Review Article

RECENT ADVANCES IN MYCOTOXIN DETECTION

Abstract

The significance of detecting mycotoxins in food and animal feeds cannot be overstated due to their harmful effects on both humans and animals, even at extremely low concentrations. Consequently, it is imperative to establish a method that is sensitive, dependable, and accurate for mycotoxin determination across various food products. To achieve this objective, numerous methods have been devised for mycotoxin analysis, including: HPLC, Lateral flow assay, Biosensors, Spectroscopic techniques, Flourescent polarization immunoassay, Smart phone recognition based immune microspheres etc.Mycotoxin-labeled urease acts as the competitive antigen, enabling colorimetric ELISA to detect multiple mycotoxins. A smartphone analysis program quickly digitizes the test results, with the brightness value calculated from the images indicating mycotoxin concentration. As mycotoxin concentration increases, the color development of the particles becomes lighter, leading to a continual increase in the average brightness value.

Keywords: mycotoxin, fungal strains, matrix extraction, fungal growth

INTRODUCTION

Mycotoxins are toxic secondary metabolites and low molecular weight compounds majorly produced by mycotoxigenic fungal strains *viz*., *Aspergillus, Penicillium, Fusarium, Claviceps* and *Alternaria*(Xie *et al*., 2022). Even low concentration of mycotoxin causes serious health impact in both human and animals. The detection of small mycotoxin molecules in such small concentrations is challenging but not impossible. The significance of detecting mycotoxins in food and animal feeds cannot be overstated due to their harmful effects on both humans and animals, even at extremely low concentrations. Consequently, it is imperative to establish a method that is sensitive, dependable, and accurate for mycotoxin determination across various food products (Krska, 1998). Analyzing mycotoxins presents challenges primarily due to three factors: the intricate nature of the sample matrix, the exceptionally low

mycotoxin levels in food, and the disparities in their physicochemical characteristics (Rahmani, *et al*., 2009). As a result, the process of mycotoxin analysis in food and feed involves several steps, encompassing sampling, sample preparation, matrix extraction, purification (Krska, 1998), and the actual measurements. Modern analytical methods of mass-spectroscopy and chromatography are well-capable of detecting mycotoxins in ppm level of concentrations. Advanced analytical methods are usually expensive and available in specialised laboratories; their use requires highly trained technical and academic personnel which makes the analysis very expensive and time consuming. Much preferable solution would be portable and easy-to-use biosensor and immunochromatographic devices suitable to express, in-field detection of mycotoxins. Many mycotoxin being vey heat stable, they are difficult to degrade by conventional techniques. Hence advanced management strategies are required for inhibition of fungal growth and degradation of mycotoxin. To achieve this objective, numerous methods have been devised for mycotoxin analysis, including: HPLC, Lateral flow assay, Biosensors, Spectroscopic techniques, Flourescent polarization immunoassay, Smart phone recognition based immune microspheres etc.

MYCOTOXINS PRESENT IN FOOD COMMODITIES

The mycotoxins produced by fungi are majorly classified as aflatoxins (AFs), ochratoxin A (OTA), patulin (PT), sterigmatocystin (STC), trichothecenes (TCTs) fumonisins (FBs), deoxynivalenol (DON), zearalenone (ZEA), alternariol (AOH), tenuazonic acid, and alternariol monomethyl ether. Table provides information about some important mycotoxins, and food commodities they are contaminating (Balendres*et al.*, 2019).

Table 1 : Information about some important mycotoxins, and food commodities

Mycotoxins	**Producer Organism**	**Commodities affected**
Aflatoxins	*Aspergillus flavus, A. parasiticus, Penicillium puberulum*	Nuts, spices, grains, milk, milk products
Zearalenone	*Fusarium roseum, F.verticillioides*	Cereals, maize, rice, beer
Deoxynivalenol	*Fusarium graminearum*	Cereals, Cereal products
Fumonisins	*Fusarium verticillioides*	Maize, Sorghum, Asparagus
Ochratoxins	*Aspergillus ochraceus,*	Cereals, wine, coffee, dried

	Penicillium viridicatum	fruits, nuts, cheese
Patulin	*Penicillium expansum*	Fruits & vegetables
T2 toxin	*Fusarium poae, Fusarium acuminatum*	Wheat, rye, maize, soybeans

HISTORICAL OVERVIEW

The toxicity of certain fungi, like mushrooms, has been recognized for a considerable period. However, it wasn't until the 1850s that the potential health risks to humans and animals from other toxic fungi came to light. During this time, researchers identified a specific illness called ergotism, which was linked to the consumption of rye and other grains contaminated with the *Clavicepspurpurea* fungus (Burfenning,1973). Subsequently, reports from Russia highlighted additional instances of mycotoxicosis affecting humans. For instance, human stachybotryotoxicosis was connected to the consumption of bread infected with *Fusarium graminearum*, while alimentary toxic aleukia (ATA) was associated with the ingestion of grains that had been infested with *Fusarium poae* and *Fusarium sporotrichioides*. While isolated cases of mycotoxicosis in domestic animals had been documented before 1960, the emergence of turkey X disease in England marked a turning point in mycotoxin research, sparking significant interest within the scientific community (Sargeant *et al*., 1961).

IMPACT OF MYCOTOXIN ON HUMAN AND ANIMAL HEALTH

Mycotoxins are widely recognized for their ability to cause severe health issues in both humans and animals when they enter the body. They employ various mechanisms to exert their harmful effects (Bennett and Klich, 2003). These adverse effects can manifest as carcinogenic, mutagenic, teratogenic, and immunosuppressive, all of which stem from the consumption of mycotoxin-contaminated food or animal feeds (Binder *et al*., 2007; Wild and Gong, 2010). Mycotoxins can negatively impact organisms through acute or chronic toxic effects, leading to complications in the central nervous system, lungs, liver, digestive system, and cardiovascular system.

In addition to the adverse health consequences for both humans and animals, mycotoxin contamination has a detrimental effect on the trade of food and animal feed (Stoev *et al*., 2002; Richard, 2007). According to the Food and Agriculture Organization (FAO) of the

United Nations, approximately 25% of agricultural crops worldwide are tainted with mycotoxins, putting nearly 4.5-5 billion people at risk of chronic exposure to these toxins. This burden is especially pronounced in less developed countries (Imes, 2011).

CONVENTIONAL MYCOTOXIN DETECTION METHODS

The conventional techniques for mycotoxin detection rely on either chromatographic or immunological methods. High-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC-MS), and gas chromatography–mass spectrometry (GC-MS), ELISA, PCR and Lateral flow assay were commonly employed. These methods offer several advantages, including excellent selectivity, high sensitivity, and a low limit of detection (LOD), all while maintaining a high throughput capacity. As a result, these chromatographic techniques have become the standard against which alternative mycotoxin detection methods are compared (Ong *et al*., 2021).

1.Chromatography

There are several chromatographic methods for measuring mycotoxins. These techniques aim to accurately determine mycotoxin levels and typically involve either liquid chromatography (LC) or gas chromatography (GC) combined with detectors like ultraviolet (UV), fluorescence (FLD), or mass spectrometry (MS). By using advanced instrument setups along with thorough sample preparation, these methods can detect a wide variety of mycotoxins with high sensitivity.

1. Thin layer chromatography (TLC)

Traditional Thin Layer Chromatography (TLC) is considered a useful preliminary method for detecting mycotoxins. It's valued for its affordability, simple equipment, and the ability to spot mycotoxins with fluorescent signals under UV light. However, it falls short in terms of accuracy and sensitivity, which makes it challenging to precisely quantify mycotoxins. While TLC is accepted as a recognized method for detecting Aflatoxins (AFs), it has largely been replaced by High-Performance Liquid Chromatography (HPLC) for quantifying mycotoxins.However, there have been advancements in TLC for detecting Ochratoxin A (OTA). Caputo et al. (2014) improved TLC detection for OTA analysis. They found that by applying just 2 µl of the sample onto the TLC plate, they could detect as little as 0.2 µg of OTA. This method offers better sensitivity than using a UV lamp and has a limit of detection as low as parts per billion (µg/kg), comparable to LC methods.

2. Liquid chromatography (LC)

Liquid chromatography (LC) has been developed as an improvement over the limitations of the thin-layer chromatography (TLC) method. TLC has constraints such as limited plate length and susceptibility to environmental factors like humidity and temperature since it operates in an open system. LC is commonly combined with various detection methods including UV absorption, amperometric detection, and fluorescence detection (FLD), often incorporating pre-column or post-column derivatization. In LC coupled with FLD, the fluorescent properties of aflatoxins (AFs) are exploited for quantification. It is widely recognized that LC-MS and LC-FLD represent the good standard techniques for mycotoxin detection (Cirlini*et al.*, 2012). However, the sensitivity, precision, and accuracy of LC-MS methods can vary depending on the specific mycotoxins, matrices, ionization techniques, and sensitivity of the employed procedures. LC-MS often yields suboptimal results for the quantitative measurement of mycotoxins due to issues like ion suppression and matrix effects.

3. Gas chromatography (GC)

Gas chromatography (GC) analysis is a separation method that relies on the separation of substances between a stationary liquid phase and a mobile gas phase. GC is commonly utilized for both qualitative and quantitative assessments of food samples. It has found extensive application in the detection and quantification of mycotoxins in food products (Turner *et al.*, 2009). Particularly, it is preferred for analyzing trichothecenes due to their limited absorption in the ultraviolet-visible range, lack of fluorescence, and varying polarity (Koch, 2004). GC is well-suited for examining substances that are thermally stable, non-polar, semi-polar, volatile, and semi-volatile, including pesticides, oils, and steroids (Scott, 1993).

Since most mycotoxins are non-volatile, a derivatization process is typically employed to enhance their volatility and improve their detectability in GC systems (Koch, 2004; Turner *et al.*, 2009). This derivatization involves reacting the hydroxyl groups of mycotoxins with specific agents, such as trimethylsilyl (TMS) for TMS esters, pentfluoropropyl (PFP), heptafluorobutyl (HFB), or trifluoroacyl (TFA) for fluorination, and acetic anhydride for acetylation, thereby forming the corresponding esters (Koch, 2004). The choice of derivatization agent depends on the specific mycotoxins under investigation and the type of detection system being employed (Langseth andRundberget, 1998). Fluorinating agents are preferred for achieving enhanced sensitivity and selectivity when analyzing type A and B trichothecenes (Valle-Algarra *et al.*, 2005).

4. High performance liquid chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a widely employed chromatographic method for mycotoxin analysis, offering a broad range of detection options. HPLC is a modern analytical technique used to analyze mycotoxins, utilizing various adsorbents tailored to the physical and chemical characteristics of the specific mycotoxins (Turner *et al.*, 2009). HPLC is primarily a quantitative technique and is suitable for cleaning up sample extracts online. It can be coupled with different detectors, each offering distinct levels of sensitivity and selectivity.

The most commonly used detectors in HPLC are ultraviolet (UV) and fluorescence detectors (FLD). UV detectors identify analytes by measuring how the sample absorbs light at different wavelengths, allowing for identification. On the other hand, fluorescence detectors rely on the presence of a chromophore in the particles for analyte identification. Some toxins, like Aflatoxins (AFs), naturally exhibit fluorescence properties, enabling direct detection by HPLC-FLD. Electrochemical and fluorescence detection are two highly sensitive detection modes used for quantitative studies in HPLC (Singh and Mehta, 2019).

2.ELISA – Enzyme Linked Immunosorbent Assay

Immunological assays, such as ELISA, have gained much popularity for mycotoxins screening due to their low cost and easy application. Mycotoxins extract can be analyzed directly in the ELISA assay and does not require cleanup procedures. Such immunoassays provide fast, economical measurements even though the number of matrices tested are limited and often lack precision at low concentrations. Commercially available ELISA kits for mycotoxin detection based on competitive assay format that uses either a primary antibody specific for target molecule or conjugate of an enzyme and required target. Complex formed will interact with a chromogenic substrate to give a measurable result. These are highly specific as well as simple to use. Development of antibodies for most mycotoxins due to their size require development of a carrier molecule to achieve immunogenicity (Turner *et al*, 2009). The occurrence of structurally related mycotoxins or matrice interference can obstruct conjugate and antibody bindingvulnerable to errors in quantifiable mycotoxin measurements. An indirect ELISA detection technique with an immunoaffinity column sample preparation using the same antibody was found to be extremely sensitive at 0.02 µg/L. In order to recover high sensitivity, most researchers concentrated on modifying the normal ELISA protocol. ELISA formats (such as direct, indirect, competitive, and sandwich) are recognized as an excellent and accurate for

screening the mycotoxins, but the procedure is somewhat time-consuming, not ideal for field testing and requires specialist plate readers.

3. Lateral Flow Strip

Lateral flow immunoassay (LFI) is especially suitable for on-site monitoring of mycotoxin contamination in food. A particular antibody with a specific label is mixed within a liquid sample and then flows through a membrane. Its first point of contact is with an antigen that has been coated onto the membrane (referred to as the test line or T-line). When the sample does not contain the target substance (as seen in a negative sample), the labeled antibodies attach to the coated antigen, concentrating at the T-line, which becomes visible and detectable. Conversely, when the target substance is present in the sample at a concentration below the lower detection limit (in a positive sample), the labeled antibody binding sites become saturated and cannot attach to the coated antigen. As a result, the T-line remains invisible and undetectable. Typically, a second control line (C-line) is included in the process. This line is formed by secondary antibodies specific to the species and serves to capture any excess specific antibodies. The presence of the C-line confirms the proper execution of the assay, ensuring the integrity of reagents and materials. It can also be used to calculate the T/C signal ratio, helping to normalize variations between different test strips or as an internal standard for comparing the intensity of the T-line, determining whether the result is positiveor negative (Anfossi*et al.,* 2013). Even-though they are easy to use, result obtained is qualitative not quantitative.

4. Polymerase Chain Reaction

PCR is a highly advanced physico- chemical method for analysis of mycotoxins in an agriculturalcommodities. This may be applied to screen the agricultural commodities for the absence of mycotoxin producers prior to or even after processing. Negative results in this assay indicate that a sample should be virtually free of mycotoxins. Only positive samples are analyzed for presence of mycotoxins using physico- chemical standard methods. Unique DNA sequences of the respective organism have to be chosen as primer binding sites for detection of mycotoxin producing fungi. Genes involved in mycotoxin biosynthetic pathway form a perfect basis for accurate and specific detection system for mycotoxigenic strains in agricultural commodities, foods and animal feeds. Those genes are supposed to be exclusively present in organisms potentially producing mycotoxins (Konietzny, U. and Greineer, R., 2003). PCR methods for detection of aflatoxigenic Aspergilli based on the norsolerinic acid reductase

encoding gene nor 1, Versicolorin A dehydrogenase encoding gene ver 1, Sterigmatocystin o-methyltransferase encoding gene omt A and regulatory gene aflR have been described (Chen *et al.*, 2002). The target sequence for the detection of patulin producing penicillium strains is within the isoepoxydon dehydrogenase encoding gene IDH (Paterson*et al*.,2000).

RECENT ADVANCES IN MYCOTOXIN DETECTION

Many conventional techniques, while accurate and sensitive, have drawbacks like being time-consuming, costly, and reliant on complex equipment and skilled professionals. These methods may not be suitable for handling a large volume of samples or conducting on-site screenings. In contrast, rapid detection technology represents a departure from traditional laboratory methods. It often draws from various fields such as immunology, molecular biology, spectroscopy, and electrochemistry. Rapid detection offers simplicity, affordability, ease of operation, and only necessitates a portable device with a short detection timeframe. This approach aligns with the demand for real-time on-site mycotoxin screening in the realm of food safety (Li *et al*., 2021). Recent advances in mycotoxin detection includes

1. Biosensors
2. Spectroscopic techniques
3. Fluorescent polarization immunoassay
4. Phage display
5. Multicolorquantum dot nanobeads for simultaneous multiplex immunochromatographic detection
6. Aggregation induced emission
7. Smartphone recognition-based immune microspheres

1. Biosensors

Biosensors are analytical devices which measure concentration of analyte. The use of biosensors in the food industry can contribute to reducing the presence of mycotoxins by providing significant benefits such as fast, easy and inexpensive sample analysis, reproducibility, stability, accuracy and on-site testing of samples (Majer-Baranji*et al*., 2021).The transducers that are mainly used for mycotoxin detection are optical (surface plasmon resonance—SPR and fluorescence), piezoelectric (quartz crystal microbalance—QCM), and electrochemical (impedimetric, potentiometric and amperometric). Common recognition elements are peptides, enzymes, antibodies, cells, nucleic acids, but other materials such as aptamers, molecularly imprinted polymers and recombinant antibodies may also be

used. Metal nanoparticles, carbon nanotubes and nanofibers have been tested to improve the sensitivity of the biosensor. These materials are biocompatible and are characterized by special physicochemical characteristics, such as high surface-volume ratio. Interaction of these materials produces physical/chemical change, that is detected by transducer and converted to electrical signal. This signal is interpreted and converted to analyte concentration present in the sample.

TYPES OF BIOSENSORS

A. Based on receptors used

1. Immunosensors
2. Aptasensors
3. Molecularly imprinted polymers

B. Based on transducers used

1. Electrochemical biosensors
    - Impedimetric biosensors
    - Potentiometric biosensors
2. Optical biosensors
    - Surface plasma resonance
    - Planar waveguide biosensors
3. Pieozoelectrics biosensors
    - Quartz crystal microbalance

A. Based on receptors used

1. Immunosensors

A. Labelled Immunosensors

The detection of small analytes (low molecular weight molecule) is mostly conducted using a competitive immunoassay format. In this assay, the sample analyte competes with coated labelled analyte or analyte-protein conjugate for a limited number of antibody binding sites. As the analyte concentration in sample increases, more labelled analytes are displaced. In the competitive assays, the surface of electrode is coated with the saturated concentration of labelled analyte-protein conjugate and competition takes place between free analyte and immobilized labelled analyte-protein conjugate for the fixed amount of antibody named as “indirect” method. Detected amount of labelled antigen is inversely proportional to

concentration of sample analyte. Labelled non- competitive immunoassay follows sandwich format. Enzymes/fluorescent material/ nanomaterial tagged secondary antibody generates signal on capturing antigen. The most common analyte-protein is found to be BSA-mycotoxin conjugate. A linker molecule such as polyethylene glycol (PEG) is employed as a spacer in between OTA and ovalbumin (OVA) conjugate (OTA-PEG-OVA) to reduce the steric effect (Jia *et al*., 2021).

B. Label Free Immunosensors

The innovation of label free immunosensors can also be classified as non-competitive and competitive. The main advantage of these label free immunosensors is simplicity and singlestage reagent less operation. Such immunosensors are often inadequate to meet the demand of sensitive detection. As concern of simplicity and speed of immunosensors, the non-competitive protocol is favoured over the competitive one. The major drawback of non-competitive label free immunosensor is false positive response due to nonspecific binding. In a label free non-competitive immunosensor, the interaction of immobilized antibodies and analyte is read out by the transducer. The transducer and mode of detection play the important role for obtaining the enhanced sensitivity of the label free immunosensor. The sensing ability of an electrochemical immunosensor can be enhanced by incorporating either a metal (PtCo NP) or metal oxide nanoparticles(such as CeO2, Fe3O4, TiO2, ZnO etc.) or conducting polymer etc into a given matrix (Chauhan *et al.*, 2016).

2. Aptasensors

The key part of the aptasensor is the aptamer, a synthetic oligonucleotide ligand (either single stranded DNA (ssDNA) or RNA containing 10–50 variable bases and are known to exhibit high specificity and strong binding affinity. Aptamers can fold into well-defined three-dimensional structures and bind to their ligands by complementary shape interactions. In comparison to the antibody, an aptamer has several advantages such as ease of synthesis and modification with a variety of chemical groups, stability, cost-effectiveness etc (Kotagiri *et al*., 2019). The aptamers can maintain their structures over repeated cycles of denaturation and show high binding affinities and specificities towards the broad range of target (macro to micro molecules, like toxins, drugs, peptides, proteins and whole cells etc.). The aptamers are considered to be a better alternative to antibodies in many biological applications.

A. Labelled Aptasensors

The signalling in aptasensors occurs due to changes in the conformation of aptamer as the target (mycotoxin) molecules are introduced into the reaction chamber. The detection can be achieved through transducer either in “signal on” or “signal off” form, depending on the format of the assay. Commonly used labels are fluorescence material (fluorescein, luminols, QDs etc), enzyme HRP, GO, ALP, electroactive compounds (ferrocene, ferro- cyanide, methylene blue (MB), Pt and Cds QDs, and other metal nanoparticles (NPs)). In the “signal on” format, target detection is based on the signal enhanced after the interaction with target. While the signal off refers to diminish in signal due to the formation of target-aptamer complex.

B. Label Free Immunosensors

In a label free aptasensor, the transducer measures direct interaction of aptamer and analyte through the change in transducer signal (Rhouati*et al*., 2016).The formation of aptamer-target complex induces different types of changes where the aptamer can undergo configurational or conformational modifications. Depending on this change label-free aptasensing strategies can be classified into: (1) structure switchable aptamer assays; (2) aptamer construct assembly/disassembly based assays (3) target-induced variation in charge transfer transistance.

1. Structure switchable aptamer assays

In apta-switching detection strategies, aptamers are first folded into a stable three - dimensional structure involving weak and non-covalent bonds, hydrogen bonding, van der Waals interactions and hydrophobic effects. Thenthe switchable event occurs by aptamer destabilization or attachment to a complementary strand. Finallythe conformational change is directly translated into a measurable signal.

2.Aptamer construct assembly/disassemblybased assays

This category of label-free aptamer-assays is based on the modification of the sensor’s configuration. After formation of the aptamer-target complex, an association or a dissociation of the biosensing construct is induced. The association or assembly leads to the formation of a sandwich structure with a secondary aptamer. On the other hand, the dissociation or disassembly results in the release of a DNA strand. Both mechanisms cause changes on the surface electrode by generating a signal that can be translated by different detection methodologies.

3. Target-Induced Variation in Charge Transfer Resistance

This aptasensing format is the simplest one, because it is based on the direct monitoring of the interaction between the aptamer and its target. It does not depend on a conformational change of the aptamer structure or configurational change of the aptamer construct. In this kind of detection scheme, the binding of the target forms a resistant barrier on the sensing surface, blocking the electron transfer to the electrode. This change in charge resistance is proportional to the amount of the target in the sample.

3. Molecularly Imprinted Polymers

MIPs are reliable bioreceptor based analytical method for mycotoxin detection. These are more stable over varying conditions like temperature, pH, organic solvents etc. Easier to be produced at relative lower cost and can be used for several times. Designed to mimic natural recognition entities like antibodies and biological receptors with specificities similar to antibody-antigen interactions. During molecular imprinting, cross-linked polymers are formed by free-radical co-polymerization of functional monomers and a cross-linker in the presence of an analyte (like mycotoxins) serving as template(Wang *et al.*, 2022). After removing the original template, resulting in threedimensional network contains specific recognition cavities complementary in shape and size with target. It can recognize particular target molecule mimicking biological activity of natural receptors. As for mycotoxins, template is harmful and expensive (Leibl *et al*., 2021).

B. Based on transducers used

A. Electrochemical Biosensor

Electrochemical biosensors operate by detecting alterations in electrical signals generated through chemical interactions between recognition elements immobilized on electrodes and the target analytes. These sensors can be classified into various categories based on the type of detectable electrical signals, includingpotentiometric and impedimetricmethods.

(1) Impedimetric Sensors

The electrochemical impedance spectroscopy (EIS) technique has been developed to identify mycotoxins. This technique records the alterations observed in the interface between the electrode and the redox probe(Vidal *et al*, 2013). Three electrodes constitute an impedimetric sensor, the working, the reference and the counter electrode. Samples were dropped onto detection area of electrode. Impedence measured by using portable detector.

Results were shown on LED screen of detector. Impedimetric sensors have been successfully tested for AFB1, AFM1, OTA and PAT (Mazaafrianto*et al.,* 2018).

(2) Potentiometric Sensors

The potentiometric sensors employ ion-selective electrodes. For this technique, two (working and reference) or three (working, reference and counter) electrode systems might be employed. The information on the recognition event is provided by the changes in circuit potential between the working and reference electrodes. For the mycotoxin determination in foods, differential-pulse voltammetry (DPV), cyclic voltammetry (CV), and square-wave voltammetry (SWV) have been used. Working principle based on target induced dissociation of aptamer from linker on electrode surface. Potentiometric sensors have been successfully tested for AFB1 in corn powder, for OTA in grape juice and red wine, for PAT in juiceand for ZEN in maize.

Development of flexible Dispense-Printed Electrochemical immunosensor for Aflatoxin M1 detection in milk (Abera *et al.,* 2019)

In this work, flexible biosensors were fabricated using dispense printed electrodes and functionalized with single walled carbon nanotubes (SWCNTs), subsequently coated with specific antibodies to improve their sensitivity. Then the immunosensor used for testing AFM1 present in buffer solution and spiked milk sample using a chronoamperometric technique. The milk was defatted by centrifuging at 6000 rpm for 15 min at 4˚C for analysis. Fat and cream were discarded and skimmed milk was collected for the experiment. Analysis of AFM1 in skimmed milk was carried out by spiking with known concentration of AFM1 (0, 0.01, 0.02, 0.05, 0.1, 0.5 and 1μg/L).

The sensor's response in terms of generated current varied depending on the integration of the antibody antigen (analyte) for different concentrations of AFM1. To assess HRP (horseradish peroxidase) activity as a mediator for H_2O_2, TMB (3,3' ,5,5'-tetramethylbenzidine dihydrochloride) was chosen due to its excellent detection properties. The analysis was conducted using chronoamperometry at a provided potential of −100 mV, employing AFM1–horseradish peroxidase conjugate (AFM1–HRP) in the immunoassay. The HRP-catalyzed oxidation of TMB resulted in a color change to blue, with the intensity of this change being inversely related to the concentration of AFM1. The current generated varied inversely with the concentration of AFM1; lower AFM1 concentrations led to increased conjugate attachment

with the antibody, resulting in greater TMB oxidation and higher current. Conversely, higher AFM1 concentrations reduced conjugate attachment, diminishing TMB oxidation and resulting in lower current. The sensor's limit of detection (LOD) was 0.02 µg/L for buffer samples and 0.0259 µg/L for milk samples, with a detection range of 0.01 to 1 µg/L for both. This sensor holds promise for safeguarding consumer health, as it offers the advantages of affordability, speed, ease of use, making it suitable for applications at milk collection points or within milk processing lines.

B. Optical Biosensor

Optical sensors depend on changes in optical signals produced by transducers in response to molecular recognition events occurring on the sensing element.Optical biosensor systems also offer numerous opportunities for simultaneously detecting multiple mycotoxins (Yin *etal*., 2022).

Surface Plasmon Resonance

Optical biosensing is emerging technology alternative to traditional analytical techniques due to its high specificity, sensitivity, fast screening and cost-effectiveness. Optical biosensors also allow direct detection in real time. Important methods in the class of optical biosensors are surface plasmon resonance (SPR) and fluorescence resonance energy transfer. SPR is an uncomplicated, innovative analytical method, which gives fast results with high sensitivity. Moreover, through this technique, a label-free detection is performed as well as qualitative and quantitative analysis of multiplexed pollutants in real-time. (Rehmat *et al.*, 2019).

C. Piezoelectric Biosensors

Piezoelectric biosensors represent a category of analytical instruments that operate based on the principle of recording affinity interactions. In these sensors, a piezoelectric platform or crystal serves as the sensing component, and it operates by detecting changes in oscillations caused by the attachment of a mass to the surface of the piezoelectric crystal.

Quartz Crystal Microbalance (QCM)

QCM-based biosensors have been investigated in both mycotoxin analysis and pathogen monitoring. The QCM transducer has a gold-plated crystal quartz, which by sending an electrical signal, modifies the resonant frequency. On the surface of quartz there is a sensory layer of interest through which mass change and specific vibrations are caused. QCM-based

biosensors have been tested for AFB1 in peanut, pistachio, rice, and wheat and for OTA in red wine(Tang e*t al.,* 2018).

A Portable, Label□Free, Reproducible Quartz Crystal Microbalance Immunochip for the Detection of Zearalenone in Food Samples (Liu *et al.,* 2021)

This study developed a portable, stable, and consistent Quartz Crystal Microbalance (QCM) immunochip for the rapid, cost-effective, and sensitive measurement of ZEN (zearalenone) levels in real food samples. The QCM immunochip demonstrated precise and reproducible results when analyzing ZEN in various food samples, including corn, wheat flour, soy sauce, and milk. The results obtained from this QCM immunochip showed a strong correlation (R2 = 0.9844) with those obtained through the HPLC–MS/MS method.

The QCM immunosensing chip, designed for quantitative ZEN analysis in real food samples, is portable and reusable, following an immunosuppression format. It utilized a Type-922 QCM device from Princeton Applied Research (Oak Ridge, TN, USA) to monitor the oscillation frequency response. The chip's specificity for ZEN analysis was confirmed by testing it against five ZEN structural analogues (α□zearalenol, β□zearalenol, α□zearalanol, β□zearalanol, and zearalanone), as well as other mycotoxins such as AFB1, OTA, FB1, and DON, at various concentrations. The final choice was an anti-ZEN antibody concentration of 100 μg mL−1 for ZEN detection and a 0.05 mol L−1 NaOH solution for regenerating the QCM chip. The entire measurement process, including sample pretreatment (approximately 20 minutes), QCM measurement (5 minutes), and regeneration (5 minutes), could be completed within 30 minutes.

This newly proposed QCM immunochip demonstrated acceptable accuracy (76.6–92.5%) and high sensitivity (Limit of Detection: 0.37 μg L−1) when applied to selected food samples. It can be reused at least six times, and each analysis can be completed in just 5 minutes, highlighting its advantages of rapid, accurate, sensitive, and cost-effective detection. Furthermore, the construction and operating principles of this QCM immunochip can be adapted for analyzing other target substances in various fields.

2. Spectroscopic Techniques

Spectrometry uses the target molecules in the sample to absorb ultraviolet or visible light to produce fluorescence and determine its molecular structure. It has excellent detection sensitivity and specificity of detection.

A. Surface-Enhanced Raman Spectroscopy

SERS has been explored as a rapid screening method with the potential for on-site measurement with a portable device. As an advanced Raman spectroscopic technique, it enhances the molecular fingerprint of the mycotoxin of interest in the presence of roughened metal particles and surfaces with nano-scale dimensions. SERS can be used for rapid and simple analysis of OTA in food systems such as wines by applying on-site measurements with portable devices. The only limitation is the need for a facile extraction method in order to achieve acceptable reproducibility (Nilghaz*et al*., 2022).

B. Hyperspectral Imaging

HSI is an emerging technology that works with the combination of the spectral and spatial information of the samples. The image capturedcontains spectral information at a specific wavelength range for each pixel (Xing *et al*., 2017).Thusit bases on three axes: two spatial axes (X, Y) and a third spectral axis (λ), which results in tri-dimensional information called a hypercube. The desired regions of the samples are selected from the data cube. Then, the spectra corresponding to those regions are extracted and used for further chemometric calibrations.Single kernel analysis would enable the discrimination of the highly contaminated kernels to establish a mitigation strategy, overcoming the contamination heterogeneity of cereal batches. This document is a detailed review of the HSI recently published studies that aimed to discriminate fungi and mycotoxin contaminated single cereal kernels. The most relevant findings showed that fungal infection and mycotoxins levels discrimination accuracies were above 90% and 80%, respectively(Femenias*et al*., 2022)

3. Fluorescent Polarization Immunoassay

Fluorescence polarization immunoassay (FPIA) is one of the well-established homogeneous competitive immunoassays for quantification of target analytes. FPIA has a short measurement time and it is easy to implement, it is widely used in food analysis, clinical and biomedical applications.FPIAs are conducted using portable fluorescent polarizer analyzer with a microdevice for quantification of target analytes. FPIA is based on the competitive binding reaction between the target analyte and the fluorescent labeled target analyte (tracer) to an antibody. In FPIA, the amounts of tracer molecules and antibody molecules are fixed as constant. When the analyte molecule concentration is low, most of the tracer molecules bind to the antibody so that P becomes high. On the other hand, when the analyte concentration is high, most of the analyte molecules bind to the antibody so that the free tracer molecules are

still present and P becomes low. The required process for the measurements is mixing of analyte, tracer and antibody solutions. Therefore, FPIA does not require antibody immobilization and washing steps that lead to handling complexity and a long measurement time as in heterogeneous immunoassays(Wakao *et al.*, 2019).

4. Phage Display

Mycotoxins are toxic in nature, the necessity of pure toxin in synthesis of mycotoxin conjugates with protein molecules that may cause a toxicity risk to the manufacturers, users, and the environment. From a safety standpoint, it is advantageous to use a nontoxic chemical as an immunochemical reagent in place of toxic compound to enhance laboratory and environmental safety. Another disadvantage of using the conjugate is the release of analyte moiety from the conjugate that may give false signal to transducer. The mycotoxin conjugates can be replaced by mimotopes. Mimotopes are the peptides sequence of amino acids which show good affinity towards toxins, mimotopes selected from random phage displayed peptide libraries. Researchers have synthesized the mimotopes for various mycotoxins viz. OTA, ZEN, DON and applied for the fast and accurate detection of mycotoxins (Yan *et al.*, 2019).

5.Multicolor Quantum Dot Nanobeads For Simultaneous Multiplex Immunochromatographic Detection

The immunochromatographic assay (ICA) stands as one of the most extensively employed point-of-care testing (POCT) tools, finding broad applications in clinical diagnostics, environmental analysis, and food safety screening due to its simplicity, speed, affordability, and ease of use. Utilizing a single ICA test strip to simultaneously detect multiple target substances in a single sample can significantly reduce the overall analysis time, conserve sample volume, and cut down testing expenses in comparison to using multiple individual test strips. Recently, several multiplexed ICA strips for concurrent mycotoxin determination have emerged. These multiplexed ICA strips employ various colored nanoparticles as markers, including gold nanoparticles and amorphous carbon nanoparticles. Furthermore, multiplexed ICA strips based on quantum dots (QDs) have also been developed for monitoring multiple target analytes simultaneously. Quantum dot nanobeads (QBs) represent innovative fluorescent nanomaterials consisting of polymer nanobeads embedded with numerous QDs. QBs exhibit heightened fluorescent intensity (FI) and enhanced resistance to environmental variations, such as pH and

ion strength, compared to QDs. Consequently, QBs contribute to further enhancing the sensitivity and stability of ICA (Duan *et al.*, 2019).

6. Aggregation Induced Emission

AIE is a group of fluorescent dyes glows faintly in the dilute solution state and their fluorescence is notably enhanced in the aggregation state.Intense dyes's fluorescence may be the result of Restricted Intramolecular Rotations in the aggregate state. The most commonly used AIE dyes are 9,10-distyrylanthracene(DSA), Silacyclopentadiene (silole), Tetraphenylethene (TPE)(Janik *et al.*, 2021). AIE dye based aptasensor successfully developed for OTA detection in wine and coffee and AFB1 in peanut oil and broad bean sauce.

Label-free fluorescent aptasensing of mycotoxins via aggregation-induced emission dye

The key design of the aptasensor is the synergetic utilization of enzymatic digestion process and AIE dyes (DSAI). The enzymatic digestion process would leverage the response of aptamers towards target molecules. Exo. I was a nucleic acid enzyme that could catalyze the removal of nucleotides from single-stranded DNA initiated from the 3′ terminal. When target molecules OTA existed in the solution, the aptamer would be bound with the target OTA, and could resist the enzyme digestion process. Thus, the binding event of target OTA would result in a larger reservation of aptamer sequences. The positively charged DSAI would confer high binding ability to the negatively charged aptamer sequences. Due to the AIE effect, the OTA-binding aptamer could be efficiently lighted up by the binding and aggregation of DSAI molecules. In the absence of the target OTA, however, aptamer sequences could be digested by Exo □. It would not result in the aggregation of DSAI, thus lead to a low fluorescence signal (Yulin et al., 2019).

Ultrasensitive Lateral Flow Immunoassay for Fumonisin B1 Detection Using Highly Luminescent Aggregation-Induced Emission Microbeads (Xu *et al.*, 2023)

A fluorescent microbead-based method known as Lateral Flow Immunoassay (LFIA) has gained significant attention for its rapid and precise application in monitoring food safety. Nevertheless, traditional fluorescent microbeads face limitations due to the quenching effect caused by the aggregation of loaded fluorescent molecules, resulting in weak signals and inadequate sensitivity in LFIA. In this research, we incorporated a green-emitting fluorophore with aggregation-induced emission (AIE) characteristics into polymer nanoparticles using an

emulsification technique, forming exceptionally bright fluorescent microbeads referred to as AIEMBs. These AIEMBs were then utilized as signal indicators in a competitive LFIA (AIE-LFIA) to swiftly and highly sensitively detect fumonisin B1 (FB1) in real corn samples.

The AIEMBs were initially modified with anti-FB1 monoclonal antibodies (mAbs) and employed as AIEMB probes in a competitive LFIA (AIE-LFIA) for rapid FB1 detection. In the absence of FB1 in the sample, the AIEMB probes were captured by the immobilized antigen (BSA-FB1) on the test (T) line and by goat anti-mouse IgG on the control (C) line, generating two vivid green bands on the test strips. Conversely, when FB1 was present, the binding of AIEMB probes to BSA-FB1 on the T line was inhibited, resulting in a dimmer or invisible T line. The brightness of the T line gradually decreased with increasing FB1 concentration from 0 to 100 ng/mL, becoming invisible at an FB1 concentration of 3.15 ng/mL. The prepared green-emitting AIEMBs outperformed AuNPs as reporters in competitive LFIA for FB1 detection, offering improved sensitivity, accuracy, and reliability. Utilizing these AIEMBs as signal indicators, a highly sensitive competitive AIE-LFIA for rapid FB1 screening was developed, achieving a limit of detection (LOD) of 0.024 ng/mL for detecting FB1 in real corn samples. This LOD is approximately ten times lower than that of conventional AuNP-LFIA.

This study demonstrates the feasibility of using AIEMBs as signal probes to enhance the sensitivity of competitive LFIA and presents a versatile approach for quickly and accurately screening small molecules such as mycotoxins, pesticide residues, and other chemical hazards.

7. Smartphone Recognition-Based Immune Microspheres

Microsperes is avery small, hollow/solid round particle made from glass, ceramic or polymer. Upconversionnanopartilces are doped on microspheres. On excitation with 980nm, microsphere emit different colour. The system can quickly complete the competitive reaction and digital analysis of the results. (1) Multiple detections are based on the immune competition method. This section uses a direct immune-competitive approach based on hydrogel solid phase particles. The solid-phase carrier hydrogel particles can be prepared into different topologies. Theoretically, a type of particle coating with a mycotoxin monoclonal antibody can be used as a solid-phase carrier and can realize the function of encoding the analyte. Large-scale preparation of coding hydrogel is the basis of high-throughput simultaneous detection. This is essential for the rapid completion of multi-target inspections.

(2) Competitive ELISA is adaptable for naked eye detection of analytes. Colorimetric ELISA with an acid-base indicator, in principle, through the conjugated catalysis of the label (e.g., urease, alkaline phosphatase) and the corresponding substrate to produce a slight pH change in the solution system, is a method for visual identification of various harmful substances. (3) Smartphone analysis program for quickly digitized test results. The detection method completely gets rid of the use of complex equipment. With the introduction of smartphones that integrate image acquisition and analysis and processing functions, field detection is easy to implement (Yang *et al.*, 2020).

Rapid, simultaneous detection of mycotoxins with smartphone recognition-based immune microspheres (Zhang*et al.*, 2021)

A smartphone-based immunoassay system utilizing hydrogel microspheres has been developed for the simultaneous and rapid detection of two mycotoxins. This efficient detection system comprises three key steps:

1. Rapid Separation: It begins with the swift separation of unbound substances following a direct competitive reaction, utilizing hydrogel solid-phase carrier particles.
2. Efficient Detection: The system employs the catalytic capabilities of enzymes for efficient detection.
3. Fast Image Capture and Analysis: Image capture and analysis are performed rapidly using smartphone software.

The feasibility of this intelligent rapid detection system was validated using OTA (Ochratoxin A) and ZEN (Zearalenone) as model mycotoxins. The system combines direct competitive enzyme immunoassay with smartphone image processing to create an intelligent detection system. A special type of particle coating, armed with a mycotoxin monoclonal antibody, serves as a solid-phase carrier capable of encoding the analyte. Mycotoxin-labeled urease acts as the competitive antigen, enabling colorimetric ELISA to detect multiple mycotoxins. A smartphone analysis program quickly digitizes the test results, with the brightness value calculated from the images indicating mycotoxin concentration. As mycotoxin concentration increases, the color development of the particles becomes lighter, leading to a continual increase in the average brightness value.

The entire detection process takes just 30 minutes, and results are analyzed in a mere 10 seconds. The detection limits for OTA and ZEN are impressively low at 0.7711 ng L^{-1} and

1.0391 ng L−1, respectively. The recovery rates for both mycotoxins fall within the range of 76.72% to 122.05%. This detection system significantly shortens the time required for analysis and offers a portable and practical solution for food safety monitoring. Importantly, this smartphone-based rapid detection system is versatile and can find applications not only in food pollutant detection but also in environmental monitoring, disease diagnosis, and various other fields.

CONCLUSION

Detecting mycotoxins in food is very challenging due to their low levels in samples and their diverse properties. This has resulted in the development of many different testing methods for individual mycotoxins or specific groups of mycotoxins in various types of foods.To address the growing complexity of food samples and the increasing number of mycotoxins we're concerned about, we need faster and more accurate testing methods. This demand has led to the creation of rapid screening methods that use techniques like ELISA, biosensors (such as protein chips and antibody-coated electrodes), spectroscopy, fluorescent polarization immune assays, and aggregation-induced emission. New techniques are being developed to accurately and precisely identify mycotoxins without the need for time-consuming sample preparation and cleanup.Because some mycotoxins can have stronger effects when they occur together, efforts have been made to detect and measure multiple groups of mycotoxins at the same time. Methods for simultaneously determining several categories of mycotoxins are highly desirable. This not only speeds up the testing process but also significantly enhances food safety by checking for various types of mycotoxins at once.

FUTURE THRUST

The forthcoming challenge lies in the precise identification of modified mycotoxins within the food supply chain, the assessment of the toxicity associated with numerous mycotoxins within a single sample, and the establishment of regulations pertaining to emerging, novel mycotoxin variants. The future developments in rapid immunoassay technology for the detection of multiple mycotoxins will emphasize the following aspects:

- Developing quantitative immunoassay platform for simultaneous detection of hazard mycotoxins in the fields.
- Using single dilution factor for determination of diverse mycotoxins at the same time.

- Simplifying sample preparation to fast analysis.
- Reporting the signals to ubiquitous smartphone or portable inexpensive device.
- Transmitting assay results to widely accessible smartphones or affordable portable devices.
- Utilizing 3D printing and microfluidic devices for straightforward and accurate analysis.
- Amplifying signals using nanomaterials or eco-friendly, cost-effective novel molecules.
- Minimizing reliance on antibodies as recognition elements by adopting animal-free alternatives like bacteriophages, aptamers, and molecular imprinting polymers to reduce costs and address ethical concerns.

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