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Immunochemical methods for rapid mycotoxin detection: evolution from single to multiple analyte screening. A review.

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Abstract

This review focuses on recent developments in immunochemical methods for detection of mycotoxins, with a particular emphasis on the simultaneous multiple analyte determination. This includes high-throughput instrumental analysis for the laboratory environment (microtiter plate ELISA, different kinds of immunosensors, fluorescence polarization immunoassay and capillary electrophoretic immunoassay), as well as rapid visual tests for on-site testing (lateral-flow, dipstick, flow-through and column tests). For each type of immunoassay perspectives for multiple analyte application are discussed and examples are cited.

Introduction

Mycotoxins are toxic secondary fungal metabolites with deleterious effects for humans and animals. Some mycotoxins have been proven to be carcinogenic while others are nephrotoxic, hepatotoxic or immunosuppressive. Many kinds of food and feed can be contaminated with mycotoxins since they can be formed in commodities before as well as after harvest. Commodity circulation around the world, irregular storage and a lot of (other) uncontrolled reasons cause mycotoxin contaminations. Therefore many countries have set regulations with maximum levels for the major mycotoxins in different commodities.

Two main groups of methods exist for mycotoxin analysis: laborious methods for determination of mycotoxins with high sensitivity and precision, and screening methods for rapid detection in a non-laboratory environment. The first group of methods is particularly represented by liquid chromatography in combination with mass spectrometry or fluorescent (Fl) detection. Recently advanced chromatographic methods are more and more shifted to multiple mycotoxin detection. Indeed, several mycotoxins co-occur and can simultaneously contaminate foodstuffs: aflatoxin B1 (AfB1), fumonisin B1 (FB1) and zearalenone (ZEA) in Korean corn (Park et al. 2002), FB1, other *Fusarium* mycotoxins and AfB1 in corn from China (Chu and Li, 1994), fumonisins and aflatoxins in Brasilian corn (Ono et al. 2001), AfB1 and ochratoxin A (OTA) in spices (Fazekas et al. 2005). Simultaneous determination of multiple mycotoxins makes mass spectrometric detection more popular. Multiple mycotoxin determination however causes changes in extraction, clean-up and preconcentration procedures. This requires compromises to establish optimal conditions during extraction and sample preparation. Instead of immunoaffinity columns (IAC) for single and few analyte determination, MycoSep® columns (Berthiller et al. 2005; Biselli and Hummert, 2005), C_{18} sorbents (Sørensen and Elbaek, 2005) and re-extraction with hexane (Rundberget and Wilkins, 2002) were used for multiassay clean-up. At present the determination of 16 mycotoxins in fungal cultures (Delmulle et al. 2006), 18 mycotoxins and

metabolites in bovine milk (Sørensen and Elbaek, 2005), 9 mycotoxins in cheese (Kokkonen et al. 2005) and 39 mycotoxins in wheat and maize (Sulyok et al. 2006) are described.

Absolute leaders in the second group of analytical methods for mycotoxins are immunochemical methods with high sensitivity and selectivity provided by specific antibodies. Application of immunoassays for individual mycotoxin rapid screening is now a common analytical practice. Main trends for research in this field are sensitivity improvement, matrix effect reduction, simplification and shorter time of analysis. Immunomethods for rapid detection of individual mycotoxins are summarized in recent reviews concerning immunochemical methods (Maragos 2004; Zheng et al. 2006) and analytical methods in general (Krska et al. 2005; Gilbert 1999). Besides, reviews were published devoted to the determination of single mycotoxins or groups of related toxins, such as ochratoxin A (OTA) (Visconti and De Girolamo 2005), trichothecenes (Krska et al. 2001; Koch 2004; Schneider et al. 2004), aflatoxins (Maragos 2002), citrinin (Xu et al. 2006).

By analogy with chromatographic methods, the new trend is the development of multi-mycotoxin screening methods. This review summarizes immunochemical methods for rapid mycotoxin analysis with focus on multiple analyte determination. State of the art and perspectives for multiple mycotoxin detection are described in comparison with existing multi-methods for other groups of low molecular analytes. Both instrumental and non-instrumental immunochemical methods are presented.

Instrumental methods

To obtain quantitative or semi-quantitative results immunochemical methods, based on relatively simple equipment (microplate reader, luminometer, capillary electrophoresis system, etc.) are usually used. Contrary to chromatographic methods, immunoassay is usually combined with a simple extraction, pre-concentration and clean-up procedure, or often without pre-concentration

and clean-up. The most frequently used approaches for mycotoxin immunoassay are binding of specific antibodies to a solid support (direct competitive enzyme-linked immunosorbent assay (ELISA) format) or coated antigens (indirect competitive ELISA format). These formats are realized in all non-homogenic assays: microtiterplate immunoassays and sensors. Homogenic assays are represented by fluorescent polarization immunoassay and capillary electrophoretic immunoassay.

Microtiter plate enzyme-linked immunosorbent assays

The microtiter plate ELISA format is commonly used as a rapid test for mycotoxins. ELISA tests are commercially available for the determination of individual mycotoxins (Aflatoxin B_1, (AfB1) aflatoxin M_1 (AfM1), deoxynivalenol (DON), OTA, ZEA, T-2 toxin, citrinin) in some foodstuffs as well as for groups of mycotoxins, e.g. aflatoxins (B_1, B_2, G_1, G_2), fumonisins (B_1, B_2 and B_3) and trichothecenes. They are useful tools for screening and quantification and offer benefits with respect to speed and sensitivity. Adaptability of the standard ELISA procedure for individual or groups of related mycotoxins determination depends on the cross-reactivity of the antibodies. For rare mycotoxins in special matrices ELISAs are under development. An ELISA was developed for satratoxin G and other macrocyclic trichothecenes associated with indoor air (Chung et al. 2003).

Different approaches are used for simultaneous determination of several analytes. The multiple label method for simultaneous analyte detection is not widespread. The possibility to determine two different analytes in one tube using a direct ELISA format was shown for the thyroid hormones thyroxin and triiodothyronine using conjugates with two different enzyme labels – alkaline phosphatase and β-D-galactosidase (Blake et al. 1982). But this approach is not prevalent, mainly because the optimal conditions for enzymatic reactions are different.

At present two main ways for multiassay with ELISA exist: by using antibodies with suitable cross-reactivities for the determination of groups of related compounds and also by application of antibodies to different compounds in separate rows in one ELISA plate. Determination of the total concentration of related compounds (total aflatoxins, total fumonisins) can be realized using one antibody with high cross-reaction to all representatives of the target group of mycotoxins. Recently, a competitive direct and indirect ELISA format was developed using a monoclonal antibody specific for an acetylated form of DON, but also showing sensitivity and cross-reactivity for nivalenol (NIV), DON and some DON derivatives (Maragos et al. 2006). Also, application of several antibodies with different cross-reaction allows to differentiate and to quantify the individual concentration of cross-reacting analytes using different mathematical models. This approach was developed for the determination of triazine herbicides and their metabolites (Wortberg at al. 1995, 1996; Jones et al. 1996; Muldoon et al. 1993; Bhand et al. 2005) and also for the determination of 2,4-dinitro-phenol and 4-nitrophenol (Nistor et al. 2004). We suppose that this approach can also be successfully realized for AfB1 determination in the presence of other aflatoxins, DON in the presence of NIV, T-2 toxin in the presence of HT-2 toxin, etc.

In the literature we did not find any example of determination of multiple non-cross-reacting mycotoxins by ELISA using the second multi-assay approach (application of antibodies for different compounds in separate rows of one ELISA plate). For other compounds however we did. A single qualitative ELISA format, based on the application of three different polyclonal antibodies (anti-azaperol, anti-propionylpromazine and anti-carazolol) was developed for the rapid screening of sedatives including butyrophenone, azaperone, phenothiazines acepromazine, propionylpromazine, chlorpromazine and the β-blocker carazolol (Cooper et al. 2004). An analogous approach was used in a multi-antibiotic ELISA for screening five banned antimicrobial growth promoters. In this test kit four polyclonal antibodies were applied: anti-bacitracin, anti-olaquindox and anti-virginiamycin were used for the detection of the

corresponding antibiotics while the anti-spiramycin antibody was also used for tylosin as this antibody showed high cross reactivity with tylosin (Situ and Elliot 2005; Situ et al. 2006).

Immunosensors

Immunosensors are devices based on the detection of analyte-antibody interactions. When biological molecules specifically interact, changes in physicochemical parameters are generated and are electronically sensed (Marco et al. 1995; Patel, 2002; Tothill, 2001). To transform this interaction to an analytical signal suitable for analyte concentration measurement, three main groups of sensors have been developed: luminescent/colourimetric sensors, surface plasmon resonance sensors and electrochemical sensors. They are mainly based on different ELISA formats and use automated flow devices. Usually the term biosensor implies reversibility or real-time readout. The analytical cycle includes steps of competition (not for the non-competitive format), immunocomplex capture, display (for enzyme labels), and sensor regeneration, all operations being carried out automatically (Gonzalez-Martinez et al. 2001). As for other rapid immunoassays, the sensor is usually combined with a simple methanol (or acetonitrile) - water-extraction of mycotoxins from food samples.

Colourimetric and luminescent sensors. Colourimetric and luminescent sensors are based on the visible or UV light transformation into the analytical signal. These sensors use different supports – microbeads, waveguides or optical fibers.

A colourimetric sensor was developed for AfB1 detection using the direct competitive ELISA principle with anti-AfB1 antibodies bound to polymethylmethacrylate microbeads as the solid support. As enzymatic label alkaline phosphatase was used. The colour development was measured with a spectrometer by reading the absorbance across the beads at 620 nm. This method could detect AfB1 down to a level of 0.2 ng/mL in artificially contaminated food

materials, which is comparable to the sensitivity of a microtitre plate ELISA (Garden and Strachen, 2001).

Fluorescent sensors for mycotoxin determination are more frequently described. They have been realized for mycotoxin detection in three different formats: a direct competitive format, based on the fluorescence of a special label, an indirect competitive format with labelled fluorescent antibodies and a non-competitive format, based on the native fluorescence of the mycotoxin molecules. In comparison with the colourimetric sensor, fluorescent labelling speeds up the assay time by eliminating the need for substrate addition and colour development.

A sensor, based on the competitive direct ELISA format was developed for FB1 determination in corn. Monoclonal antibodies were covalently bound through a heterobifunctional silane to an etched 800 μm core optical fiber. The analytical signal was generated by the fluorescence intensity of fluorescein isothiocyanate (FITC) -labelled fumonisin derivatives. This sensor showed a working range of 10 - 1000 ng of FB1/mL, a midpoint of dynamic range of 70 ng/mL, and a limit of detection of 10 ng/mL. (Thompson and Maragos, 1996; Maragos and Thompson, 1999). A completely automated flow-through immunosensor with fluorescence detection, based on the direct competitive ELISA format, was developed for ZEA detection. For standard solutions the immunosensor showed a dynamic range from 0.019 to 0.422 ng/mL and a detection limit of 0.007 ng/mL. Corn, wheat, and swine feed samples were analyzed with this device after extraction of ZEA using accelerated solvent extraction (Urraca et al. 2005).

The possibility to apply immunosensors with horseradish peroxidase (HRP) as a fluorescent label for multi-analyte format was tested for the simultaneous determination of the insecticide carbaril, the herbicide atrazine and the antifouling agent irgarol 1051 as model targets. It was shown that this approach with one fluorescent label allowed to only determine the total concentration of analyte (Gonzalez-Martinez et al. 2001).

Fluorescent biosensors using the indirect competitive ELISA principle were developed for AfB1 (Strachan et al. 1997) and ZEA (Carter et al. 2000). FITC fluorescence intensity was measured. The immunosensor allowed detecting AfB1 down to a level of 4 ng/g in reference food materials such as nut puree, peanuts, pistachio. This approach is suitable for both fluorescent and non-fluorescent analytes and can be applied for multiple mycotoxin assays if separate zones of beads with conjugates for different analytes are used.

An array biosensor system was developed for simultaneous, multiplexed detection of OTA, FB1, AfB1 and DON. NeutrAvidin slides patterned with biotinylated analogs of the mycotoxins allowed realizing a one-step indirect competitive assay in the flow cell. A biotinylated secondary antibody lane was also added on the planar waveguides as a positive control. The slides were then exposed to a mixture of various combinations of mycotoxin standards and Cy5-labelled anti-mycotoxin antibodies. The ability to run both sandwich and competitive immunoassay formats on a single waveguide surface was also shown. This allowed the user to monitor for both large and small molecular weight food contaminants simultaneously (Sapsford et al. 2006). An analogous array biosensor was developed using a single waveguide for the detection of multiple toxins (staphylococcal enterotoxin B, ricin, cholera toxin, botulinum toxoids, trinitrotoluene, and also FB1). For the different analytes various immunoassay formats were used. For FB1 detection biotinylated FB1 was immobilized on the waveguide surface. FB1 in solution competed with the immobilized FB1 for binding to the Cy5-labelled fluorescent antibodies. Decreased fluorescence indicated the presence of fumonisin in the samples. For the large analytes the sandwich format was used (Ligler et al 2003).

Non-competitive formats suppose binding of target mycotoxins without competition with a labelled molecule. No competition between reagents and absence of washing steps simplify the assay. The main restriction here is the presence of native fluorescence of mycotoxins. Because the fluorescence of the mycotoxin itself is detected, the response of the sensor is directly proportional to the analyte concentration. This approach was used for AfB1 detection (Maragos

and Thompson, 1999). Carter et al. (1997) compared the sensitivity of several fiber-optic approaches for AfB1 detection in peanut extract with ELISA: indirect competitive ELISA showed an LOD at 0.1 ng/mL, the same format, transferred to a fiber-optic detection system had an LOD for AfB1 at 5 ng/mL. Replacement of the enzymatic alkaline phosphatase label by FITC allowed to increase the sensitivity up to 1 ng/mL. The native fluorescence-based sensor was found to be the most sensitive format with an LOD of 0.05 ng/mL. Since many mycotoxins have their own fluorescence (aflatoxins, OTA, ZEA, citrinin) this approach could be expanded for their simultaneous determination. Separate signals could be obtained by using specific locations of the antibodies on the waveguide or by spectral selection. The disadvantage of this approach, however, is the sensitivity of the signal to the refractive index of the sample solution.

To our knowledge chemiluminescent sensors have not yet been applied for multiple mycotoxin analysis, however they show good potentials for multianalyte detection. Chemiluminescence could provide high sensitivity, while no excitation source or additional optics are necessary. A parallel affinity sensor array (PASA) using chemiluminescent labels (peroxidase/luminol) was developed for detection of triazine herbicides. Reagents such as antibodies or hapten conjugates were immobilized on a glass slide, which formed a biochip. Direct and indirect competitive immunoassay formats were developed with detection limits at 20 ng/L terbutylazine (Weller et al. 1999).

For the simultaneous determination of cross-reacting analytes the same approach as for microtiterplate ELISA was used, e.g. the application of different antibodies and mathematical analysis of results. Five separately immobilized antibodies with different cross-reactivity towards s-triazines were used in combination with neural network to identify and estimate amounts of atrazine, terbuthylazine and ametryn (Samsonova et al. 1999). An approach for identification of cross-reacting analytes on the base of PASA was described for triazines as a model substance class. Samples of atrazine, terbuthylazine and simazine at a concentration level of 0.1 mg/L, and deethylatrazine at 0.3 mg/L were quantified (Winklmair et al. 1999).

Thus, although luminescent sensors allow spectral discrimination of compounds, separate disposition of immunoreagents for individual compounds serves as more practical use for miniaturization, process standardization, and consequently shows more perspectives for multiple mycotoxin determination.

Surface plasmon resonance sensors. Surface plasmon resonance (SPR) is an optical phenomenon used to measure changes on the surface of thin metal films (usually Au or Ag) under conditions of total internal reflection (Homola et al. 1999). This principle allows the direct detection of the biological interaction without labelling any of the interactants. In contrast to fluorescence sensors, based on different kind of equipment, almost all present-day SPR sensors for mycotoxins are based on Biacore sensors (Biacore AB, Uppsala, Sweden). Immunoassay formats for the SPR sensor are similar to the ones described above: indirect competitive ELISA, in which antigen is attached to the metal surface, direct competitive ELISA or non-competitive format with attached antibodies. Because most mycotoxins have a low molecular mass, the mass change caused by binding to the surface could be too small to result in a significant change in refractive characteristics. Therefore, for mycotoxin quantification indirect methods with antigen-modified surfaces have mostly been used.

Sensitivity of SPR sensors and microtiter plate ELISAs were compared for AfB1 using the same immunoreagents (polyclonal antibody and AfB1-BSA conjugate). In contrast to ELISA (12 – 25000 ng/mL) the SPR sensor (3.0 – 49 ng/mL) had a more sensitive, but narrow, linear range of detection (Daly et al. 2000). Van der Gaag et al. (1999) developed an assay for the detection of AfB1 in spiked grain samples based on commercially available monoclonal antibodies with a detection level of 0.2 ng/mL.

For SPR sensors, as for other sensor types, one of the critical characteristics is the regeneration process. If the antibody has high affinity, the regeneration solution cannot completely remove the bound antibody from the sensor surface immobilized with hapten–protein

conjugates. Application of single chain antibody fragments instead of antibodies for AfB1 determination allowed to simplify the regeneration procedure and to make regeneration conditions less stringent (Dunne et al. 2005). A similar approach allowed to detect AfB1 at a level of 3 ng/g in grain sample extracts (Daly et al. 2002).

For DON rapid screening in wheat samples polyclonal antibodies were used. Additional clean-up of extracts using MycoSep™ columns was necessary (Schnerr et al. 2002). Authors mention that in beer DON could be analysed after degassing, without other preparations. Similar sensitivity was reached for DON determination with monoclonal antibody. Wheat samples were only filtered and diluted. No significant loss of surface activity was observed after more than 500 injection and determination cycles (Tudos et al. 2003). Fumonisin molecules have a larger molecular weight than other mycotoxins. This allows using the non-competitive format with bound antibody for direct fumonisin determination. This approach was realized with a home-made sensor by Mullett et al. (1998) with a detection limit of 50 ng/mL.

For the simultaneous determination of AfB1, ZEA, DON and FB1 with LODs of 0.2, 0.01, 0.5 and 50 ng/g, respectively, an inhibitive sensor format with four serial connected flow cells was developed (van der Gaag et al. 2003). Different procedures were used for immobilizing mycotoxins on the sensor chip. DON was immobilized with BSA as a carrier protein, for FB1 aminocaproic acid was used as a spacer, while AfB1 and ZEA were immobilized after chemical activation. Mycotoxins were extracted with acetonitrile–water (90/10 v/v), cleaned with a Mycosep 224 MFC column and 10 times diluted. Before injection into the sensor, the sample extract was mixed with the four mycotoxin-specific antibodies. Extraction and clean-up of the sample required approximately 15 min. Additionally, the measurement took 10 min, including regeneration of the sensor chip surface, making a total of approximately 25 min for the simultaneous determination of four mycotoxins in a single sample.

Contrary to luminescent sensors and common immunoassay methods, the SPR approach does not require special labels and therefore sorption by non-specific interactions could occur

and interfere under conditions of real sample analysis. This demands additional clean-up steps, which make the analysis more complicate and extensive. Besides, since no signal enhancement is included in SPR, the test sensitivity is relatively low compared with enzyme or fluorescence immunoassays.

Electrochemical sensors. Electrochemical immunosensors for mycotoxins are usually based on the competitive ELISA principle. Electrochemical transducers allow detecting redox labels directly. In the indirect competitive ELISA format the immunosensor strip, coated with analyte-protein conjugate was incubated with a small volume (few μL) of sample (or standard) solution and specific antibody. The amount of antibody that reacted with the immobilised analyte, was evaluated using a secondary antibody labelled with alkaline phosphatase. The enzyme activity was detected by adding 1-naphthyl phosphate substrate resulting in the production of the electrochemically active product, 1-naphthol. In the direct competitive ELISA format the working electrode was coated with antibody. Competition was run between analyte and analyte-enzyme conjugate. Finally activity of the enzymatic product 1-naphthol was detected.

An indirect competitive ELISA based screen-printed electrode was developed for simple and fast measurement of AfB1 in barley using differential pulse voltammetry. LOD for AfB1 determination with this sensor was 30 pg/mL. It was near to the LOD of a spectrophotometric ELISA with the same immunoreagents (20 pg/mL) (Ammida et al. 2004, 2006). Screen-printed electrodes were also developed for OTA determination in wine (Alarcon et al. 2004) and wheat (Alarcon et al. 2006). Direct and indirect competitive ELISA formats were compared and the direct format was shown as more sensitive: working range was at 0.05–2.5 ng/mL in comparison with 0.1–7.5 ng/mL in the indirect format. A similar sensor, based on direct competitive ELISA, was used for AfM1 detection in milk. The screen-printed electrode exhibited linearity between 30 and 240 pg/mL for direct AfM1 detection in milk following a simple centrifugation step but without dilution or other pretreatment steps. The total assay time was about 75 min (Micheli et al.

2005). Screen-printed carbon electrodes in direct competitive ELISA format was used for AfB1 detection. Using this electrochemical immunosensor, a calibration plot for AFB1 was obtained over the concentration range 0.15 to 2.5 ng/mL, giving a detection limit of around 0.15 ng/mL in buffer solution (Pemberton et al. 2006).

While redox labels are attached to reagents, immobilised at the sensor surface, enzyme activity leads to soluble products. This is an obstacle for detection at multiple sites (for multiple analytes) in one reaction vessel. This problem may be overcome by the use of microelectrode arrays or other miniaturised transducer structures (Brecht and Abuknesha, 1995). Pemberton et al. (2006) mentioned that an array configuration of the AfB1 sensor is suitable for use in conjunction with 96-well microtiter plates; indeed, each step of the immunoassay was performed by dipping the electrodes into microwells. These results represent the initial studies towards the development of an automated instrument for multi-analyte determination using immunosensor arrays for mycotoxin detection. Therefore, it can be concluded that electrochemical sensors in array format are a high promising approach towards rapid, automated multi-mycotoxin analysis. One of the goals of the European FP6 project BioCop is the development of electrochemical sensors for rapid trichothecene analysis and first results can already be found on the website (www.biocop.org).

Fluorescence polarization immunoassay

Contrary to the methods described above, fluorescence polarization immunoassay (FPIA) is a homogeneous method; it functions without attachment of immunoreagents to solid surfaces. In this format, the analyte is labelled with a fluorophore, usually fluorescein. Free analyte and labelled analyte (tracer) compete for specific antibody-binding sites in solution. The analytical signal is the value of fluorescence polarization of the fluorescein label, which corresponds to the rate of its rotation in solution. Free tracer is a small mobile molecule with a high rate of rotation.

Tracer bound to antibody has a lower rate of rotation and, consequently, a higher fluorescence polarization value. Therefore the polarization value is inversely proportional to the amount of free analyte present in the sample (Figure 1).

As separation of free and antibody-bound analyte is not necessary in FPIA and equilibrium time is usually very short, the test procedure is relatively simple, without any washing steps. Polarization measurement may be performed using portable equipment. Sometimes kinetics of interactions can be relatively slow and incubation time can greatly affect the assay sensitivity. For DON determination with monoclonal antibodies the midpoints for the competition curves ranged from 0.03 µg/mL with a 15 s incubation to > 1µg/mL with a 12 min incubation (Maragos et al. 2002). Using another antibody clone with new DON-fluorescein tracer improved the kinetics, resulting in shorter interaction time (Maragos and Plattner, 2002). With the same antibody clone Lippolis et al. (2006) developed a method for the rapid quantification of DON in durum wheat kernels, semolina, and pasta at levels foreseen by existing international regulations. A background signal was observed in both spiked and naturally contaminated samples, strictly depending on the testing matrix. After subtracting the background DON level, accurate quantification of DON was possible at levels greater than 0.10 µg/g for all matrices. Comparative analyses of naturally contaminated samples performed by FPIA and HPLC-Fl methods showed a good correlation ($r > 0.995$) (Lippolis et al. 2006). FPIA also allowed screening FB1 in maize samples with an LOD at 0.5 µg/g (Maragos et al. 2001). FPIA for aflatoxin determination with monoclonal antibodies was developed for naturally contaminated corn, sorghum, peanut butter and peanut paste. The assay could be used as a screening method for aflatoxin analysis in the range of 5 - 200 ppb and showed good agreement with HPLC-Fl. (Nasir and Jolley, 2002). Dynamic range of FPIA for OTA determination in barley was from 5 to 200 ng/mL with midpoint at 39 ng/mL and LOD at 3 ng/mL (Shim et. al. 2004). Maragos and Kim (2004) developed FPIA for ZEA in maize. The assay could be used to detect as little as 0.11 µg/g maize within 10 min, including extraction time.

The analytical signal in FPIA is measured like a mean characteristic of the solution (namely the fluorescence polarization value). Therefore it cannot be used for simultaneous detection of several individual analytes in a single tube. FPIA only allows detecting a signal, related to the presence of one or more compounds without discrimination. In this respect FPIA was used for urine screening for multiple drugs of abuse: cocaine metabolite, amphetamine, and several barbiturates (Colbert and Childerstone, 1987). However FPIA has potentials for use as an array multi-immunoassay with separate tubes for simultaneous parallel detection of several analytes.

Capillary electrophoretic immunoassay

Capillary electrophoretic immunoassay (CEIA) allows combining separation of analytes from each other and from sample matrix, with high specificity of antibodies and sensitive detection such as laser induced fluorescence detection (LIF) (Bao, 1997; Yeung et al. 2003). Like FPIA, CEIA is performed without a supporting phase, e.g. in solution. Besides, CEIA uses a similar set of reagents as for FPIA, e.g. specific antisera and fluorescein-labeled tracers. The assay procedure includes the incubation of extract or liquid sample with antibody solution and tracer and then application of a tiny aliquot (nL to pL) of this mixture onto the capillary. Separation of the unbound fluorescent tracer and the antibody-tracer complex occurs as a result of the electrophoretic forces. LIF detection allows obtaining peak areas proportional to the fluorescein labelled analyte concentrations. In the case of absence of free analyte, all tracer is bound to the antibody, so the signal (peak area) of non-bound tracer is minimal, while the signal of the complex is maximal. If analyte is present in the sample, the peak area of the non-bound tracer increases, while the signal of bound tracer decreases.

The combination of CE with immunoassay was used for the analysis of fumonisins in maize. Fumonisin standards could be analyzed with this technique with a total analysis time of 6 min, 2 min of which were required for washing the capillary between analyses. In optimal

conditions the midpoint of dynamic range was between 500 and 1700 ng/mL of FB1. The technique was able to detect FB1 in corn samples spiked with high levels of FB1 (> 10,000 ng/g) (Maragos, 1997). The author concluded that sensitivity in this format highly depended on the relative affinity of the antibody for fumonisin and the tracer under conditions of high electric field strength.

The advantage of CEIA is the integration of the separation step in this technique. So, a simultaneous multi-component analysis is possible due to the high resolving power of capillary electrophoresis. Standard or sample is added to a mixture of specific antibodies and tracers. This approach was applied in the clinical assay area for detection of some drugs and drugs of abuse. Simultaneous determination of methotrexate and vancomycin was described by Suzuki et al. (2003). Multianalyte capillary electrophoretic immunoassay was developed for methadone, opiates, benzoylecgonine (cocaine metabolite) and amphetamines (Caslavska et al. 1999; Thormann et al. 2000). For morphine and phencyclidine determination in urine samples cyanine dyes were used as fluorescent labels: morphine was derivatized with Cy5 , phencyclidine with Cy5.5 (Chen and Evangelista, 1994).

As evidenced by the foregoing, CEIA provides good possibilities for multiassay and its expansion in the food control area e.g. for multiple mycotoxin determination can be expected.

Non-instrumental methods

In the last decade there has been a continuous growth in development of rapid methods for mycotoxin analysis. Moreover, non-instrumental rapid screening techniques which could be used outside the laboratory environment, at the place of sampling, are becoming more and more important. Indeed, results are expected immediately, so that commodities can be further processed without delay. Additionally, quantification of the toxin concentration is not always

necessary as the first step of an analytical survey, and a simple presence/absence test can be sufficient.

The non-instrumental estimation of results is based on visual evaluation. So, different visual labels are used, such as enzymes for catalytic enzymatic reactions, colloidal gold, fluorescent labels and liposomes, encapsulating a visible dye. As in instrumental-based methods, the basic immunoassay formats used in non-instrumental tests for determination of mycotoxins are direct or indirect competitive ELISAs. Non-instrumental tests usually give qualitative results as positive/negative (Yes/No), characterizing the presence (or absence) of the target analyte in concentrations higher than the fixed cut-off level. The cut-off level can be established on the basis of either noticeably reducing of colour development or complete colour suppression. Some tests assume semiquantitative estimation on the basis of the colour intensity. To make interpretation of results easier, special control zones have been included in the majority of present-day tests. All rapid tests with visual estimation of results are combined with a simple sample preparation procedure: extraction with methanol or methanol / water (or buffer) mixture, filtering and dilution with buffer.

Commercially available test kits for mycotoxin determination are presented on the website of AOAC International (www.aoac.org) and of the European Mycotoxin Awareness Network EMAN (www.mycotoxins.org). In this section three types of membrane-based tests and one type of gel-based tests are examined.

Lateral flow tests

Lateral flow tests, also called immunochromatographic strip (ICS) tests, are unique rapid and user-friendly test formats which need neither instrument nor additional chemicals or handling steps. Usually, the lateral flow strip consists of 3 parts: conjugate pad, porous membrane and absorbent pad. After application on the conjugate pad, the liquid components of the assay move along the membrane by capillary flow to the absorbent pad. Lateral flow devices

can be made in several forms depending upon which reagent is labelled (the antigen or the antibody) and the type of the label.

The most popular label for the ICS test is colloidal gold. Particles of colloidal gold with a 40 nm diameter provide binding zones with red colour. For assay realisation colloidal gold-labelled specific antibodies are deposited on the conjugate pad (Figure 2). The test line (or lines in the case of multiassay) is coated on the membrane with analyte-carrier protein (such as BSA) conjugate. To simplify the result interpretation, a control line is used as a secondary zone, coated with antibody, above the test line. Liquid sample material (or sample extract), applied on the sample pad, dissolves colloidal-gold labelled specific antibodies and carries them along the membrane. When target analyte is present in the sample, the specific antibodies bind it. The whole complex migrates along the membrane and binds with the secondary antibodies on the control line. If the analyte is absent, the labelled specific antibodies bind to the analyte-BSA conjugate on the test line. So, absence of analyte results in red colour both for test line (lines) and control line. In the presence of target analyte (analytes) above the cut-off value only the control line is red.

Lateral flow devices with colloidal gold labels are commercially available for aflatoxins, DON, T-2 toxin, OTA and ZEA. Assay sensitivity can be improved by using a portable hand-held instrument for quantification. Further, ICS tests with colloidal gold labels are described in literature. A one-step lateral flow dipstick was developed for FB1 using polyclonal antibodies. Matrix effects were tested for corn, barley, peanuts, oats, rice and sorghum, and interference was completely eliminated by a 15-fold dilution of the sample extract with buffer solution (Wang et al. 2006). For OTA detection an ICS based on monoclonal antibodies was developed with a detection limit of 500 ng/mL in buffer solutions (Cho et al. 2005). The same format was used for AfB1 detection in pig feed with colloidal gold labeled monoclonal anti-AfB1 antibodies. In optimal conditions the limit of detection, resulting in no colour on the test line, was at 5 ng/g AfB1 (Delmulle et al. 2005). Comparison of membrane-based immunoassays with colloidal gold

labels to a rapid variant (20 min) of a microtiter ELISA with HRP label for FB1 determination was realized by Wang et al. (2006), and this in similar conditions with the same immunoreagents. Lateral flow and flow-through formats had a visual detection limit of 1.0 ng/mL, so only two times higher than the ELISA format (0.5 ± 0.2 ng/mL).

A colloidal carbon - antibody conjugate, which resulted in black lines, was tested as label for sporidesmin A ICS determination, but its sensitivity was less (25 ng/mL) than for colloidal gold conjugate (up to 4 ng/mL) (Collin et al. 1998). Labelling with polystyrene microspheres was proved unsuitable for use in ICS due to non-specific attachment of microspheres to protein bands on the ICS membrane (Collin et al. 1998). A two-step procedure for AfB1 detection was developed using a label with visible dye sulforodamine B, encapsulated into dipalmitoylphosphatidyl derivatives liposomes. Firstly the ICS test was inserted into a test tube with standard solution. After 3 min it was transferred to another test tube with AfB1 labeled with liposome. The second step was needed because liposomes are instable in the presence of organic solvent, usually used for extraction of mycotoxins (Ho and Wauchope, 2002).

A lateral flow device using an enzymatic marker, e.g. a lateral flow ELISA, needs additional steps (washing steps) but allows improving sensitivity. It was shown that at the same conditions of immunoassay, enzymes in the presence of chromogenic substrate could provide a 10 times better sensitivity than with colloidal gold labels (Zhang et al. 2006).

To our knowledge, ICS tests for multiple mycotoxin determination have not yet been developed up to this moment, but multi-analyte ICS exist for two insecticides. For simultaneous determination of carbaril and endosulfan, the sample solution was incubated with colloidal gold labeled anti-carbaril and anti-endosulfan antibodies and pipetted on the sample application site. After the liquid migrated toward the two test lines and the control line, different intensities of colour on the test lines could be visually observed (Zhang et al. 2006). This gives confidence in the possibility to also apply multianalyte ICS tests for mycotoxin detection.

Dipstick tests

Dipstick or strip or dot-ELISA assay is usually based on a membrane with a spot (or spots) of the specific antibody and needs several assay steps. According to the direct competitive ELISA scheme, the dipstick with attached antibody is placed in different solutions containing the sample, the analyte-enzyme conjugate and then the chromogenic substrate.

A two step dipstick was established for 15-acetylDON (15-acDON). The dipstick with one antibody zone was applied to a mixture of sample solution and 15-acDON-HRP conjugate, and then it was transferred to the chromogenic substrate solution. A separate dipstick was used as negative control. The detection limit in buffer solution was 5 ng/mL – more than 10 times higher than with a microtiter plate ELISA (0.35 ng/mL). At this toxin level the dot colour development was sufficiently reduced in comparison with the negative control. No colour development was detected at 20 - 25 ng/mL (Usleber et al. 1993). A similar dipstick was established for T-2 toxin in wheat. In optimal conditions a visible LOD was at 0.25 ng/mL in buffer solution. Colour development was completely suppressed at 3 ng/mL T-2 toxin. It was possible to make visually a clear distinction between the negative control and a wheat extract spiked with 12 ng/g T-2 toxin (De Saeger and Van Peteghem, 1996). To integrate the negative control into the dipstick, anti-HRP antibody was used. A dipstick for FB1 determination contained two lines: one with anti-FB1 antibodies and a control line with anti-HRP antibodies. The anti-HRP antibodies bound FB1-HRP in a non-competitive format. After substrate application, colour developed on two lines (Schneider et al. 1995a).

More multianalyte dipsticks for mycotoxin determination were reported in comparison with other non-instrumental immunoassays. On one dipstick five specific antibody solutions (anti-AfB1, T-2 toxin, 3-acDON, roridin A and ZEA) were spotted onto spatially distinct zones of the membrane. To perform the competitive dipstick assay, a mix of the five toxin-HRP conjugates was added to the mycotoxins solution and the dipstick was incubated in it for 30 min.

For each dipstick, a separate negative control dipstick was prepared with five spots and put into a negative control tube. The detection limits for spiked wheat samples were 30 ng/g for AFB1, 100 ng/g for T-2 toxin, 600 ng/g for 3-acDON, 500 ng/g for roridin A and 60 ng/g for ZEA (Schneider et al. 1995b). Authors mentioned that the sensitivity of this multimycotoxin dipstick was less than for single analytes. This was connected with two reasons. First: to simplify the interpretation of results only a complete spot colour suppression was scored positive. Second: immunoreagent concentrations were chosen to obtain equal colour intensities for all five negative spots. Therefore, it was necessary to make some tests less sensitive compared with the single analyte test, e.g. by using a higher concentration of the toxin-enzyme conjugate .

For simultaneous screening of FB1, AfB1 and ZEA a line immunoblot assay was developed by binding the relevant antibodies to segmental sections of the membrane. The proposed method allowed evaluating the concentration level of each mycotoxin as a result of the application of three different dilutions for each specific antibody. Detection limits were determined by visual comparison of positive strips and negative controls as 500, 0.5 and 3 ng/mL for FB1, AfB1 and ZEA, respectively (Abouzied and Pestka, 1994).

Simultaneous determination of cross-reacting related compounds in dipstick format was demonstrated for zearalenone and aflatoxin families. An immunoblot approach, called ELISAGRAM, combined sensitivity and selectivity of ELISA with the capacity of thin layer chromatography (TLC) to separate structurally related compounds. In the first step separation by TLC allowed to discriminate the individual mycotoxins. The next step was blotting the TLC plate with an antibody-coated membrane. The following steps, including the application of conjugate, washing buffer and substrate were as in a common dipstick assay scheme (Pestka, 1991). This approach was used for separation and determination of four aflatoxins (AfB1, AfB2, AfG1 and AfG2) and two zearalenones (ZEA and α-zearalenol). So, different formats of dipsticks give potentials to simultaneously determine both cross-reacting and non-cross-reacting mycotoxins.

Flow-through tests

Flow-through or immunofiltration assay (IFA) or enzyme-linked immunofiltration assay (ELIFA) for mycotoxins utilizes a direct competitive ELISA format, where specific antibodies are attached to a membrane. The membrane is placed on an absorbent body, which inhibits the immediate back-flow of fluids that could obscure results. The sample is added to the upper surface of the membrane where it flows through the membrane into the pad of absorbent material while analyte binds to the antibody spot on the membrane. Then the analyte-enzyme conjugate is added and bound by the remaining not-bound antibodies. The last step is the chromogenic substrate application to obtain coloured product in the presence of enzyme. Colloidal gold application as a visual label for IFA allowed avoiding chromogenic substrate application (Wang et al. 2006). But contrary to lateral flow tests, colloidal gold is not commonly used as a label for flow-through assays.

IFA for OTA detection in wheat was established using the format described above. An OTA concentration of 4 ng/g in spiked wheat completely suppressed the colour development (De Saeger and Van Peteghem, 1999). For T-2 toxin the detection limit was 50 ng/g. A collaborative study of five laboratories showed that these flow-through kits can be used for the screening of wheat, rye, maize and barley for the presence of OTA and T-2 toxin (De Saeger et al., 2002). Application of this format to AfM1 detection in both liquid and powdered milk was also described. However, immunoaffinity columns had to be used to clean-up the milk samples (Sibanda et al. 1999). IFA with internal control spots (anti-enzyme antibody) were applied for T-2 toxin detection with LOD at 50 ng/g in rye, wheat, barley and maize (Sibanda et al. 2000); for OTA with LOD at 4 ng/g in roasted coffee (Sibanda et al. 2002), at 8 ng/g in green coffee (Sibanda et al. 2001), for fumonisins with LOD at 1000 ng/g in maize (Paepens et al. 2004), with LODs determined as complete colour suppression. An IFA with LOD, determined as colour

intensity reduction, was developed for detection of FB1 at 40-60 ng/g in corn-based food (Schneider et al. 1995a), and also for sporidesmin A with LOD at 1 ng/mL (Collin et al 1998).

Schneider et al. (1995a) compared dipstick and flow-through formats for FB1 and concluded that both tests showed the same sensitivity in buffer solutions (7.5 – 10 ng/mL). These tests however were 50 times less sensitive than the corresponding microtiter plate ELISA with instrumental detection. For sporidesmin A determination with polyclonal antibodies it was also shown that a direct competitive ELISA was more sensitive (LOD 0.2 ng/mL) than IFA (LOD 1.0 ng/mL), ICS (LOD 4 ng/mL) and dipstick (1 ng/mL). The lower sensitivity of a competitive immunoassay using visual evaluation is mainly the result of the high antibody density at the membrane dot required for sufficient colour development for a negative sample (Collin et al. 1998).

To increase the sensitivity of IFA some modifications were applied to flow-through tests. In particular, to avoid limitations related to volumes of washing buffer, reagents and sample another construction of a membrane-based test was proposed, in which the absorbent body was not fixed to the reaction membrane. Between steps it was possible to wash with a stream of washing buffer from a wash bottle. Adsorbent body replacement allowed applying several portions of sample resulting in preconcentration of target analyte in the antibody spot. No limitation in volume allowed to use signal amplification with biotinylated tyramine and avidin-HRP conjugate for AfB1 determination. Signal amplification decreased the limit of detection from 5 to 0.25 pg/spot (0.2 to 0.01 ng/mL) with densitometric detection (Pal and Dhar, 2004). For the simultaneous screening of several samples for AfB1 they used four membrane strips with four separate antibody zones for each. A similar test with 36 spots was used for membrane-based IFA for T-2 toxin detection (Pal et al. 2004). To eliminate matrix interferences a clean-up step was included in the assay. This allowed using less diluted sample extracts, which resulted in an improvement of the detection limit. For AfB1 determination in groundnuts, wheat, corn, chilli, soybean and poultry feed samples a cleaning solution, containing trifluoroacetic acid or

propionic acid or sodium bicarbonate, was applied over the immunosorbent areas of the membrane as a part of the assay procedure (Pal et al. 2005). To improve the quality of immunoreagent spots on the membrane, a new spotting method was developed by Saha et al. (2006).

A prototype eight-well immunofiltration test device was developed for the simultaneous determination of seven mycotoxins: AfB1, FB1, T-2 toxin, roridin A, DON, diacetoxyscirpenol and OTA. Membranes coated with different anti-toxin antibodies were fixed to each well. Under the membranes a cellulose layer support was attached as a filter pad. The assay procedure was similar to one for a single mycotoxin determination, except that a mixture containing the respective toxin–enzyme conjugates was used for all wells. Detection limits in spiked wheat samples were at 10 ng/g (AfB1), 50 ng/g (OTA), 3500 ng/g (DON), 100 ng/g (T-2 toxin), 5 ng/g (diacetoxyscirpenol), 250 ng/g (roridin A), and 50 ng/g (FB1), respectively (Schneider et al. 2004). As mentioned before for other types of multianalyte tests with visual detection, there was a loss of sensitivity in comparison with single analyte determination, particularly because the demands in assay simplification and easy interpretation did not allow the use of optimal reagent concentrations.

Tandem column tests

Flow-through kits have some advantages such as being easy-to-perform and rapid (assay time 5-25 min). Compared with ICS tests, flow-trough tests usually show better sensitivity. For some matrices however they need additional clean-up steps before analysis, such as an immunoaffinity clean-up for AfM1 detection in milk (Sibanda et al. 1999), or a solid-phase clean-up for OTA detection in roasted coffee (Sibanda et al. 2002).

Integration of solid-phase clean-up and immunoassay in one device was obtained by the clean-up tandem immunoassay column, which contained a clean-up layer and a detection

immunolayer. As a clean-up layer aminopropyl derived silica was used. The purpose of the clean-up layer was to reduce the extract colour intensity and to minimize matrix effects. The detection immunolayer was a gel bound with specific antibodies and its aim was to bind and preconcentrate the analyte. The clean-up layer could be positioned above or below the detection immunolayer. Results were obtained as detection immunolayer colour development (for negative result) or no colour development (for positive result) after performing a direct competitive immunoassay. The analysis procedure itself could consist of seven (Lobeau et al, 2005; Lobeau et al. 2007), five (Goryacheva et al. 2006) or three steps (Goryacheva et al., 2007a), including washing steps. Clean-up tandem immunoassay columns allowed to detect OTA in strongly coloured foodstuffs such as roasted coffee (Lobeau et al. 2005), cocoa (Lobeau et al. 2007) and spices (Goryacheva et al. 2006). Cut-off levels for OTA detection for each matrix were established according to EU acting or discussing limits: 2 ng/g for cocoa, 5 ng/g for roasted coffee and 10 ng/g for spices. Assay sensitivity could be varied by changing antibody and conjugate dilutions, and also volume of extract or liquid sample. This column design had no limitation for volumes of liquid sample or extract, reagent and washing buffers. It allowed, in particular, to use high extract volumes to improve assay sensitivity.

For multiple mycotoxin screening the number of detection immunolayers was increased. For each detection immunolayer a specific antibody for the separate analyte was bound to the gel before placing it into the column with the clean-up layer. This approach was realized for the simultaneous detection of AfB1 and OTA in *Capsicum* spp. spices, nutmeg, ginger, black pepper and white pepper. According to acting and discussing EU limits, cut-off levels were chosen at 5 ng/g for AfB1 and 10 ng/g for OTA (Goryacheva et al. 2007b). As for membrane-based multiple immunoassays with visual detection, to prevent incorrect interpretation of results, time and intensity of developed colour should be about the same for both analytes. Preliminary results showed that for a future perspective the proposed multiassay design could be applied for up to four analytes (unpublished data).

Conclusions

In this manuscript we have reviewed a number of rapid immunochemical methods for mycotoxin determination. Focus was put on the trend towards multiple analyte determination. It was clear that more attempts were made for other groups of low molecular contaminants. Therefore, besides the few existing multi-mycotoxin rapid methods (Table 1), other possible approaches and designs were discussed. Development of rapid multiple mycotoxin methods will allow to reduce costs and further shorten analysis time. We expect great potential from array immunosensors systems, even towards multiple food contaminants detection. Many efforts should be made on method validation for non-instrumental multiple mycotoxin tests. A great and important challenge is the development of high quality antibodies or synthetic alternatives against many different mycotoxins.

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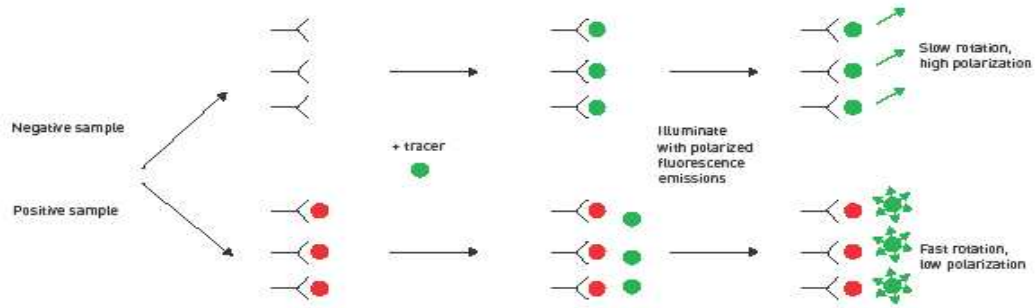
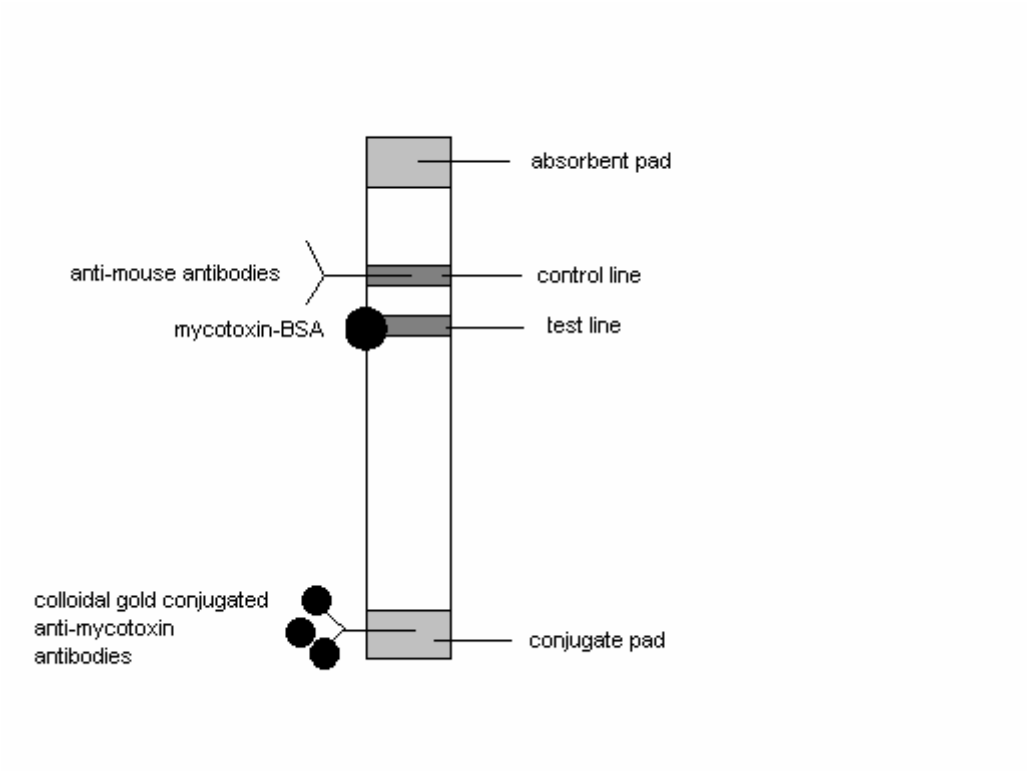
Figure captions

Figure 1. Principle of fluorescence polarization immunoassay

Figure 2. Principle of a lateral flow test





Immunochemical techniques	Mycotoxins detected	Sample matrix	Limit of detection	Reference
Fluorescent sensors	ochratoxin A fumonisin B1 aflatoxin B1 deoxynivalenol	buffer	10 µg/mL 20 µg/mL 50 ng/mL 10 µg/mL	Sapsford et al. 2006
Surface plasmon resonance sensors	aflatoxin B1 zearalenone deoxynivalenol fumonisin B1	*not specified*	0.2 ng/g 0.01 ng/g 0.5 ng/g 50 ng/g	van der Gaag et al. 2003
Dipstick tests	aflatoxin B1 T-2 toxin 3-acetyldeoxynivalenol roridin A zearalenone	wheat	30 ng/g 100 ng/g 600 ng/g 500 ng/g 60 ng/g	Schneider et al. 1995b
	fumonisin B1 aflatoxin B1 zearalenone	buffer	500 ng/mL 0.5 ng/mL 3 ng/mL	Abouzied and Pestka, 1994
Flow-through tests	aflatoxin B1 fumonisin B1 T-2 toxin roridin A deoxynivalenol diacetoxyscirpenol ochratoxin A	wheat	10 ng/g 50 ng/g 100 ng/g 250 ng/g 3500 ng/g 5 ng/g 50 ng/g	Schneider et al. 2004
Tandem column tests	aflatoxin B1 ochratoxin A	*Capsicum* spp. spices, nutmeg, ginger, black and white pepper	5 ng/g 10 ng/g	Goryacheva et al. 2007b

Table 1. Overview on existing immunochemical multi-mycotoxin rapid methods.