occurrence, and risk level, laboratory analysis must be completed. Mycotoxin analysis can be conducted with a variety of methods including the more simplistic ELISA to the more advanced liquid chromatography-tandem mass spectrometry. The use of analytical technologies that detect mycotoxin type and level also allow for the development of large databases of information, in turn providing information back to producers to help them make better decisions about the quality and safety of feedstuffs.

Conclusions and Applications: Testing feedstuffs and feeds for mycotoxins is critical for understanding local and global prevalence and risk. Many technologies are available that provide rapid and accurate quantification of numerous mycotoxins in feedstuffs and complete rations. Mycotoxin testing programs that monitor mycotoxin prevalence can be valuable for reducing the effect of mycotoxins on animal performance and health around the world.

Key words: analytical, feed quality, monitoring, multimycotoxin assays, mycotoxins

INTRODUCTION

Quality and safety of agricultural commodities are concerns worldwide and are challenged by the frequent occurrence of mycotoxins in feedstuffs. The presence of mycotoxins can affect crop quality, human health, and animal production, which in turn can influence global economies (D'Mello et al., 1999; Bueno et al., 2015). Mycotoxins (from Greek *mykes*, "fungus," and *toxikon*, "poison") are nonliving, invisible, secondary metabolites produced by some fungal species. Secondary metabolites are chemical compounds considered to be unessential for short-term fungal development but important for long-term survival (Geisen et al., 2017). A variety of fungal organisms can produce mycotoxins, most notably those in the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps*, and *Alternaria*. One mold species may produce several different mycotoxins, and several mold species can produce the same mycotoxin. Mycotoxin production is often dependent on physical and environmental conditions such as moisture, temperature, and oxygen presence but also may be influenced by biological factors, such as insect damage and plant variety, or chemical factors, such as pesticide or fungicide exposure (Tola and Kebede, 2016).

The most common route of mycotoxin exposure is through the consumption of contaminated feeds, making crop quality a critical factor in the development of mycotoxicosis in animals. Due to significant effects of mycotoxins on the agricultural industry, programs that monitor mycotoxins and assess risk are necessary. Although mycotoxin management encompasses many steps, an important first step for ensuring feed safety is the use of sound analytical methods that detect mycotoxin type and concentration. There are many methods available for analysis of mycotoxins, ranging from ELISA to liquid chromatography-tandem mass spectrometry (**LC-MS/MS**), the latter of which is becoming more and more popular due to the ability of this technique to report sensitive and simultaneous information on multiple mycotoxins (Jackson et al., 2012; Shephard et al., 2013; Berthiller et al., 2017). The aims of this review are therefore to provide a summary of available techniques relevant to the agricultural industry for mycotoxin analysis, as well as provide results on mycotoxin occurrence worldwide based on data obtained from these technologies. This summary of mycotoxin contamination in feedstuffs is important for producers wanting to better manage mycotoxins and improve safety.

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SAMPLING FOR MYCOTOXINS

Detection of mycotoxin occurrence and concentration in foods and feeds destined for human and animal consumption is important. However, before analyzing samples for mycotoxins, proper sampling techniques must be put in place to obtain representative results. Regarding regulatory and quality assurance activities, the correct decision about a lot can only be made if the mycotoxin concentration can be determined with accuracy and precision (Whitaker et al., 2005). It is often the case that the mycotoxin content of the lot is estimated by completing analysis on only a small sample of the total. If the sample does not accurately represent the lot, then that feedstuff or feed may be classified incorrectly, resulting in either economic loss for the producer or health consequences for the animal.

Limiting the inherent variability of a collected sample is critical for obtaining accurate evaluation of the mycotoxin occurrence, but mycotoxins are rarely uniformly distributed throughout a lot. It is likely that within one lot, some subsamples may contain a high concentration of mycotoxins and other subsamples may contain little or no mycotoxins (Miraglia et al., 2005). Obtaining a proper sample that accounts for this uneven mycotoxin distribution is necessary to reduce sample variance. Thus, sampling needs to be completed randomly with collection of incremental samples throughout the lot.

It is estimated that sampling variance may account for over 75% of the total variability in mycotoxin analysis of a contaminated commodity, whereas sample preparation may account for about 16% of the variance and analytical determination for only about 8% of the variance (Miraglia et al., 2005). To minimize the variance from a sample, sampling procedures should include methods for collecting multiple small subsamples taken from many different locations that are evenly distributed throughout the lot (Whitaker et al., 2005). The correct number of subsamples to collect can be based on whether the commodity is static or moving, as well as the matrix type (Miraglia et al., 2005). The European Commission states that for lots of cereal products less than 50 t, 10 to 100 incremental samples should be collected based on the lot weight (European Commission, 2006). Total weight of the final composite sample should be between 1 and 10 kg. For lots greater than 50 t, a minimum of 100 subsamples should be taken incrementally from the lot, with a final aggregate weight of 10 kg. In the case of closed system lots, such as those on a ship or in silos, it may be acceptable to sample only a portion of that commodity, but the quantity sampled should be at least 10% of the lot.

When collecting subsamples, the most effective method is to follow an automatic sampling procedure that takes multiple small portions at period intervals from a moving stream that are combined into a final sample (Davis et al., 1980). The moving stream of grains should be sampled frequently at allotted intervals throughout the entire time the lot is moving, collecting small amounts at each point to avoid accumulating too large a sample. Research conducted by Mallmann et al. (2014) showed that automatic sampling can significantly reduce sampling variance by 5.6 times and total variance by 3.9 times. Using this automatic technique reduces variability between samples because it is applied to a moving lot with continuous collection. Although not as ideal, probe sampling can be used for stationary lots that cannot be moving when sampled. Probe sampling may be best used for recently blended lots, such as from harvesting or feed manufacture. Procedures for collecting probe samples from carriers and bags are provided in the USDA *Grain Inspection Handbook* (USDA-GIPSA, 2013).

Incrementally collected subsamples will likely need to be further blended and subdivided to reach a final sample size desired for mycotoxin analysis. The final smallest sample to be used for analysis is the test sample (Rahmani et al., 2009). To reduce sample size, the entire collection of incremental samples should be ground, blended, and properly divided using an approved mill such as a USDA Agricultural Marketing Service mill or vertical cutter mixer (Whitaker, 2003). This process is designed to minimize variation as the sample is reduced from kilogram weight to gram weight that will be analyzed for mycotoxins. The discussed proper sampling techniques must be used for all official controls; however, all individuals looking to gain knowledge of mycotoxin contamination of a commodity or finished feed should aim to follow these steps as much as possible.

TOOLS TO ASSESS MYCOTOXIN PRESENCE

Mycotoxins are stable chemical compounds that once formed are generally unaffected by harvesting, storage, and processing of feedstuffs (Turner et al., 2009). Mold can continue to produce mycotoxins from the time the host plant is growing in the field to the time that food or feed is consumed. Furthermore, mold and mycotoxin distributions are not uniform within a feedstuff or feed, which can result in areas of lower mycotoxin prevalence and hot spot areas, or localized regions of high mold growth and mycotoxin concentrations that may be the result of moisture condensation, insects, or microbial growth (Nesic et al., 2015).

Following accurate sample collection, the first step in assessing risk of contamination in a commodity should be the use of laboratory techniques that accurately determine mycotoxin concentrations. Historically, mycotoxin detection has been completed on only a limited number of key crops, for a handful of mycotoxins, with differing analytical methods and extraction techniques (i.e., solid-phase extraction, immunoaffinity columns), each developed specifically for a mycotoxin subgroup. More recently however,

laboratory techniques and instrumentation have advanced, and access to mycotoxin standards have dramatically improved, including the availability of stable isotope standards. Additionally, new technologies and methodologies have been developed that allow for sensitive, selective, and accurate multiplex analysis of hundreds of mycotoxins at once in numerous feedstuffs as well as extending its applicability to complex diet composition (i.e., complete rations). The information gained from the analytical results can help producers to minimize mycotoxins entering the diet, therefore helping to reduce risk to animal health and performance.

ELISA

ELISA technology has been available for several decades and is one of the more frequently used methods for detection of a limited number of mycotoxins. This process can be used for screening as a semiquantitative or quantitative approach for the detection of controlled mycotoxins in specific ingredients (Zheng et al., 2006; Bueno et al., 2015). The basic process of ELISA includes a competitive assay format that uses antibodies, enzymes, and a target molecule (i.e., the mycotoxin). After the mycotoxin is extracted from the feedstuff matrix using extraction reagents, either solvent based or aqueous based, the sample extract and an enzyme-mycotoxin conjugate are mixed and exposed to antibody-coated wells or strips. The enzyme-mycotoxin conjugate then competes with the mycotoxins from the sample for a limited number of antibody binding sites. After washing, another enzyme substrate is added that forms a color reaction. The greater the amount of mycotoxin present in the sample, the lower the binding of the enzyme-mycotoxin conjugate with the antibody and the less signal that is generated. Comparing the known optical density of a standard with that of the test sample then provides an interpretation of the concentration of mycotoxin present in the sample.

ELISA technologies are used in multiple different types of mycotoxin assessment techniques. Some forms of ELISA must be used in strictly a laboratory setting, such as if using microtiter plates, whereas other forms of ELISA technology can be used directly at the feed mill or on farm. Of these techniques, lateral flow tests may provide the highest level of portability because all reagents are incorporated into the lateral flow dipstick (Bueno et al., 2015). In this method, a sample extract is added to a sample pad, and any mycotoxin present binds to the antimycotoxin antibody gold particle complex in the conjugate pad (Zheng et al., 2006). Once in contact, the solutions move up the immunochromatography test strip by capillary migration, and they reach a test zone that contains a mycotoxin protein conjugate. This mycotoxin protein conjugate can capture any remaining free antimycotoxin antibody gold particles that were not originally bound with the sample mycotoxin to form a visible line at the test zone. A control zone is always included that indicates the validity of the test performed. Once the sample has incubated with the test strip for the allotted amount of time recommended by the manufacturer, the results are then determined with the use of a reader device. These machines vary by company but can range from a stationary device to an application on a mobile phone. Results using lateral flow tests can generally be obtained in about 10 min, making them a quick option for mycotoxin detection. Commercially available ELISA kits have been developed for several of the key mycotoxins that can affect the agricultural industry, including aflatoxins, ochratoxins, deoxynivalenol (**DON**), zearalenone, T-2/HT-2 toxins, and fumonisins (Bueno et al., 2015).

Most commercial ELISA kits for mycotoxin detection provide quantitative or semiquantitative results depending on factors such as the manufacturer, the mycotoxin being tested, or the feedstuff analyzed. Generally, ELISA kits provide accurate and reproducible results, although there may be some loss of sensitivity due to the reduced incubation time that is characteristic of most rapid test kits (Zheng et al., 2006). Additionally, there can be matrix effect challenges due to the interaction between the components of the feedstuff and the test components (Li et al., 2014). Furthermore, these tests are designed only for feedstuffs and not complete rations, limiting their use in the field.

Another challenge with ELISA systems is that when mycotoxin structures are similar, these antibody-based techniques are prone to cross-reactivity (Tangni et al., 2010). Furthermore, the amount of cross-reactivity between mycotoxins can vary between commercial testing kits due to varying affinities based on the source and production of the antibody. The occurrence and variation in cross-reactivities could distort the result obtained for a particular mycotoxin, providing either an over- or underestimation of mycotoxin content. Investigation of several commercial kits for DON analysis showed that many test kits can have high cross-reactivity with 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, and some may also cross-react with nivalenol and fusarenon X (Li et al., 2014). On one hand, this cross-reactivity could lead to an overestimation of the mycotoxin being tested, whereas on the other hand, the cross-reactivity may give some, although unknown, indication of multiple mycotoxin contamination and the incidence of a mycotoxin subgroup.

The use of ELISA-based technology has allowed for high throughput, simple and inexpensive detection methods that can be used by nonscientific personnel (Turner et al., 2009). Furthermore, the use of ELISA in lateral flow tests also allows for a rapid, portable, and user-friendly technique when testing for mycotoxins as a screening method directly at the mill or farm. As a result, a greater proportion of the agricultural industry can have access to mycotoxin detection systems for the management of mycotoxins.

TLC Separation

The use of TLC can provide a simple and low-cost method for mycotoxin analysis that provides qualitative or semiquantitative results (Bueno et al., 2015) following preferably a thorough sample extraction. The disadvantage of this technology is that it does not provide an identification of the mycotoxin and may not provide sensitive or accurate measurements of mycotoxin contamination, although it does allow for high reproducibility of results. Research has demonstrated that the results are more accurate when the mycotoxin compounds fluoresce under UV light.

Gas Chromatography Separation

The process of gas chromatography (**GC**) involves separating components by their relative affinity for a stationary column and an inert gas (Bueno et al., 2015). Analytes separated on the column are eluted with the inert gas and are detected by physical or chemical methods coupled with GC such as flame ionization detection, electron-capture detection, or mass spectrometry. This method of analysis can be quite sensitive for mycotoxin analysis but does require preliminary cleanup of the extracts. Additionally, due to the conditions during the gas phase, only nonpolar, volatile analytes and high-temperature stable compounds can be analyzed (Bueno et al., 2015), and it has been mainly applied to trichothecenes or fumonisins. Most mycotoxins are not volatile and need to be derivatized to increase their volatility, their thermal stability, and chromatographic separation for analysis by GC (Turner et al., 2009) or to increase their response in the detector. Due to the need for this extra derivatization step, GC requires more complex sample preparation procedures that could affect throughput in a commercial application.

Liquid Chromatography Separation

Liquid chromatography (**LC**) such as HPLC coupled with UV, a diode array detector, or a fluorescence detector can be used as a reliable method for mycotoxin detection (Bueno et al., 2015). Simply, compounds can be eluted on a normal or reverse-phase column that separates mycotoxins over time based on their polarity under a pressurized liquid mobile phase flow (Turner et al., 2009). The eluted compounds from the column are then measured by the detector based on UV or fluorescent emission wavelengths specific to each mycotoxin analyzed and compared with commercially available standards for identification. Furthermore, the dose-dependent signal response of calibrant solution allows quantification of specific mycotoxins in the sample (Bueno et al., 2015). Once injected into the column, the mycotoxin detection process by HPLC takes a variable amount of time, from 4 to 50 min, with an average of about 20 min. This process is very accurate and less prone to matrix effect coming from the sample, but careful selection of solvent mobile phases and conditions is needed to optimize mycotoxin separation and prevent coelution of mycotoxins or other potential interferences, which generally involve the necessity of performing physical or chemical extraction beforehand to isolate further the compound of interest. Unlike with other methods, such as GC, LC can be applied to ionic, polar, and nonpolar molecules.

More recently, advancements in the LC technology have taken place. First, ultra-pressure liquid chromatography systems working at greater pressure and lower flow rates are now offering means of decreasing dramatically the elution time, while improving peak shapes. This process could help further separate coeluting compounds and enable greater throughputs. Second, many laboratories are now switching to mass spectrometry detection, which offers extended detection capabilities especially in terms of number of analytical targets, rather than using UV or fluorescent detection. One factor for this switch is the ability, when coupled with LC, to detect multiple mycotoxins with simplified chromatographic workflows including "masked" and "emerging" mycotoxins based on their molecular weight-to-charge ratio.

Tandem Mass Spectrometry Detection

Liquid chromatography coupled with tandem mass spectrometry provides one of the most robust and sensitive methods of analysis of a large number of mycotoxins. Most LC-MS/MS methods use either atmospheric pressure, chemical ionization, or preferentially electrospray ionization sources coupled to single or triple quadrupole mass spectrometers (Bueno et al., 2015). To accurately determine mycotoxin types and concentrations, a matrix match strategy or isotopically labeled standards are required to measure and correct for matrix effect (Jackson et al., 2012). Alternatively, internal standards including stable isotopes can be used to normalize the response (signal suppression or enhancement) of their nonlabeled counterpart but can also be used for chemically related analytes and to improve recovery of mycotoxins, thereby improving accuracy and precision. Furthermore, unlike GC, the use of LC coupled to MS/MS can be used for detecting those mycotoxins that may not be thermally stable or volatile. Additionally, LC-MS/MS with multiple reaction monitoring modes and fast polarity switching instruments can target specifically a variety of analytes at very low concentrations, making it one of the most sensitive methods available (Jestoi, 2008). Furthermore, this method enables maximal throughput without requiring thorough extraction or sampling preparation, which makes it a very attractive approach to commercial analysis.

The use of LC-MS/MS allows for the determination of numerous mycotoxin compounds including mycotoxins that have little UV-visible absorbance or fluorescence emission such as fumonisins, which generally require derivatization, the "masked mycotoxins" (i.e., conjugated forms of toxins) and "emerging mycotoxins" (Bueno et al., 2015). Conjugated mycotoxins are those that are not de-

tected by conventional analysis (examples ELISA, HPLC) because the mycotoxin has been chemically changed by the host plant so that the mycotoxin is conjugated to a more polar substance (Berthiller et al., 2013). Conjugated mycotoxins may have a lesser toxicity, whereas other times the risk from these mycotoxins is unchanged or in some cases the risk may be increased. Examples of these mycotoxins can include deoxynivalenol-3-glucoside and zearalenone-14-β-D-glucopyranoside (Z14G) among many others (Berthiller et al., 2013). Conjugated mycotoxins may elude analysis because of their changed physicochemical properties, leading to a lack of recognition by testing methods other than LC-MS/MS (Berthiller et al., 2013). Regardless of technology used to detect conjugated forms, data on many of these mycotoxins are still limited due to a lack of commercially available standards with the exception of that for deoxynivalenol-3-glucoside.

Emerging mycotoxins are another group of mycotoxins of importance. These mycotoxins have been defined as those that are neither routinely analyzed or legislatively regulated but which are seen to be rapidly increasing in incidence due to the advent in detection methods (Gruber-Dorninger et al., 2017). This category now includes many fungal metabolites that may have significant toxicological roles in the agricultural industry but also compounds that even if lacking known toxicological properties could have an influence on the microbiome due to their antibiotic properties, which in turn may affect digestive performance. Although some of the emerging mycotoxin could be detected by analytical methods such as HPLC, they are susceptible to interference to co-eluting compounds (Sorensen et al., 2008). As a result, the use of LC-MS/MS is the best method for accurate detection of these emerging mycotoxins due to its sensitivity and reliable quantitation of target mycotoxins.

IMPORTANCE OF ASSESSING MYCOTOXIN CO-CONTAMINATION

Whether parent, conjugated, or emerging compounds, not being able to detect these mycotoxins can lead to an underestimation of the total mycotoxin content in a feedstuff or feed as these mycotoxins add to the total risk to performances and health status observed in the animal. Surveys of feedstuffs and complete rations show that mycotoxin co-contamination and conjugated and emerging mycotoxins are common and need further consideration (Smith et al., 2016). Although the toxicity of the combination of mycotoxins is difficult to predict based on their individual toxicities, it is known that mycotoxin interactions could lead to additive, synergistic, or antagonistic effects on toxicity (Oh et al., 2012; Alassane-Kpembi et al., 2017).

Alassane-Kpembi et al. (2017) reported results of mycotoxin interactions from more than 80 publications. In this summary, the researchers concluded that mycotoxin interactions are complex and that additive, synergistic, and antagonistic relationships can vary by mycotoxin type and even concentration. An interesting example of these complex interactions is observed between several members of the type B trichothecene family, such as DON, 15-acetyl-deoxynivalenol, 3-acetyl-deoxynivalenol, nivalenol, and fusarenon X. At mycotoxin concentrations causing 50% cytotoxicity to proliferating Caco-2 cells, DON and 3-acetyl-deoxynivalenol are observed to have an additive effect, whereas they have a synergistic effect at lower concentrations of each mycotoxin (Alassane-Kpembi et al., 2013). The combinations of DON and nivalenol or fusarenon X also resulted in synergy at lower concentrations of mycotoxins. Other types of mycotoxins may have varied interactions. In a study completed with *Penicillium* mycotoxins, researchers observed that the effects on the bovine macrophage cell line varied by mycotoxin type, concentration, and combination (Oh et al., 2012). Mycotoxins such as citrinin, ochratoxin A (OTA), patulin, and penicillic acid caused cytotoxic effects individually on the proliferation of the bovine macrophage cell line, but mycotoxin concentration was important. Furthermore, some mycotoxins such as citrinin and OTA or OTA and patulin had significant additive effects on reducing proliferation of the bovine macrophage cell line. A synergistic effect between OTA and penicillic acid was also observed (Oh et al., 2012).

The interaction effects of DON with other mycotoxins is not just observed in the laboratory but has also been documented within the animal. Pigs consuming DON were observed to have increased levels of intestinal enterocyte apoptosis compared with controls, and the consumption of DON with nivalenol resulted in a synergistic effect on enterocyte apoptosis (Cheat et al., 2016). The complex nature of multiple mycotoxin combinations is also shown in broilers challenged with *Eimeria* spp. causing coccidiosis (Grenier et al., 2016). In this research, chronic feeding of DON and fumonisin B1 at concentrations generally considered not hazardous still resulted in metabolic and immunological disturbances and contributed to the severity of coccidiosis. Interactions between DON and fumonisin B1 were observed but were dependent on the health or performance variable measured, with antagonism observed in 3, additivity in 9, and synergism in 2 measured endpoints (Grenier et al., 2016).

As such, the knowledge of presence and type of mycotoxin interactions in a feedstuff or feed are important in assessing risk. Although information on a wide variety of mycotoxins is limited with simpler techniques such as ELISA, the use of advanced LC-MS/MS with its speed, detection limits, and capability has allowed for detection of multiple mycotoxin compounds (Gruber-Dorninger et al., 2017).

APPLICATIONS

Analysis of toxicity and occurrence are essential for mycotoxin risk assessment. Knowledge of mycotoxin incidence

on a local, regional, or global scale is required for producers to make decisions on purchasing, formulations, and management. There are many types of mycotoxin testing methods, from ELISA to LC-MS/MS, each providing a different purpose and benefit for assessing mycotoxins. For instance, ELISA technologies are quick, require little training for their use, and are portable, whereas LC-MS/MS can provide highly scientific and accurate quantification on numerous mycotoxins simultaneously including parent, conjugated, and emerging mycotoxins. Surveys of commodities show that mycotoxin co-contamination and conjugated and emerging mycotoxins are common and need consideration (Smith et al., 2016; Alassane-Kpembi et al., 2017). If livestock producers or their advisors test for only 1 or 2 mycotoxins, they may get an underestimation of mycotoxin risk. However, the use of LC-MS/MS methods can demonstrate multimycotoxin risk and help producers to tailor mycotoxin management programs.

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