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Aflatoxin Measurement and Analysis

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1. Introduction

Aflatoxin is a group of secondary metabolites produced by fungi *Aspergillus* species, such as *A. flavus* and *A. parasiticus*; in particular, *A. flavus* is common in agriculture. *A. bombycis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* are also aflatoxin-producing species, but they are encountered much less frequently (Bennett and Klich, 2003).

Aflatoxin contamination can be occurred very widely. They can be found in over a hundred kinds of agro-products and foods, such as peanut, corn, rice, soy sauce, vinegar, plant oil, pistachio, tea, Chinese medicinal herb, egg, milk, feed etc.,. Also some of them in animal organism can be detected. Besides these, aflatoxin can spread and accumulated in environment, for example, river and agricultural field.

Aflatoxins are highly toxic, mutagenic, teratogenic, and carcinogenic compounds, a group of difuranocoumarin derivatives, consisted of a coumarin and a double-furan-ring of molecule usually. Aflatoxin B₁, for example, its toxicity is ten times of potassium cyanide, 68 times of arsenic and 416 times of melamine. Furthermore, their carcinogenicity is over 70 times than that of dimethylnitrosamine and 10000 times of Benzene Hexachloride (BHC). And International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) accepted that aflatoxin should be classified as a Group 1 carcinogen in 1987, and then AFB₁ is classified as Group 1 (carcinogenic to humans) by the WHO-IARC in 1993 (Li, Zhang & Zhang, 2009). According to the nearest researches by University of Pittsburgh, aflatoxin may play a causative role in 4.6–28.2% of all global HCC cases (Liu and Wu, 2010).

To protect agricultural environment, estimate quality of commercials of agro-products and food, and safeguard safety of consumers' health and lives, over seventy countries setup maximum limits in agro-products, and analytical methods for determination of aflatoxin, play a great role for monitoring and estimation of the contaminants.

There are a variety of well established methodologies reported for analysing aflatoxins in many different foodstuffs, such as thin layer chromatography, high-performance liquid chromatography, ultra-pressured layer chromatography, immunoaffinity chromatography-high-performance liquid chromatography, near infrared spectroscopy and immunoassay

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methods. We here will not only demonstrate current such analytical methods for aflatoxins, but also illuminate tomorrow's trends on analysis of aflatoxins. To help readers understand them well, some basic information of these methods were also presented, including principle of developing, choosing and using these methods.

2. Pretreatment of sample

2.1 Immunoaffinity or multipurification column

The immunoaffinity column (IAC) occupies a special place among the immune analytical approaches, being used many years as a method of sample purification and concentration in the aflatoxin analysis (Scott & Trucksess, 1997). The principle of the IAC is that an antibody (polyclonal or monoclonal) recognized the analyte is immobilized onto a solid support such as agarose or silica in phosphate buffer, all of which is contained in a small column.

The clean-up procedures are completed in four steps (Figure 1):

Condition. The column is initially conditioned with phosphate buffered saline (PBS) and reaches room temperature.

Loading of the sample. The crude sample extract is applied to the IAC containing specific antibodies to aflatoxin at slow steady flow rate of 2-3 mL/min. Gravity or vacuum system can be used to control flow rate. The aflatoxin binds to the antibody and is retained in the IAC. The crude sample extract must be in aqueous solution because organic solvents can damage the antibody and can interfere with the antibody-aflatoxin interaction. The binding strength of the antibody-aflatoxin will influence recovery of the IAC. The specificity of antibody is important to remove the structurally closely compounds which can cause interferences in the quantitation of aflatoxin. The capacity of the IAC (the total number of antibody sites available for binding aflatoxin) is also important as overloading the column will lead to poor recovery (Senyuva & Gilbert, 2010).

Washing. The column is washed with washing solution (water or phosphate buffered saline) to remove impurities. After washing completely, the IAC is blown to dryness by N₂ stream.

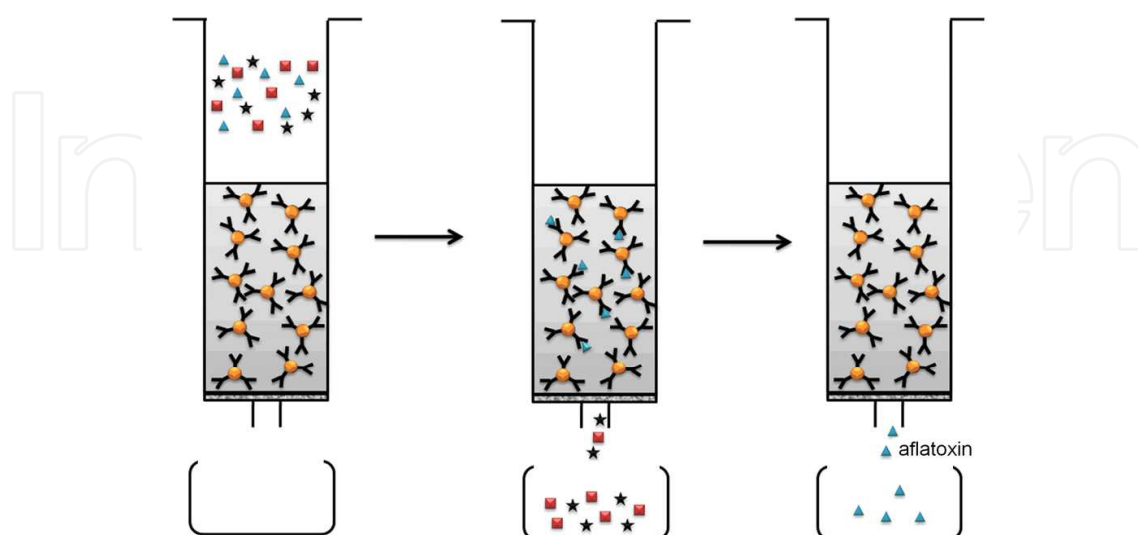


Fig. 1. Scheme of aflatoxin immunoaffinity column for sample pretreatment (clean-up and enrichment).

Elution. By passing a solvent such as acetonitrile through the IAC, breaking the antibody-aflatoxin bond, the captured aflatoxin is removed from the antibody and thus eluted from the column. The big volume of sample loading and the small volume of solvent eluting make the analyte concentrate. The eluate containing aflatoxin is then further developed by addition of fluorescence enhancer or directly measured by HPLC method.

The principle of solid phase extraction (SPE) columns is a variation of chromatographic techniques that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. The columns contain different packing materials, ranging from silica gel, C-18 (octadecylsilane), florisil, phenyl, aminopropyl, ion exchange materials, both anionic and cationic, and molecular imprinted polymers (Giraudi et al, 2007; Jornet et al, 2000; Mateo et al, 2002; Vatinno et al, 2008; Yu & Lai, 2010; Zambonin et al, 2001). The generally procedure is to load the sample into column, retain the analyte, wash away impurities, and then elute the analyte. A MycoSep multifunctional cleanup column has been developed for one step clean-up of aflatoxin (Figure 2). The MycoSep clean-up column is pushed into a test tube (containing the sample), forcing the sample to filter upwards through the packing material of the column. The interferences adhere to the chemical packing in the column and the purified extract, containing the aflatoxin of interest, passes through a membrane (frit) to the surface of the column. The method is rapid, simple and economical due to the fact that the clean-up of aflatoxin from the column is a single pass procedure using the extract solvent as the eluting solvent. The column has a long shelf-life because it contains no biological reagents, and can be stored at room temperature. However, unlike immunoaffinity columns, the MycoSep clean-up column cannot concentrate the analyte during the clean-up procedure, and also the recovery may vary depending upon the complexity of the food samples (Zheng et al, 2006).

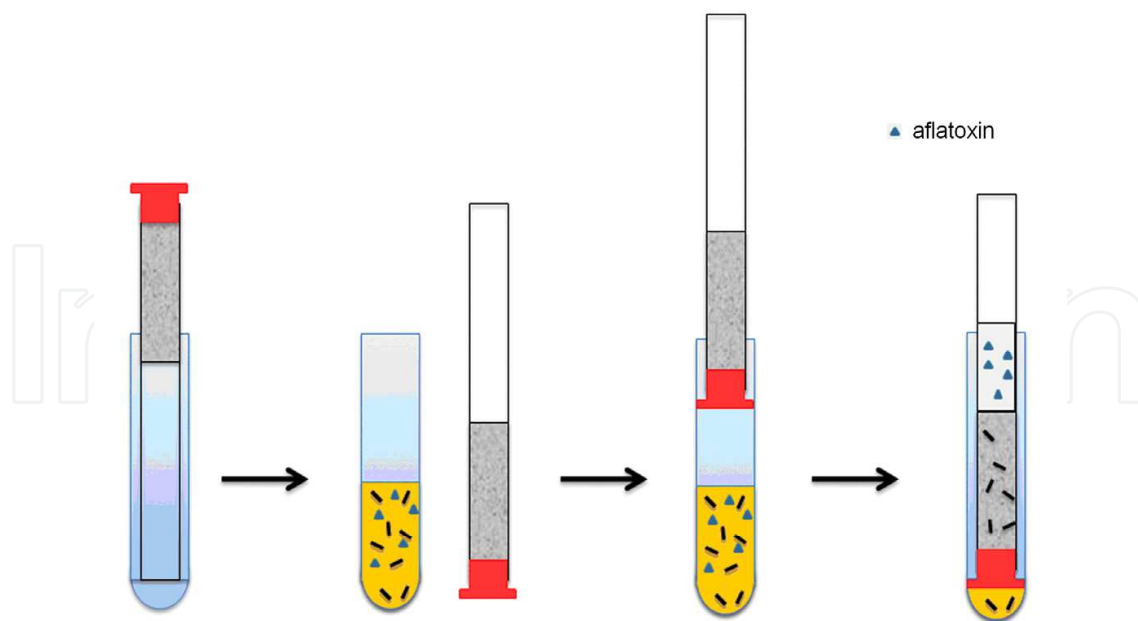


Fig. 2. Scheme of aflatoxin multifunctional cleanup column for sample pretreatment (clean-up).

2.2 How to simplify current protocol

The selection of pretreatment methods for samples depends mainly on two aspects: one is the analytical methods adopted, another is samples to be analyzed. The former is more important with great differences according to the kinds of analytical methods. Complexity, time consuming and cost are the main factors contributed the popular degree by operators and practicability in on-site use. Among these factors complexity degree is most concerned for the exposure hazards of aflatoxins.

Sample pretreatment for instrumental analysis (e.g., HPLC, GC, LC/MS and GC/MS) is very tedious, expensive and time consuming, and needs well equipped laboratories to accomplish it, e.g., frequently involving in large-scale equipment, large sample volumes, extensive extraction or derivatization steps (Tang et al., 2008), complicate clean-up and concentration, and multiple centrifugation, etc. While for immunoassay (for instance, enzyme-linked immunosorbent assay, ELISA) it is usually easier, cheap and rapid generally without derivation but still need clean-up and concentration. How to simplify current pretreatment protocol is a question to extend the methods for aflatoxins detecting outside the laboratory. As an alternative, lateral-flow immunochromatographic assay combines chromatography with immunoassay with less interference due to chromatographic separation, offers the advantages of most simple, cheap and time-saving, requiring only a simple extraction step (Tanaka et al., 2006) or even no need for extraction (e.g., detection of aflatoxin M1 in milk). Therefore, the pretreatment protocol of sample can be simplified by adopting suitable analytical methods, e.g., immunochromatographic assay.

3. Sample analysis

3.1 High fidelity methods

3.1.1 HPLC (UPLC) with fluorescence detector

Since the late 1960's, High Performance Liquid Chromatography (HPLC) had developed, HPLC is by far the most reported chromatographic method using a variety of detection strategies. It was developed rapidly in recent years, about 80% of the world organic compounds (health food efficacy composition, nutritional fortifiers, vitamins, protein etc.) use HPLC for separation and determination. The assessment of the quality of foods using this method provides an acceptable, accurate, and alternative method to establish guidelines and to evaluate the status of aflatoxins in contaminated foods.

HPLC analysis of aflatoxins

HPLC have high efficiency, high sensitivity (HPLC-FLD with as low as 0.1 pg (ng kg⁻¹) detecting limit (Herzallah, 2009) and high resolution. And the chromatographic column can be used repeatedly. So modern analysis of components relies heavily on HPLC employing various adsorbents depending on the physical and chemical structure of different components.

The most commonly found detectors for HPLC are fluorescence detectors (FLD), which rely on the presence of a chromophore in the molecules. A number of toxins already have natural fluorescence (e.g. aflatoxins) and can be detected directly by HPLC-FLD. Determination for aflatoxins by HPLC with fluorescence detections is often the method of the choice. The use of the HPLC in determination of aflatoxins and their metabolites showed higher levels of accuracy and lower detection limits when using CN activate Solid Phase

extraction (SPE-CN) or immunoaffinity column (IAC) combined with application of FLD (Brera et al, 2007; Edinboro, & Karnes, 2005; Jaimez & Fente, 2000)

Chromatography columns were the most important part of the HPLC, normal and reversed-phase columns were used for separation and purification of toxins depending on their polarity. Reversed-phase C18 columns with methanol-water or acetonitrile-water mobile phases, is most commonly used for aflatoxins in most laboratories.

Modern analysis of mycotoxins relies heavily on HPLC employing various adsorbents depending on the physical and chemical structure of the mycotoxins. The use of the HPLC in determination of aflatoxins and their metabolites showed higher levels of accuracy and lower detection limits when using SPE-CN or IAC regardless of the HPLC detectors used. Zhao used UPLC for determinations of Aflatoxins B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂), and the detection limits (S/N = 3) for B₁, B₂, G₁ and G₂ were 0.32, 0.19, 0.32 and 0.19 µg kg⁻¹, the corresponding quantification limits (S/N = 10) were 1.07, 0.63, 1.07 and 0.63 µg kg⁻¹, respectively (Fu et al, 2008).

Fluorescence enhancement methods of aflatoxins

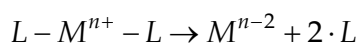
Derivative with a suitable fluorophore can enhance the natural fluorescence of aflatoxins, which can improve the fluorescence detection sensitivity. The present needs for HPLC fluorescence detection of aflatoxins determination in food and feedstuffs are an emphasis on the improvement of the sampling and extraction steps to lead to more accurate determinations, and further investigations of non-destructive pre-column or post-column derivative methods appears to be a large unexplored field. Some aflatoxins like aflatoxin B₁, aflatoxin G₁, because of its low signal or its easy quenching signals, several derivation reagents were used during the detection procedure.

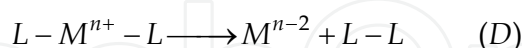
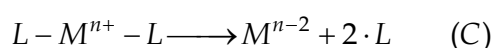
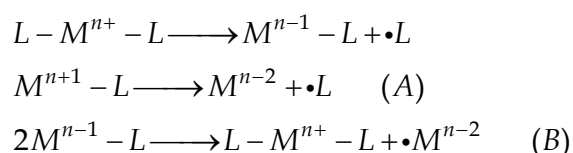
There are mainly three kinds of derivatizations: TFA, halogen, and its derivatives, metal ions (Hg²⁺), cyclodextrine and its derivatizations. The enhancement mechanisms varies with different kinds of derivatizations.

AFB₁ derivative method is mainly based on hydrolysis of the second furan ring in acidic solution, and AFB₁ is transformed into B_{2a}, which makes a fluorescent greatly enhanced. This mechanism is commonly used by TFA, halogen, and its derivatives (PBPB) etc. (Francis et al., 1988; Joshua, 1993; Braga et al, 2005)

Dr. Ma (2007) had studied on the metal ions (Hg²⁺) enhancement for aflatoxins and proposed the probably mechanism was that AFB₁ can be chelated with Hg²⁺, the propose of the complexes fluorescence can be enhanced, the speculate metal complexes electronic transition occurred ligand AFB₁ to employed by Hg (II), the charge transfer transition metal ions, namely ligand-to-metal charge transition (LMCT) transition. LMCT transition with high energy, and its absorb is in the UV area, LMCT transition is occurred against bonding σ orbital, electronic horizontally inspire with ligand AFB₁ oxidation and reduction of metal, occurred by electron reaction. Metal ions are two ligand simultaneously electronic warp reduction. Speculation that ligand AFB₁ is probably in the form of $\cdot L$ base separation formed $2 \cdot L$ or formed new molecular $L - L$ or $L - M^{2+} - L$, reactant system rigid structure to strengthen or conjugated system increased, fluorescent intensity was greatly enhanced (Ma, 2007).

The main reaction procedure may be described by the next response equations:





The proposed mechanism of inclusion allows explaining data previously reported on fluorescence emission enhancement for AFB1 in presence of β -cyclodextrines (β -CDs), the region of AFB1 exhibiting the most hydrophobic character is constituted by the methoxy group and by the portion of the coumarinic and cyclopentanone ring opposite to the carbonyl groups. However, the methoxy group alone is probably too small to produce a good fitting, displacing all water molecules placed within the β -CD cavity. The hydrophobic portion of coumarinic and cyclopentanone rings cannot be included into β -CD for steric reasons. β -CDs and AFB1 main composed a Host and guest system in this way β -CD can protect AFB1 from come into contact with some reagents which can lead to fluorescence signals quenching, and in this way it is consistent with the observed enhancement of AFB1 fluorescence emission in presence of β -CDs, and this system may explained by Hydropathic analysis. The inclusion of the bifuranic system of AFB1 into the β -CD cavity allows for fluorescence enhancement due to the protection of the fluorophore from the quenching and also in this case a variation in the circular dichroism spectrum. The affinity of AFTs to β -CD is rather low, being the calculated binding constants for the AFT: CD complexes around 10-3 M. Although the enhancement of AFTs native fluorescence, due to inclusion into CDs, has already been successfully employed in HPLC analysis for increasing the sensitivity, the low affinity of the formed complex cannot lead to a specific chemosensor for mycotoxin detection in a complex matrix such as food (Manetta et al, 2005).

Derivatisation can also be performed by employing either pre- or post-column. Bromine (Br_2), TFA (trifluoroacetic acid) are common used for pre-column derivative; Post-column reaction with iodide or bromide by an electrochemical cell (Kobra Cell) or addition of bromide or pyridinium hydrobromide perbromide (PBPB) (Akiyama et al, 2001; Stroka et al, 2003) to the mobile phase coupled with fluorescence detection has yielded sensitive determinations of aflatoxins: these reactions and others have been extensively reviewed, like β -cyclodextrine, is also used for post-column derivatisations. Aghamohammadi showed the methods which are based on the enhanced fluorescence of AFB1 by β -CD in 10% (v/v) methanol-water solution, For concentrations ranging from 0 to 15 $\mu\text{g kg}^{-1}$ of AFB1 in pistachio samples as prediction set, the values of root mean square difference (RMSD) and relative error of prediction (REP) using multiple linear regressions (MLR) were 0.328 and 4.453%, respectively were observed (Aghamohammadi & Hashemi, 2007).

AFB and AFG were commonly derivated in most experiment because of its low and easy quenching signals. A. Cepeda et al., (1996) was also studied using of cyclodextrin (CD) inclusion compounds showed an analytical method based on the incorporation post-column of a CD solution that promotes the greatest enhancement of AFB and AFG fluorescence (Figure 4).

From the figure 4 the different chromatograms we can see that with the addition of CD and its derivatives AFB1, AFB2 and AFG1, AFG2 were obtained greatly fluorescence enhancement.

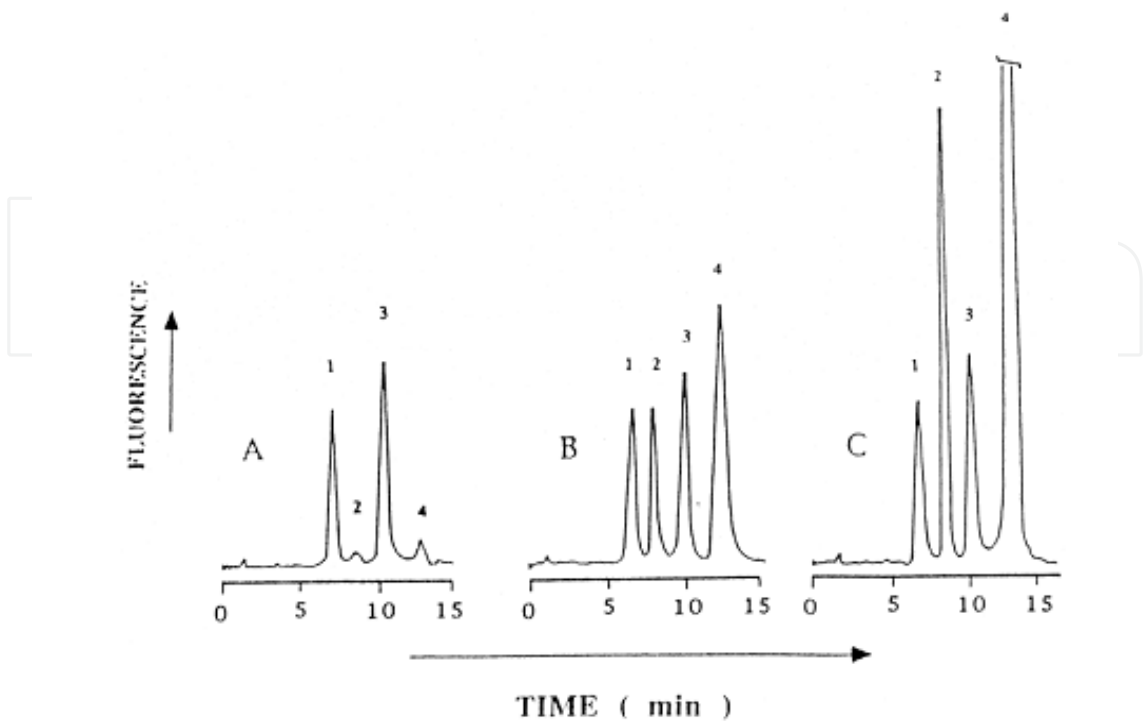


Fig. 3. Comparison of the different chromatograms: (A) without CD; (B) with addition of 10⁻² M CD; (C) with addition of 10⁻² M DM-CD. Peaks: 1 =AfG2; 2=AfG1; 3=AFB2; 4=AFB1.

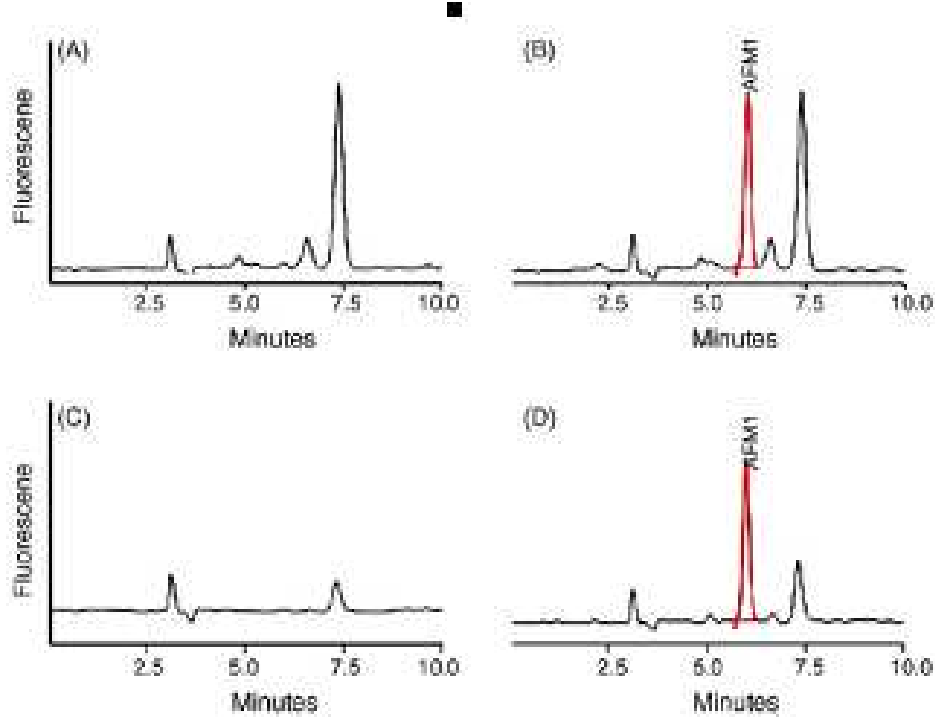


Fig. 4. Chromatograms of AFM1-free milk (A); milk spiked with AFM1 at 200 ng kg⁻¹ (D); mobile phase, acid/acetonitrile/2-propanol deionized water (2: 10: 10.78), flow rate was 1.2 ml min⁻¹

Besides AFB and AFG, fluorescence enhancement for sensitive detection could also be used for AFM1 analysis. Anna Chiara Manetta (2005) reported HPLC method with fluorescence detection by using pyridinium hydrobromide perbromide as a post-column derivatising agent had been developed to determine aflatoxin M1 in milk and cheese. The detection limits were 1 ng kg⁻¹ for milk and 5 ng kg⁻¹ for cheese. The calibration curve was linear from 0.001 to 0.1 ng injected. The method included a preliminary C18-SPE clean-up and the average recoveries of Aflatoxin M1 from milk and cheese, spiked at levels of 25–75 ng kg⁻¹ and 100–300 ng kg⁻¹, respectively, were 90 and 76%; the precision (RSD) ranged from 1.7 to 2.6% for milk and from 3.5 to 6.5% for cheese.

Chromatograms (Figure 5) and the data result showed that use of CD for detect AFM1 can significantly improve the detection sensitivity.

3.1.2 HPLC-MS-MS

High performance liquid chromatography (HPLC) combined with fluorescence detection is proved to be very accurate and has been extensively studied in different materials. However, in order to improve detection limits of AFB₁ and AFG₁, a tedious pre- or post-column derivatization must be done in conventional HPLC methods (Huang et al, 2009; Tassaneeyakul et al, 2004). These problems have been successfully solved in the present study by introducing HPLC-MS method.

As shown in Figure 6, a HPLC-MS system was equipped with an autosampler, the HPLC system, the ionization source (which interfaces the LC to the MS) and the mass spectrometer. There are various types of ionization sources that can be used as the interface between the HPLC and the mass spectrometer. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the two most common ionization sources. For both ESI and APCI, the ionization occurs at atmospheric pressure, so these sources are often referred to as atmospheric ionization (API). As shown in Figure 7, there are several types of mass spectrometers available for interfacing with HPLC. Single quadrupole mass spectrometer (Figure 7a) is a common system used for the HPLC-MS, this system can provide a mass spectrum for each chromatographic peak that elutes from the LC column and is analyzed by the MS system. Time-of-flight (TOF) mass spectrometer (Figure 7b), which has the added capability of providing a higher mass resolution spectrum from each component that is assayed. The triple quadrupole MS-MS system (Figure 7c) and ion-trap mass spectrometer (Figure 7d) are important tools in quantitative analysis and qualitative analysis. HPLC-ESI-MS/MS has become the most emerging analytical tool for the determination of aflatoxins and their metabolites (Cavaliere et al, 2007; Sulyok et al, 2010; Huang et al, 2010). Single quadrupole mass spectrometer (Nonaka et al, 2009) and ion-trap mass spectrometer (Cavaliere et al, 2006) were also used in the determination aflatoxins. LC-MS provides decisive advantages in performing identification as well as determination of analytes at trace levels.

Matrix effects, however, limit the potential of LC-MS. Molecules originating from the sample matrix that coelute with the compounds of interest can interfere with the ionization process in the mass spectrometer, causing ionization suppression or enhancement, which is the so-called matrix effect (Fan et al, 2011). Ion suppression (or enhancement) might be encountered due to matrix components that co-elute with the analyte of interest. If available, internal standards can often successfully amend these effects. Other possible strategies including the use of matrix matched standards or very careful validation of certain toxin/matrix combinations to exactly sample can determine the matrix effect.

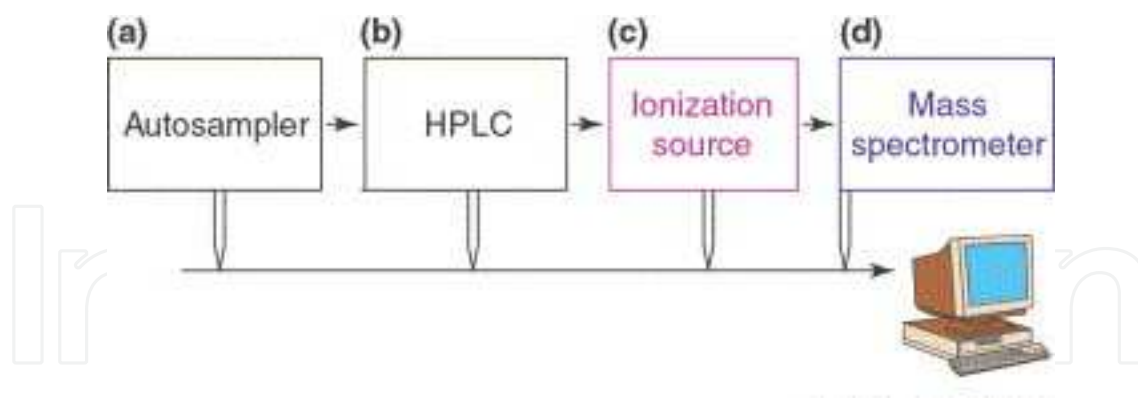


Fig. 5. The elements of an LC-MS system. (a) Autosampler; (b) HPLC; (c) ionization source; (d) Mass spectrometer.

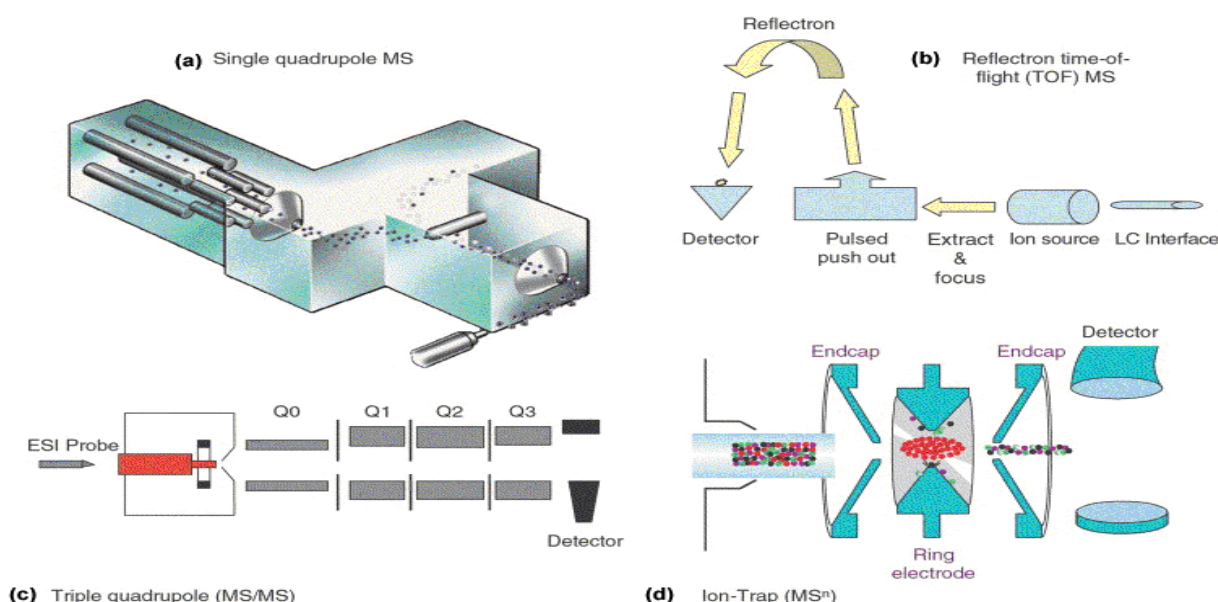


Fig. 6. Types of mass spectrometers which can be used in LC-MS. (a) Single quadrupole MS; (b) Time-of-flight MS; (c) Triple quadrupole MS; (d) Ion-Trap MS

In general, all aflatoxins exhibit good ESI ionisation efficiency in the positive ion mode with abundant protonated molecules $[M+H]^+$ and sodium adduct ions, but practically no fragmentation in the full scan spectra (Blesa et al, 2003; Ventura et al., 2004). The formation of sodium adduct ions can easily be suppressed by the addition of ammonium ions to the mobile phase leading to a better MS sensitivity (Cavaliere et al, 2006). Reports about the utility of APCI interfaces are inconsistent and ionisation efficiencies in this mode seem to be highly dependent on the aflatoxin subgroup and the APCI interface geometry (Abbas et al, 2002). In this respect, only the structurally related sterigmatocystin offers strikingly better sensitivity with an APCI interface in the positive ion mode than with ESI (Scudamore et al, 1996), and consequently only Abbas et al. applied APCI for the detection of AFBs in the low ppb range (Abbas et al., 2002). According to recent

investigations, atmospheric pressure photoionization (APPI) seems to be a more reliable alternative to ESI. Since this interface offers strikingly lower levels of chemical noise and ion suppression than ESI it was found to be two to three times more sensitive (Cavaliere et al., 2006). The product ion spectra of the protonated aflatoxin species contain a number of abundant product ions reflecting bond cleavages and rearrangement reactions of the polycyclic ring system along with loss of water, carbon monoxide and carbon dioxide (Cavaliere et al., 2006). Despite this favorable fragmentation behaviour, only the quantitative single stage of LC-MS can not meet the EU criteria concerning unambiguous compound identification in residue analysis (Zllner & Mayer-Helm, 2006). In this respect, Cavaliere et al. demonstrated that the QTrap technology opens a new dimension of MS analyte confirmation and quantification. Its operation in the quadrupole linear ion trap configuration (enhanced product ion scans) produces complete product ions mass spectra even close to the LOQ which guarantees accurate analyte quantification simultaneously to unambiguous analyte confirmation (Cavaliere et al., 2006).

Cavaliere et al. compared the calibration curves set up in standard solution and in sample matrix and found close similarity of both slopes, proving that the influence of matrix components on the analyte signal was negligible and matrix effects could be excluded. Alternatively, Edinboro and Karnes infused post-column the aflatoxin analyte into a blank sample injection. As they did not find any dips in the baseline they concluded that ion suppression was absent in the analyte elution zone (Edinboro & Karnes, 2005).

Direct comparison of LC/MS and LC-FL revealed in most cases good correlation of quantitative results (Blesa, et al., 2003) though LC/MS method robustness and sensitivity seem to be inferior to LC-FL. In this context, Vahl and Jorgensen reported large variations of the recovery rates in different spices. They attributed this observation to severe matrix effects that are not compensated by the applied internal standard AFM1 and by a calibration curve set up in standard solution (Vahl & Jrgensen, 1998). Besides, Blesa et al. demonstrated in peanut samples that LC/MS is less sensitive than LC-FL (Blesa et al., 2003) though this can be partly explained by the use of single quadrupole instrumentation in the SIM mode that is inferior to a tandem MS and SRM recording (Cavaliere et al., 2006; Vahl et al., 1998).

3.2 Rapid assay methods

3.2.1 Portable tester

Due to high toxicity and extensive pollution of aflatoxins, some special portable tester and corresponding assay techniques were developed for rapid, sensitive, quantitative and convenient on-site determination of aflatoxins. The rapid tester device is based on chromatography and fluorescence spectrometric technologies, including clean-up and concentration with an immunoaffinity column, derivatization for fluorescence enhance and fluorescence excited at 360 nm. Ma et al. (2007) developed a rapid method for detecting aflatoxin B1 with an immunoaffinity column and portable rapid tester (Li et al., 2005; Li et al, 2006; Ma et al, 2007), which was obtained from Beijing Chinainvent Instrument Tech. Co. Ltd. (Beijing, China). Using the assay method developed, the results of showed the linear range of the method was 0.3–25 lg/kg, the average recovery was above 90% with CV being under 5%, the LOD for AFB1 from peanut and its related products was 0.3 lg/kg, the time for whole test process was about 45 min and the cost of detection was lower than other instruments and methods. Chiavaro et al. (Chiavaro et al, 2005) detected AFB1 and AFM1 in

pig liver with portable tester obtained from VICAM (Watertown, MA, USA). The detection limit was 1.0 mg/kg for AFB1 and AFM1. Mean recoveries were $80.7 \pm 9.0\%$ for AFB1 spiked at 1.0–9.7 mg/kg levels and of $76.7 \pm 6.6\%$ for AFM1 spiked at 1.0–5.5 mg/kg levels. Considering its low price, portability and reliable quantification, the rapid tester dedicated to aflatoxins is suitable to use in the field, particularly in Third World countries.

Nowadays, the light sources of rapid tester are mainly LED, Xenon light for fluorescence assay. Due to the lack of fluorescence intensity, the aflatoxin has to be derivatized to enhance fluorescence using toxic and environmentally unfriendly solvents such as bromine. To address this issue, a laser is applied as excitation resource of portable tester. This light resource can provide steady light and can induce aflatoxins at ppt level without enhancer derivatization. Although the price of laser is higher than LED and Xenon light, the advantages of laser resource will make it have more widely applicable and a bright future.

3.2.2 Biosensor

Immunosensors are designed to improve sensitivity and to simplify determination. There are at least four classification of immunoassay at present: optical, electrochemical, piezoelectric (PZ) and micromechanical (Raman Suri et al., 2009), all of which depend on Abs and sensitive components. Two kinds of immunosensor have been developed for determination of aflatoxin (i.e. electrochemical and optical).

Competitive and non-competitive assays have both been used to develop electrochemical immunosensors for determination of aflatoxins. One type of electrochemical immunosensor is based on competitive ELISA. In this assay system, specific Ab or Ag (hapten-protein conjugate) is immobilized on the electrode, and enzyme conjugate is free. After competitive reaction, a different density of enzyme due to different concentration of analyte will bind to the electrode. Finally, the binding enzyme density can be shown by current produced from the catalytic oxidation reaction of the enzyme with substrates. Many such immunoassays have been described for aflatoxins (Ammida et al, 2004; Micheli et al, 2005; Parker & Tothill, 2009; Tan et al, 2009; Vig, et al, 2009) and they all had high sensitivities (LOD 0.01–0.4 ng/mL). With a non-competitive immunoassay, the formation of the Ab–Ag complex by a simple one-step immunoreaction between the immobilized enzyme–Ab conjugate and analytes in sample solution introduced a barrier of direct electrical communication between the immobilized enzyme and the electrode surface, so local current variations could be detected by the enzyme bioelectrocatalytic oxidation reaction with substrates. Sun et al. (Sun et al, 2008) and Liu et al. (Liu et al, 2006) developed such immunoassays for aflatoxin B1, whose linear ranges of detection were 0.1–12 ng/mL and 0.5–10 ng/mL, respectively. Using no enzyme and substrate, Owino et al. (2007) developed a non-competitive immunoassay with an LOD of 100 mg/L for aflatoxin B1 through a variation of electrochemical-impedance spectroscopy.

Optical immunosensors developed for determination of aflatoxins include mainly surface plasmon resonance (SPR) and some array devices. SPR, which is a well-known physical phenomenon, is surface electromagnetic waves that propagate in a direction parallel to the metal/dielectric (or metal/vacuum) interface. Since the wave is on the boundary of the metal and the external medium (air or water for example), these oscillations are very sensitive to any change of this boundary, such as the adsorption of molecules to the metal surface (El-Sherif, 2010). For biomolecular-interaction analysis, SPR sensors are valued for

their ability to monitor molecular binding without labels and in real-time (Amarie et al, 2010). In a SPR of antibody-antigen interaction system, specific antibodies are immobilized on a sensitive optical component (i.e. layer of Au on a glass surface). When the antibodies capture analytes specifically, SPR occurs through the sensitive component. The angle of SPR is increased in line with the increase in the amount of analyte binding to the Au. Based on SPR method, immunoassays for aflatoxin B1 have been described by Daly et al. (2000) and Wang et al. (Wang & Gan, 2009), and their linear ranges were 3.0–98.0 ng/mL and 0.3–7.0 ng/mL, respectively. An outstanding characteristic of these immunoassays depends on a one-step reaction of Ab and analyte with a non-competitive format. To increase the sensitivity of detection, Wang et al. (Wang et al, 2009) developed a novel biosensor using long-range surface-plasmon-enhanced fluorescence spectroscopy. In this system, the binding of fluorophore-labeled molecules to the sensor surface is probed with surface plasmons and the emitted fluorescence light is detected. This approach takes advantage of the enhanced intensity of electromagnetic field occurring upon the resonant excitation of surface plasmons, which directly increases the fluorescence signal. Using this novel sensor, they obtained the lowest reported LOD for aflatoxin M1 (0.6 pg/mL). Solid-array sensors often depend on a competitive assay format. Specific Abs or Ags are immobilized on a solid surface (e.g., waveguide surface) and fluorescence-labeled conjugates are presented in the competitive system. Using an indirect competitive procedure, Sapsford et al. (2006) developed such an immunoassay for aflatoxin B1 with LODs for AFB1 0.3 ng/mL in buffer, 1.5 ng/g and 5.1 ng/g in corn, and 0.6 ng/g and 1.4 ng/g in nut products. Array sensor is a good tool for multiple compounds. For determination of aflatoxin B1 and ochratoxin A in the same operation, Adányi et al. (Adányi et al, 2007) devised a solid-array sensor with a sensitive detection range of 0.5–10 ng mL⁻¹ using a competitive detection method.

3.2.3 Microplate reader

Microtiter plate and reader-based immunoassays mainly use competitive assays. Microtiter plates should have the features of binding proteins uniformly (e.g., Ags or Abs against aflatoxins or secondary Abs). 96-well polystyrene is used most commonly (Table 1). Microtiter readers can report optical absorbance or intensity of chemiluminescence or fluorescence, and they often contain data processing software that can build assay standard curves and equations and report amounts of analytes. In the past, most immunoassays developed were microtiterplate and reader based (Zhang, Li, Zhang, et al, 2009; Li, Zhang, Zhang, et al., 2009; Guan, Li, Zhang, et al, 2011). Some new materials (e.g., magnetic nanoparticles) have been used in aflatoxin-ELISA (Radoi et al, 2008). ELISA is the rapid test method most used today. ELISA kits have been commercial and used widely for aflatoxins in foods and agricultural products. Chemiluminescence immunoassay (CLIA) developed based on ELISA. Generally, chemiluminescence immunoassay can reach higher sensitivity than ELISA. With 384-well black polystyrene microtiter plates, a secondary Ab labeled with HRP and a luminol-based substrate, Magliulo et al. (2005) reported a chemiluminescence immunoassay for aflatoxin M1 in milk, that the limit of quantification was 1 ppt, so they thought that the developed method was suitable for accurate, sensitive, high-throughput screening of aflatoxin M1 in milk samples with a reduction of costs and increased detectability, as compared with previously developed immunoassays.

Fluorescence labels were also developed in ELISA format for analysis of aflatoxins, which is called Time-Resolved Fluoroimmunoassay (TRFIA). The labels used in this assay are lanthanide chelates such as Eu, Tb, and Sm. Lanthanide chelate labels offer the potentially significant advantage of a strong fluorescence with long decay time. As the measurement time is extended, the background noise is substantially reduced when the short-lived, non-specific background interference has disappeared. Moreover, the labels have a large Stock shift between the excitation and emission wavelength. The advantages of lanthanide chelate labels greatly increase the sensitivity of TRFIA. Huang et al. (2009) developed a TRFIA method for aflatoxin B1 using Eu3+ chelates as label. The sensitivity of the method was 0.02 µg/L and dynamic range of 0.02–100 µg/L. The intra- and inter-batch coefficient of variation was 3.2 and 7.3%, respectively, and the average recovery rate was 88.1%.

The advantage of microtiter plate-based immunoassays may be that they can be used to detect a large number of samples with a 96-well or 384-well plate at one time. These methods are used as quantitative or semi-quantitative assays for high through-put screening of aflatoxin samples.

Type	Label	Plate	Microplate reader
ELISA	HRP	Polystyrene, 96 well, clear	Absorbance, 450 nm
CLIA	HRP	Polystyrene, 96/384 well, black	Chemiluminescence, CCD
TRFIA	Lanthanide chelate	Polystyrene, 96 well, black	Fluorescence, 613 nm

Table 1. The parameters of immunoassay based on microplate

3.2.4 Lateral flow strip

Lateral flow strip assay is a new immunochromatographic technology combining chromatography with immunoassay and has attracted great interest in recent years. Nanoparticles are usually selected as the detector reagent, e.g., nanogold (Au) is most applied. A lateral flow strip comprises three membrane pads: absorbent pad, conjugate-release pad, sample pad and a nitrocellulose (NC) membrane, as shown in Figure 8. With capillary action, test buffer containing analytes is introduced to the absorbent pad from the bottom of the strip. After reaching the Au conjugate-release pad, the Au-labeled Ab can bind analytes specifically. The complex is then transferred by the flow to the nitrocellulose membrane and reacted with the immobilized Ag for the generation of signals. If the test buffer contains analytes, the complex migrates along the membrane and binds to the secondary Abs on the control line and no red signal can be observed on the test line. If the analyte is absent, some the Au-labeled Abs bind to the immobilized Ag (aflatoxin-protein conjugate) on the test line and the rest of the Au-labeled Abs flow to and bind control Abs (Li, Zhang, & Zhang, 2009).

Lateral flow strip assay has many advantages, such as:

- 1. requiring only a sample extraction step before use;
- 2. simplicity of procedure with single step, e.g., only adding test solution to the sample pad on the strip;
- 3. rapid on-site detection within a few minutes (5-15 min);
- 4. concentration levels of target analytes can be observed directly with the naked eyes;
- 5. user-friendly format no need for skill personnel;
- 6. less interference due to chromatographic separation; and
- 7. low cost

Because of these advantages, lateral flow strip assay has become one of the commercial and widely-used immunoassays for rapid determination of mycotoxins, such as ochratoxin A (Lai et al, 2009; Liu, Tsao, Wang, & Yu, 2008; Wang, Liu, Xu, Zhang, & Wang, 2007; Cho et al., 2005), deoxynivalenol (Kolosova, De Saeger, Sibanda, Verheijen, & Van Peteghem, 2007; Xu et al., 2010; Kolosova et al., 2008), T-2 Toxin (Molinelli et al., 2008), zearalenone (Kolosova, De Saeger, Sibanda, Verheijen, & Van Peteghem, 2007), fumonisin B1 (Wang, Quan, Lee, & Kennedy, 2006), aflatoxins (Sun, Zhao, Tang, Zhou, & Chu, 2005; Sheibani, Tabrizchi, & Ghaziaskar, 2008) and so on.

The visual detection limit (VDL), defined as the minimum concentration producing the color on the test line significantly different or weaker to that on the test line of negative control strip without aflatoxin (Li, Wei, Yang, Li, & Deng, 2009; Zhou et al., 2009), was used to express the sensitivity of the lateral flow strip assay. The visual detection limit of published conventional lateral flow strip assay for aflatoxins are summarized in Table 2.

References	Aflatoxins	VDL ^a (ng/g)
(Delmulle, De Saeger, Sibanda, Barna-Vetro, & Van Peteghem, 2005)	AFB1	2.0
(Sun, Zhao, Tang, Zhou, & Chu, 2005)	AFB1	0.5
(Shim et al., 2007)	AFB1	0.1
(Zhang, Li, Zhang, Zhang, 2011)	AFB1	0.03
	AFB2	0.06
	AFG1	0.12
	AFG2	0.25

^a The VDLs here were selected out from the original as defined above.

Table 2. VDLs of published conventional lateral flow strip assay for aflatoxins.

Challenges in test strip production include adjusting the flow properties of the test strip and, as already mentioned, reducing matrix background interference by optimization of multiple parameters including (Krska & Molinelli, 2009):

- 1. type and pore size of analytical membrane;
- 2. type and concentration of blocking agent for blocking membrane binding sites after spraying of reagents;
- 3. type of buffer, pH range and ionic strength; and
- 4. use of surfactants and modifiers for pre or post treatment of test strip materials

Similar to ELISA, optimization with a selection of reagents (concentrations), materials and assay conditions is necessary.

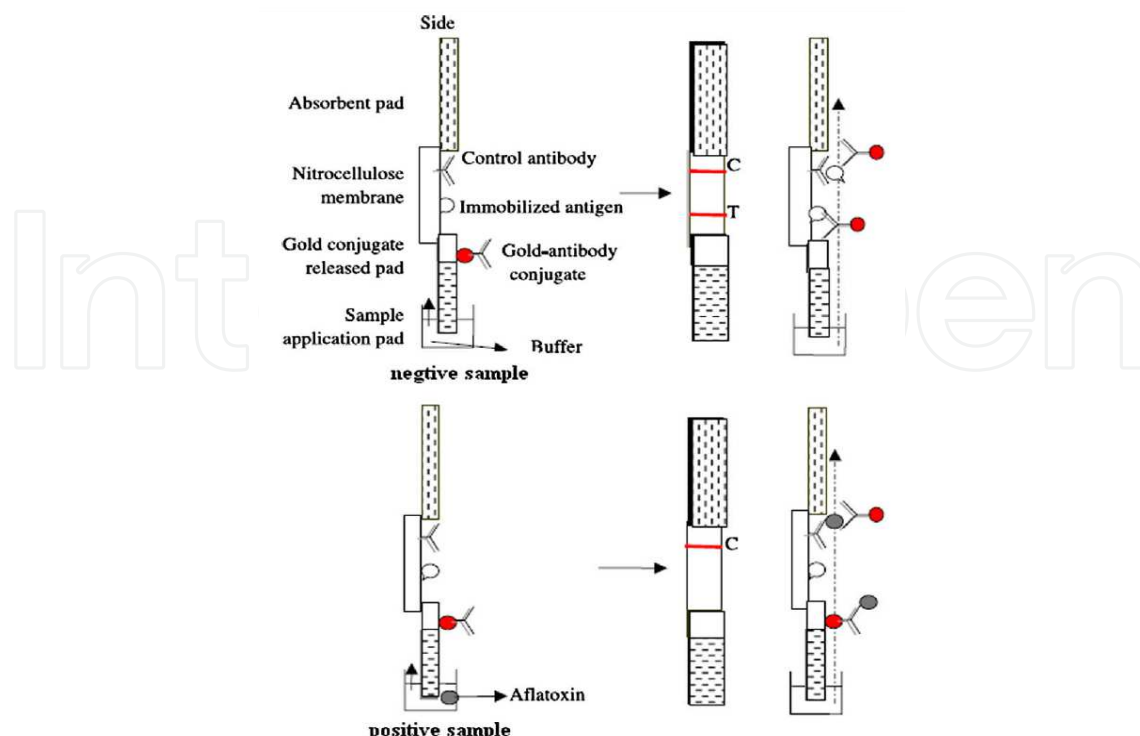


Fig. 7. Construction of lateral flow strip, which comprises three pads (from top to bottom): absorbent pad, gold-conjugate release pad and sample application pad, and a nitrocellulose (NC) membrane. The sample is introduced by capillary action from the bottom of the strip. On reaching the gold-labeled antibody pad, the antigen-Ab reaction takes place. The binding complex is then transferred by the flow to the NC membrane and then reacted with the immobilized antigen to generate signals. Signals generated from the sample without aflatoxin (negative sample) and with aflatoxin (positive sample) are shown in panels (Sun, Zhao, Tang, Zhou, & Chu, 2005).

3.3 Other methods

Besides the above, both of layer chromatography (TLC) and generic fluorospectrophotometry are two traditional methods for determination of aflatoxin content. And there are several standard methods published previously (http://www.aoac.org/omarev1/2005_08.pdf; Van Egmond and Jonker, 2004). Recently they were used by fewer and fewer laboratories with occurring of so many modern equipments and protocols. Maybe, lack of automatism and high possibility to be harmful to operators and environment are the main reason. They are not described with more details here.

4. New trends

4.1 Quantitative strip assay

As description above, lateral flow assays are currently widely used in a wide range. However, most of the strip tests developed are qualitative tests (Molinelli, Grossalber, & Krska, 2009) with a simple yes/no response to the levels of the target analytes. Although the conventional quantitative analysis may be suitable for verifying certain analyte (e.g., for a pregnancy test), it is not adequate when the level of an analyte is important (Liu et al.,

2007), e.g., most clinical decision for illness progression require known concentrations of pathogens; the countermeasures for contaminated foods and feeds need be taken according to the contamination level. A trend can be seen towards (semi-) quantitative strip tests driven by a strong demand from industry (Molinelli, Grossalber, & Krska, 2009). To meet the requirement, two kinds of approaches have appeared depending on the need of detector or not. With advanced nanotechnologies, a few methods have integrated chromatographic separation and electrochemical (Wang, Quan, Lee, & Kennedy, 2006), fluorescence (Sun, Zhao, Tang, Zhou, & Chu, 2005) or optical detectors (Sheibani, Tabrizchi, & Ghaziaskar, 2008) for rapidly quantitative detection. Compared with conventional strips which just based on visual judgment, these approaches offer a greater sensitivity and dynamic range as well as a better quantitative capability (Kim, Oh, Jeong, Pyo, & Choi, 2003). However, these approaches can lead to environmental pollution from heavy metal (e.g., mercury, Hg), or may suffer from optical interference (e.g., photobleaching), the rising costs due to the use of detector, and the complex software for imaging and analysis (Liu et al., 2007); all of these potential problems limit their well application on spot. As detector-free approaches, a one-step competitive ICA for semiquantitative determination of lipoprotein (a) in plasma is developed (Lou, Patel, Ching, & Gordon, 1993), the dose ranges can be simply encoded to different numbers of a colored ladder bar that had fully developed color on the assay strip, and a pH sensitive dye is used as the end-of-assay indicator. A potential problem could arise that the time of end-of-assay with a pH sensitive indicator may vary from people to people and cause a disparity in result determination. Subsequently, a dipstick test determined microalbuminuria in patients with hypertension (semi-) quantitatively by comparing the colored singal with a standard color chart (Gerber, Johnston, & Alderman, 1998) such as with pH paper. However, the color indication of the assay is not self-confirmative, and may also show an error in matching intensity (Cho & Paek, 2001).

According to the description above, although problems exist in two kinds of approaches, the detector-free methods seem to have more potential on-site application value considering convenience, low-cost and no interferences from instrument itself. To overcome the disadvantages of published detector-free methods, a novel strategy for detector-free (semi-) quantitative strip (DFQ-strip) assay is proposed just like a novel “ruler” for content measurement of target analyte. The illustration design of the DFQ-strip was shown in Figure 9. The DFQ-strip consisted of five parts similar as the traditional ones with three pads (sample, conjugate release, and absorbent pads), a NC membrane and a plastic backing plate. On NC membrane, three scale lines defined as SL- I , II and III constituted the measuring bar which played a role as a ruler. After reaction different number of scale line appeared indicating the concentration (range) of analyte, in other words, every scale line’s disappearing represented a concentration (expressed as threshold level) playing a role as scale on the ruler, while the visual detection limit played a role as an unlined out scale. As a detector-free approach, the strategy spurned the traditional method with just one test line for one analyte or multi-test line for multianalyte, three scale lines were designed to offer multiple dynamic ranges for one analyte. Therefore, compared with the traditional qualitative tests, the DFQ-strip assay not only expresses yes/no response but also offer the content (range) of target analytes. For a negative sample, three color bands (scale lines) are formed in the test zone of DFQ-strip (figure 10a) and the color intensity is graded with the weakest color in SL- I and deepest color in SL-III. For positive samples, with migration, the free probe became less and less, which is more and more favorable for the competition of analyte. The intensity of the color is inversely proportional to the analyte concentration in

sample. Thus, during the competitive reaction, SL- I will disappear fistly, and then SL- II and SL-III at last. Consequently, a positive sample, in accordance with the amount of analyte in sample, will result in three, two, only one weaker red band or no color mark in test zone compared with those of negative control (figure 10b). But, similar as the traditional strip assay, in any case, if no red line appears at the control zone, the test result is considered invalid (figure 10c).

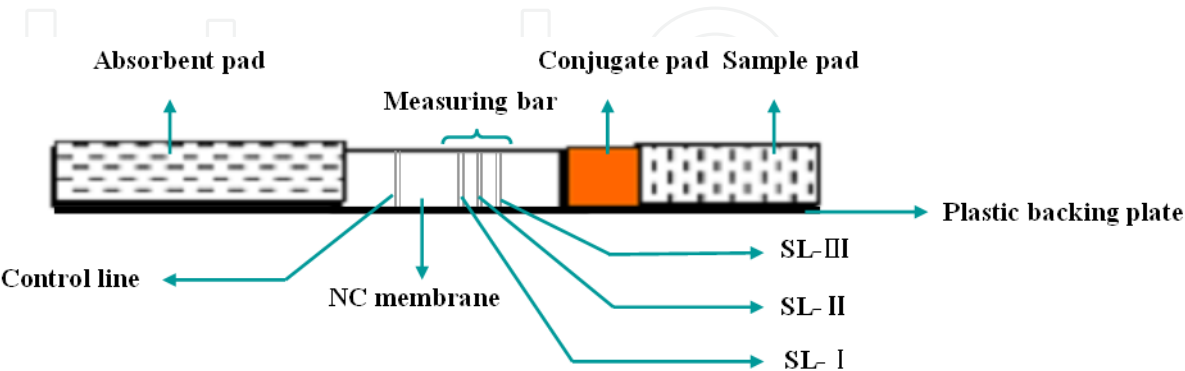


Fig. 8. Illustration of the DFQ-strip design. The DFQ-strip consisted of five parts similar as the traditional ones with three pads (sample, conjugate release, and absorbent pads), a NC membrane and a plastic backing plate and the differences lay in lines on NC membrane. There were four lines, one control line and three scale lines on NC membrane. The measuring bar which played the role as a ruler was comprised by SL- I , SL- II and SL-III.

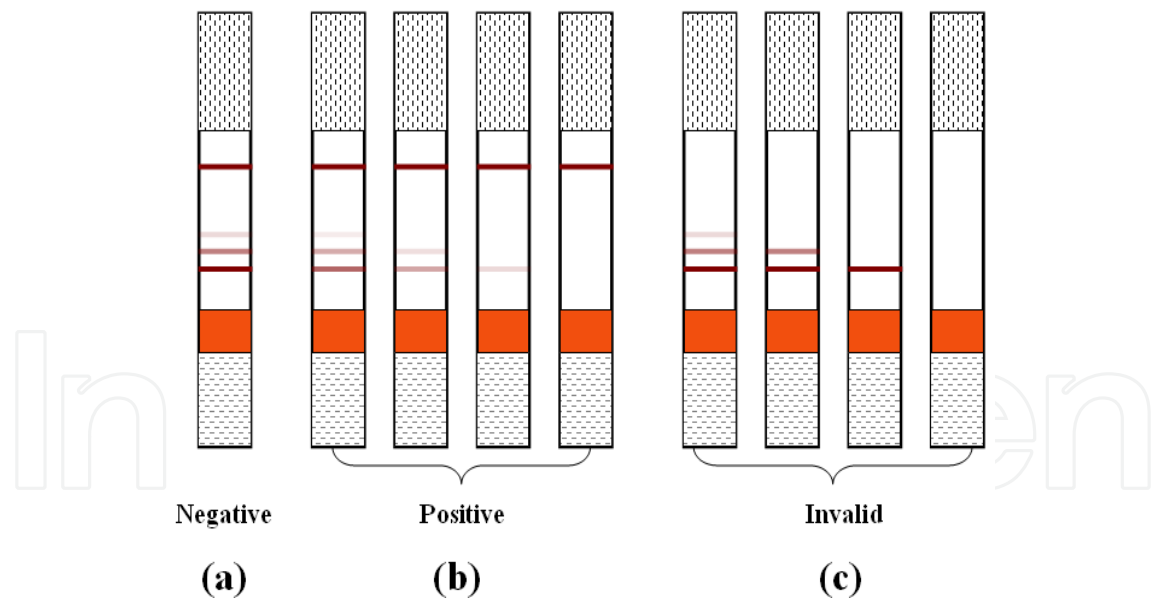


Fig. 9. Illustrations of DFQ-strip assay results for negative, positive and invalid.

A comprehensive model system of DFQ-strip is constructed taking aflatoxin B1 as target analyte. The visual detection limit (VDL, unlined out scale) of the DFQ-strip assay was 0.06 ng/mL, and the threshold levels (scales) for SL-I, II and III were 0.125, 0.5 and 2.0 ng/mL, respectively (the data will be published recently). Moreover, all results supported the feasibility of the idea with high sensitivity, precision and accuracy, multiple dynamic ranges, as well as good (semi-) quantitative capability, stability. Besides, this DFQ-strip

assay had good practicability, great application value for toxic or harmful substances (e.g., mycotoxins) in-situ monitoring but still possessed the advantages of conventional strips such as procedure simplicity, rapid operation, immediate results, low cost, and no requirement for skilled technicians or expensive equipment, etc. The strategy is proposed as an alternative idea for sensitive, rapid, convenient and (semi-) quantitative detection of analyte on site.

4.2 Green immunoassay

Aflatoxin standards and their derivate have been considered as high poison. So there is high possibility that these compounds using in analytical processes induce second contamination of environment. How to reduce or eliminate the use of hazardous substances? For this, green immunoassay strategies will be introduced as the below.

On the one hand, nontoxic surrogates of aflatoxin can be designed in ELISA system. As is known, aflatoxin calibration curves must be used for every plate to reduce differences in plate-to-plate variability and improve accuracy. Furthermore, the pure toxin, used as the calibrator, is hazardous to operators and the environment. According to the reaction principle of antibody and hapten, we can design some mater, such as second antibody, which can also bind the active area of the specific antibody against aflatoxin. Such compounds will act as calibrator and be named as surrogate.

There are usually four steps for development of a green immunoassay with nontoxic surrogates:

- to prepare specific antibodies (the first antibody) against targets;
- to produce F(ab')₂ fragments of the target antibody
- to produce anti-idiotypic antibodies (the second antibody) to the target;
- to establish an calibrator curve for detection.

As an example developed in our laboratory, a green enzyme-linked immunosorbent assay (ELISA) to measure aflatoxin M1 (AFM1) in milk was developed and validated with a surrogate calibrator curve. Polyclonal anti-idiotypic (anti-Id) antibody, used as an AFM1 surrogate, was generated by immunizing rabbits with F(ab')₂ fragments from the anti-AFM1 monoclonal antibody (mAb). The rabbits exhibited high specificity to the anti-AFM1 mAb, and no cross-reactivity to either of the other anti-aflatoxin mAbs or the isotype matched mAb was observed. After optimizing the physicochemical factors (pH and ionic strength) that influence assay performance, a quantitative conversion formula was developed between AFM1 and the anti-Id antibody ($y = 31.91x - 8.47$, $r = 0.9997$). The assay was applied to analyze AFM1 in spiked milk samples. The IC₅₀ value of the surrogate calibrator curve was 2.4 µg mL⁻¹, and the inter-assay and intra-assay variation was less than 10.8%; recovery ranged from 85.2 to 110.9%. A reference high-performance liquid chromatography method was used to validate the developed method, and a good correlation was obtained ($y = 0.81x + 9.82$, $r = 0.9922$).

On the other hand, how can we develop some immunoassay using no target standards? In our previous review (Li et al, 2009), noncompetitive immunoassay format was described. And this immunoassay's signal can be found stronger and stronger with increasing of target content, which means "no signal, no target". This kind of noncompetitive immunoassay is especially suggested for fast screening of samples without any use of the toxin standards, although, when developing this assay, toxin standards will have to be used for evaluation of sensitivity. Recently, some noncompetitive immunoassays, such as SPR assay and sandwich

assay for other small chemicals were developed. However, there are still no reports for analysis of aflatoxins in agro-products or in environment.

5. Outlook

Facing so many kinds of current analytical methods for aflatoxin, how can we choose them for our sample analysis?

Actually, each method has its own features. In our opinion, there are three classes of analytical methods, (1) High fidelity method, (2) qualitative rapid method and (3) quantitative rapid method.

1. High fidelity method means they have been authorized with high sensitivity, accuracy and precision, and especially means traditional chromatographic technology with high extent of efficiency and intelligentization. Considering its mature and vive methodologies, there has been many standard method set by governments or international organizers. Usually, such methods have been using to make impartiality data for inspection reports. For analysis of aflatoxin, HPLC with detector of mass or fluorescence belongs to high fidelity method. Disadvantageously, these methods depend on very expensive instruments which can only be sited on some special room. The room also needs to meet some special requirement of environment. Usually, their process need to spend so many organic solvent and total cost of sample measurement is relatively high.
2. Qualitative rapid method means it needs few time to finish a test process and it can only tell operator "positive" or "negative" data. A typical such method is nanogold particle-based immunochromatographic assay. Usually, these methods especially fit for screening of a great number of samples or on-site analysis. The main advantage is simple, rapid, convenient, detector-free and low-cost for sample analysis. Its main disadvantage is lack of content details and it is generally not considered to make data for inspection report on agro-products.
3. Quantitative rapid method means it can be used to get content details with high sensitivity; however it has lower accuracy and/or precision than that of high fidelity method some time. Here, it especially means quantitative immunoassays including ELISA, portable tester-based immunoassay, immunosensor and so on (Li, zhang & zhang, 2009). These methods have been considering as important valuable complement for high fidelity method (HPLC-MS/FLD). They have attractive features including high sensitivity and specificity, simple operating, short time consuming, the possibility of analysis of difficult matrices without extensive pre-treatment, and low costs. According to the previous discussion, these technologies facing the following challenges,
 1. preparation of more specific antibodies against aflatoxins via inducing of novel structural hapten, screening of mAb or rAb, or mending antibody of engineering,
 2. exploring of non-animal antibody preparation techniques, such as development of rAb or some simulative antibodies mentioned above,
 3. researches on use of novel labels, such as sensitive nanoparticles (quantum dots, gold particle, magnetic beads, etc),
 4. development of noncompetitive immunoassays with one reaction step for faster, simpler and more sensitive assay, and
 5. legalization of immunoassay methods. Comparing the amount of immunoassay kinds, there are only few methods have been constituted as test standard. In China,

for example, there are just some ELISA standards for determination of aflatoxins. So, we think legalization of immunoassay methods may become one of important task in the future.

The second and the third above maybe become the main research trends. And, rationally, we predict immunoassay devices such as portable fast tester special for aflatoxins will be used in wide fields.

For analytical works, our aims need to be clear firstly, which means “why the samples need to be determination of aflatoxins?” Generally, there are three kinds of aims: (1) For justifying only with or without target contaminants; (2) For getting qualitative extent of contamination with low or high content of aflatoxin; (3) For quantitative evaluation on contaminant in samples. And then, to reach the aim, an appropriate method need to be chosen with the principle of saving (time and/or cost) and speed of measurement.

With developing of analytical technologies, sensitivities of methods will be enhanced. To meet requirement of on-site assay, many novel analytical devices, representing automatization, minization and high throughput, will be developed and improved. It means that tomorrow analytical methods will be of simplification, intelligentization and portability. Also, future assay protocols will use fewer and fewer poison chemicals including toxin standards and organic solvent. These methods will make great importance on analysis of aflatoxin, to protect agricultural environment, to estimate quality of commercials of agro-products and food, and to safeguard safety of consumers’ health and lives.

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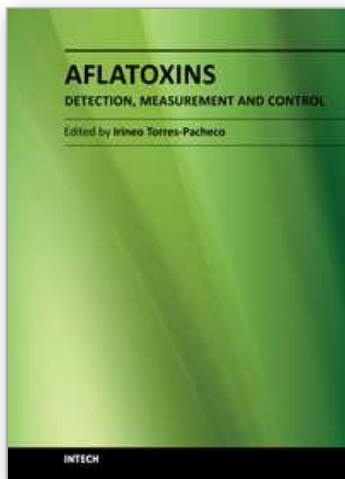
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Aflatoxins - Detection, Measurement and Control

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities. The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical perspective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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