MYCOTOXINS: AN OVERVIEW ON THEIR QUANTIFICATION METHODS

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(Received March 19, 2010)

Mycotoxins are secondary metabolites produced by several filamentous fungi that grow on various food and feed. These compounds elicit a wide spectrum of toxicological effects, representing a health risk for both, humans and animals. Mycotoxin analysis is an important tool in controlling fungal contamination of food and feed. This article reviews the techniques used for their determination, and the advantages and limitations of each method are critically discussed.

Key words: mycotoxins, analytical, biological and immunological methods.

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi species, mainly *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* etc. (1-3). The most common are aflatoxins, citrinin, ochratoxins, fumonisin and patulin. The chemical structures of mycotoxins are very complex and diverse (Table 1), the majority of them being synthesized from small molecules (acetate, pyruvate etc). Contamination with mycotoxins has been reported in a large number of commodities, such as cereals, legumes, fruits, vegetables, wine and beer. Mycotoxins exert a broad range of toxic properties (Table 1) and represent an economic and health risk.

Concern about safety of human foods and animal feeds has led to an increased development of accurate and suitable analytical methods for mycotoxin identification and quantification. All these methods are standardized and the level of mycotoxin contamination in food and feed is strictly regulated by specific organization (FDA, USDA, EPA and AOAC).

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Table 1

Mycotoxins and their physiological effect

Mycotoxins	**Producing organism**	**Chemical structure**	**Effect on mammalian cells**
Aflatoxins (B1, B2, G1, G2, M1, M2)	*Aspergillus*	Difuranocoumarin derivatives	Carcinogenic
Citrinin	*Penicillium*	Benzopyran derivate	Nephrotoxic
Fumonisin	*Fusarium* *Alternaria*	Isoflavonoid compounds	Carcinogenic Hepatotoxic
Trichothecenes	*Fusarium* *Trichoderma*	Sesquiterpenoid compounds	Cytotoxic Immunosuppressive
Ochratoxin	*Aspergillus* *Penicillium*	Dihydroisocoumarin derivatives linked to phenylalanine	Carcinogenic Nephrotoxic Hepatotoxic Teratogenic
Patulin	*Penicillium* *Aspergillus*	Unsaturated heterocyclic lactones	Carcinogenic Immunotoxic Genetoxic
Zearalenone	*Fusarium*	Phenol resorcyclic acid lactone	Estrogenic activity Potential carcinogenic and teratogenic

The present paper describes the methods that are currently used for mycotoxin detection and the advantages and disadvantages of each technique are critically discussed.

PRE-TREATMENT METHODS

The vital step for a right choice of detection procedure is the extraction and clean-up methods to remove the mycotoxins from the type of matrices. Several methods are used depending on the chemical structures of the mycotoxins and the biological matrix. These include liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), solid phase extraction (SPE), solid phase microextraction (SIME) etc. (4, 5).

SPE is by far the most popular technique currently used for analysis of fumonisin, aflatoxin B1, patulin, ochratoxin in food and feed. The technology is based on chromatographic columns containing different bonding phases, ranging from C-18 (octadecylsilane), silica gel, anionic and cationic exchange materials to immunosorbents and molecular imprinted polymers (MIPs). In addition to cleaning, SPE methods can also be used to pre-concentrate samples. Compared to LLE, SPE is faster and requires less solvent. There are, however, some disadvantages of SPE, such as the lack of a universal column for all toxins, slow binding kinetics (MIPs), high cost and single use of bonding phases (*i.e.*, immunosorbents).

DETECTION METHODS

CHROMATOGRAPHIC TECHNIQUES

Thin layer chromatography (TLC) is a method still broadly used for quantitative and semi-quantitative measurements of mycotoxins with detection by fluorodensitometry or visual procedures (0.01 ppm detection limit). TLC based on silica gel, F254 fluorescent silica gel or silica gel impregnated with organic acid has been reported to be applied for detection of common mycotoxins (aflatoxins, citrinin, fumonisin) (6). Although it costs less, is simple and suitable for rapid screening, the lack of automation has led to TLC being replaced by other techniques.

Gas chromatography (GC) is a technique applicable to the compounds that are volatile and thermostable. Detection is achieved by linking the system to mass-spectrometry (MS), flame ionization or Fourier transform infrared spectroscopy. Most mycotoxins are not volatile and therefore need to be derivatised by chemical reactions such as silylation or polyfluoroacylation in order to be quantified. The method has been used to measure trichothecenes in fungal cultures in tandem with MS (7).

Due to its limitation to volatile and thermostable compounds, GC is not a technique suitable for commercial purposes.

High performance liquid chromatography (HPLC) is widely accepted as an official method for the determination of toxins. It is applied in conjunction with UV, fluorescence, amperometric or spectrofluorimetric detection. Both normal and reverse-phase HPLC are used for separation and purification (8, 9). A number of mycotoxins already have natural fluorescence (ochratoxin, citrinin) and thus can be detected directly by HPLC-fluorescence (HPLC-FD) (10). Others, such as fumonisin, require derivatisation that can be performed by employing o-phthalylaldehyde or 9-(fluorenylmethyl) chloroformate.

Spectroscopy is often used and nowadays MS with electrospray or pressure chemical ionization interface is a configuration allowing an increase in sensitivity compared to HPLC-FD (11). The limit of detection of zearalenone measured by HPLC-MS in maize was reported to be as low as 120 ng/kg. HPLC, coupled to triple quadrupole MS, for determination of eleven mycotoxins in three food matrices was developed by Beltran *et al* (12). The limit of detection varied between 0.1 and 1 μg/kg in all the tested matrices.

As an analytical tool, HPLC offers the advantage of a high resolution, limit of detection, with the possibility to be coupled to multiple detection automated systems.

Although accurate and specific, most of the chromatographic assays are expensive, time-consuming and require expensive equipment and clean-up procedures.

PHYSICO-CHEMICAL METHODS

Capillary electrophoresis (CE) is an electrophoretic method leading to a fast separation of components based on charge and mass dependent migration in electrical fields. In combination with fluorescence detection, CE allows the detection of mycotoxins at trace levels. A flow system coupled to CE for the screening of aflatoxins in feed samples has been reported (13).

BIOLOGICAL METHODS

Biosensors have emerged as a rapid, sensitive, practical and convenient method for mycotoxin analysis. They consist of a recognition element, commonly of biological origin, that produces a quantifiable response in a signal transduction element when in contact with the target analyte. Most signal transduction mechanisms are optical (colorimetric, fluorescence, enhanced chemiluminescence), electrochemical or surface plasmon resonance (14, 15). Tissue biosensors, optical immunosensors, enzyme sensors, electrochemical sensors, quartz crystal, array and plasmon resonance biosensors have been applied to detect ochratoxin, aflatoxins, fumonisin and deoxynivalenol in different commodities. For example, zearalenone and its derivatives were detected in milk products with a yeast whole-cell bioluminescent sensor (genetically modified *Saccharomyces cerevisiae*), allowing detection at nanomolar concentrations (16).

Compared to other traditional analytical techniques, biosensors offer the possibility to monitor a large number of samples thus being a very convenient tool, that can also be automated, for screening toxins in routine analysis. The main limitation is regeneration of the receptor surface. The improvement of their specificity, sensitivity, reproducibility and stability are important requirements for future applications on a large scale.

IMMUNOLOGICAL METHODS

These are binding assays based on monoclonal or polyclonal antibodies raised against toxins (antigens), which can be performed as immunoaffinity column-based analysis (IAC) or enzyme-linked immunosorbent assay (ELISA) (17).

The principle of the ELISA test is the antigen-antibody reaction. The competitive assay format, in which the toxin competes with the enzyme conjugated to the toxin for specific immobilized antibodies, is often used in commercial available kits (Ridascreen, Aflatoxin Cup, etc.) (Fig. 1A). Bound enzyme conjugate converts the substrate into a colored, fluorescent or chemiluminescent active product. Another commonly used assay format is based on the competition between free and immobilized toxin for the binding sites for the toxin on the specific antibodies (Fig. 1B). Secondary antibodies labeled with an enzyme (*i.e.*,

peroxidase, alkaline phosphatase) added to the system will interact with a chromogenic, fluorogenic, chemiluminescent or electrochemical substrate to give a measurable result. Both direct and indirect competitive ELISA assays were used to measure common mycotoxins in fungal cultures (18).

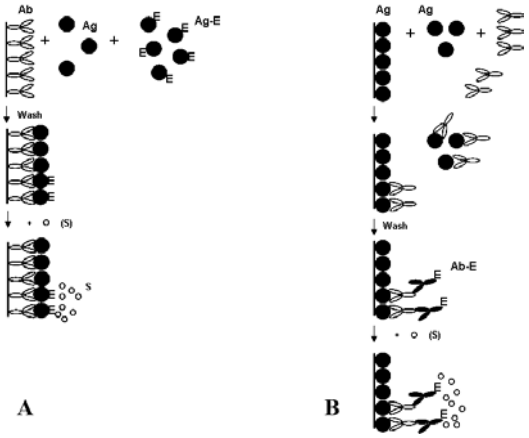

Fig. 1. – Direct (A) and indirect (B) competitive ELISA assay.

The critical point in using immunoassays is the stability of enzyme, the tracer element which is the most sensitive reagent. Several methods are available for its stabilization, including immobilization to a solid phase, chemical modification or addition of stabilizing agents (19).

Some examples of protocols that employ classical ELISA assay for mycotoxin determination in food commodities are presented in Table 2.

Table 2

ELISA protocols used in mycotoxins determination

Toxin	Protocol
Aflatoxins	Sequential injection immunoassay
Aflatoxin from milk (M1)	Competitive ELISA
Deoxynivalenol	Competitive ELISA (Veratox, Neogen Corp., Lansing, MI)
Fumonisin	ELISA commercial kit (Ridascreen, R-Biopharm, Darmstadt, Germany)
Ochratoxin	Indirect ELISA
Trichothecenes and zearalenone	Commercial ELISA kits (R-Biopharm, Darmstadt, Germany)
Trichothecenes	Commercial ELISA assay (QuantiToxTM Kit, Envirologix INC., Portland, Maine)
Zearalenone	Indirect ELISA

ELISA was combined to other techniques such as electrochemical sensors (20-22) and surface plasmon resonance (15) in order to give protocols with higher sensibility. Yuan *et al*. (15) reported a rapid and highly sensitive competitive immunoassay coupled to SPR for ochratoxin quantification in cereals and beverage. The toxin was immobilized on a monolayer surface containing gold nanoparticles through its ovalabumin conjugate with polyethylene glycol linker. By applying gold nanoparticles for signal enhancement the limit of detection was improved from1.5 ng/ml to 0.042ng/ml.

An enzyme linked immunomagnetic assay based on magnetic beads as solid phase and screen printed electrodes as sensing platform for HT-2 and T2 – toxins of trichothecenes family – detection was developed (23, 24). The use of an antibody clone with 100% cross-reactivity towards both toxins led to their simultaneous and highly sensitive detection.

To provide a highly sensitive system, *chemiluminescent* (CL) and *bioluminescent* (BL) reactions are exploited in immunoassays. These involve either the use of components of CL (luminol) and BL (luciferase) reactions as labels, or the use of the reactions to monitor an enzyme label or its products. CL and BL methods have been developed for many enzymes, *e.g.*, alkaline phosphatase, horseradish peroxidase (HRP), Renilla luciferase, xanthine oxidase. The most successful enzyme assay is the enhanced CL for HRP involving luminol, or isoluminol, hydrogen peroxide and p-iodophenol as enhancer. A direct CL method for alkaline phosphatase and adamantyl 1,2-dioxetane phenyl phosphate substrate is also very sensitive. The CL ELISA has a similar sensitivity to that obtained by ELISA for ochratoxin analysis in wheat (25).

Fluorescence polarization immunoassay (FP) is a method based upon the competition between free and fluorescein tracer-toxin for toxin specific monoclonal antibodies in solution. In this case, the detection does not involve an enzymatic reaction. Moreover, the separation of the bound and free label is not required. Such assay has been developed for aflatoxin and deoxynivalenol quantification in grains (26).

Application of immunoassays, and especially ELISA, became widespread because of the sensitivity, specificity, rapidity, in screening different commodities, relatively low cost and simplicity. Moreover, the detection limit is lower than those obtained with instrumental methods. A comparative study performed with ELISA and HPLC showed that the immunoassay tended to slightly underestimate the toxin content compared to HPLC, but it was more rapid than a screening tool. Today, ELISA is mostly used for rapid monitoring in industry, whereas chromatographic methods are applicable for research purposes.

CONCLUSIONS

In conclusion, although a broad range of detection techniques for mycotoxins are now available, new methods are still required, mainly for measuring multiple toxins with high sensitivity from a single matrix.

Acknowledgements. The authors acknowledge support from the CNMP–PNCDII Program, 61-045 and 61-030 /2007 projects.

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