CHAPTER 4.

Analysis of mycotoxins

Summary

The analytical methods for mycotoxin determination used in fully developed countries require sophisticated infrastructure, stable electricity, ready availability of supplies, and qualified and experienced technicians for instrument maintenance. Simple and appropriately validated tools analogous to those used for the management of contaminated bulk commodities at the grain elevator level are needed at the rural level in developing countries. These tools are needed to promote public health and to manage emergency situations in subsistence farming communities with an immediate and severe problem of mycotoxin contamination of food grains, with the goal of working towards feasible reductions in exposure. Two general analytical approaches that require less infrastructure are described here. The first approach is thin-layer chromatography (TLC), which has been used for more than 50 years to analyse mycotoxins. The advantages of TLC include simplicity and proven reliability. Accuracy may be improved by using precision spotters to apply precise amounts of sample to TLC plates and optical readers. The costs of these refinements to TLC are far lower than those of gas or liquid chromatography systems. The disadvantages of TLC include the need for stable supplies of solvents and standards as well as safe conditions for their storage. The second approach described here is based on immunological methods using anti-mycotoxin antibodies. These tests are available as kits, have the necessary standards built in, use little or no organic solvent, and are generally easy to use. The disadvantages of these methods include the need to refrigerate the kits before use and the limited shelf-life. It has been proposed that companies and development agencies could be solicited to develop packages of kits, sampling equipment (e.g. grinders), and training models for deployment in the many areas where mycotoxins are a chronic problem.

1. Introduction

Determination of mycotoxin concentrations in staple crops is a challenging exercise because of the problems associated with sampling heterogeneously distributed compounds (see Chapter 3) and the fact that the analytical methods need to have low limits of detection, generally in the mg/kg (ppm) or µg/kg (ppb) range, depending on the individual mycotoxin being analysed.

Analysis at these levels needs to be very specific to avoid analytical interferences and can produce large uncertainties. Given the many advances in analytical science since the discovery of the aflatoxins in the 1960s, it is not surprising that a wide array of analytical methods have been used for mycotoxin testing; most fall into the general categories of either chromatographic methods or immunological methods based on antibody technology (for a tutorial review, see Shephard, 2008). All these analytical methods require solvent extraction of the mycotoxin of interest from the matrix, followed by key analytical steps, which, for chromatographic determination, typically involve extract clean-up and concentration before final determination. Another component of the complexity of mycotoxin analysis is the fact that the varied chemical structures of mycotoxins mean that specific methods are required for individual toxins. This constraint is now being overcome by the use of expensive and sophisticated mass spectrometric methods. Because of this plethora of methods and their individual characteristics, when selecting a method for mycotoxin analysis, one should consider the purpose for which the results are needed, the matrix to be analysed, the detection limit required, and the expertise and infrastructure available.

2. Analytical methods used in developed countries

For the survey and control of mycotoxin levels and the implementation of mycotoxin regulations in developed countries, several official analytical methods have been validated by interlaboratory collaborative studies conducted under the auspices of international bodies such as AOAC International and the European Committee for Standardization (CEN). Most official methods are based on high-performance liquid chromatography (HPLC) with various detectors, and the most recent of these methods use immunoaffinity columns (IACs) for sample extract clean-up before the HPLC analysis. More recently, the advances made in coupling mass spectrometry to HPLC have enabled analytical chemists to combine analytical steps with a confirmatory test by measuring the mass spectrum of the HPLC peak. The highly specific nature of mass spectrometry has also been used to avoid extract purification and to develop multitoxin methods, which can be applied to mixtures of mycotoxins in one analytical experiment. However, in addition to these official methods, rapid screening methods have been developed for situations where quick decisions are required, such as at granaries, silos, and factories, and such methods can be adapted for transfer to developing countries. Most of these rapid methods are based on immunological principles and use antibodies raised against specific mycotoxins. They include quantitative enzyme-linked immunosorbent assays (ELISAs), fluorometric methods, lateral flow devices, and a range of tests that give a yes/no result for contamination above or below a set control level. Whereas a full review of these methods lies outside the scope of this chapter, many recent reviews exist of methods for the analysis of mycotoxins in fully developed countries or in export certification laboratories set up in countries to certify bulk commodities for export (Krska *et al.*, 2008; Solfrizzo *et al.*, 2009; Maragos and Busman, 2010; Shephard *et al.*, 2012).

3. Analytical methods useful in developing countries

The techniques used in developed countries require sophisticated infrastructure, stable electricity, ready availability of supplies, and qualified and experienced maintenance technicians. The facilities are expensive to build, require highly trained personnel, and generally have a low throughput unless staff numbers are large and spare instruments are available. The difficulties in meeting the challenges associated with mycotoxin testing in Africa, namely a lack of political commitment, infrastructure and trained personnel, sustainable supplies, instrument maintenance and repairs, and laboratory quality control, have been discussed by Waliyar *et al.* (2008). For these reasons, the extent to which such methods can be transferred to developing countries depends on the country's exact stage of development and the importance attached to analytical determinations of mycotoxins. Usually, developing countries rely on less sophisticated methods, such as thin-layer chromatography (TLC) and antibody-based methods. TLC remains a useful tool in developing countries, can be semi-automated with sample spotters and ultraviolet (UV) scanners for detection, and has the advantage of testing several samples simultaneously. Its potential use in rural settings is discussed in Section 4.1.

The wide range of commercial immunological methods has found broad application in laboratories that lack sophisticated instrumentation or in which access to such instrumentation is limited by high demand. Of these, quantitative or semiquantitative ELISAs, which do not require sample extract purification, are widely used and have the advantage of handling many samples in a

single experiment. The purification of sample extracts via IACs has also been commercialized for direct fluorescence measurements using proprietary calibrated fluorometers. In recent years, the technique of fluorescence polarization (FP) immunoassay has been successfully applied to mycotoxin determination. Rather than measuring the total fluorescence, FP measures the orientation of the fluorescence emission, which is related to the rate of molecular rotation. The advantage of FP is that it is performed entirely in the extract solution (Lippolis *et al.*, 2006). Lateral flow devices can provide a yes/no result for contamination above or below a set control level, and they have also been commercialized with optical readers to provide quantitative results. It needs to be recognized that all antibody-based methods are liable to cross-reactivities and matrix effects, but they are extremely useful as a first line of analysis. Where it is possible, problematic samples can be confirmed by HPLC methods.

4. Analytical methods useful in rural areas

Methods of analysis suitable for use at the rural level are still a challenge to analytical scientists. Citing Sashidhar (1993), Fernández-Surumay *et al.* (2000) commented (in a rural Latin American context) that methods used in fully developed countries "require highly qualified personnel, as well as sophisticated equipment in advanced laboratories.... [therefore,] simpler methods must be developed that do not require such infrastructure, are easier to manipulate, and at the same time do not compromise the quality of the analysis." A decade earlier, in commenting on mycotoxin analysis in developing countries, Coker (1991) wrote: "It is therefore imperative that the development of efficient, cost-effective sampling and analysis methods is pursued with considerable urgency." Unfortunately, the agenda set by these authors has not yet been addressed.

Appropriate and useful tools analogous to those used for the management of contaminated bulk commodities at the grain elevator level are needed at the rural level in developing countries. These tools are needed to promote public health and to manage emergency situations in rural areas with an immediate and severe problem of mycotoxin contamination of food grains. This is not a question of meeting Codex standards, but rather of working towards feasible reductions in exposure.

Analytical methods applied in rural settings need to relate to a comprehensive risk management strategy designed to address and reduce exposure to relevant mycotoxins. Therefore, the methods must be rapid and easy to perform and should require a minimum of local or transportable infrastructure. Methods should have a wide analytical range because determinations at the rural level mostly require a focus on removing or managing contaminated lots as opposed to determining small differences in contamination that might be relevant for compliance with a regulatory limit. For this purpose, TLC and some immunological methods would be suitable.

4.1 Thin-layer chromatography

Cognizant of the constraints in developing countries, Sashidhar (1993) described a portable mycotoxin analysis kit housed in a large fibreglass (or metal) suitcase-sized package as a suitable method for use at the village market level. The approach used was a simple, reliable, and inexpensive TLC method. The main components of the kit were a portable sample grinder, a robust domestic blender for toxin extraction, a TLC tank, and solvents. Chromatography was carried out using strips of silica-coated plastic sheets for dipsticks and visualization with a handheld UV lamp. The author reported a detection limit of 10 ppb for aflatoxin B_1.

TLC methods are useful for the key toxins discussed in this book: aflatoxins and the *Fusarium* mycotoxins fumonisins, deoxynivalenol, and zearalenone (Lin *et al.*, 1998; Schaafsma *et al.*, 1998; Shephard, 1998; Shephard and Sewram, 2004). AOAC International has approved several TLC methods for aflatoxins in groundnuts and maize as well as for ochratoxin A in barley, deoxynivalenol in wheat, and zearalenone in maize (Table 4.1). These procedures are more accurate and reliable if carried out with precision spotters and optical readers (Nawaz *et al.*, 1992); these units are relatively expensive but are far less costly and are easier to maintain than HPLC or gas chromatography (GC) instruments, particularly because of the absence of precision pumps and, in the case of GC, a constant supply of carrier gas.

Like all chemical analyses, TLC requires trained staff; individuals with college-level education need several weeks of intensive training in chemical analysis to perform reliable TLC analyses. Experience has been gained in providing such training in Asia and Africa (FAO, 1990; Boutrif, 1995; Cardwell, 1996). Potential problems with TLC analysis include the acquisition of the essential mycotoxin standards, the preparation of fresh standards in solution, and the stability of the resulting solutions. Pure standards as solids are expensive and are perishable unless stored under very carefully controlled conditions. The preparation of standards in solution requires access to an accurate balance as well as weighing conditions with appropriately conditioned air (ca. 25 °C, 30–40% relative humidity).

Table 4.1. AOAC International official TLC methods for some mycotoxins in cereals

Mycotoxin	AOAC method	Commodity	Remarks
Aflatoxins	968.22	Groundnuts and groundnut products	CB method
	970.45	Groundnuts and groundnut products	BF method
	998.03	Groundnuts	Alternative BF method
	993.17	Maize and groundnuts	
	975.37	—	Aflatoxin B_1/aflatoxin G_1 confirmation method
	985.17	—	Aflatoxin B_1 confirmation method
Ochratoxin A	973.37	Barley	
Deoxynivalenol	986.17	Wheat	
Zearalenone	976.22	Maize	

BF, United States Food and Drug Administration Best Foods; CB, United States Food and Drug Administration Contaminants Branch; TLC, thin-layer chromatography.

More typically, standards are purchased as certified solutions or access to a UV spectrophotometer is required for accurate determination of toxin concentration. The stability of standards in solution is limited unless they are kept refrigerated or frozen. Therefore, the challenges of TLC are training, solvent supplies, acquisition of standards, standard preparation, and standard stability.

4.2 Antibody-based methods

As noted previously, many primary testing laboratories in both developed and developing countries use antibody-based methods to assay mycotoxins. The sampling, grinding, blending, and extraction steps are similar to those for TLC, except that solvent use is much reduced and solvents are usually less expensive and less hazardous. Several studies have examined antibody-based tests and have shown that commercially available products from several companies are quite effective (Schaafsma *et al.*, 2009). A comparison of TLC and antibody-based methods showed that the training needs are similar for the two kinds of systems but that for antibody-based methods the equipment and supply costs are lower, and the problems associated with standards are eliminated. Cross-reactivity of related mycotoxins occurs with most ELISA methods and precludes their use as regulatory tools (Tangni *et al.*, 2010). Although antibody-based tests can also suffer from the occurrence of various matrix effects, especially if they are used inappropriately or in matrices for which they were not validated, they could supply the rapid analyses needed in rural settings. The local development of antibodies and immunoassay kits has been proposed to obviate the commercial costs, but care needs to be exercised in adequately validating locally developed kits. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has developed a simple, robust, versatile, and cost-effective ELISA for the determination of aflatoxins in groundnuts (ICRISAT, 2009).

After appropriate validation, the United States Department of Agriculture Grain Inspection, Packers and Stockyards Administration (GIPSA) has approved several antibody-based tests for use as first-action tools at silos. Several companies produce very good dipstick methods based on antibodies with simple-to-use and relatively inexpensive readers. It would seem plausible to build on the portable mycotoxin analysis kit of Sashidhar (1993), replacing the TLC method with antibody-based dipsticks. The sensitivity of these methods can be improved by using nanoparticles to support the capture antibody (Posthuma-Trumpie *et al.*, 2009; Maragos and Busman, 2010).

5. Conclusions

The capacity to perform mycotoxin analysis is needed to manage emergency situations and to promote public health. When a rural region has an immediate and severe problem of mycotoxin contamination of food grains, appropriate risk management practices (see Chapter 7) need to be implemented. Suitable portable and robust analytical equipment and methods are needed to identify the most severely affected rural areas and to provide feedback on the effectiveness of the management practices instituted. In addition to mycotoxin analysis for managing emergency situations, general public health can be promoted by regular monitoring of mycotoxin in rural areas, which may also identify priorities for improved agronomy, crop varieties, greater crop diversity, and improved storage. Many commercial ELISAs are available for mycotoxins, each with its strengths and weaknesses, including sensitivity, cross-reactivity, and shelf-life. Evaluating such tests for applicability for field use in rural areas of developing countries would be a useful and important project.

References

Boutrif E (1995). FAO programmes for prevention, regulation, and control of mycotoxins in food. *Nat Toxins,* 3:322–326, discussion 341. doi:10.1002/nt.2620030430 PMID:7582637

Cardwell KF, ed. (1996). *Proceedings of the Workshop on Mycotoxins in Foods in Africa, November 6–10, 1995, Cotonou, Benin.* Croydon, UK: International Institute for Tropical Agriculture. Available at http://www.fao.org/inpho_archive/content/documents/vlibrary/new_else/x5422e/x5422e00.htm#Contents.

Coker RD (1991). Recent developments in methods for sampling and analysis of mycotoxins. In: Champ BR, Highley E, Hocking AD, Pitt JI, eds. *Fungi and Mycotoxins in Stored Products: Proceedings of an International Conference held at Bangkok, Thailand, 23–26 April 1991.* Canberra: Australian Centre for International Agricultural Research (ACIAR Proceedings Series, No. 36), pp. 115–122. Available at http://aciar.gov.au/publication/pr036.

FAO (1990). *Manuals of Food Quality Control. 10. Training in Mycotoxins Analysis.* Rome: Food and Agriculture Organization of the United Nations (FAO Food and Nutrition Paper 14/10). PMID:2126246

Fernández-Surumay G, Negrón-González G, Isea-Fernández G, Sánchez-Camarillo E (2000). Reporte de análisis cuantitativo de aflatoxinas por el método ELISA en muestras de materias primas de alimento balanceado para aves provenientes de una planta ubicada en el municipio Mara del estado Zulia, Venezuela [Report of quantitative analysis of aflatoxins by the ELISA method in samples of raw ingredients of balanced feed for poultry from a factory located in Mara municipality of Zulia State, Venezuela]. *Rev Cient Fac Cienc Vet Univ Zulia*, 10:63–68.

ICRISAT (2009). Action against aflatoxins: ICRISAT's approach to protecting human health. Patancheru, India: International Crops Research Institute for the Semi-Arid Tropics. Available at http://www.icrisat.org/what-we-do/SASA/sasaindex_august.html.

Krska R, Schubert-Ullrich P, Molinelli A *et al.* (2008). Mycotoxin analysis: an update. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 25:152–163. doi:10.1080/02652030701765723 PMID:18286405

Lin L, Zhang J, Wang P *et al.* (1998). Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *J Chromatogr A*, 815:3–20. doi:10.1016/S0021-9673(98)00204-0 PMID:9718702

Lippolis V, Pascale M, Visconti A (2006). Optimization of a fluorescence polarization immunoassay for rapid quantification of deoxynivalenol in durum wheat-based products. *J Food Prot*, 69:2712–2719. PMID:17133816

Maragos CM, Busman M (2010). Rapid and advanced tools for mycotoxin analysis: a review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 27:688–700. doi:10.1080/19440040903515934 PMID:20155533

Nawaz S, Coker RD, Haswell SJ (1992). Development and evaluation of analytical methodology for the determination of aflatoxins in palm kernels. *Analyst*, 117:67–74. doi:10.1039/an9921700067 PMID:1605348

Posthuma-Trumpie GA, Korf J, van Amerongen A (2009). Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem*, 393:569–582. doi:10.1007/s00216-008-2287-2 PMID:18696055

Sashidhar RB (1993). Dip-strip method for monitoring environmental contamination of aflatoxin in food and feed: use of a portable aflatoxin detection kit. *Environ Health Perspect*, 101 Suppl 3:43–46. PMID:8143644

Schaafsma AW, Limay-Rios V, Paul DE, Miller JD (2009). Mycotoxins in fuel ethanol co-products derived from maize – a mass balance for deoxynivalenol. *J Sci Food Agric*, 89:1574–1580. doi:10.1002/jsfa.3626

Schaafsma AW, Nicol RW, Savard ME *et al.* (1998). Analysis of *Fusarium* toxins in maize and wheat using thin layer chromatography. *Mycopathologia*, 142:107–113. doi:10.1023/A:1006937720711 PMID:9926423

Shephard GS (1998). Chromatographic determination of the fumonisin mycotoxins. *J Chromatogr A*, 815:31–39. doi:10.1016/S0021-9673(98)00187-3 PMID:9718704

Shephard GS (2008). Determination of mycotoxins in human foods. *Chem Soc Rev*, 37:2468–2477. doi:10.1039/b713084h PMID:18949120

Shephard GS, Berthiller F, Burdaspal PA *et al.* (2012). Developments in mycotoxin analysis: an update for 2010–2011. *World Mycotoxin J,* 5:3–30. doi:10.3920/WMJ2011.1338

Shephard GS, Sewram V (2004). Determination of the mycotoxin fumonisin B_1 in maize by reversed-phase thin-layer chromatography: a collaborative study. *Food Addit Contam*, 21:498–505. doi:10.1080/02652030410001670175 PMID:15204551

Solfrizzo M, Alldrick AJ, Van Egmond HP (2009). The use of mycotoxin methodology in practice: a need for harmonization. *Quality Assur Safety Crops Foods*, 1:121–132. doi:10.1111/j.1757-837X.2009.00021.x

Tangni EK, Motte JC, Callebaut A, Pussemier L (2010). Cross-reactivity of antibodies in some commercial deoxynivalenol test kits against some fusariotoxins. *J Agric Food Chem*, 58:12625–12633. doi:10.1021/jf103025e PMID:21087038

Waliyar F, Siambi M, Jones R *et al.* (2008). Institutionalizing mycotoxin testing in Africa. In: Leslie JF, Bandyopadhyay R, Visconti A, eds. *Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade*. Wallingford, UK: CAB International, pp. 359–368.