Immunochemical methods for mycotoxin analysis: from radioimmunoassay to biosensors

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Introduction

Immunochemical methods have been used in diagnosis of diseases both for humans and animals for many years. However, wide application of these methods for analytical purpose was not initiated until the nineteen fifties when Yalow developed a radioimmunoassy for insulin[1]. In addition to the specific ***antibody***, the modern immunochemical methods use a sensitive ***marker*** for the analyte and a good ***method*** to separate the bound and free analyte in the specific antigen (Ag) and antibody (Ab) interaction. The assay systems became a new highly sensitive, specific and simplified versatile tool for the analysis of many biologically active substances, including low molecular contaminants such as mycotoxin. In the last four decades, new approaches to make sensitive markers have been made. For example, by conjugating the enzymes to the Ag or Ab to amplify the signal, more sensitive and versatile enzyme-immunoassay systems have been developed. With the availability of sensitive instruments, more fluorescence-tagged markers are now being used. Simplified and effective methods for the separation of free and bound species are now available. In addition to using animals for Ab production, monoclonal Ab technology was introduced. Such developments have led to a wide application of immunoassays for the analysis of contaminants in foods and agricultural products. Recent development of novel separation techniques and sensitive detection systems have led to the development of several immunochemical based biosensors. Thus, a new dimension of immunoassay system is now emerged. Whereas many types of immunoassays are now available for mycotoxins, most approaches are based on the competition of binding between unlabeled toxin in the sample and labeled toxin in the assay system for the specific binding sites of Ab molecules. Because of limited space, this review will focus only on important developments and applications of immunoassays for mycotoxins. For details, the readers should consult the original papers and recent reviews both on the overall analytical methods[2-9] and immunoassays for mycotoxins[10-18].

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Development in preparation of immunochemical reagents for mycotoxin analysis

Preparation of immunogens Mycotoxins are low-molecular-weight haptens and they are not immunogenic. They must first be conjugated to a protein/polypeptide carrier before subsequent use in immunization for antibody production. Mycotoxins with reactive group can conjugate to a protein directly. However, introduction of a reactive group is necessary for most mycotoxins[8-10, 17]. Whereas water-soluble carbodiimide and mixed anhydride methods are most commonly used for the conjugation, other methods, including Mannich condensation method in the presence of formaldehyde, cross-linked with glutaraldehyde and the activated ester method such as the formation of N-hydroxysuccinimide (NHS) esters, 1,1'-carbonylimidazole and m-maleimidobenzoyl-N-hydroxysuccinimide ester, have also been used. Recent developments on methodology for the conjugation of agricultural and food-related haptens have been shown in several reviews[14, 15, 17]. In view of wide application of immunochemical methods for low molecular weight compounds, most of these reagents are now commercially available.

Production of antibodies (Ab) Both polyclonal (pAb) and monoclonal (mAb) antibodies have been used for immunoassays. While rabbits are still commonly used as the animal species for the production of pAb, other animals such as goats, pigs and sheep have also been used. Useful pAb can also be recovered from eggs (IgY) after immunizing hens. Another approach to generate pAb involves immunizing BALB/c mice with immunogens and then collecting the antibodies from ascites fluid. With the maturity of hybridoma techniques, cell lines capable of continuously generating mAb with unique specificity against various mycotoxins are now available[14-17]. Rapid progress in antibody cloning has also led to the application of this technology in agriculture and foods. Several laboratories have initiated work in cloning the antibodies against aflatoxin (AFB) and zearalenone (ZE) with some success[17, 19-22]. Because the affinity of the cloned antibodies was not as high as the original mAb, such antibodies are still not widely used.

An alternative approach for preparing immunochemical reagents is through generating anti-idiotype antibodies (Ab2). Ab2 for AFB_1[23, 24], FmB1[25] and T-2 toxin[26] have been generated and they can be as the surrogate for production of Abs against mycotoxins. For example, instead of using AFB-BSA conjugate as the immunogen, the Ab2 for AFB has been used as the immunogen to generate anti-anti-idiotype antibodies (Ab3) with specificity similar to the original antibody (Ab1)[25]. It should be reiterated that the antibodies generated by any of these methods described above should be well characterized for their specificity.

Antibodies against the following mycotoxins have been generated: AAL Toxin (pAb), aflatoxin B_1 (AFB), (pAb & mAb); AFG, AFB_{2a}, AFQ, AFB-DNA, AFM (pAB, mAb), citrinin (pAb), cyclopiazonic acid (CPA, mAb, pAb), ergot alkaloids (mAb, pAb), fusarochromanone (pAb), fumonisin B1 (FmB1, mAb, pAb); kojic acid (pAb), ochratoxin A (OA) (mAb, pAb); patulin, (pAb), paxilline related (mAb, pAb), PR-toxin (pAb), rubratoxin B (pAb), secalonic acid (pAb), sporidesmin (mAb, pAb), sterigmatocystin (pAb), diacetoxyscirpenol (pAb), deoxynivalenol (DON, mAb, pAb), FX, DOVE, AcDON, nivalenol (NIV), roridin A, T-2 toxin and its metabolites HT-2, T-2-tetraacetate, 3'-OH-T-2, dep-T-2, (pAb), versicolorin A (pAb) and zearalenone (mAb, pAb)[14, 17]. In addition, Abs against specific groups of fungi and key enzymes involved in the biosynthesis of AFB and TCTC have been produced and these Abs have been using in cloning genes involved in the biosynthesis of these toxins. Details regarding

their specificities can be seen from the original papers and from several reviews[14, 17]. Whereas most of these Abs are very specific for the respective mycotoxins, they do cross react with the analogues to some degree. Thus, one should clearly understand the cross-reactivities of the generated antibody to the analogues of the parent mycotoxin before selecting an Ab preparation in the immunoassay.

Preparation of assay markers The availability of an effective marker plays an important role in developing of immunoassay protocols to be used both in characterizing Ab and developing of assay protocols. Radioimmunoassay (RIA) was developed in the early work. Tritiated, carbon C-14 and I-125 labelled mycotoxins or mycotoxin derivatives were used as the markers. The sensitivity of RIA is greatly dependent upon the specific radioactivity of the original radio-nuclides used. Methods for the preparations of different radioactive mycotoxins and their derivatives appeared in several reviews.

Enzyme-linked immunosorbent assay (ELISA) is one of the most common methods used for the determination of mycotoxins in foods. While horseradish peroxidase (HRP) is most commonly used in the ELISA, other enzymes such as alkaline phosphatase and others have also been used[14, 17]. Conjugation of mycotoxins to a marker enzyme (most commonly) or to antibodies was generally done via the periodate oxidation with subsequent reductive alkylation method or by cross-linking using glutaraldehyde. Water-soluble carbodiimide and NHS methods have also been used. To alleviate the problem that the antibodies may have non-specific cross-reaction with the residues in the approximate linking-bridge region for the protein/hapten with the enzymes, methods or carrier proteins different from those used in the preparation of conjugates for immunization are often used in the immunoassays. With the availability of instrumentation, fluorescence-tagged markers are used both in heterogeneous and homogeneous systems. The most common approach involves the preparation of a fluorescein isothiocyanate (FITC)-tagged marker.

Development of immunoassay protocols for mycotoxins

Radioimmunoassays (RIA) RIAs are commonly used in most earlier work for mycotoxin analysis and have been used for the analysis of AFB in corn, wheat, peanuts, milk, serum, and eggs as well as for DON in corn and wheat, OA in serum and kidney, nivalenol NIV in barley, PR toxin in cheese, and T-2 toxins in corn, wheat, serum, and urine. Generally, RIA can detect 0.25-0.5 ng of purified mycotoxin in each analysis. Because of the sample matrix interference, the lower limit for mycotoxin detection in food or feed samples is about 2-5 µg/kg. Higher sensitivity can be achieved by using iodinated-mycotoxin marker (0.004-0.1 ng/assay), by clean-up of the sample, and by using radioactive markers of high specific activity[17, 27]. As newer solid-phase matrices and more immunochemical reagents became available, more efficient methods for the separation of free and bound toxin were developed[17, 27]. Thus, separation can be achieved by a simple filtration or centrifugation step. Although RIA is very simple, sensitive and specific, the use of radioactively labelled mycotoxin hinders its wide applications. Nevertheless, this method is still used by some laboratories. For example, an improved RIA was recently developed for AFM in milk[28].

Enzyme immunassay (EIA) Enzyme immunoassay involves use of an enzyme as a marker to detect the immuno-complex. Since an amplification is incorporated into EIA, the assay is more sensitive than RIA and also avoids the problems encountered in handling radioactivity. Depending on

whether or not the immunocomplex is separated from the free Ag, several types of EIA formats are available. The most common EIAs used for mycotoxin are the *direct competitive* ELISA (dc-ELISA) and *indirect competitive* ELISA (idc-ELISA). Both types are heterogenous, involving the separation of free and bound Ag-Ab. Solid-phases such as microtiter plates, cellulose, nylon beads/tubes, nitrocellulose membrane, polystyrene tubes/balls, and modified magnetic beads, etc. have been used.

1) Direct competitive ELISA (dc-ELISA) In this assay, specific ***Abs*** against mycotoxins are coated on the solid phase such as an ELISA plate. The sample or mycotoxin standard solution is generally incubated simultaneously with enzyme-toxin conjugate or incubated separately in two steps. The amount of tagged-enzyme bound to the plate is then determined by incubation with a chromogenic substrate solution. The resulting color/fluorescence, which is inversely proportional to the mycotoxin concentration present in the sample, is then measured instrumentally or by visual comparison with the standards. In this assay, the mycotoxin-enzyme conjugate (marker) and free mycotoxin compete for the same binding site on the solid-phase antibody. Excluding the time for sample preparation, *dc-ELISA* generally can be completed in 0.5-2 hours. In general, *dc-ELISA* is approximately 10-100 times more sensitive than RIA and as little as 2.5 pg of pure mycotoxin can be measured. Since a clean-up step is usually not necessary, many samples can be analyzed within a relatively short period. It can detect 0.05 - 50 μg/kg of mycotoxins in foods and feeds[3, 4, 14, 17]. Like RIA, the sensitivity of ELISA can be improved with a clean-up treatment for the sample[14, 17]. By selecting better Ab and toxin-enzyme conjugate, the entire *dc-ELISA* procedure can be completed in less than one hour[12, 13, 14, 17, 27]. *dc-ELISA* is one of the most common protocols currently being used for immunoassay of mycotoxins. The application and sensitivity of *dc-ELISA* for selected mycotoxins in different commodities is summarized in Table 1[14, 17].

2) Indirect competitive ELISA (or double antibody ELISA) In the indirect competitive (idc-ELISA), a ***mycotoxin-protein*** (or polypeptide) conjugate is first prepared and then coated to the microtiter plate. After incubation with specific rabbit (or other type) Ab in the presence or absence of the homologous mycotoxin, the amount of rabbit-Ab bound to the plate coated with mycotoxin-protein conjugate is then determined by reaction with goat anti-rabbit (or anti-other type) IgG-enzyme complex (which is commercially available) and by subsequent reaction with the substrate. Thus, toxin in the samples and toxin in the solid-phase compete for the same binding site with the specific Ab in the solution. The *idc-ELISA* has also been widely used for the analysis of a number of mycotoxins[14-17] with a sensitivity comparable to or slightly better than the dc-ELISA. idc-ELISA requires less Ab (100 times less) and does not require preparation of a toxin-enzyme conjugate. However, it takes more analytical time (2 hours). To optimize the assay, selection of secondary antibody-enzyme conjugate in the *idc-ELISA* is important. For example, in a mAb-based ELISA for AFM_1, the HRP-labeled anti-mouse antibody was 50 times more sensitive than in the alkaline phosphate-labelled system. Improvement of *idc-ELISA* can also be made by using affinity purified mycotoxin-conjugate and flourescent substrate. Two modifications have been made to shorten the assay time for idc-ELISA. One involved the conjugation of Ab to an enzyme, which is then used in the ELISA instead of using a second antibody-enzyme conjugate, and the other involved premixing the Ab with the second antibody-enzyme conjugate before the assay[14-17]. The application and sensitivity of *idc-ELISA* for mycotoxins in different commodities are also shown in Table 1[14, 17].

Table 1. Sensitivity of direct and indirect competitive ELISA for selected mycotoxins[a,b]

Mycotoxins	Foods/Feeds	Detection limits (μg/kg) or (μg/L)	
		dc-ELISA	idc-ELISA
AFB/AFs	C, F, S P, Pb, Rs, Wh	1-10 (1)[c]	0.25-5
AFM	M, Ch	0.10 (0.01)	0.005
CPA	C, P, MF	(0.05-0.1)	-
DAS	C, Wh	-	300 (1)
DON	C, Wh	1000 (10)	1000 (10)
3-AcDON	B, R	16	1
15-Ac-DON	Wh	50-100	-
Fm	C, MF, M, BR	10-500	200 (50)
H-Fm	C	5-10	-
NIV	B	-	(30)
OA	B, K, MF, S, Wh,	30 (1-2)	0.06-50 (1)
ST	S, U, W	-	0.01-5 (0.05)
T-2	C, M, S, U, W, Wh	2.5-50 (1)	5 (0.2-1)
HT-2	U	-	(0.5)
Type A TCTC	C	50-100	-
ZE	C, W, MF	50 (10)	1-60

[a] For detailed references, see Chu[14, 17].

[b] Abbreviations used: B, barley; BR, beer; C, corn; Ch, cheese; F, figs; H-Fm, hydrolyzed Fm; K, kidney; M, milk; MF, mixed feed; P, peanuts; Pb, peanut butter; R, rye; Rs, raisins; S, serum; Wh, wheat; U, urine.

[C] Values in parenthesis are for samples that had been subjected to a clean-up treatment before immunoassay.

3) Considerations in improvement of both dc- and idc-ELISA The efficacy of both dc- and idc-ELISA for the analysis of mycotoxins has been studied extensively by comparing data obtained with HPLC and TLC methods. Whereas good correlation has been found in most immunoassays[13-17], problems do exist for some other assays. For example, data obtained from ELISA of Fms were always higher than those obtained from chemical analysis[29]. This problem was attributed to the cross-reaction of the Abs with some structurally related compounds. Once high affinity Abs were used, the non-specific interaction was minimized[17, 29].

In addition to select Ab with appropriate affinity to the mycotoxin/mycotoxin-marker and an enzyme-marker with high enzyme specific activity and stability, efficacy of both ELISAs can be improved by: (i) using an alternate substrate, (ii) avoiding matric interference, and (iii) avoiding excess extraction solvent. For example, biotin-avidin interaction has been used to amplify signal. Tetramethylbenzidine and fluorescent substrates have been used to enhance the sensitivity. As mentioned above, the sensitivity can always be improved with a clean-up for the sample. Nevertheless, such treatment is not necessary if high sensitivity is not required for a certain assay. The threshold of solvent concentration affecting ELISA should always be determined in the presence of sample matrix prior to the assay. Excess solvent may not only interfering ELISA, it may also carry more interference substances from the sample. For example, more dilution was necessary for corn samples extracted with acetonitrile than those extracted with methanol in the ELISA of hydrolyzed FmB1. However, neither 10 % methanol nor acetonitrile affect the assay significantly in a mAb-based ELISA for

FmB1[14, 17, 29, 30]. Although ELISAs could run in a system containing as high as 20-30 % of methanol [14, 17], samples containing 7-10 % methanol were used in most assays.

Immunoscreening (IS) methods Progress in developing rapid IS methods for mycotoxins has been made in the last decade. By shortening the incubation time through adjusting the Ab and enzyme concentrations in ELISA, the assay now can be completed in less than 30 min.[2-17]. For simple operation, the Ab is immobilized on a paper disk or other membrane which is used directly as a strip or mounted either on a plastic card (card screen test), on a plastic strip (as dipstick), in a plastic cup, or in a syringe, or onto polystyrene beads instead of microtiter plate. The reaction is carried out on the wetted membrane disk. After reaction, the absence of color (or decrease in color) at the sample spot indicates the presence of toxin in the sample. The reaction is generally very rapid and takes less than 10-15 minutes to complete. Such IS methods have be made available for AFB, DON, 3-acetyl-DON, FmB, OA, T-2 Toxin, ZE and *Penicillium islandicum* in different commodities[17]. A multiple testing strip with detection limit of 0.5, 500, and 3 ng/mL for AFB_1, FmB1 and ZE, respectively was also developed[31]. Based on the catalyzed reporter deposition and using a synthesized electron rich protein with multiple phenolic groups as blocking agent, a Super-CARD ELISA system was developed for AFB[32]. The sensitivity of the assay increased about 5-fold and was completed in about 15 minutes.

Another screening test is the immunoaffinity method, which was originally designed for mycotoxins such as AF, OA, and ZE that fluorescence[2, 3, 14, 17, 33, 34]. In this assay, sample extracts diluted in phosphate buffer are applied to the affinity columns in which specific antibody was covalently bound to the solid-matrix. After washing to remove the unbound materials, the specific mycotoxin is then eluted from the column with the appropriate solvent system and then subjected to other chemical analyses. For mycotoxins with native fluorescence such as AF, OA and ZE, the toxin level in the eluate could be directly determined fluorometrically or be determined after derivatization to enhance the fluorescence[17, 34]. For FmB and DON screening, it is necessary to introduce a fluorophore to the materials eluted from the immunoaffinity column (IAC)[13, 14, 34]. The sensitivity to the IAC screening tests for AFB_1, FmB1, OA and ZE is 2 µg/kg, 1 mg/kg, 5 µg/kg and 0.2 mg/kg, respectively.

The application of various IS tests for mycotoxins has been shown in several reviews[2-6, 9-17, 34]. Kits for ELISA and IS tests are commercially available[14, 17] and all of them permit monitoring of mycotoxins semi-quantitatively. The effectiveness of these kits for screening mycotoxins in the field was examined by FSIGS as well as documented in many studies[7, 9, 14, 17, 34]. Collaborative studies for some ELISA protocols and IS methods in various commodities have been conducted and some of them have been adopted by the AOAC International as first action[7, 9, 14, 16, 17, 34-38].

Complementing chemical analyses by immunochemical methods

Immunoaffinity methods With the availability of Abs against most compounds discussed above, immunoaffinity columns (IACs) were made by conjugating them to a solid-phase matrix[34]. These columns are then used either in a screening test as discussed above or as a clean-up column for subsequent chemical analysis. Since we first used this approach in the RIA[33], the IAF columns have gained wide application as a clean-up tool for a number of mycotoxins and are not limited to fluid samples[14, 17]. Immunoaffinity columns for a number of mycotoxins are also now commercially

available. Affinity columns for AFB, AFM, OA, FmB, CPA, and ZE have been made as the cleanup column before HPLC, including incorporation of such column for automation, or TLC analysis of these mycotoxins in foods[17, 34, 39]. IACs have also been made for the concentration of AFB-DNA adducts, AF metabolites, and AFB-albumin adducts from body fluids before any other chemical or immunoassays are done. A number of collaborative studies indicate that this technique is an efficient method for clean-up of AF[14, 17, 34-38]. Applications of IAC for various mycotoxins are summarized in Table 2. Details on this technique have been extensively reviewed[2, 6, 7, 9, 14, 17, 38].

Table 2. Immunoaffinity chromatography of mycotoxins[a]

Mycotoxins	Commodities	Analysis
AFM	M[b]	Fl[c]
AFB	F, P	TLC
AFB	Cff, P, nuts, figs, etc	HPLC/PsCD
AFs	C,P,Ct,F,Pb, BR	Fl/Br; HPLC/PsCD
AFL	S, U	HPLC
AFQ_1	U	HPLC
AF-adducts	T, S, U	HPLC, ELISA
AF-albumin	S	HPLC, ELISA
CPA	C, P, feed	ELISA
FmB, HFms	C, starch	HPLC; LC/MS
OA	Cff, T, W, C, So	HPLC, LC/MS
OA	Cff, C, So	CE
ZE	M, U, C	ELISA, HPLC, MS

[a] For detailed references: See Chu[17].
[b] Commodities tested: BR, beer, Cff, coffee; post column derivatigation; T, animal tissues; So, sorghum; other abbreviations are described in tables 1.
[C] Methods for final analysis: Fl/Br and Fl represent fluorometric analysis of the solution eluted from the column with and without treatment with bromine solution, respectively.

Combination of immunoassay with other chromatographic methods Immunoassay has proved to be an effective tool as a post-column monitoring system in HPLC[40]. This is especially useful for the analysis of compounds with no specific absorption such as the trichothecene (TCTC) mycotoxins. In the analysis of various type A TCTC mycotoxins, the sample extract with no clean-up treatment was subjected to HPLC with a C-18 reversed-phase column. Individual fractions eluted from the column were analyzed by ELISA using “generic” antibodies. Thus, an ELISA chromatogram was obtained. This approach can not only identify each individual group A TCTC, but can also determine their concentration quantitatively. As little as 2 ng of T-2 toxin and related TCTCs as well as their metabolites can be monitored by this method[41, 42]. A combination of HPLC and ELISA technology proved to be an efficient, sensitive, and specific method for the analysis of TCTC[41, 42], ST[43] and AAL[44]. Likewise, ELISA has been used in combination with TLC and fractions collected from TLC were analyzed immunochemically[45, 46]. In another approach, the chromatogram obtained from high performance TLC was blotted onto a nitrocellulose membrane coated with antibody. After incubation with mycotoxin-enzyme conjugate and substrate, the color was developed[47]. This method has good sensitivity for multiple mycotoxins, but the need for a large amount of antibody limits its wide application.

Combination of immunofluorescence and capillary electrophoresis (CE) This assay is based on separation of the competition between binding of the bound- and free-mycotoxins by CE and using a fluorescein-tagged toxin, (e.g.FL-FmB) as the marker[48, 49]. In the analysis of FmB, purified FL- FmB was subjected to CE. Addition of purified mAb to FL-FmB before separation resulted in ***quenching*** of fluorescence and decreasing the intensity of the FL-FmB peak as the formation of mAb-FL-FmB complex. When FmB is present, it competes with FL-FmB for binding of mAb and causing an increase in FL-FmB peak. The IC_{50} of unlabeled FmB was highly dependent upon the Ab concentration and ranged from 58 to 4170 ng/ml (at 15-75 µg/ml of antibody). The method is rapid and requires only 6 min for complete analysis of FmB standard. Since both AFB and OA exhibit fluorescence, Ab was not used in the CE for these mycotoxins. Instead, IACs were used as the cleanup tools in the assay[50, 51].

Development of immunochemical based biosensors

Although development of An/Ab-based biosensors for mycotoxin was initiated in the late nineteen eighties, application of this technology only emerged in recent years. The principles used for designing the biosensors are similar to various immunoassay discussed earlier. For example, the idc-ELISA principle was used in the so-called "hit-and-run" assay[52] for T-2 toxin. A column packed with gel conjugated with T-2 toxin was equilibrated with FITC-labeled Fab fragment of IgG (anti-T-2 toxin). After injection of a sample containing T-2, the FITC-Fab was eluted from the column. Likewise, ribonuclease-labeled Fab was also used as the marker[53] in this system. In a homogeneous immunoassay for T-2 toxin, liposomes and complement[54] were used. However, the sensitivity of these systems were lower than the regular ELISAs for mycotoxins. With the availability of sensitive detection systems, high affinity Ab and improvement in preparation of marker ligand, several biosensors developed in the last few years have shown some promise for wide applications[18, 55] (see Table 3).

Table 3. Sensitivity of immunosensors for selected mycotoxins[a, b]

Immunosensors[a]	Detection	Detection limit (µg/kg or µg/L)		
		AFB	DON	FmB
FOEW	Fl & EW	-	-	10 (10-1000)[b]
LIA-flow type	Fl & Liposome	-	-	(1-1000)
LIA-strip type	Fl, Liposome, visual screening	20	-	-
TRFIA	Fl, Europium complex	0.01 (0.001-2)	-	-
API	Fl	4.0	-	-
IAFB	Fl	0.1 (0.1-50)	-	-
FPA	Fl, FP	(5-200)	(1000-20,000)	100
SPR	SPR, BIAcore	3 (3-98)	2.5 (130-10,000)	50

[a] Abbreviations used: FOEW, fiber-optic-evanescent-wave; LIA, liposome immunosensors; TRFIA, Time-resolved fluoroimmunoassay; API, automated particle-based immunosensor; IAFB, immunoaffinity fluorometric biosensors; FPA, fluorescence polarization assay; SPR surface plasmon resonance. Also see tables 1 & 2.

[b] Values in parenthesis are the assay range.

Biosensors based on direct competitive immunoassays Similar to the dc-ELISA, the Abs are coupled in the solid-phase and a marker conjugated to mycotoxin are used in the following biosensors.

1) Fiber-optic-evanescent-wave (FOEW) immunosensor In this system, Abs are bound to an optical fiber and an evanescent wave effect was utilized to excite the flourescent-tagged toxin near the surface of the Ab-fiber as the tagged toxin bound to the fiber[55-58]. In the assay, toxin in the sample competes with the labeled toxin for binding with Ab, resulting in a decrease of signal. The assay involves: (i) saturation of Ab binding sites with the labelled mycotoxins, (ii) displacement of the labeled toxin by the toxins in the samples, and (iii) resaturation of Ab binding sites with the marker. mAb and-FITC-FmB1 were used in the FmB1 assay[57, 58]. The sensor was also capable detecting AFB in absence of Abs non-specifically. Since sample clean-up was necessary, the non-specific problem could be overcome by using an IA column. Thus, the FOEW method could be applied to other mycotoxins such as OA & ZE[18, 57-58]. Similar to FOEW, optrodes containing an immobilized reagent were developed for AFB detection[59]. AFB_1 was detected using either a competitive ELISA or a native fluorescence-based format in less than one hour.

2) Liposome immunoanalysis (LIA) In flow-injection (FILIA), the Abs are immobilized via protein A in a capillary immunoreactor column. Mycotoxin, such as FmB1, in the sample competes with the FmB1-tagged liposomes encapsulated with sulforhodamine B (SRB) dye for a limited number of Ab binding sites. In this flow-injection system, 35 % MeOH was used for the regeneration of antibody binding sites after each measurement which allows the immunoreactor to be used for up to 70 sequential sample injections without any loss of reactivity. It took less 11 min for each run without preconcentration of the sample[60, 61]. Similar to FILIA, the Abs were immobilized in a zone of a plastic-backed nitrocellulose strip (strip-LIA) which has been used for AFB assay[62]. When the AFB contaminated sample and AFB-liposomes passed through the strip, competition between their binding with Ab occurred with a decrease in color in the zone. The system is capable of detecting 20 ng of AFB_1 and could be used as a screening test.

Biosensors based on indirect competitive immunoassays Several biosensors with different monitoring systems are all based on cd-ELISA principle in which the mycotoxin or mycotoxin derivatives are coupled to the solid-phase.

1) Time-resolved fluoroimmunoassay (TRFI) In this system, AFB-BSA was coated to in the solid-phase and Europium ion (Eu) complex of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid-labeled goat anti-rabbit-IgG was used as the marker[63]. In another system, purified Ab labled with diethylenetriamine pentaacetic acid -Eu^{3+} was used[64].

2) Miniaturized ELISA (mini-ELISA) This is a flow-through set-up system using a pretreated activated fused silica capillary to serve as a reaction cartridge and can detect T-2 toxin down to a level of 4 ng/g of reference sample. The toxin, e.g. T-2 toxin, was conjugated to the solid-phase and anti-mouse-urease conjugate was used as an enzyme marker to produces a pH-shift that can be detected by a miniaturized ion sensitive field effect transistor (ISFET) array[65]. Mab against T-2 was used in the test. The main advantages of the system are small sample volumes, short cycle times and an excellent stability of the regenerable receptor layer.

3) Automated particle-based immunosensor (API) This is a kinetic exclusion assay with AFB_1-BSA coated to polymethylmethacrylate beads (98 um) and FITC-labelled goat-anti-rabbit Ab as the marker. In the assay, the beads were first pumped to a capillary flow cell. The sample or calibrated

standard solutions that had been incubated with antibodies were then allowed to pass through the cell for a period of 120 seconds, followed immediately with the labelled secondary antibody (120 sec.). Finally, the flow cell was washed with buffer to remove excess label. The fluorescence during each step of the reaction was recorded using a simple fluorimeter. It takes about 8 min to complete the cycle. The amount of Ab, thus the toxin, can be calculated by measuring the difference in voltage from the sensor between the beginning and at the end of the assay[66].

Immunoaffinity fluorometric biosensors (IAFB) Based on the IA principle and advanced electro-optical and miniaturized fluidic system, a highly sensitive, versatile and fully automatic biosensor has been developed for analysis of mycotoxins containing fluorescence. In the AFB assay, the assay can be completed in less than 2 minutes with a 1 ml sample volume and the sensor could be used for about 100 times without refurbishment[67]. Whereas The IAFB can be used for other mycotoxins, derivatization is necessary for mycotoxins with no fluorescence.

Homogeneous biosensors (HS) Separation of the bound and free species is not necessary in the HS. One of this type of biosensors is "*Fluorescence polarization (FP) assay*". A sensitive instrument is used to measure the *fluorescence polarization* (FP) for the Ag-Ab interaction and a fluorescence tagged mycotoxin is needed for such assays[68-72]. Because of its inhibition of the binding of tagged-mycotoxin with specific Ab, a decrease of signal occurs when mycotoxin is present in the sample. The assay takes about 5-15 minutes including extraction (5-10 min. in natural and spiked samples). FITC-tagged FmB[69] and fluorescein-tagged AFB[68, 70] and DON[68, 71, 72] were used for the analysis of FmB, AFB and DON, respectively. Sensitivity of these assay depends greatly on the affinity of the Abs and the type of markers used.

Surface plasmon resonance (SPR) Several types of SPR immunosensors were developed. In the analysis of FmB1[73], pAbs against FmB adsorbed onto a thin gold film were coupled to a glass prism. The output beam of a planar light-emitting diode is focused through the prism to excite SPR at the surface of the gold film. Thus, when a sample containing FmB is added to a cell on the outside of the gold film, the angular profile of reflected light intensity shifts. This changes the resonance angle and the reflected beam intensity at a selected angle, both of which are proportional to the FmB concentration. Less than 10 min. was needed to run one sample.

For the analysis of AFB and DON, the principle of idc-ELISA and a Biocore system were used. AFB-BSA was conjugated on the dextran gel surface. Competition between immobilized AFB-conjugate and free AFB in solution for binding to Ab injected over the surface occurs[74]. DON-biotin conjugate bound to the streptavidin was on the SPR sensor surface[75]. For repeated analysis, an effective approach to remove the bound Ab is necessary. Whereas selection of an effective reagent is essential, Abs with an adequate affinity also play a key role. For example, only a pAb against AFB was effectively regenerated by a solution consisting of 1 M ethanolamine in 20 % (v/v) acetonitrile at pH 12.0 among two different Abs tested[74]. Using the same inhibition assay principle, a miniaturized SPR device allows detecting multiple mycotoxins in a single measurement of longer analytical time (25 min). However, cleanup treatment of sample was necessary[76].

Concluding remarks

From the above review, it is apparent that immunoassays have gained wide acceptance as

analytical tools for mycotoxins Antibodies against almost all the important mycotoxins are currently available. Sensitive, simple, and specific immunoassays have been established for the analysis of various mycotoxins. Several immunoassay techniques have been adopted as first action by the AOAC. Immunoscreening methods have been widely accepted as a simple method for screening for AFB and several other mycotoxins. The immunoaffinity columns have become a popular cleanup tools in conjunction with other chemical methods. Kits for above techniques are commercially available. Immunochemical methods have also been used in various toxicological studies[8, 9, 13, 17, 77-80] as well as in cloning genes involved in the biosynthesis of AFB and TCTC[17]. Anti-idiotype and anti-anti-idiotype Abs against several important mycotoxins have been produced and they have been effectively used in the ELISA as well as used as An to generate Ab against original mycotoxins.

Although most immunoassays are very effective, the sensitivity of some assays is still very low because of low affinity Abs used in the assay. Future efforts to generate high affinity Abs against some mycotoxins, e. g. DON, should be continued by using better immunogens, selecting hybridoma cell lines capable producing high affinity Abs, and by cloning the genes for such cell lines. Recent advances in hybridoma and cloning technology in conjunction with improved sensitive rapid methods such as SPR (BIAcore) in selecting of clones would help in achieving this objective[22, 76]. Other approaches including structural modulation[81, 82] to understand the Ab-antigen interaction for cloning would assist point mutation to generate new clones. For example, selected mimotope phage preparations from the phage-displayed random peptide libraries have shown to be effective substituting for AFB in ELISA[21]. Hopefully, a new generation of Abs could be made available. Better labeling techniques, including fluorescent-labeled Abs/mycotoxins, should be tested. By employing advanced microarray and nano-technology fluidic systems, a new generation of immuno-biosensors can be made. With newer methods being used in the production of hapten-protein conjugates[15, 17], cloning of antibodies[14, 17, 19-22] as well as advances in development in biosensor areas[83-85], immunoassays for mycotoxins will be advanced to another new era. I hope that this review will not only generate more interest in using immunochemical methods but will also stimulate additional research to simplify the assay procedure as well as to increase the sensitivity and specificity of the assay in alleviating matrix interference problems.

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