Chapter 2
Molecular Mechanism of Detection of Aflatoxins and Other Mycotoxins

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2.1 Introduction

To date, we do not know how to detoxify chemically or physically crops and foods that are contaminated by mycotoxins in ways that retain their edibility. Our safety, therefore, relies on our ability to detect, quantify and avoid them.

Because most of the people and animals under threat by mycotoxins live in third-world countries, the fast, simple to perform and interpret, and inexpensive, yet sensitive methodologies for the detection of mycotoxins are the main issue of this chapter. These methods are easy to use, and do not require expertise in mycology or toxicology. Thus, those methodologies that involve heavy expensive equipment, sophisticated labs and infrastructure and require highly trained experts will be reviewed more briefly.

2.2 Detection of Aflatoxigenic Fungi

Aspergillus, *Penicillium*, *Fusarium* and *Alternaria*, species that often contaminate foodstuffs and feedstuffs, produce most of the mycotoxins that threaten humans and animals, and cause heavy losses of crops. Each genus comprises many species. The identification of toxigenic fungi, therefore, requires proficiency in mycology.

People and livestock in the developed, affluent countries enjoy aflatoxin-safe food and animal feed thanks to strictly enforced regulatory measures. The latter rely on a plethora of sensitive and accurate methods for the detection and quantization of aflatoxins and other mycotoxins. These assays require the use of sophisticated, expensive scientific equipment, and highly trained professional

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personnel to operate it. Fungal contaminations of crops and foods, however, are widespread in less developed countries (Jelinek et al. 1989).

I thus applaud Rodney Bothast and Dorothy Fennel who developed the *Aspergillus* differential medium (ADM), a diagnostic medium that enables the identification and enumeration of aflatoxigenic *Aspergillus* (Bothast and Fennel 1974). Inexpensive reagents, an autoclave and a simple 365 nm UV lamp (Hara et al. 1974) are sufficient for the identification of aflatoxigenic fungi by laboratory technicians who are not specialists in mycology.

Common media such as czapek, sabouraud dextrose or yeast extract sucrose (Difco) can support the growth of *Aspergillus*. Addition of methyl-β-cyclodextrin (Wacker, Munich) (Fente et al. 2002) or of a combination of methyl-β-cyclodextrin plus bile salts (0.6% Na-deoxycholate) (Rojas-Durán et al. 2007) enhances the natural fluorescence of aflatoxins, allowing detection of aflatoxigenic colonies after 3 days (Fente et al. 2002) or 36 h (Rojas-Durán et al. 2007a, b) of incubation.

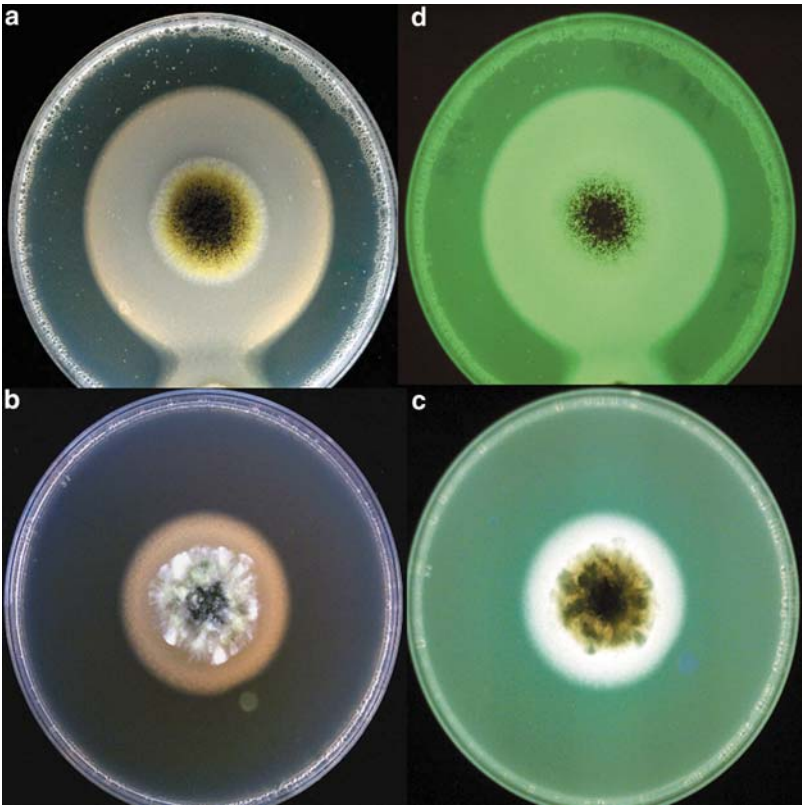

Fig. 2.1 A non-aflatoxigenic strain (**a**, **b**) and aflatoxigenic strain (**b**, **c**) of *A. niger* visualized under visible light (**a**, **b**) and under 365 nm UV light (**c**, **d**). The rim of the white ring around the colony of the aflatoxigenic strain displays faint blue fluorescence (Rojas-Durán et al. 2007). With permission of TR Rojas-Durán

ADM contains per liter 25 g tryptone, 20 g yeast extract, 0.5 g ferric citrate and 25 g agar. The high concentration of iron is required for the production of pigments. ADM prevents the sporulation-dependent appearance of secondary colonies on the plates, thus allowing more accurate counting and assessment of the level of infection. Colonies of *Aspergillus* produce a bright yellow-orange pigment, and blue (AFB_2, AFB_2) or green (AFG_2, AFG_2) fluorescent halos appear around aflatoxigenic colonies upon exposure to UV light (Bothast and Fennel 1974; Fente et al. 2002; Fig. 2.1).

2.3 Examination of Fungal Colonies under UV Light

The detection of AFs as judged by fluorescence of fungal colonies is not easy, in that non-aflatoxigenic strains of *Aspergilli,* such as *A. parasiticus* and *A. niger*, fluoresce under UV (Figs. 2.1 and 2.2) (Rojas-Durán et al. 2007a, b). This could be interpreted erroneously to be due to the presence of AFs.

A more diagnostic test for the presence of AFs in fungal colonies is the room temperature phosphorescence of AFs that lasts ca 0.5 s after switching off the UV light (Rojas-Durán et al. 2007a, b). Non-aflatoxigenic *Aspergilli* do not phosphoresce, whereas AF-producers do (Fig. 2.2).

Fluorescence and phosphorescence are not the only outcomes of exposure of AFs to UV light. AFB_2 and AFB_2 are activated by 365 nm UV light, resulting in AFB_2–8,9-oxide. Binding of the latter to DNA at the N^7 position of guanine residues yields 8,9-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_2 (Fig. 2.3). The structure of the AF-DNA photoadduct (Israel-Kalinsky et al. 1984; Shaulsky et al. 1990; Stark 2007) is identical to the AF-DNA adduct that is formed *in vivo* from ingested

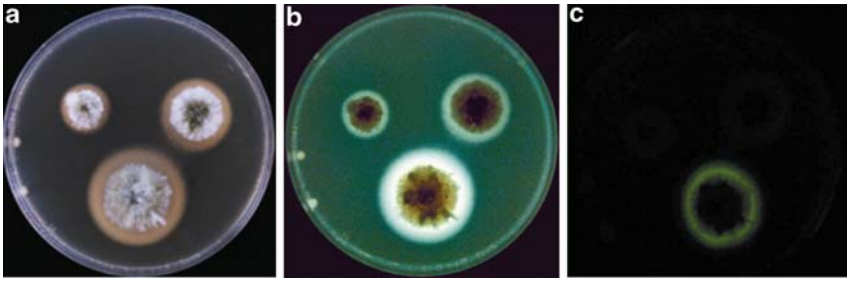

Fig. 2.2 Two non-aflatoxigenic (top colonies) and one aflatoxigenic (bottom colony) strains of *parasiticus* visualized (**a**) under visible light; (**b**) 365 nm UV light. The ring around the aflatoxigenic strain displays blue fluorescence; (**c**) room temperature phosphorescence was photographed with a digital camera with a 2.5 s exposure after switching-off the UV lamp. Phosphorescence persists for *ca*. 2 s, (Rojas-Durán et al. 2007). With permission of TR Rojas-Durán

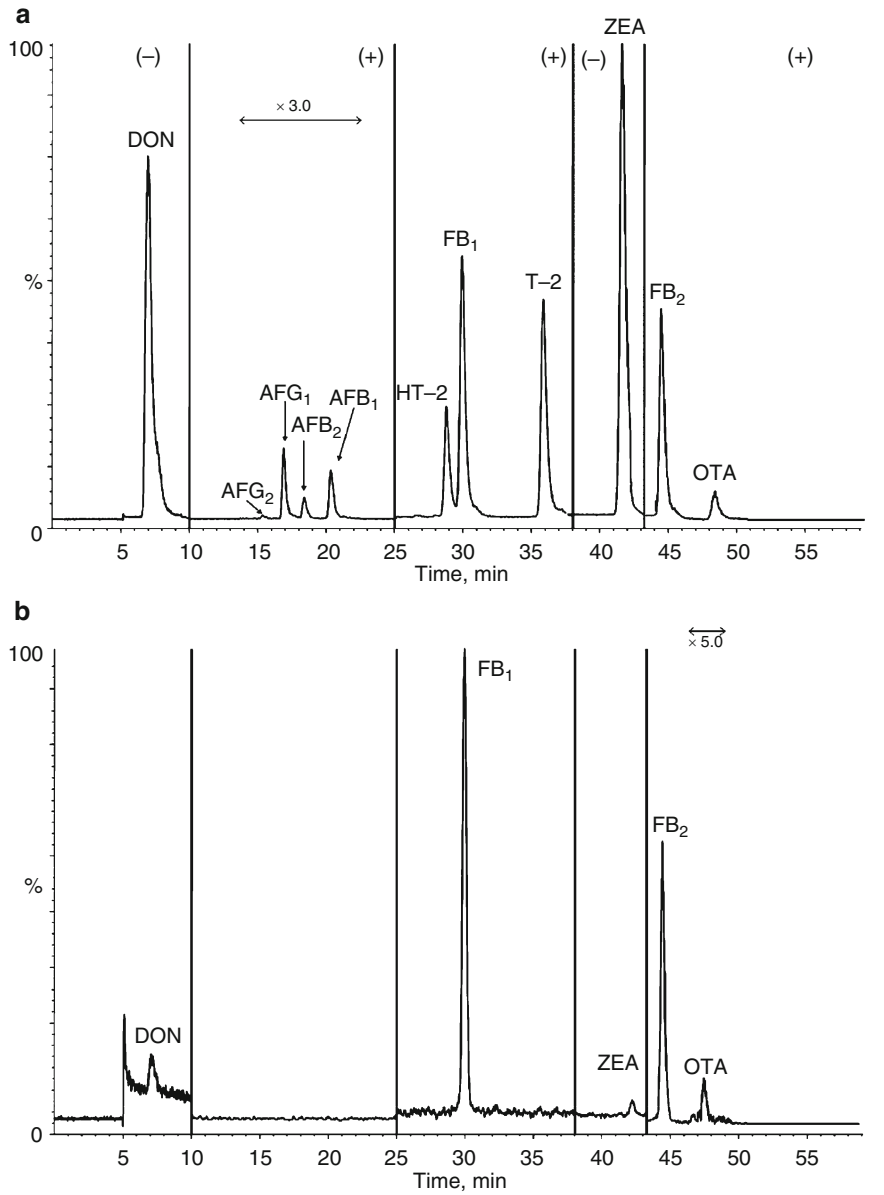

Fig. 2.3 HPLC-tandem mass spectrometry of multiple mycotoxins. *Panel A*: a water-methanol extract of 20 g maize that was purified on a AOFZDT2^{TM} immunoaffinity column, and spiked with (in ppb) DON 500; AFG_2 2; AFB_2 2; AFG_2 6; AFB_2 20; FB_2 500; FB_2 250; HT-2 200; T2, ZEA (zearalenone) 200; OTA 20. Presented is the ion chromatogram of the spiked extract. *Panel B*: ion chromatogram of a similar extract that was contaminated naturally with (in ppb) DON 5; FB2 72; FB2 26; ZEA 0.5; OTA 0.3. The lines at 5, 20, 25, 38 and 44 min represent switches of polarity of the ion source (Lattanzio et al. 2007). With permission of VM Lattanzio

AFB_2 by cytochrome P450, mainly in the liver (Essigmann et al. 1977; Croy et al. 1978; Stark et al. 1979). Formation of AF-DNA adducts is considered as the initiating step of AF-induced carcinogenesis. Covalent DNA- and protein adducts are also responsible for acute toxicity.

2.4 Detection of Aflatoxins in Corn Kernels Contaminated with Aflatoxigenic Fungi

It is possible to detect corn kernels that are contaminated by aflatoxigenic fungi, and to estimate roughly the level of AFs in a corn sample. Exposure of infected corn kernels that contain AFs to 365 nm UV light results in intense blue-green fluorescence of aflatoxin-containing kernels. More than four fluorescent kernels in a 5-pound sample of corn (approximately 6,000 kernels) indicates that the level of aflatoxins is at least 20 ppb, i.e., the FDA action level for AFs in human foods. The presence of less than four fluorescent kernels per 5-pound sample, though, does not mean that the sample is not contaminated with AFs. (Munkvold et al. 2005). This convenient but crude assay should be followed by the identification of AFs and determination of their levels with portable kits, and by confirmation of their identity in the laboratory.

2.5 Detection of Mycotoxigenic Fungi by Polymerase Chain Reaction

2.5.1 Aflatoxins

At least 25 genes are involved in the biosynthesis of AFs and its regulation (Bhatnagar et al. 2006). Primers pertaining to sequences of *afl-2*, *aflD*, *aflM* and *aflP*, (*apa-2*, *nor-2*, *ver-2*, *omt-2*, respectively) (Shapira et al. 1996; Geisen 1996; Chen 2002) have been used to detect and identify aflatoxigenic strains of *A. flavus* and *A. parasiticus* among isolated colonies, or in DNA extracts from in foodstuff and feedstuff.

Briefly, DNA of *Aspergilli* is used as template for the amplification of genes involved in AF biosynthesis. Sequencing of the amplified fragments confirms the identity of AF biosynthetic genes. However, the mere presence of the genes reflects only the potential of the fungus to produce aflatoxin. AF production depends on temperature, humidity, composition of the growth medium, growth phase and age of the culture. A recent application of reverse transcription-polymerase chain reaction (RT–PCR) for the characterization of aflatoxigenic *Aspergilli*, relies on the presence of mRNAs pertaining to AF biosynthesis genes.

Table 2.1 Genes used for the identification of aflatoxigenic fungi by PCR and RT-PCR. The table is based on Chang et al. (1993), Shapira et al. (1996), Geisen (1996), Sweeney et al. (2000), Chen et al. (2002), Bennett and Klich (2003), Yabe and Nakajima (2004), Scherma et al. (2005), Bhatnagar et al. (2006), Lee et al. (2006), Degola et al. (2007), Kale et al. (2007)

Gene	Synonym	Enzyme	Step in AF biosynthesis pathway
apa-2	afl-2		Polyketide to norsolorinic acid? Regulator of AFB2 biosynthesis
aflD	nor-2	Norsolorinic acid reductade	Norsolorinic acid to averantin
aflI	avfA	Averufin oxidase	Averufin to versiconal hemiacetal acetate
aflM	ver-2	Versicolorin A dehydrogenase	Versicolorin A to demethylstrigmatocystin
aflO	omtB	*O*-methyltransferase	Demethylstrigmatocystin to sterigmatocystin
aflP	omtA	*O*-methyltransferase	Sterigmatocystin to *O*-methylsterigmatocystin
aflQ	ordA	Oxidoreductase	*O*-methylsterigmatocystin to aflatoxin B2
aflR		Transcription factor containing a zinc cluster DNA binding motif	Positive regulator of AFB2 biosynthesis
aflS	aflJ	Transcription factor	Positive regulator of AFB2 biosynthesis

RT–PCR is indicative of the presence of the aflatoxigenic fungus and of the AF biosynthetic enzymes.

Multiplex RT–PCR containing 4–5 primer pairs of various combinations of *aflD*, *aflO*, *aflP*, *aflQ*, *aflR* and *aflS* (*aflJ*) were used to detect toxigenic fungi (Sweeney et al. 2000; Scherma et al. 2005; Degola et al. 2007). The genes, their enzyme products and their functions in the AF biosynthetic pathway are shown in Table 2.1 Non-aflatoxigenic strains lack one or some AF biosynthesis genes (Shapira et al. 1996) and their mRNA products (Degola et al. 2007). PCR methodologies for rare *Aspergilli* such as *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamar* that produce AFs have yet to be developed (Bennett and Klich 2003).

2.5.2 *Other Mycotoxins*

A real-time PCR assay for ochratoxigenic *Aspergillus* includes primers pertaining to the β-ketosynthase domain of a polyketide synthase from *A. carbonarius* (Selma et al. 2007). The PSK4 gene of *Fusarium graminearum* is involved in the synthesis of fumonisins and can be used to detect *Fusaria* that produce zearalenone (Lysøe et al. 2006). A recent review describes in detail PCR methods for the detection of fungi that produce aflatoxins, T2 toxin and DON, fumonisins and patulin (Niessen 2007).

Table 2.2 Limits of detection and quantization of fluorescent mycotoxins. The reproducibility and precision stem from the direct measurement of the compound without derivatization or ionization, and from the high sensitivity of fluorimetry. Non-fluorescent mycotoxins such as trichothecenes can be derivatized with fluorescent chromophores (Visconti et al. 2005) before HPLC. Less popular is post column derivatization (Waltking and Wilson 2006)

Toxin	LOD (ppt) ($ng\ kg^{-1}$ or $ng\ l^{-1}$)	LOQ (ppt)	Reference
Aflatoxin B_2	70	220	Nguyen et al. (2007)
Aflatoxin M_2	6	25	Marilena Muscarella et al. (2007)
Sterigmatocystin	500	NT	Versilovskis et al. (2007)
Citrinin	220	350	Nguyen et al. (2007)
Ochratoxin A	80	250	Nguyen et al. (2007)

2.6 Detection and Determination of Mycotoxins in the Analytical Laboratory

2.6.1 High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) of extracts of commodities, foods and feeds is the most prevalent and sensitive current method for the identification and quantization of mycotoxins. Uncanny sensitivity and precision in the detection of ppt (parts per trillion) concentrations of the fluorescent mycotoxins AFB_2, AFB_2, AFG_2, AFG_2 and sterigmatocystin, citrinin and ochratoxin A can be achieved by careful preparation and concentration of extracts of grain/fruit samples, followed by HPLC in an apparatus equipped with a fluorescence detector (Table 2.2), (Nguyen et al. 2007).

Often, the mycotoxins extracted from field samples undergo clean-up using commercial immunoaffinity columns before their analysis by HPLC. The columns are available for all the important mycotoxins: AFB_2, AFB_2, AFG_2, AFG_2, AFM_2, ochratoxin A, T2 toxin, deoxynivalenol (vomittoxin), citrinin, fumonisins FB2, FB2, FB3, zearalenone, patulin and moniliformin. Multiplex columns are available for AFs, ochratoxin A and zearalenone (http://www.vicam.com/products/mycotoxin.html). The rationale beyond the multiplex columns and for multiplex detection methods is the frequent production of more than one mycotoxin by a single fungus, and the frequent contamination of crops or silage with several species of fungi (Wang et al. 1995; Logrieco et al. 2007; González Pereyra et al. 2008).

2.6.2 HPLC-Mass Spectrometry (LC-MS) and HPLC-Tandem Mass Spectrometry (LC-MS-MS)

These are the ultimate methods for the identification/confirmation of the identity of mycotoxins, including those which do not fluoresce or do not absorb visible

UV light. Such methods allow the identification and sometimes the quantization of many mycotoxins in a single sample (Fig. 2.3) (Lattanzio et al. 2007). The techniques require much care and precision. Despite the fact that LC-MS and LC-MS-MS are the most sensitive methodologies for the detection of mycotoxins, it is difficult to achieve complete ionization in every measurement because the degree of ionization is finicky and is complicated by trivial details. Thus, whereas the method of choice for quantifying trichothecenes and fumonisins is LC-MS, mycotoxins such as AFs, OTA, patulin and ZRN can be accurately quantified by HPLC with detectors other than MS. The reader is referred to the excellent recent review of (Sforza et al. 2006) that deals with LC-MS techniques for the analysis of all of the important mycotoxins. LC-MS methodologies involve the most expensive apparatuses and require the service of high-level professionals. The cost of the complete system could be prohibitive.

2.6.3 Enzyme-linked immunosorbent assay Kits

Detection and quantization of mycotoxins with commercial kits is invariably based on the competition enzyme-linked immunosorbent assay (competition ELISA).

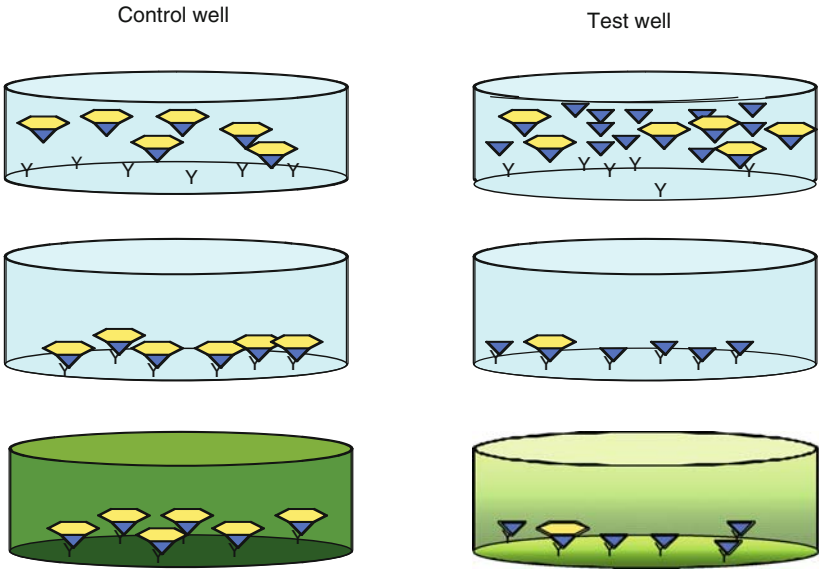

Fig. 2.4 Competition ELISA kit for the quantization of mycotoxins: for detailed explanation, see text. *Hexagon-triangles* enzyme-mycotoxin conjugate, *triangles* free mycotoxin, *Y* anti mycotoxin antibody. *Top row*: apply enzyme-mycotoxin conjugate in the control well and test wells, and a sample of the mycotoxin extract in the test well only, and incubate. *Middle row*: wash wells. *Bottom row*: add the chromogenic substrate

Table 2.3 Limits of detection and range of quantization of mycotoxins in competition ELISA

Mycotoxin	LOD (ppb)	Detection range (ppb)	Reference
AFB_2 AFB_2 AFG_2 AGB_2	2.5	4–40	Zheng et al. (2005b)
AFB_2	0.005		Delmulle et al. (2005)
AGM_2	0.05	0.05–0.76	Sibanda et al. (1999)
Ochratoxin A	2	2–40	Zheng et al. (2005a)
Deoxynivalenol, nivalenol	80	NT	Yoshizawa et al. (2004)
T2-toxin	30	NT	Yoshizawa et al. (2004)
Patulin	20	NT	de Champdoré et al. (2007)
Zearalenone	2	2–200	Wang et al. (2006)
Fumonisin B_2	0.24	0.24–0.9	Quan et al. (2006)
Fusarin	2	NT	Maragos et al. (2008)

Wells in the ELISA microtiter plate contain a bound antibody against a mycotoxin. The detecting reagent is a covalent complex of this mycotoxin and an enzyme, usually horseradish peroxidase or alkaline phosphatase.

The reagent is mixed with a sample of the mycotoxin extract and the mixture is placed in the well. In the control well (absence of mycotoxin in the sample), the mycotoxin-enzyme conjugate can saturate the bound antibody, and addition of a chromogenic substrate results in the development of color. In the test well, free mycotoxin molecules in the extract compete with the conjugate on the bound antibody. The higher concentration of mycotoxin, the less the conjugate can react with the bound antibody, leading to fainter color development (Fig. 2.4).

The assay is quick, easy to perform and requires an affordable ELISA microplate reader (Table 2.3). The sensitivity of competition ELISA approaches that of the LC-MS method. Immunoaffinity columns can be used here to purify and concentrate mycotoxin samples. The commercial ELISA kits, the analytes, the manufacturing companies and the primary matrix for extraction and preparation of samples are summarized in a table published on the Web by the American Organization of Anaytical Chemists (AOAC) (http://www.aoac.org/testkits/TKDATA5.htm).

2.6.4 Lateral Flow Immunochromatography

This is also called rapid one-step assay of mycotoxins. It has become the most popular method for the rapid identification of mycotoxins and rough estimation of their concentration.

A typical lateral flow immunochromatography (LTF) strip is comprises the following (Fig. 2.5, top to bottom): a loading pad where a sample of the extract is applied; a zone containing colored particles (e.g., latex, gold) coated with a mouse monoclonal anti-mycotoxin antibody; a zone of nitrocellulose membrane that allows the migration of the particles together with the mycotoxin sample; a test

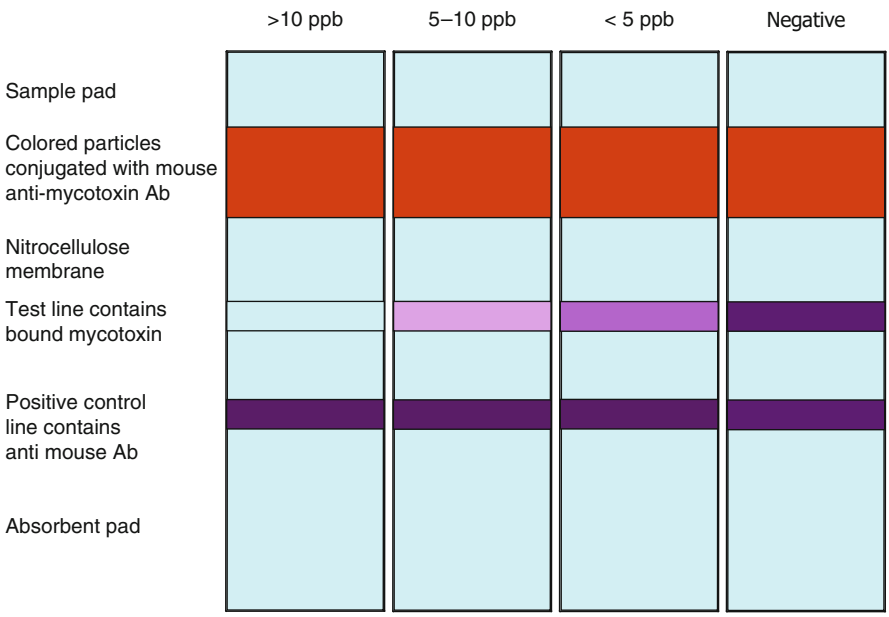

Fig. 2.5 Rapid one-step assay [ROSA]. See text for explanations

line that contains immobilized mycotoxin; a positive control line that contains a secondary anti-mouse antibody, and an absorbent pad.

A sample of the extract that contains the mycotoxin in question is applied and migrates along the strip. Upon reaching the conjugate zone, the mycotoxin binds the anti mycotoxin–particle complex. Free and mycotoxin-containing particles now migrate to the test line. The immobilized mycotoxin captures only the free particles that form a visible colored line, whereas mycotoxin-containing particles continue to migrate.

In the presence of a mycotoxin in the sample at a concentration higher than the cut-off point of the strip (saturation of the particles with mycotoxin), the mycotoxin-containing particles will fail to bind to the test line, and vice versa. Thus, the intensity of the color in the test line is inversely proportional to the concentration of the mycotoxin.

Upon reaching the positive control line, both free and mycotoxin-containing particles can bind the anti-mouse antibody, thus forming a strongly colored line regardless of the presence or absence of mycotoxin. The sensitivity of LTF is very high (Table 2.4), and is comparable to those of sophisticated methodologies such as LC-MS-MS and surface plasmon resonance (SPR) (see below). The use of fluorescent reagents can bring the LOD to 50–200 ppt, as has been shown with other toxins (Kim et al. 2003). The highest sensitivity in the detection of a mycotoxin by LTF was 5 ppb of AFB_2 in pig feed, using a commercial immunaffinity column for the purification and concentration of the extract (Delmulle et al. 2005).

Table 2.4 Limit of detection and range of quantization of mycotoxins assayed by LTF and by surface plasmon resonance (*a*), (Sibanda et al. 1999). The SPR assay was designed as an inhibition assay

Mycotoxin	Rapid one-step assay (ROSA)		Surface plasmon resonance	
	LOD (ppb)	Detection range (ppb)	LOD	Range
Total aflatoxins	1–2	0–250	NT	NT
AFB_2	0.005 (a)	NT	0.2	2
AFM_2	0.05	0–0.7	NT	NT
Ochratoxin A	2	0–7	0.2	3
T2-toxin	35	75–500	NT	NT
DON	0.5–2	0–6,000	0.5	500
Zearalenone	5	0–2,400	0.02	200
Fumonisin B_2	50	0–5	50	2,000
Reference	http://www.helica.com/food-safety/aflatoxin http://www.charm.com http://www.r-biopharm.com http://www.romerlabs.com/pdts_kits.html http://www.charm.com/content/b Logcategory		Van der Gaag et al. (2003)	

2.7 New Biosensors

The reader is referred to the reviews (Zheng et al.2006; Prieto-Simón et al. 2007) where the clear descriptions and explanations of the physico-chemical principles of new devices and methodologies that involve biosensors are accompanied with illustrations. The new methods are at the experimental stage.

2.7.1 SPR

This is a measurement of mass concentration changes that occur at the sensor (microbalance) surface due to binding of molecules. It is useful mainly for molecules larger than 20 kDa. The advantages of this method are: use of unlabeled/unmodified mycotoxins and minute sample size. A biosensor array based on SPR has made it possible to assay several mycotoxins simultaneously (Van der Gaag et al. 2003). SPR is a very sensitive method (Table 2.4)

2.7.2 Fluorescent Polarization Immunoassay

This is a very rapid test. Three minute extraction and 2 min assay were sufficient to detect 2 ppm of DON (Lippolis et al. 2006), 500 ppb of FB2 (Maragos et al. 2002) and 500 ppb of zearalenone within 20 min (Maragos and Kim 2004). A fiber optic immunosensor was used to detect 20 ppb of FB2 (Thompson and Maragos 2006).

2.7.3 Molecular Imprinting

It is a method by which small molecules surround a molecule of e.g., mycotoxin that is bound on a solid support. After polymerization of the surrounding molecules and washout of the mycotoxin, a pseudo receptor is formed. The selective binding of other molecules of the same mycotoxin to the imprinted polymer is enhanced as compared to binding to a non-imprinted polymer. Ochratoxin A was successfully used to imprint such a polymer (Yu et al. 2007).

2.7.4 Arrays of Biosensors

Biosensor arrays are designed to perform the simultaneous assays of many mycotoxins. The frequent production of several mycotoxins by a single fungus, and the contamination of crops with several toxigenic fungi are some of the reasons for the development of the arrays. The current arrays are experimental as of yet. All of them rely on immuno-competition reactions (Sapsford et al. 2006). The array for the detection of AFB_2 is based on a 96-well microtiter plate coupled to a multichannel electrochemical immunosensor. The limit of detection was 30 ppt (Piermarini et al. 2007). The limit of detection of arrays based on fluorescent antibodies against DON, AFB2 and ochratoxin are at the ppb level (Ngundi et al. 2005, 2006a). The arrays are regenerable (Ngundi et al. 2006b).

2.7.5 Electronic Noses

These sensors are being developed to identify volatile biomarker compounds emitted from grains. Air is pumped from a container with a sample of grains. The volatiles are analyzed by an array of chemical sensors that operate at various temperatures. Every grain has its typical signature of volatiles. Every fungal contamination has its own signature of "off odors" (new patterns of volatiles deviating form the normal ones). The signature depends also on the presence of mycotoxins in the contaminated grains. For example, samples with normal odor have no detectable ochratoxin A and average DON contents of 26 ppb (range 0–80), whereas samples with off-odor had average OTA contents of 76 ppb, (range 0–934) and DON contents of 69 ppb (range 0–857) (Olsson et al. 2002). Electronic noses could differentiate between closely related Penicillia used in cheese production (Karlshøj et al. 2007a), and detect patulin in apples (Karlshøj et al. 2007b).

2.7.6 *Straight from the Baker's Oven: Solid State SsDNA Odor Sensors*

ssDNA 22-mers of ssDNA containing a fluorescent chromophore and dried onto a solid support can interact non-covalently with volatiles, which results in increase in fluorescence. This ability depends on sequence but is unrelated to coding properties. The mechanism of change in fluorescence is unclear, and involves minute changes in the 3D structure of the oligonucleotide (White et al. 2008). The sensor is not monospecific; for example, a single sequence detects 6 ppb of dinitrotoluene, a prescursor of TNT that exsists in land mines, 30 ppm of a precursor of the nerve gas sarin, and ca 34,000 ppm of propionic acid (White et al. 2008). Arrays of sensors, however, can identify the odor signature of volatiles. High throughput microarray techniques enable the screening of hundreds of thousands of oligonucleotide sensors. Volatile precursors of AFB_2, ochratoxin A and DON (Zeringue et al. 1993; Olsson et al. 2002) could be used to identify toxigenic fungi by such odor sensors.

2.8 Conclusion

Mycotoxin detection is a major problem in developing countries where contaminated food commodities may readily reach food stores and homes. The development of sophisticated kits for the detection of minute amounts of mycotoxins is the most important step towards safer foods and feeds in these countries.

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Online Resources

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http://www.r-biopharmrhone.com/pro/afla/afla.html,http://www.charm.com
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