SPECIAL GUEST EDITOR SECTION

Multiplexed Biosensors for Mycotoxins

CHRIS M. MARAGOS
U.S. Department of Agriculture, Agricultural Research Service, Mycotoxin Prevention and Applied Microbiology Research Unit, 1815 N. University St, Peoria, IL 61604

Significant progress has been made in the development of biosensors that can be used to detect low-MW toxins produced by fungi (mycotoxins). The number of formats that have been investigated is impressive and is an indication of the importance attached to finding easy-to-use, accurate, and rapid methods for detecting these toxins in commodities and foods. This review explores the details of multiplexed biosensors based on many formats, including multiplexed immunoassays, suspension arrays, membrane-based devices (flow-through and immunochromatographic), and planar microarrays. Each assay format has its own strengths and areas that need improvement. Certain formats, such as multiplexed immunochromatographic devices, are well developed and relatively easy to use, and in some cases, commercial products are being sold. Others, such as the suspension arrays and microarrays, are laboratory-based assays that, although more complicated, are also more amenable to a larger scale of multiplexing. The diversity of such efforts and the multitude of formats under investigation suggest that multiple solutions will be found to satisfy the need for multiplexed toxin detection.

Immunoassays are widely used to detect individual mycotoxins in a wide variety of commodities, foods, and biological samples. The development of immunoassays for mycotoxins began in the 1970s with radioimmunoassay and ELISAs. Since then, many immunoassays specific for mycotoxins have been developed. Numerous commercial test kits, including many that have undergone third-party validation, are available. Over time, immunoassays have become easier to use and faster, with improved performance characteristics such as sensitivity and solvent tolerance. As our understanding of mycotoxins has improved, and as monitoring programs have been established, the need has arisen to test for larger numbers of toxins in a cost-effective manner. This has driven the development of both single-toxin and multitoxin tests, including biosensors. In the common vernacular, the distinction between "immunoassay" and "biosensor" has tended to erode. Biosensors are characterized by a biological element (recognition element) that interacts with a target analyte. In the case of immunosensors, the recognition element is an antibody or an antibody fragment. Other elements, such as receptors or aptamers, have also been shown to be feasible and are discussed elsewhere in this issue of the *Journal of AOAC INTERNATIONAL*. In addition, there are fully synthetic recognition materials, such as molecularly imprinted polymers, that may also be used. However, the latter are not biosensors because the recognition element is not biologically derived. In this review, most of the examples of biosensors are immunosensors, but in many cases, the underlying format would also be amenable to another recognition element. Biosensors are further characterized by the ability to transform the binding event into a change in a physical property that is detectable (signal transduction). Many biosensors use reagents with labels (enzymes, colored particles, fluorophores, etc.) to facilitate either signal transduction or detection. Lastly, biosensors are characterized by incorporating a detector to quantify the signal (optical, electrical, acoustic, etc.). Given the breadth of research in this area, a number of reviews of the subject have been published that cover different aspects of mycotoxin immunoassays and biosensors (1–9).

Although the variety of biosensors is astonishing, many fall into two basic types: those in which the recognition element is immobilized onto a solid support (well, membrane, fiber, etc.) and those in which a toxin or toxin–protein conjugate is immobilized. The two formats are often referred to as antibody-immobilized or antigen-immobilized, or (less accurately) as "direct" or "indirect" assays. There are also immunosensors for which neither reagent is immobilized. However, multiplexed assays employing the latter have not been widely described for mycotoxins. Of the two immobilization formats, the antigen-immobilized format is, by far, the most commonly used in multiplexed biosensors for mycotoxins (Figure 1). The reasons can be attributed to the nature of the toxins themselves (primarily their low MW) and the detection technologies associated with the biosensors. One advantage of biosensors is their potential for reuse, a characteristic that distinguishes them from single-use ELISA kits. Toxin–protein conjugates tend to hold up better under multiple cycles of use than do antibodies, because generally the activity of antibodies decreases with each cycle of regeneration (cleaning of the surface) between assays. Certain of the label-free sensors also respond better when the toxin or antigen is immobilized, rather than the antibody, an aspect that is discussed in more detail later. The presence or absence of a label is central to the performance of the assay and is a convenient way to classify the many types of biosensors. For this review, the assays are classified into three groups, namely, those using enzymatic labels, those using nonenzymatic labels, and those that are label-free.

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Corresponding author's e-mail: chris.maragos@ars.usda.gov
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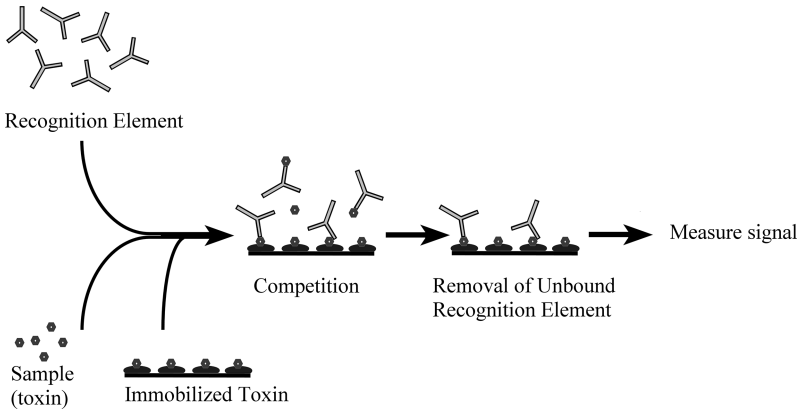

Figure 1. Immunoassay using the antigen-immobilized format. The antigen may be the toxin itself or a toxin–protein conjugate. Certain assay platforms permit real-time measurement of the binding of the primary antibody. For others, such as ELISA, a label (enzyme, quantum dots, fluorophore, colloidal gold, etc.) is attached to the primary antibody either covalently or noncovalently (i.e., with a labeled secondary antibody). The latter requires additional steps to permit binding and then removal of excess reagent (not shown).

Assays with Enzymatic Labels

ELISAs Arranged in Parallel

ELISAs are readily amenable to multiplexing, which can take several forms. To measure the responses from individual toxins but still have a multiplex format, the responses from the individual assays must be discriminated from one another. This discrimination can be accomplished in several ways. The simplest is to conduct the individual assays in separate chambers, e.g., by having a separate ELISA for each toxin in its own microwell and conducting multiple ELISAs in parallel. In this fashion, multiple toxins can be tested on the same microtiter plate. An advantage of arranging ELISAs in parallel is convenience. Even though such an arrangement does not reduce the number of assays conducted, it permits the use of pre-existing tests. The concept could be applied, e.g., to established and validated commercial test kits without changing the reagents used in the kits. Urusov et al. (10) developed a kinetic ELISA for aflatoxin B_1 (AFB_1), ochratoxin A (OTA), and zearalenone (ZEA) in maize and processed poultry products. Toxin–protein conjugates were immobilized into separate wells of a microplate. A streptavidin–polyperoxidase label was used to detect biotinylated mycotoxin antibodies. This format essentially entailed three ELISAs conducted in parallel and took 25 min. The LODs were 0.24, 1.2, and 3 μg/kg for AFB_1, OTA, and ZEA, respectively.

ELISAs based on electrochemical, rather than optical, detection can also be multiplexed in this fashion. Piermarini et al. (11) reported an antigen-immobilized immunoassay for AFB_1, with detection using a microplate reader equipped to measure 96 screen-printed electrodes. The conversion of α-napthylphosphate to 1-naphthol by alkaline phosphatase was detected by intermittent pulse amperometry. Another study used immobilization of an aflatoxin conjugate onto magnetic beads (rather than the electrode itself) and capture of the beads onto the electrode surface (12). Although these two studies tested only for aflatoxin, the formats would seem to be amenable to multitoxin detection. The conduct of ELISAs in parallel has certain advantages, and it seems a simple matter to divide a sample extract among the test wells for the various toxins. It does, however, introduce the possibility of confusion during the handling of the assays, an aspect that might be addressed with automation.

ELISAs Arranged in Arrays

It is possible to multiplex ELISAs without dividing the sample extract into separate chambers. However, to assign the responses to each individual toxin, some form of discrimination between the responses of the individual assays is needed. This has been accomplished by separating the individual reactions on microarray plates or chips, or by conducting assays on microbeads and discriminating the type of beads. An example of the former is an assay for AFB_1 and fumonisin B_1 (FB_1) standards in water using antigens immobilized onto the surfaces of microarray plates or chips (13). More recently, Oswald et al. developed a microarray for the detection of aflatoxins, OTA, fumonisins, and deoxynivalenol (DON) in cereal grains (14). The assay used immobilized derivatives of the toxins rather than immobilized toxin conjugates. The chemiluminescent product from the enzymatic reaction was detected with a charge-coupled device camera. A significant advantage of the technique was automation. Assays took 11 min. With oat extract, the chips were reused 50 times, although a lengthy regeneration step was required. The working ranges in oat extract, defined as the toxin concentrations causing between 20% inhibition (IC_{20}) and 80% inhibition (IC_{80}) are shown in Table 1 (10, 14, 15–48). An intraday study determined good intermediate precision for the method. Recovery rates, determined at two levels for each toxin in oats, wheat, rye, and maize, were generally good. The method was compared to a fluorescence polarization immunoassay (FPIA) method, an ELISA method, and an LC-MS method for DON using certified reference materials and naturally contaminated samples. Although some outliers were observed, agreement among the four methods was generally good. Similar instrumentation was also used in a single-toxin assay for OTA in green coffee extracts (49). A commercial multimycotoxin array was recently released by Randox (Crumlin, United Kingdom). The array can quantify up to 10 mycotoxins, including paxilline; fumonisins; OTA; AFB_1; aflatoxins B_2, G_1, and G_2; diacetoxyscirpenol; DON; T-2 toxin; ZEA; and ergot alkaloids. The assays use a format with mycotoxin antibodies immobilized and are discussed in greater detail in a separate article in this issue. Enzymatic conjugates convert substrate to a chemiluminescent product. After sample extraction and dilution, the extract is applied to the surface of the chip and

Table 1. Selected multimycotoxin biosensors applied to commodities and foods[a]

Label	Format	Detection	Matrix	Toxins and their LOD[b]	Detection range or cutoff value[c]	Analysis time[d]	Reference
Enz[e]	AgI, planar microarray[f]	Lum[g]	Cereals	AFB_1: 0.9 μg/kg OTA: 1.1 μg/kg FB_1: 159 μg/kg DON: 40.5 μg/kg	AFB_1: 2.4–11.4 μg/kg OTA: 1.8–15.5 μg/kg FB_1: 150–2555 μg/kg DON: 72.6–1160 μg/kg	19 min	(14)
Enz	AgI, suspension microarray	Lum	Cereals	AFB_1: 1.19 pg/mL FB_1: 0.60 pg/mL OTA: 0.73 pg/mL	AFB_1: 0.001–1 ng/mL FB_1: 0.001–1 ng/mL OTA: 0.01–1 ng/mL	(over 1 h)	(15)
Enz	AgI, microwell ELISA	Color[h]	M and poultry products[i]	AFB_1: 0.24 μg/kg OTA: 1.2 μg/kg ZEA: 3 μg/kg	AFB_1: 0.25–10 μg/kg OTA: 2–400 μg/kg ZEA: 5–500 μg/kg	25 min	(10)
Enz	AbI, test strip[j]	Color	W[k]	AFB_1: 30 μg/kg T-2: 100 μg/kg 3-acetyl-DON: 600 μg/kg RA: 500 μg/kg[l] ZEA: 60 μg/kg	AFB_1: 30 μg/kg T-2: 100 μg/kg 3-acetyl-DON: 600 μg/kg RA: 500 μg/kg ZEA: 60 μg/kg	(35 min)	(16)
Enz	AgI, LFD[m]	Lum	M flour	FB: 6 μg/kg[n] AFB_1: 1.5 μg/kg	FB_1: 6–15 000 μg/kg AFB_1: 1.5–500 μg/kg	30 min	(17)
Enz	AgI, membrane surface	Color	M	NA[o]	AFB_1: 20 μg/kg ZEA: 60 μg/kg DON: 1000 μg/kg OTA: 20 μg/kg FB_1: 250 μg/kg	10 min	(18)
Enz	AbI, membrane flow-through	Color	Cereal grains and silage	NA	OTA: 2.5–25 μg/kg ZEA: 50–125 μg/kg FB_1: 1000–2500 μg/kg	15 min	(19)
Enz	AbI, membrane flow-through	Color	P cake, M, cassava flour[p]	NA	OTA: 3 μg/kg AFB_1: 5 μg/kg DON: 700 μg/kg ZEA: 175 μg/kg	30 min	(20)
Enz	AbI, column	Color	P cake, M, cassava flour	NA	OTA: 3 μg/kg FB_1: 1250 μg/kg DON: 1000 μg/kg ZEA: 200 μg/kg	30 min	(20)
Enz	AbI, column	Color	Spices	NA	AFB_1: 5 μg/kg OTA: 10 μg/kg	ND[q]	(21)
FL[r]	AbI, microwell SAM[s]	FL	Cereals	DON: 3.2 μg/kg ZEA: 0.6 μg/kg AFB_1: 0.2 μg/kg T-2: 10 μg/kg FB_1: 0.4 μg/kg	DON: 2–29 ng/mL ZEA: 0.06–0.23 ng/mL AFB_1: 0.03–0.26 ng/mL T-2: 2–24 ng/mL FB_1: 0.08–0.89 ng/mL	ND	(22)
FL	AbI, microwell DAM[t]	FL	Cereals	ZEA: 1.8 μg/kg AFB_1: 1 μg/kg	ND	ND	(22)
FL		FL	Cereals	ZEA: 0.02 ng/L AFB_1: 0.01 ng/L	ZEA: 0.06–0.85 ng/L AFB_1: 0.03–0.21 ng/L	ND	(23)
AuNP[u]	AgI, LFD	Color	W	NA	DON: 1500 μg/kg ZEA: 100 μg/kg	10 min	(24)
AuNP	AgI, LFD	Color	W, O, M[v]	NA	In μg/kg: ZEA: 280 (M) 80 (W, O) T-2/HT-2: 400 (M,W, O) DON: 1400 (M, W, O) FB: 3200 (M)	30 min	(25–27)
AuNP	AgI, LFD	Color	P, M, rice	AFB_1: 0.25 ng/mL OTA: 0.5 ng/mL ZEA: 1 ng/mL	AFB_1: 1 ng/mL OTA: 2 ng/mL ZEA: 4 ng/mL	20 min	(28)
AuNP	AgI, LFD	Color	M, W	AFB_1: 0.03 μg/kg ZEA: 1.6 μg/kg DON: 10 μg/kg	AFB_1: 1 μg/kg ZEA: 50 μg/kg DON: 60 μg/kg	15 min	(29)

Table 1. (*continued*)

Label	Format	Detection	Matrix	Toxins and their LOD[b]	Detection range or cutoff value[c]	Analysis time[d]	Reference
AuNP	AgI, LFD	Color	M, W	ZEA: 0.35 ng/mL FB_1: 5.23 ng/mL	ZEA: 0.94–7.52 ng/mL FB_1: 9.34–100.45 ng/mL	15 min	(30)
AuNP	AgI, LFD	Color	M, W, feedstuff	NA	ZEA: 6 ng/mL FB_1: 50 ng/mL	15 min	(31)
AuNP, silver	AgI, LFD	Color	M	NA	FB_1: 2 ng/mL DON: 40 ng/mL	>10 min	(32)
FL	AgI, suspension	FL	Feeds, rapeseed meal	Shown in calibration plots	IC_{50} values (buffer): OTA: 0.29 ng/mL AFB_1: 0.33 ng/mL ZEA: 0.39 ng/mL FB_1: 1.6 ng/mL T-2: 2.2 ng/mL DON: 6.7 ng/mL	ND (>100 min)	(33)
FL	AgI, suspension	FL	M, P	AFB_1: 0.42 ng/kg (M), 0.64 (P) DON: 193 (M), 3.73 (P) T-2: 0.50 (M), 0.32 (P) ZEA: 0.45(M), 77 (P)	AFB_1: 0.07–593 µg/kg (M), 0.04–253 (P) DON: 0.71–1525 (M), 0.16–2284 (P) T-2: 0.01–9.59 (M) 0.01–11.02 (P) ZEA: 0.01–55.35 (M), 0.18–35.72 (P)	4 h	(34)
FL	AbI, suspension	FL	W, M	AFB_1: 0.01 µg/kg OTA: 1.5 µg/kg FB_1: 36 µg/kg T-2: 27 µg/kg ZEA: 1.6 µg/kg DON: 76 µg/kg	Reported as figures	ND	(35)
FL	AbI, suspension	FL	W, M, feeds	OTA: 0.7 µg/kg (M), 3.4 (W) ZEA: 5.8 µg/kg (M), 32 (W) FB: 170 µg/kg (M), 1270 (W)	Reported as figures	50 min	(36)
FL	AbI, suspension	FL	M, W, feeds	ZEA: 0.51 ng/mL FB_1: 6.0 ng/mL DON: 4.3 ng/mL AFB_1: 0.56 ng/mL	ZEA: 0.73–6.8 ng/mL FB_1: 11.6–110.3 ng/mL DON: 8.6–108.1 ng/mL AFB_1: 1.1–14.1 ng/mL	2 h	(37)
FL	Aptamer-immobilized, planar suspension	FL, SPCMs[w]	Rice, M, W	OTA: 0.25 pg/mL FB_1: 0.16 pg/mL	OTA: 0.01–1 ng/mL FB_1: 0.001–1 ng/mL	1.5 h	(38)
FL	AgI, planar suspension	FL, SPCMs	M, P, W	AFB_1: 0.5 pg/mL FB_1: 1 pg/mL CIT: 0.8 pg/mL[x]	AFB_1: 0.001–10 ng/mL FB_1: 0.001–10 ng/mL CIT: 0.001–1 ng/mL	3 h	(39)
FL	AbI, planar suspension	FL	B, malted B[y]	NA	AFB_1: 1.6–2460 µg/kg DON: 440–>4240 µg/kg FB_1: 55–3850 µg/kg OTA: 0.2–25.2 µg/kg T-2: 26–2528 µg/kg ZEA: 10–736 µg/kg	2 h	(40)
FL	AgI, planar microarray	FL	B, M meal, W	DON: 1 µg/kg (M), 180 (W), 65 (B) OTA: 1 µg/kg (M), 60 (W), 85 (B)	Reported as figures	>15 min	(41)
FL	AgI, planar microarray	FL	W	ND	OTA: 0.1–10 ng/mL FB_1: 5–200 ng/mL	>1.5 h	(42)
FL	AgI, planar microarray	FL	Drinking water	AFB_1: 0.01 ng/mL AFM_1: 0.24 ng/mL[z] DON: 15.5 ng/mL OTA: 15.4 ng/mL T-2: 0.05 ng/mL ZEA: 0.01 ng/mL	AFB_1: 0.04–1.69 ng/mL AFM_1: 0.45–3.90 ng/mL DON: 20.2– 69.2 ng/mL OTA: 35.7–363 ng/mL T-2: 0.11–1.81 ng/mL ZEA: 0.08–7.47 ng/mL	4 h	(43)

Table 1. (*continued*)

Label	Format	Detection	Matrix	Toxins and their LOD[b]	Detection range or cutoff value[c]	Analysis time[d]	Reference
FL	AgI, planar microarray	FL	P	AFB_1: 4 pg/mL OTA: 4 pg/mL ZEA: 3 pg/mL	AFB_1: 4–9000 pg/mL OTA: 4–3000 pg/mL ZEA: 3–20 000 pg/mL	2 h	(44)
FL	AgI, planar microarray	FL	W	ND	DON: 400–3000 µg/kg OTA: 3–10 µg/kg T-2: 70–300 µg/kg ZEA: 50–300 µg/kg	20 min	(45)
None	AgI, planar microarray	iSPR[aa]	M, W	DON: 84 µg/kg (M), 68 (W) ZEA: 64 µg/kg (M), 40 (W)	Reported as calibration plots	14 min	(46)
None	AgI, planar microarray	iSPR	P	AFB_1: 8 pg/mL OTA: 30 pg/mL ZEA: 15 pg/mL	Reported as calibration plots	>95 min	(47)
None	AgI, double 3-plex	SPR[bb]	B	DON: 26 µg/kg ZEA: 6 µg/kg T-2: 0.6 µg/kg OTA: 3 µg/kg FB_1: 2 µg/kg AFB_1: 0.6 µg/kg	DON: 26–3200 µg/kg ZEA: 16–160 µg/kg T-2: 0.6–290 µg/kg OTA: 13–320 µg/kg FB_1: 10–1200 µg/kg AFB_1: 3–260 µg/kg	15 min	(48)
None	AgI, planar array	iSPR	B	DON: 64 µg/kg ZEA: 96 µg/kg T-2: 26 µg/kg OTA: 160 µg/kg FB_1: 13 µg/kg AFB_1: 10 µg/kg	DON: 192–4800 µg/kg ZEA: 224–8000 µg/kg T-2: 80–3800 µg/kg OTA: 320–5700 µg/kg FB_1: 48–3800 µg/kg AFB_1: 48–8000 µg/kg	15 min	(48)

[a] Although there are many more biosensors, this table reports only those used for multimycotoxin detection. It includes only reports dealing with one or more food matrices.

[b] Care should be used in interpretation of LOD here. In some cases, this is the IC_{10} or IC_{20}; in some cases, it was determined from the variability of control signals; and in some cases, it was reported in the primary literature, but the basis for the statistic was not.

[c] For quantitative assays, the detection range is the dynamic range over which the assay was reported to function. For qualitative assays, this is the threshold or "cutoff value" at which the assay is configured to give a fully "positive" response (e.g., complete inhibition of color development).

[d] Analysis times should be used with caution because some articles reported the time for the whole analytical procedure, whereas some reported only the time for the final (determinative step). When no value was provided, it was estimated from the length of the steps.

[e] Enz = Enzymatic.

[f] AgI = Antigen-immobilized.

[g] Lum = Chemiluminescence.

[h] Color = Colorimetric.

[i] M = Maize or "corn."

[j] AbI = Antibody-immobilized.

[k] W = Wheat.

[l] RA = Roridin A.

[m] LFD = Lateral flow device.

[n] FB = B fumonisin.

[o] NA = Not applicable. For qualitative or semi-quantitative tests sensitivity is indicated in the adjacent column by the cutoff value or the lower limit of the detection range, which may or may not equal the LOD.

[p] P = Peanut.

[q] ND = Not described.

[r] FL = Fluorescence.

[s] SAM = Single-analyte multiplex.

[t] DAM = Dual-analyte multiplex.

[u] AuNP = Gold nanoparticle.

[v] O = Oats.

[w] SPCMs = Silica photonic crystal microspheres.

[x] CIT = Citrinin.

[y] B = Barley.

[z] AFM_1 = Aflatoxin M_1.

[aa] iSPR = Imaging SPR.

[bb] SPR = Surface plasmon resonance.

incubated for 30 min, followed by addition of the conjugate(s) and incubation for 1 h. The chips are washed, the substrate is incubated for 2 min, and the signal is measured. Regeneration of the chips was not described. The manufacturer reports that the assay can be used with a wide variety of matrixes and that it is currently undergoing evaluation in the AOAC accreditation program.

Although spatial separation is commonly used, other mechanisms also exist to discriminate between assays for the individual toxins in a multiplexed system. Xu et al. (15) developed a method for detecting AFB_1, FB_1, and OTA based on a suspension array using SPCMs. The format was similar to the schematic in Figure 2B, with toxin antigens immobilized. To allow discrimination between the toxins, each antigen was immobilized onto a different type of SPCM. Unlike Figure 2B, the beads were not captured before the addition of the secondary antibody (labeled with horseradish peroxidase; HRP). The SPCMs with attached immunocomplexes were categorized based on their colors or reflectance and then transferred to wells of a 384-well plate. An optical reader was used to detect a chemiluminescent product of the enzymatic reaction (Table 1). Recovery rates ranged from 72.9 to 113.9% for AFB_1, 63.5 to 115.6% for FB_1, and 77.7 to 121.6% for OTA in wheat, maize, and rice. Results from 12 naturally contaminated samples also compared favorably to an ELISA reference method.

Immunochromatographic Devices with Enzymatic Labels

Membrane-based immunoassays are among the most common mycotoxin tests. Two early attempts in this area involved conducting ELISAs with mycotoxin antibodies spotted onto membranes. Abouzied and Pestka (50) multiplexed ELISAs for AFB_1, FB_1, and ZEA on a nitrocellulose membrane. Similarly, Schneider et al. (16) recognized the potential for test strips, and developed a dipstick test for AFB_1, T-2 toxin, 3-acetyl-DON, roridin A, and ZEA in wheat. The format involved immobilizing the antibodies as distinct spots on a treated nylon membrane. The test strip was immersed in a mixture of the relevant toxin–HRP conjugates and the corresponding toxins. These reports demonstrated the potential for detecting multiple mycotoxins simultaneously with membrane-based assays. An antigen-immobilized variation of this format was recently used to detect AFB_1, ZEA, DON, OTA, and FB_1 in cereal samples (18). In this case, the toxin–protein conjugates were immobilized as

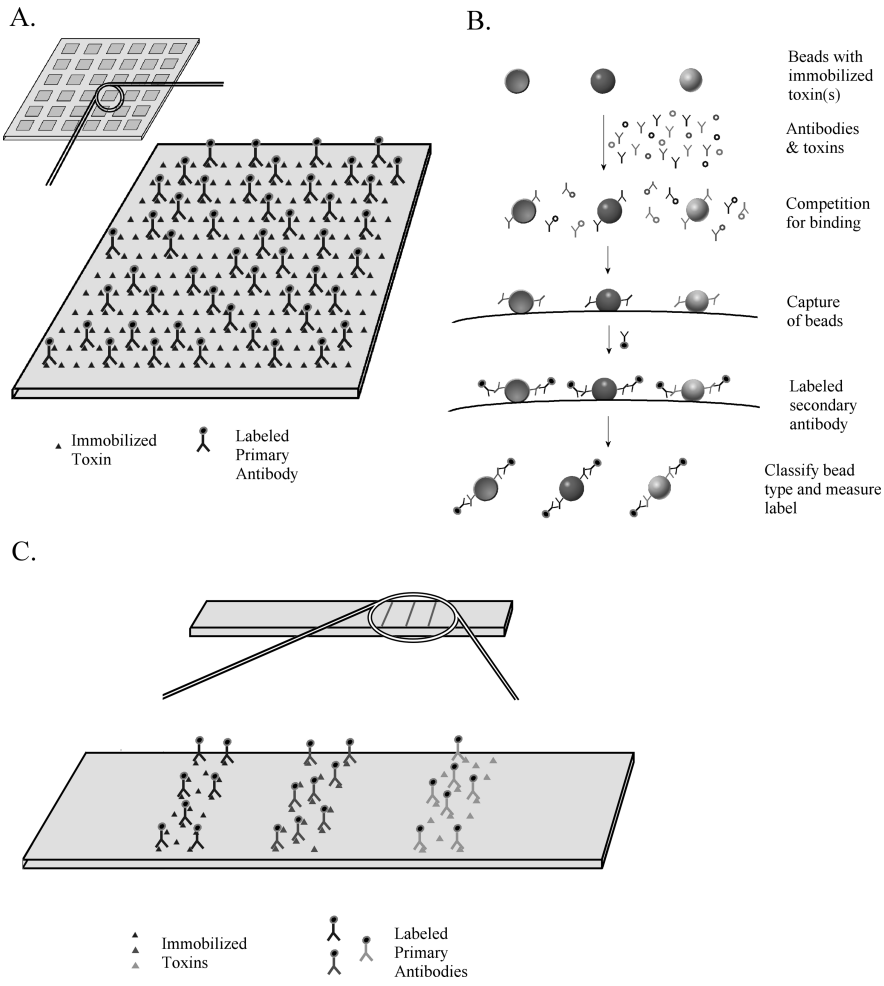

Figure 2. Four types of multiplexed mycotoxin assays, all using an antigen-immobilized format. (A) Planar microarray, (B) suspension microarray, and (C) lateral flow immunoassay (LFIA).

spots on a membrane, separated by a rubber "fence" to provide individual reaction zones. This qualitative test was applied to spiked and naturally contaminated maize samples and compared to results from (quantitative) commercial ELISA test kits.

Two common forms of membrane-based assays are those in which the solutions pass through the membrane perpendicular to the surface (flow-through tests) and those in which solutions pass through the membrane parallel to the surface. The latter are immunochromatographic devices that use capillary action to move fluids through the membrane, which often is a thin layer or strip of nitrocellulose. In addition to providing separation between various elements of the assay, such as bound and unbound antibodies, movement through the device helps to filter out large particulates and to separate smaller matrix components. These aspects of filtration and chromatography enable immunoassays to be conducted on relatively complex matrixes with minimal sample preparation. The combination is a powerful and widely used format for immunoassays, often referred to as LFIA (Figure 2C). The tests themselves are commonly referred to as LFDs or, more colloquially, as dipstick tests. The application of LFIA to mycotoxins and phycotoxins was recently reviewed (6).

Modern LFDs are constructed to take advantage of the separation potential afforded by lateral flow through the device. As described in a later section, most also incorporate nonenzymatic labels for detection. An exception is a recent study in which enzyme-based LFIA was used for detection of AFB_1 and FBs in maize (17; Table 1). Recoveries ranged from 80 to 115%. When applied to naturally contaminated maize flour, the assay showed good agreement with a reference method.

Flow-Through Assays with Enzymatic Labels

As previously mentioned, immunoassays can be configured so that the solutions pass through the membrane perpendicular to the surface. The format is amenable to multitoxin detection, which has been demonstrated in several reports. Burmistrova et al. developed an antibody-immobilized assay for OTA, ZEA, and FB_1 in cereal grains and silage (19). The assay had cutoff values (values giving complete inhibition of color development) that varied depending on the toxin and the matrix tested (Table 1). Although qualitative, responses from these assays compared well to an LC-tandem MS (LC-MS/MS) method. Rather than using flow through a membrane, immunoassays can also be constructed on columns of microbeads. One such device was constructed for AFB_1 and OTA in spices (21). Antibody toward each of the toxins was immobilized in different regions of the column, which also included a layer of aminopropyl-derived silica for cleanup before the detection zones. The absence of color at one or more of the antibody bands indicated the presence of toxin. A comparison of two different flow-through formats (membrane and column-based) was reported (20). The membrane-based assay, using immobilized mycotoxin antibodies, was developed for OTA, AFB_1, DON, and ZEA. The assay was applied to spiked and naturally contaminated samples of peanut cake, maize, and cassava flour (Table 1). With maize samples, the false-negative rates and the false-positive rates for all four toxins were 0%. The column-based (gel-based) assay was constructed for OTA, FB_1, DON, and ZEA (20). As with the earlier work (21), the format was essentially an ELISA conducted within an immunoaffinity column (Table 1). With maize, the false-negative rates were low (0% for DON and FB_1, 5% for ZEA and OTA), and false-positive rates for all four toxins were 0%. Comparing the gel-based and membrane-based assays, the authors indicated a preference for the membrane-based assay, primarily because of its greater ease of use.

Assays with Nonenzymatic Labels

Although enzymatic labels may have an advantage in sensitivity (in that the enzyme can continue to amplify signal over time), nonenzymatic labels generally have the advantage of speed. The difference, of course, is the incubation step required to provide the enzyme with substrate and convert it to product for detection. There is a wide variety of nonenzymatic labels, including colored, fluorescent, or magnetic materials, and others. Well-known examples include colloidal gold, quantum dots, and R-phycoerythrin (R-PE). Substantial research has gone into simplification of immunoassays with such labels, using formats analogous to those described previously for the ELISA-type assays, including microwell-based devices, LFDs, suspension arrays, and microarrays.

Microwell-Based Assays

Several multitoxin immunoassays have been developed that are performed in microwells and use colored or fluorescent labels. In such cases, samples are tested in parallel wells of a plate, with each assay type (i.e., toxin) in a different well. This has been referred to as a SAM. An example is the recent report of an antibody-immobilized assay for DON, ZEA, AFB_1, T-2 toxin, and FB_1 using luminescent quantum dots (22; Table 1). In comparisons with ELISAs, the SAM-based assay was 3- to 12-fold more sensitive. With naturally contaminated cereal samples, good correlations were seen for DON, FB_1, ZEA, and T-2 tested by the SAM method and by LC-MS/MS. The same group also conducted a DAM with two toxins (ZEA and AFB_1) having different quantum-dot labels (22). The assays could be combined in a single well by having quantum dots with different λ_{em}'s and double-scanning of the plate. The DAM assay demonstrated slightly poorer sensitivity than the corresponding SAM assays for ZEA and AFB_1. The same group continued investigations with the use of quantum dots encapsulated in silica-coated liposomes (SLQDs; 23). Again, antibodies for ZEA and AFB_1 were coimmobilized into microwells in a DAM format. Toxin–protein conjugates were immobilized (separately) onto the SLQDs, and the toxins and conjugates competed for the immobilized antibodies. Comparing the IC_{50} values of the assays using SLQDs to the assays using quantum dots revealed an improvement in sensitivity of 4-fold for ZEA and 6-fold for AFB_1. Good recoveries were observed for ZEA and AFB_1 in wheat and maize spiked over the range 0.5–10 μg/kg. Good correlations were seen between naturally contaminated samples tested by the method and by LC-MS/MS. These results suggest that there may be significant advantages to using SLQDs as labels for future immunoassays.

LFDs with Nonenzymatic Labels

To eliminate the steps associated with the enzymatic reaction, most reported LFDs have used nonenzymatic labels such as colloidal gold, quantum dots, or fluorescence. Many

single-toxin LFDs have been developed, but space does not permit their inclusion here. Almost all of the multiplexed LFDs have used an antigen-immobilized format and have used reagents labeled with colloidal gold. The gold colloids are in the nanometer size range, and they are often referred to as AuNPs. In an early work, assays for DON and ZEA were combined on an LFD using primary antibodies labeled with AuNPs (24). Spiking studies with wheat indicated that the LFD gave a clear response to DON and ZEA at the indicated cutoff levels (Table 1) within 10 min. The application of a commercial LFD for multiple toxins in wheat, oats, and maize was investigated (25). The cutoff values were 80% of the European Union (EU) maximum permitted levels. Good agreement was observed between the qualitative and quantitative results from LC-MS/MS, with a false-positive rate of less than 13% and a false-negative rate of 0%. The same group developed an experimental design and in-house validation protocols for multimycotoxin qualitative methods used on cereals (26). The protocol was further applied to an LFD for the determination of ZEA, T-2 and HT-2 toxins, DON, and fumonisins in wheat and maize. The in-house validation design was developed with three steps: (*1*) estimation the precision of the method, (*2*) establishment of the cutoff values, and (*3*) assessment of the rate of false-positive responses. The validation was further extended through the completion of an interlaboratory study of the method in wheat and maize (27), making this the most thorough, published validation of a multimycotoxin LFD. In this study, which involved 12 laboratories, the total SD of the responses varied from 10 to 27% for the analyte/concentration/matrix combinations included in the study. This indicated good ruggedness in use of the assay between different laboratories. Despite unacceptably high false-positive rates for T-2 and HT-2 toxins, the rates were below 10% for ZEA, DON, and fumonisins.

Multitoxin LFDs have recently been a very active area of research (28–32, 51; Table 1). Li et al. developed an LFD for AFB_1, OTA, and ZEA in maize, peanuts, and rice (28). For a combined solution of OTA, ZEA, and AFB_1 (1, 2, and 0.5 ng/mL, respectively), the false-negative rate was 5%, and the results were in good agreement with a reference ELISA method. Song et al. developed an LFD for AFB_1, ZEA, and DON in cereals (29). The color that developed at each of the test lines was evaluated visually (for qualitative analysis) and with an optical density reader (for semiquantitative analysis). Using the semiquantitative approach, the dynamic ranges in maize (IC_{20}–IC_{80}) were reported to be 0.3–15 μg/kg (AFB_1), 9–186 μg/kg (ZEA), and 27–597 μg/kg (DON). In fortified maize and wheat, good agreement was seen between the measured values and the fortified values. Two additional reports described the development of an LFD for FB_1 and ZEA in maize, wheat, and feeds (30, 31). The first of these was a qualitative test applicable to all three matrixes, whereas the second was a quantitative test for the same toxins in maize and wheat using a photometric strip reader (Table 1). Very recently, a multi-LFD was described for detection of 20 mycotoxins from five different groups in cereal food samples (ZEAs, DONs, T-2s, AFs, and FBs; 51). Although AuNPs provide significant color for visual detection, silver can be used to further enhance the signal. The process coats the AuNPs in silver, enlarging the particles and changing the color from red to black, and has been applied to a DON/FB_1 test strip (32). In spiked maize, the treatment improved sensitivity by roughly 2-fold. The technique may be useful in cases in which the improved sensitivity is needed and the additional steps are not an impediment.

Suspension Arrays with Flow Cytometry–Based Detection

Microbead-based suspension arrays can also be constructed with nonenzymatic labels using either toxin/antigen-immobilized or antibody-immobilized formats. An antigen-immobilized version is depicted in Figure 2B. For the signals from the beads to be accurately attributed to the appropriate toxins, the beads must be distinguished from one another. Encoding of the beads can be accomplished in several ways, such as with different colors or by fluorescence emission at multiple wavelengths. An early example of a multiplexed suspension assay was developed for FB_1 and OTA (52). To allow the microbead (microsphere) types to be distinguished, they were manufactured with two fluorescent dyes in various proportions. This is the so-called multianalyte profiling (xMAP) technology. Immobilized onto the microspheres were FB_1 or OTA. The antibodies were tagged with R-PE, which is highly fluorescent and has an emission maximum different from the microspheres. It is unfortunate that the presence of maize or oat matrix caused issues and led to low recoveries. The following year, Peters et al. reported a multiplexed flow cytometric method for AFB, OTA, DON, FB, ZEA, and T-2 in feed, using the xMAP technique (33). Six toxin–BSA conjugates were immobilized onto the microspheres. The format was similar to that shown in Figure 2B, with super-paramagnetic beads that were captured for the washing steps. Although components of the feed matrix caused detrimental effects on certain of the assays, the results with Food Analysis Performance Assessment Scheme reference samples suggest significant potential for the technique as a screening tool. Peters et al. also suggested that finding a suitable multimycotoxin cleanup might improve the applicability of the technique. A variation of the antigen-immobilized format using xMAP was also investigated for four groups of mycotoxins in maize and peanuts (34). Although matrix effects were observed and the assays were time consuming (4 h), they were quite sensitive (Table 1).

An alternative format for the suspension assay, using immobilized antibodies, was described for six groups of mycotoxins in wheat and maize (35). The reporter molecules were the corresponding toxins labeled with R-PE. The operational aspects of the assay were not fully described, so it is unclear how long it actually took. The reader is referred to the original article for estimates of the dynamic ranges for each of the toxins. The antibody-immobilized approach was also investigated for OTA, FB, and ZEA in cereals and cereal-based feed (36). Results with fortified wheat and maize using matrix-matched calibration curves indicated overestimation by 1.5-fold for ZEA in maize, 4-fold for FB_1 in maize, and 3-fold in wheat. OTA tended to be underestimated. Significant overestimations were also seen for fumonisins in naturally contaminated wheat, maize, and feed samples, a result not attributable to the FB_2 and FB_3 contents of the samples, as measured by LC-MS/MS. Rather than attaching the fluorescent label directly to the toxin(s), a variation on the antibody-immobilized suspension assay used R-PE attached to toxin–protein conjugates (37). The assays

were well characterized with regard to LOD and dynamic range (Table 1). Recoveries from maize were good, and a comparison to LC-MS/MS using a small number of commercial samples indicated generally good agreement, without the matrix effects that have challenged other suspension assays.

Suspension Arrays with Planar-Based Detection

Although many of the microbead-based suspension assays use flow cytometers for classification of the beads and interrogation of the fluorescent signal, other means of performing these acts have been described, including the use of instruments that measure from planar surfaces such as slides. One such assay incorporated SPCMs for detection of AFB_1, FB_1, and CIT (39). Toxin–protein conjugates were immobilized onto the SPCMs, and the antitoxin antibodies were labeled with fluorescein. Upon completion of the competition and washing steps, the SPCMs were transferred to the surface of a glass slide. The different kinds of SPCMs were distinguished from each other by using reflectance spectroscopy or bright-field microscopy. The assays were very sensitive (Table 1), and spiking studies indicated good recoveries from peanuts, maize, and wheat. An antibody-immobilized suspension assay for six groups of mycotoxins using planar array detection has also been reported (40). Paramagnetic microspheres were used so that they could be immobilized on a surface with a magnetic field. A transportable system was used to illuminate the beads and detect their fluorescence. Barley matrix had a strong effect on the calibration curves, and matrix-matched calibration was used. The system was capable of detecting all six toxins at the EU maximal levels for unprocessed cereals.

Recently, a novel format of suspension assay, using aptamers rather than antibodies, was applied for the detection of OTA and FB_1 in cereal samples (38). The aptamers were immobilized onto SPCMs, and a DNA strand with sequence complementary to part of the aptamer was labeled with a fluorophore. A complex of aptamer–complementary DNA–fluorophore, immobilized on the SPCMs, was exposed to the sample. In the presence of toxin, the complementary DNA–fluorophore was displaced from the SPCMs, reducing the amount of bound fluorophore. After washing, the SPCMs were transferred to the surface of a glass slide, and fluorescence was determined. Recoveries from spiked rice, maize, and wheat were good, and the method compared favorably with ELISA for detection in naturally contaminated samples.

Planar Arrays

Planar arrays using nonenzymatic labels have been investigated for some time. One such device used evanescent waves to excite fluorophores near the sensor surface (53, 54). The device was used to develop a regenerable array for OTA and DON in barley, maize meal, and wheat (41). The assay was based on an antigen-immobilized format, with detection of Cy5-tagged (fluorescent) antibodies, similar to that depicted in Figure 2A. Significant matrix effects were observed, with greater variability in the negative controls leading to large differences in the LODs (Table 1). DON was determined in naturally contaminated wheat using matrix-matched calibration, and the results generally agreed with an HPLC reference method. An automated version of the assay demonstrated increased LODs relative to the manual version. Using the same general assay format, the investigators also described a multiplexed assay for OTA, AFB_1, FB, and DON in buffer (55). A similar format was used to develop a microarray for DON and AFB_1 in buffer (56). Unlike the aforementioned device that used an evanescent wave to excite the fluorescent label, the latter array used a fluorescence scanner. The antigen-immobilized approach was also pursued in an assay for detection of OTA and FB_1 in wheat (42). A modification was to include the use of a labeled secondary antibody. A similar format was developed for measurement of six mycotoxins in drinking water (43). To improve the uniformity of antigen immobilization and to suppress the nonspecific adsorption of other proteins onto the surface, a sensor coated with poly(ethylene glycol) methacrylate-co-glycidyl methacrylate (POEGMA-co-GMA) was developed (44). The sensor was used to detect AFB_1, OTA, and ZEA. The surface modification permitted elimination of the traditional "blocking" step with inert protein. The array was quite sensitive for AFB_1, OTA, and ZEA in solution (Table 1), and recoveries from spiked peanuts were reported to be good. Rather than an antigen-immobilized assay, Mak et al. reported on a very different type of antibody-immobilized assay for AFB_1, ZEA, and HT-2 in buffer (57). The format was described as a sandwich-type assay. Intriguingly, the investigators appear to have used the same (monoclonal) antibodies to bind to different portions of the toxins to make the sandwich. Because of the small size of the mycotoxins, forming such a complex would be highly unusual. The signal from magnetic nanoparticles (used to label the antibodies) was detected using a giant magnetoresistive sensor, with a detection limit of 50 pg/mL reported.

Recently, a device analogous to that described earlier by Ngundi et al. (41) was made commercially available. This device, the Bayer Quality Analyzer (Bayer Technology Services, Leverkusen, Germany), was used by Tittlemier et al. to detect DON, OTA, ZEA, and T-2 in wheat (45). As with the earlier device, the Bayer instrument uses an evanescent wave in a planar waveguide in an antigen-immobilized format to excite fluorophores near the surface of the chip. In this case, the chip (Micro-Laboratory) is disposable and contains all the reagents needed for the immunoassay (similar to many LFDs). Notably, the technology does not require washing steps, which contributed to the relative rapid assay times (20 min). A two-stage evaluation was performed: a multiday evaluation of accuracy and precision, and an evaluation of performance with 35 wheat samples from Canadian Grain Commission monitoring and research projects. The accuracy and precision of the method for OTA and DON with in-house reference materials and certified reference materials were good, particularly for OTA in the range of 4.2–7.0 μg/kg and DON in the range of 610–1300 μg/kg. Performances for DON, OTA, ZEA, and T-2 were also evaluated with fortified wheats. Generally, good recoveries were reported, but with significant variation (RSDs from 13–41%). The authors concluded that the system performed well in the concentration ranges relevant for the EU regulatory limits of OTA and OTA in wheat, with potential issues at concentrations above and below those ranges. The manufacturer's Web site indicates that chips are also available for DON, ZEA, OTA, AF, and FB in maize (http://www.efmo.org/pdf/BQA.pdf). This advanced system has clear potential for further application in settings in which routine analysis of multiple mycotoxins is required.

Label-Free Assays

A major advantage of using label-free technologies is the avoidance of the time-consuming and oftentimes hazardous procedures for their production. Eliminating the use of labels also avoids impact that the label might have on the relative specificity of the recognition element (58). In many cases, the molecular recognition event can be monitored as it occurs (in real time), which has the potential to be translated into more rapid assays. Recently, a label-free electrochemical aptasensor was described for FB_1 (59). A glassy carbon electrode was coated with AuNPs to which a capture DNA was immobilized. The immobilized DNA was used to capture an FB1 aptamer. The conductivity of the complex was improved by the addition of a graphene sheet–thionine, nanocomposite that attached to the aptamer and could be displaced by FB_1. Thus, FB_1 binding reduced the electrical conductivity of the sensor, which was the basis for detection by cyclic voltammetry. The device was extremely sensitive for FB_1 in phosphate-buffered saline (LOD, 1 pg/mL) and had a very large dynamic range (up to 1 μg/mL). Although applied to a single toxin, the technology would appear amenable to multiplexing.

The label-free technology that has perhaps seen the greatest research effort is SPR. As early as 2003, a multitoxin method was reported (60), and the technology was recently reviewed (58). That review covers many of the variations of SPR. The authors noted that, as of 2014, there was a lack of available devices for detection of multiple mycotoxins simultaneously. The article provides an excellent review of principles of SPR and the extensive literature for detecting single "families" of toxins, which cannot be covered here. Early work with SPR showed the potential for multiplexed detection. Van der Gaag et al. measured AFB_1, ZEA, FB_1, and DON in four serially connected SPR flow cells (60). Derivatives of AFB_1, ZEA, and FB_1 were immobilized onto the surface, as was a DON–protein conjugate. The samples (unfortunately, not identified) were extracted with aqueous acetonitrile and cleaned up over an SPE column, and then diluted and mixed with antibodies before injection into the sensor. Detection limits for the four toxins were 0.2 ng/mL (AFB_1), 0.01 ng/mL (ZEA), 50 ng/mL (FB_1), and 0.5 ng/mL (DON). Despite this early promise, there were no further reports describing application to foodstuffs. Later, Kadota et al. used SPR for the simultaneous detection of nivalenol and DON using an antibody that recognized both toxins (61). Although detecting both toxins simultaneously, this was not a true multiplexed assay because the sensor response yielded an integrated signal, rather than a signal directly attributable to each toxin. However, the technique was useful for detecting both toxins in wheat and compared well to an LC-MS/MS method. A multichannel SPR instrument was used by Meneely et al. for the simultaneous detection of DON and HT-2/T-2 toxins in parallel (62). The DON and HT-2/T-2 toxin determinations were performed in separate channels. Reproducibility and relative responses of calibration curves in wheat extract for both toxins over a 9 day period were reported. R values, obtained from measurements of the IC_{50} values of the assays, were very good for both toxins (10.3 and 6.6% for DON and HT-2, respectively). Recoveries from spiked wheat, wheat-based breakfast cereals, and maize-based baby food were good for both toxins, ranging from 78 to 118%. Excellent correlations were also observed between the SPR results and an LC-MS/MS reference method.

Traditional SPR has been constrained somewhat by performing assays in individual channels, often with a reference channel to control for matrix effects. However, SPR has been combined with imaging technologies that allow multiple sites (regions of interest) within a flow cell to be rapidly interrogated. The result is iSPR (or SPRi), whereby SPR is performed at multiple locations in a flow cell, such as spots on an array. The technique was applied to the detection of DON and ZEA in maize and wheat (46). The number of samples tested was extremely small (two of each), and matrix effects were observed. However, when matrix-matched calibration was used, results compared favorably to LC-MS/MS. More recently, the technique has been attempted with modifications that include using a POEGMA-co-GMA polymer "brush" and a secondary antibody labeled with AuNPs (47). The POEGMA-co-GMA brush was described in an earlier section of this review (44; *see* Table 1). The same three toxins were used as in the earlier work (AFB_1, OTA, ZEA). The use of a secondary antibody with AuNPs increased the signal about 45-fold relative to that from the primary antibody alone. Although still very sensitive, the iSPR assay was approximately 2- to 7-fold less sensitive than the fluorescence-based microarray. Not shown in Table 1, the calibration plots in the source article give an indication of dynamic range. The impact of matrix effects on the technique was not reported.

Recently a comparison was made between a "traditional" SPR method and an iSPR method for the same set of six mycotoxins (48). The SPR method was constructed to allow testing of two chips in parallel, with each chip having four regions arranged serially to allow for measuring three toxins along with a control; hence, it was termed a double 3-plex assay. The detection ranges of the DON, ZEA, T-2, and FB_1 assays using the double 3-plex format allowed measurement at the EU regulatory limits (Table 1). Measurements of inter- and intraday precision for spiked barley samples indicated significant potential for use of the double 3-plex assay as a semiquantitative screening assay. Much of the knowledge gleaned from the double 3-plex assay was used in the design of a prototype iSPR method that included a portable instrument. As described in Table 1, assays with the iSPR were about 2- to 50-fold less sensitive than with the traditional SPR, which was attributed, in part, to differences in surface chemistries between the two types of chips and differences in sensitivities between the two instruments. The results suggest the clear potential of the technique for further development.

It is unfortunate that space does not permit full discussion of all of the potential technologies that could be applied to multitoxin analysis, and the author apologizes for not being able to include all of the reported biosensor formats. However, three newer technologies must be briefly mentioned. The first of these is a cantilever-based biosensor that was developed for fumonisins but looks amenable to multiplexing (63). The second is a multiplexed fluorescence resonance energy transfer aptasensor for OTA and FB_1 (64). Third, a multiplexed FPIA was very recently developed for detection of DON, T-2 toxin, and FB_1 in maize (65). The latter involves a homogeneous immunoassay format and is, therefore, unlike many of the other sensors discussed herein.

Conclusions

As indicated by the number of articles in Table 1, research on multiplexed sensors for detection of mycotoxins has expanded rapidly within the past 5 years. The term biosensor, as used

here, includes many different assay formats. Because of the many formats, discussion of the various sensors is necessarily complex. Because the detection principle (optical, electronic, etc.) is fundamental to understanding the formats, the biosensors were categorized according to the type of label that was used in the assay (enzymatic, nonenzymatic, or no label). The detection technology and the type of label used are key parameters that determine performance characteristics such as speed, ease of use, sensitivity, robustness, and whether the assay is qualitative or quantitative. Given such diversity of format, generalizations are difficult, and for every generalization, there is likely an exception. However, certain traits are common among the various platforms. Biosensors that have been developed using enzymatic labels tend to be sensitive because of the potential for significant amplification provided by the enzyme. They also tend to use commonly available optical readers that are relatively inexpensive. The downside to assays using enzymatic labels is that most require additional steps (washing, adding substrate, and adding stop reagent) that are avoidable with certain other formats. These steps add time, and this is one reason for the continued development of nonenzymatic labels and label-free devices. Perhaps because it is difficult to quantify, ease of use is too often ignored as a parameter for evaluating performance. Yet for field-portable tests, this is a very important criterion. Within this context, the LFDs using AuNP labels have a clear advantage, and this is one format that is the furthest along toward widespread use. One difficulty with multiplexed LFDs has been quantification, which has been addressed through the use of optical readers. Certain of the biosensor formats are more amenable to multiplexing than others. The basic reason for this is derived from the ability to discriminate between, and attribute responses to, the various toxins. Certain formats, such as suspension arrays (in which discrimination can involve many types of microbeads and, therefore, many toxins) and microarrays (in which discrimination is accomplished spatially), are well positioned to accommodate analysis of a large number of toxins. Further, they offer the potential for expansion as more analytes of interest are added. However, at this point, such formats tend to require expensive, laboratory-based equipment that requires significant technical training to operate. This is an aspect that must improve if such assays are to leave the laboratory and become field portable. Several commercial devices that were described represent excellent steps in this progression. Given the intense interest in developing improved multiplexed assays for mycotoxins and the quality of existing efforts in this area, there is reason for hope that many of the aforementioned assays will make the transition into the field and become the next generation of widely used tests.

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