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Multiplex Flow-through Immunoassay Formats for Screening of Mycotoxins in a Variety of Food Matrices

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ABSTRACT

Two multi-analyte flow-through immunoassay formats for rapid detection of mycotoxins in a variety of food matrices (peanut cake, maize and cassava flour) were developed and evaluated. The selected food matrices are typical staple foods and export products for most low-income communities around the world. The assay formats included a gel-based and a membrane-based flow-through assays and were based on the principle of indirect ELISA. Using the same immunoreagents, the performance characteristic of both assays were compared. To the best of our knowledge, this is the first report on such comparison. The gel-based format was developed to screen for ochratoxin A, fumonisin B_1, deoxynivalenol and zearalenone detection at cut-off values of 3 µg kg^{-1}, 1250 µg kg^{-1}, 1000 µg kg^{-1} and 200 µg kg^{-1} respectively, while the membrane-based format can be used to screen ochratoxin A, aflatoxin B_1, deoxynivalenol and zearalenone at the following cut−offs, 3 µg kg^{-1}, 5 µg kg^{-1}, 700 µg kg^{-1} and 175 µg kg^{-1} respectively. The applicability of these assay formats was demonstrated by evaluating the performance characteristics of both tests through performing multiple experiments on different days. Both assays were further evaluated by analyzing naturally contaminated samples in the laboratory and also in the field under tropical conditions (Cameroon; West Africa). The false negative rate with both formats was less than 5 %, which is in good agreement with Commission Decision 2002/657/EC regarding the performance of analytical methods intended for screening purposes.

Keywords: immunoassay, membrane-based, gel-based, flow-through, mycotoxins

INTRODUCTION

Mycotoxins are natural, relatively small secondary metabolites, which are produced by moulds for example *Aspergillus*, *Fusarium* and *Penicillium* growing on agricultural commodities in the field or after harvest [1]. Humans as well as livestock are exposed to mycotoxins through the consumption of contaminated food and feed and through inhalation of airborne mycotoxins. Mycotoxins are potentially harzardous to both humans and domestic animals as they cause a wide range of diseases [2]. Among the potentially toxic mycotoxins identified so far, aflatoxins, fumonisins, trichothecenes, patulin, ochratoxins, ergot alkaloids and zearalenone (ZEN) continue to receive most of the attention as they pose great threat to human and animal health [1]. Selecting a suitable method for mycotoxin analysis involves using a suitable sample preparation protocol and an efficient separation and detection method. Most of the separation methods reported in literature are chromatographic ones while the detection methods include ultraviolet (UV) spectroscopy, fluorescence and (tandem) mass spectrometry. But also biosensors and immunochemical methods are valuable analytical tools [3].

Selecting a suitable method for bio-monitoring of food contaminants such as mycotoxins will in many instances greatly depend on the final objective. For instance, when large sample series have to be monitored for a group of contaminants, sample throughput will be an important criterion since speed is of the essence. In this regard, a screening method is preferred. Enzyme linked immunosorbent assay (ELISA) is widely accepted as the "gold standard" screening method [4]. As pre-requisite, such screening methods should minimize false negative results. On the other hand, when samples are suspected to contain one or more undesirable substances, method selectivity will no doubt be the main criterion because avoiding false non-compliant (positive) is of overriding importance. To add to the ever increasing demand for more selective analytical methods, there is also a growing demand to develop multiplex screening assays to replace single analyte assays. So far, most multiplex assays are mostly chromatographic based confirmatory methods such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) [2].

However, chromatographic methods are not only time consuming but are also expensive and require highly skilled personnel for their operation, which limits its usage in resource limited laboratories especially in developing countries. For this reason, attention has now turned towards the development of “rapid tests” or techniques, which make use of simple hand-held equipments for multiplex detection. Immunochemical based “rapid tests”, including but not limited to immunoassays have been widely used in a variety of fields and applications especially to safeguard the primary health and wellbeing of humans. “Rapid tests” devices also referred to as point of care (POC) devices, may be considered as simple low cost alternatives to the more sophisticated ELISA and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods [5]. Examples of some immunochemical based rapid POC devices reported in literature consist of lateral flow and flow-through test devices. These devices are well established diagnostic tools in laboratories worldwide and continue to gain more credibility because they are subjected to rigorous intra−laboratory validation before they are put on the market. Some POC devices are based on visual observation of results to discriminate between compliant and non-compliant samples and as such are generally referred to as non-instrumental methods.

Recently, there has been an upsurge in the number of literature on non-instrumental immunochemical methods for mycotoxin detection. Examples of which include lateral flow dipstick, flow-through gel-based assay (GBA) and flow−through membrane-based assay (MBA) [6-11]. The advantages associated with the use of such assay are not only limited to the fact that it gives qualitative and or semi-quantitative results in less than 30 minutes, but also that analysis can be performed on site. Most of these assays were initially designed as single analyte assays but after a period of extensive research two analyte GBA and MBA flow-through assays were later developed [10, 12-18]. There has been the growing demand to further extend the scope of these assays to include more than two analytes while maintaining a high degree of sensitivity and specificity. Of all the multiplex (two analyte based assays) GBA and MBA reported so far for mycotoxin analysis none was developed for screening of food matrices typical of low-income communities. Thus, a multiplex assay capable of screening mycotoxins in a variety of food matrices would greatly meet the target need (providing cheap and reliable analytical methods) of low-income countries and or laboratories where access

to analytical hard-ware, financial resources and skilled analytical personnel is limited. Most of these low-income communities are not necessarily poor countries but have proven in the last decade to be emerging economies with a great potential for economic growth. Immunological methods are cost-effective and adaptable to the situation in low-income countries [19].

In this study, two multiplex non-instrumental immunoassay (the gel-based format and membrane-based flow−through) formats were developed for screening of mycotoxins in a variety of food matrices (maize, peanuts, peanut cake and cassava flour). Although they have been used previously for mycotoxin detection, the two formats have not been compared directly. Here, we used the same antibodies to develop and optimize both formats and compared their performance on the same sample extracts, in assay buffer and using various food matrices. Development of field−portable assays are increasing, thus such a comparison should be of wide interest. A new coupling support (Sepharose Activated 4B Fast Flow) was used instead of the traditional Sepharose Activated 4B. In addition, direct coupling of the anti−mycotoxin antibody to the coupling support was also evaluated for the first time. For the membrane-based assay, two other membranes types (Biotrace NT and activated PES ultrabind) widely used for protein transfer and binding were also evaluated.

The MBA was developed for the detection of aflatoxin B_1 (AFB_1), ochratoxin A (OTA), ZEN and deoxynivalenol (DON) while the GBA was developed for fumonisin B_1 (FB_1), OTA, ZEN and DON detection. These toxins were selected due to their frequent occurrence in these food matrices. The performance characteristics and robustness of these assays were compared. Qualitative results obtained with these screening assays were compared with results obtained after performing a confirmatory test using LC-MS/MS. Both immunochemical assays were also evaluated for their robustness under tropical field conditions.

Experimental

Reagents and materials

CNBr activated Sepharose 4B Fast Flow and CNBr activated Sepharose 4B were purchased from GE Healthcare Bio−Science (Uppsala, Sweden). Immunodyne ABC membrane (pore size 0.45μm), Biotrace NT (pore size 0.2 μm) and activated PES ultrabind (pore size 0.45 μm) were obtained from Pall France (Saint Germain−en−Laye, France). Absorbent pads (type AP-080, 20X25 mm) were supplied by Advanced Microdevices Pvt (Ambaka Cantt, India). As flow-through device, a plastic snap-fit device from Trosley Equipment (Dover, Kent, UK) was used. Grace octadecyl (C18) solid phase extraction (SPE) cartridges were obtained from Grace Discovery Sciences (Lokeren, Belgium). Bond Elut cartridges (1 and 3 mL) and polyethylene frits (1/4 in. and 3/8 in. diameter) were supplied by Varian (Sint-Katelijne-Waver, Belgium). Water was purified using a Milli-Q Gradient System (Millipore, Brussels, Belgium). *N, N*–dimethylformamide was purchased from Acros Organics (Geel, Belgium). Methanol, HPLC-grade was purchased from VWR International (Zaventem, Belgium). Proclin 300 (5−chloro−2-methyl−4−isothiazolin-3-one plus 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added as an antimicrobial preservative to the buffers. Concentrated hydrochloric acid (HCl), sodium acetate, sodium chloride, sodium hydrogen carbonate, glycine and glycerol were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) and Tween 20 were purchased from Sigma Aldrich (Bornem, Belgium). A 0.45 μm syringe filter was obtained from Macherey-Nagel (Neumann-Neander, Germany). Ultrafree MC centrifugal devices (0.22 μm) of Millipore (Millipore, Brussels, Belgium).

Rabbit anti-mouse immunoglobulin (IgG; protein concentration: 2.7 g/L) was supplied by DakoCytomation (Heverlee, Belgium). Monoclonal anti-ZEN antibody was prepared at Ghent University, Ghent, Belgium [20]. ZEN−horseradish peroxidase (ZEN−HRP) conjugate was prepared as described in Basova et al. [17]. Cross-reactivity of the ZEN monoclonal antibody was 69% with α-zearalenol, 42% with α−zearalanol, 22% with zearalanone and none at all (<1%) with β−zearalenol and β−zearalanol. The substrate chromogenic solution used was Colorburst Blue

TMB/Peroxide (ALerCHEK, Maine, USA). Monoclonal antibodies against FB_1, AFB_1 and OTA were prepared by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary. FB_1−HRP, AFB_1−HRP and OTA−HRP conjugates were produced and characterized by the same institute. Monoclonal anti−FB_1 antibody was an IgG1 (protein content:1 g/L) with kappa light chain and an affinity constant of 1.3×10^{10} M^{-1} [21]. The anti−OTA antibody was an IgG1 with kappa light chains with a 9.3% cross-reaction with ochratoxin B (OTB) but none at all with ochratoxin α (OTα), coumarin, 4-hydroxy-coumarin and d,l−phenylalanine. The anti−AFB_1 antibody was IgG2a with 79% cross-reaction with aflatoxin M_1, 33% with aflatoxin M_2, 76% with aflatoxin B_2 (AFB_2), 55% with aflatoxin G_1 (AFG_1), 6% with aflatoxin G_2 (AFG_2). Monoclonal anti−DON antibody (clone 4) and DON-HRP were developed at USDA−ARS−NGAUR, Peoria, IL, USA and kindly provided by Dr. Chris Maragos. The cross-reactivity of the DON antibody was 429% with 15−acetyl−DON and <0.5% with nivalenol.

From a stability point of view mycotoxin-HRP conjugates and anti-mycotoxin antibodies were kept in concentrated form at -20°C. Stock solutions were prepared in phosphate buffered saline (PBS)−1% glycerol and stored at −20°C. Working solutions were made in PBS−1% BSA and used for 3 weeks. PBS (0.01 M, pH 7.4), was used to make the wash solution (PBS−Tween 0.05%), the blocking solution (PBS−casein 2%) and the assay buffer (PBS−casein 0.1%). DON and AFB_1 standards were supplied by Sigma Aldrich (Bornem, Belgium). ZEN and OTA were supplied by Enzo LifeSciences (Antwerpen, Belgium). FB_1 was supplied by Promec Unit (Tyberg, South Africa). Stock solutions of DON, OTA and ZEN (1 mg mL^{-1}) and working solutions (100 µg mL^{-1}) were prepared in methanol and stored at −20°C. Stock solution of AFB_1 (1 mg mL^{-1}) and working solution (10 µg mL^{-1}) were prepared in dimethylformamide and stored at −20°C. Stock solutions of FB_1 (1 mg mL^{-1}) were prepared in acetonitrile/water (50/50, v/v) and stored at 4 °C. All working solutions were used for 3 months.

Sample preparation and recovery experiments

The same sample preparation procedure was applied for both the GBA and MBA. One gram of blank samples was weighed into two separate extraction tubes (A and B). During the entire assay development, two sets of parallel samples were used. Tube A (set 1) was spiked to a pre-determined (cut−off) concentration of each of the target mycotoxins. Tube B (set 2) was left unspiked (control blank). Extraction solvent (2.5 mL) which comprised of methanol/water (80/20, v/v) mixture was added to each of the tubes. Extraction was performed by vigorous shaking with the hand for 7 min. The extract was let to stand for 5 min to allow sedimentation of particulate matter. The supernatant was drawn into a syringe for sample clean-up.

Clean-up of the sample extract was carried out using a custom made sample clean-up column. The design of the clean-up column was the same for both the GBA and the MBA. The preparation of the clean-up column was as follows: in an empty 3 mL Bond Elut Reservoir, a frit was placed at the bottom. Approximately 250 mg of C_{18} clean−up sorbent was filled into the clean−up column. A second frit was placed on top of the C_{18} clean−up sorbent. Prior to loading of the sample extract onto the clean−up column, 2 mL of PBS solution was used to condition the C_{18} SPE sorbent. The entire sample extract was loaded onto the clean-up column and allowed to flow-through at a flow rate of one drop per second without the application of vacuum. Then, 1 mL of the cleaned extract was diluted in 3 mL PBS. This diluted extract was drawn into a syringe and used for analysis.

For every recovery experiment, two parallel sets of samples were used. One set was spiked to a predetermined concentration prior to the start of the sample preparation protocol (begin spike), while another set was spiked after the desired sample treatment was performed (end spike). All experiments were performed in triplicate. The ratio of the peak area of the begin spike to the peak area of the end spike were used to calculate the analyte recovery.

Preparation of immunoassay gels (GBA)

Preparation of the coupled-gel and blocked-gel

Rabbit anti-mouse immunoglobulin was coupled to activated Sepharose 4B Fast-Flow to give a coupled-gel. The coupling protocol requires first the activation of the Sepharose 4B Fast-Flow powder using acidified water. Coupling was performed as follows: Sepharose 4B Fast Flow (0.6 gram results in a final volume of 1.5 mL) was brought onto a sintered glass filter and washed with 200 mL of 1 mM HCl (pH 2). Rabbit anti−mouse immunoglobulin (500 µL) dissolved in 500 µL of coupling buffer ($NaHCO_3$, pH 8.3, containing 0.5 M NaCl) was added to the activated Sepharose gel. Using an orbital shaker, the content in the sintered glass filter was swirled for 2 h at room temperature. Then, 10 mL of the coupling buffer was used to wash off excess of rabbit anti-mouse immunoglobulin. The unreacted active sites on the Sepharose activated gel were blocked by adding 15 mL of the blocking buffer (0.2 M glycine, 0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl). The coupled gel was then washed with 3 cycles of low (0.1 M Na acetate, pH 4.0 containing 0.5 M NaCl) and high pH (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) solutions. The final gel was reconstituted in 1.5 mL of PBS buffer and kept at 4°C until use. For the preparation of the blocked gel, the protocol was the same except for the fact that no rabbit anti−mouse immunoglobulin was added during the coupling protocol.

Direct coupling of the anti-mycotoxin antibodies to the Sepharose 4B Fast Flow support was also performed separately for each respective anti-mycotoxin antibody. The coupling procedure was similar to that of the blocked gel except for the absence of rabbit anti-mouse immunoglobulin. The following anti−mycotoxin antibody dilutions and volumes were used for the coupling process (500 µL (1/50 dilution) for OTA, 150 µL (1/5000) for DON, 500 µL (1/2000) for FB_1 and 600 µL (1/500) for ZEN).

Preparation of multiplex GBA column

The preparation of the multiplex assay column consisted of preparing single analyte screening gels separately. Four separate single analyte screening gels were prepared. The preparation of each single analyte screening gel layer was carried out in a 3 mL

empty SPE reservoir. The optimized protocol was as follows: a 3/8 inch frit was pushed through the inlet to the bottom of an empty Bond Elut SPE reservoir. A 225 µL portion of the blocked gel was loaded onto the empty SPE reservoir followed by 25 µL of the anti-mouse coupled gel (1:10 dilution of the coupled gel). The mixture was swirled gently for 2 min to ensure homogeneity of the gel solution. Excess of PBS was flushed out. Specific volumes of diluted anti−mycotoxin antibodies for the different toxins (50 µL (1/50 dilution) for OTA, 15 µL (1/5000) for DON, 50 µL (1/2000) for FB_1 and 60 µL (1/500) for ZEN) were added separately into the corresponding single analyte gel solutions and allowed for 10 min for coupling. This was followed by removal of the excess PBS solution. The respective anti−mycotoxin antibodies are now coupled to the Sepharose gel. Uncoupled anti-mycotoxin antibodies were removed by washing with 1 mL of the washing solution (PBS−0.05%Tween 20). After the preparation of the separate single analyte screening gels, each of the gel solutions was in turn brought into a new 3 mL SPE reservoir to form the multiplex assay screening column. The thickness of each gel layer was estimated at approximately 5 mm with each layer separated from the next by two 3/8 inch frits (Fig. 1a). Thus, a multiplex screening column for four mycotoxins was established. There was no preference in the order of the different screening zones.

Gel-based assay protocol

For every series of analysis, an experiment with a blank sample was performed to check the performance of the assay. The diluted cleaned sample extract, drawn into a 10 mL syringe was loaded onto the multiplex assay column. The sample extract was allowed to flow through the multiplex assay column at a flow-rate of about 1 drop every five seconds. Excess of unbound mycotoxins was removed by washing the assay column with 20 mL of washing solution. Fresh PBS solution (2 mL) was added. Dilutions of the respective mycotoxin−HRP conjugates prepared separately (FB_1−HRP (1/2000), OTA−HRP (1/2000), ZEN−HRP (1/2000) and DON−HRP (1/2000)) were pooled and added onto the multiplex assay column. With the help of a syringe, the pooled solution of the mycotoxin-HRP conjugates was flowed through the multiplex assay column (PBS solution). Excess mycotoxin−HRP conjugates were washed off with 15 mL of the washing solution. For colour development, 500 µL of

the HRP substrate (Colorburst Blue TMB/Peroxide) was added. The substrate solution was split in two parts of 250 μL each prior to addition. One part was introduced through the inlet of the assay column while the other part was drawn in through the outlet. The colour intensity was visually evaluated after 5 min. The whole procedure (not including preparation of coupled and blocked gels) took 30 min and was performed at room temperature.

Preparation of immunoassay membranes (MBA)

Preparation of the Immunodyne ABC membranes

Immunodyne ABC membranes were cut into rectangular shapes of 2 cm x 4.5 cm. Four circles with a diameter of 0.3 cm each were marked on the surface of each membrane. To ensure that the circles did not overlap, a distance of 0.5 cm was kept between each circle. A 1 μL portion of undiluted rabbit anti-mouse immunoglobulin was spotted within each circle (Fig. 1b). The membranes were dried for 30 min at 37°C. In order to saturate the remaining covalent sites on the membranes, the membranes were soaked in a blocking buffer (PBS−2% casein) for 15 min, followed by drying at 37°C for 30 min. A 10 μL portion of the respective diluted anti-mycotoxin-antibody (anti−AFB_1 (1/100), anti−OTA (1/500), anti−ZEN (1/10) and anti−DON (1/500)) was applied on each of the respective single analyte detection spots. The membranes were dried at 37°C for 15 min and immediately stored under vacuum in polyethylene bags for 3 days at room temperature.

Assembly of the MBA plastic cassette

A simple MBA flow−through device consisted of two components, an Immunodyne ABC nylon membrane and absorbent pads, which are enclosed within a plastic cassette. The bottom part of the plastic cassette was filled up with absorbent pads (9 layers of absorbent pads). Then, the prepared assay membrane was placed on the stack of absorbent pads. The top part of the plastic cassette was then fitted and held in position with a tape. Fig. 1c and 1d show a typical arrangement of a multiplex assay cassette.

MBA protocol

Prior to use, the assay membranes were rehydrated by adding dropwise, 500 μL of assay buffer (PBS−0.1%casein). Assay reagents were then added in sequence onto the membrane. Between each step, the reagents were allowed to flow through completely aided by the absorbent pads. The diluted sample extract was then added to the membranes. Five times 100 μL of PBS−0.05%Tween 20 was used to wash-off interfering substances. This was followed by the addition of 20 μL of the respective mycotoxin−HRP conjugates (AFB_1−HRP (1/100), OTA−HRP (1/100), ZEN−HRP (1/1000) and DON−HRP (1/1000)) onto the corresponding single analyte detection zone. The membranes were again washed with 500 μL of PBS−0.05%Tween 20 followed by 5 other washes with 100 μL of PBS. A 25 μL portion of substrate was applied on each spot for both the test and the control immunoassay membranes. The colour intensity of the individual spots was visually evaluated after 3-5 min. The whole procedure took 30 min and was achieved at room temperature.

Naturally contaminated samples

The samples used in the study were part of a much larger survey carried out in Cameroon, West Africa in the month of December, 2010. In total 200 samples of the three matrices (maize, peanuts and cassava) were sampled from three of Cameroon's five agroecological regions. At the time of sampling, samples were randomly selected and screened with the optimized flow-through assays. All screening experiments were performed in the shadow where the average field temperature was 28±1.6°C. Small portions of homogenized ground samples were used for the screening experiments.

LC-MS/MS analysis

A Waters Acquity UPLC system (Waters, Zellik, Belgium) and a Micromass Quattro micro triple quadrupole mass spectrometer were used for separation and detection of the analytes of interest. The analytical protocol described in Ediage et al. [22], was used for the analysis of naturally contaminated samples. The protocol was as follows: to a one gram portion of a homogenized sample, internal standards zearalanone (ZAN) and deepoxy-deoxynivalenol (DOM) were added at concentrations of 200 μg kg^{-1} and 10 μg kg^{-1} respectively. The sample was extracted with 25 mL of methanol/ethyl

acetate/water (70/20/10, v/v/v), during 20 min and centrifuged for 15 min at 3170g. The extract was evaporated at 40°C under a stream of nitrogen. The residue was reconstituted in 5mL of methanol/water (85/15, v/v) to which 10 mL of dichloromethane/hexane (30/70, v/v) solution was added and shaken for 10 min. After centrifugation at 3200g for 10 min, the dichloromethane/hexane phase was discarded while the methanol/water phase was kept for further clean-up. The defatted extract (methanol/water phase) was further split into two parts of 2.5 mL each for sample enrichment (clean-up). One part (of the split-up extract) was cleaned by passing it through a glass fiber filter while the second part was cleaned up using aminopropyl (NH2) cartridges, mounted on a vacuum elution manifold. The amino cartridge was first conditioned with 5 mL of the reconstitution solvent. After the conditioning step, the sample extract was loaded onto the SPE cartridge and the eluate collected in a test tube. The reconstitution solvent (1 mL) was used to wash the SPE cartridge with the intention of eluting most of the loosely trapped (through weak hydrophobic interactions) mycotoxins with the exception of fumonisin and ochratoxin A, both of which are strongly retained by the SPE absorbent. Both parts (the NH_2 SPE and glass fiber filter) of the cleaned extracts were recombined and evaporated under a gentle stream of nitrogen. The residue was dissolved in 100 µL of mobile phase consisting of methanol/water/acetic acid (57.2/41.8/1, v/v/v) and 5 mM ammonium acetate. Ultrafree MC centrifugal devices were used to further filter the resulting solution prior to injection into the LC-MS/MS system; this was performed for 15 min at 14000g.

The column used was a 150 mm×2.1 mm i.d., 5 µm, Symmetry C_{18}, with a 10 mm×2.1 mm i.d. guard column of the same material (Waters, Zellik, Belgium). The injection volume was 20 µL. The mobile phase consisted of variable mixtures of mobile phase A (water: methanol/acetic acid, 94/5/1 (v/v/v) and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) and 5 mM ammonium acetate) at a flow rate of 0.3 mL min^{-1} with a gradient elution programme. The mass spectrometer was operated in the positive electrospray ionisation (ESI+) mode. Capillary voltage was 3.2 kV and nitrogen was used as spray gas. Source and desolvation temperatures were set at 150°C and 350°C, respectively. The optimized parameters for both the precursor and product ions are as described in Ediage et al.

[22]. The method was validated according to Commission Decision 2002/657/EC [23] and Commission Regulation (EC) No. 401/2006 [24].

Assay Validation

Performance characteristics for both the GBA and MBA

In order to provide robust assays to end users, the performance characteristics of any analytical method must be defined and properly validated. So far, for qualitative analytical (screening) methods, there are no general validation procedures available and especially screening methods for mycotoxins in foods. However, according to Commission Decision 2002/657/EC, only those analytical techniques for which it can be demonstrated in a documented traceable manner that they are validated and have false compliant rate of less than 5% at the levels of interest shall be used for screening purposes. In case a non-compliant outcome occurs, the result should be verified by a complementary method (EC, 2002) [23].

Validation of immunoassays involves determining a series of analytical performance characteristics. The following seven performance characteristics are relevant to evaluate (assess) when validating qualitative screening assays for mycotoxin determination; cut-off limits, specificity, sensitivity, false positive rate, false negative rate, positive predicted value and negative predicted value. The assay cut-off limits were determined during validation studies by constructing a response (performance characteristic) curve of percentage positives (P(x)) results versus concentration. From this curve, the cut-off limits was defined as the analyte concentration which gave 100 % true positive results. A positive result generated no signal, in this case no coloration. On the other hand, a negative test results in an intense blue coloration with no difference in color intensity between both test and control. To construct such a response curve, a series of blank samples spiked at 8 different concentration levels, in 10 replicates were used. In total 80 samples of each sample matrix were used. All samples were screened with external standards to ascertain if they were blank or not. Five of the 8 concentration levels were below a pre-determined assay cut-off limits for the different analytes, 2 levels above the pre-determined cut-off limits and one level at the pre-determined cut-off limit. The pre-determined cut-off limits were obtained during pre-validation studies. The spiked concentration levels were different for each

analyte which was based on the maximum limits of each analyte in the different food stuffs as laid down by the European Commission Decision. Commission Regulation (EC) No 1126/2007 of 28 September 2007 [25] amends Regulation (EC) No 1881/2006 which sets maximum levels for certain contaminants in food stuff as regards *Fusarium* toxins in maize and maize products [26]. Furthermore, Commission Regulation (EC) No 1881/2006 of 19 December 2006, has established maximum levels for certain contaminants in food stuff including peanuts and its processed products.

From the performance characteristic curve, the uncertainty region for each analyte was also determined. This region is defined as the concentration range within which identification (detection) becomes unreliable. For practical reasons, the final analyte cut-off limits for the different analytes were obtained using single analyte assays. These cut-off limits were later reassessed and adapted with the multi-analyte screening assay.

After having known the final assay cut-off limits, the six other performance characteristics (specificity, sensitivity, false positive rate, false negative rate, positive predicted value and negative predicted value) were then determined. Parallel blank samples (40) of each of the three sample matrices (maize, peanut cake and cassava) were used. A set of 20 samples were used as control blanks (n=20 for each analyte) while the other set of 20 samples were fortified with the target analyte at the respective assay cut-off limits. Control and test experiments were always realized in parallel and each result was shown blindly to three other colleagues who visually evaluated the results. The following equations were used to calculate the other six performance parameters: sensitivity ($N_{\text{true positive}}/(N_{\text{true positive}} + N_{\text{false negative}}) \times 100(\%)$), specificity rate ($N_{\text{true negative}}/(N_{\text{true negative}} + N_{\text{false positive}}) \times 100(\%)$), false positive rate ($N_{\text{false positive}}/(N_{\text{true positive}} + N_{\text{false positive}}) \times 100(\%)$), false negative rate ($N_{\text{false negative}}/(N_{\text{true negative}} + N_{\text{false negative}}) \times 100\ (\%)$), positive predictive rate ($N_{\text{true positive}}/(N_{\text{true positive}} + N_{\text{false positive}}) \times 100(\%)$), negative predictive rate ($N_{\text{true negative}}/(N_{\text{true negative}} + N_{\text{false negative}}) \times 100(\%)$).

Results and discussion

Optimization of the sample preparation procedure

Before considering the use of any clean-up procedure, the diluted sample extract (diluted 1/3, v/v in PBS) was applied directly on the membrane. This approach, if successful would lead to a considerable reduction in the total assay time. However, this application led to blocking of the membrane and the gel, seconds after the sample extract was applied. Due to this shortcoming, this approach was not further developed. This experience demonstrated the need to eliminate smaller particulate matter, hence, the need for a sample clean-up procedure. Filtration of the sample extract prior to dilution was deemed necessary. This procedure consisted of passing 1.5 mL of the sample extract through a 0.45 µm syringe filter followed by the addition of 1.5 mL of PBS. Recovery experiments were performed along side to assess the percentage of each of the mycotoxins trapped by the filter. The results show (Table 1), that only small amounts of the analytes were trapped by the filters (DON (3%), OTA (5.4%), FB_1 (2.5%) and 0% for AFB_1 and ZEN, respectively). Adding 1.5 mL of PBS to the sample extract resulted in a cloudy and foamy solution. When this solution was brought onto the assay membrane, it led to clogging of the membrane. The high ionic strength of the PBS solution could probably have caused aggregation of proteins, glucose and fatty acids (organic soluble compounds) and hence, the formation of a colloidal solution. Colloidal particles can reach diameter of 50 µm or more [27], which are large enough to block the pores of both the Immunodyne ABC membrane (0.45 µm).

A material which can be used to trap these molecules (colloidal particles), is a C_{18} sorbent. For this reason a C_{18} custom-made clean-up column was designed as described in the section of sample preparation and recovery experiments. The filtrate obtained with the C_{18} clean-up sorbent was more translucent than the filtrate obtained from the other previously described sample treatments. Besides, dilutions of the sample extract with PBS did not lead to any cloudy or foamy solution and the entire sample extract could easily flow through the membrane. This clearly proved that the bigger particles and the molecules responsible for the formation of colloidal particles were eliminated during the sample clean-up. Table 1 also shows the percentage of

mycotoxins trapped in the custom-made C_{18} column. The results revealed significant binding of OTA and AFB_1 to the C_{18} particles. Although a significant amount of AFB_1 and OTA was lost with the custom made clean-up sorbent, this sample preparation procedure was preferred to other previously tested protocols.

Optimization of the dilution factor for the sample extract

The efficiency of extraction (recovery) using three different volumes (2.5 mL, 5 mL and 10 mL) of extraction solvent methanol/water (80/20, v/v) was evaluated. Recovery experiments were performed as described in the section of sample preparation and recovery experiments. The LC-MS/MS conditions were the same as described in Ediage et al. [22]. As expected, a lower volume will result in a much lower analyte recovery and vice versa. The recovery data for the different extraction volumes obtained for the respective analyte and matrices evaluated are shown in Table 2. Though, a higher analyte recovery was obtained with the 10 mL solution, the 2.5 mL extraction volume was preferred because it gave the highest concentration of analyte per mL of extraction solvent. This parameter was considered important since the sample extract was expected to undergo further dilution prior to analysis.

Dilution of the sample extract after clean-up and prior to analysis was necessary in order to minimize the content of organic solvent in the final sample extract. For this reason, four different dilution ratios (1/1, 1/3, 1/5 and 1/10, v/v) were evaluated. A 1/3 (v/v) dilution ratio of the sample extract in PBS gave the best optimum results. A dilution ratio (1/5 and 1/10, v/v) resulted in high analyte cut−offs limits while a low dilution ratio (1/1, v/v) generated no analytical signal. This could be due to the high proportion of organic solvent which might have contributed to a possible denaturation of the antibodies. Furthermore, preliminary experiments, revealed that 4 mL of the diluted extract was considered the maximum volume that could be brought onto the membrane without over soaking the membranes. Over soaking of the membranes would impede the flow of solvents through the membrane. Because a 3 mL column was used for the GBA, this offered a practical limitation as in general, the 4 mL diluted extract should be applied twice (2x2 mL). Larger volumes would imply longer assay time. For this reason, a 4 mL portion of diluted sample extract was considered optimum for both assays.

Gel-based assay

Development of a multiplex screening assay involved first and foremost the optimization of different parameters for a single analyte assay. The different anti-mycotoxin antibodies and mycotoxin−HRP conjugate dilutions developed and optimized for single analyte detection were evaluated and reoptimized for multi-analyte detection. The entire assay development protocol involved the optimization of key influential parameters such as the choice of the coupling support, optimization of washing solutions and washing volumes and the thickness of the single analyte gel layer.

Choice of the coupling support and indirect coupling approach

Considering the fact that all the GBAs reported in scientific literature only made use of CNBr activated Sepharose 4B as coupling support [10, 11, 15, 18 and 28], it was deemed necessary to compare another variant of this coupling support such as CNBr activated Sepharose 4B Fast Flow, which to the best of our knowledge has not been reported in the field of mycotoxin analysis. The CNBr activated Sepharose 4B Fast Flow is widely used in chromatographic applications for separation and purification of compounds. Both coupling agents have the same average bead diameter (90 µm) but differ in the number of cross linkers as well as the antibody coupling capacity. Sepharose 4B Fast Flow has a higher number of cross linkers than Sepharose 4B. The coupling capacity of the later is twice higher than that of the former. However, since only small amounts of rabbit anti-mouse are often recommended for coupling, the difference in coupling capacity was not considered as one of the major factors which could significantly influence the performances of the different gel types. The nature and strength of the cross linkers was however considered as the most influential parameter. Both gels were evaluated in parallel experiments while keeping all other conditions constant. The results obtained with Sepharose activated 4B Fast Flow were more reproducible unlike those obtained with the use of Sepharose 4B. This difference could be explained by the high network of cross linkers (lysine residues) within the Sepharose 4B Fast Flow gel. The lysine residues interact strongly with other electrophilic residues on the coupled rabbit anti-mouse immunoglobulin through

molecular interactions such as hydrogen bonds, electrostatic and Van Der Waals forces.

Because of the consistency of the results obtained with Sepharose activated 4B Fast Flow, this coupling support was selected as coupling medium. Using control blanks and fortified blank samples, all parameters were optimized so as to obtain equal colour development in all the four detection layers within 3-7 min following the addition of the Colorburst Blue TMB/Peroxide solution. Direct coupling of the respective anti–mycotoxin antibody onto these coupling supports gave similar results with the indirect coupling approach (first coupling of the rabbit anti-mouse antibody, then the anti-mycotoxin antibody). However, much higher concentrations (a factor of 10 or more) of these anti–mycotoxin antibodies were required for coupling compared to the concentrations used following the indirect coupling approach. The direct approach did not improve the overall sensitivity of the assay. Moreover, the use of high concentrations of the anti-mycotoxin immunoglobulin could seriously compromise the cost-effectiveness of such assays to be used in low-income communities. For this reason the direct coupling approach was not further developed.

Optimization of the washing solution and washing volumes

PBS with or without additives (casein, Tween 20) was investigated as washing solution. Washing with 10 mL PBS and PBS–(0.01%, 0.03%, 0.05%) casein did not result in complete removal of the excess mycotoxin–HRP conjugates and as a result all assays (blank samples as well as fortified samples) gave very high colour intensities. Similarly, large volumes of PBS–0.05%casein solution (30 mL, 40 mL) did not improve the performance of this washing solution. Higher percentages of casein could not be used because this could lead to an increase in the viscosity of the washing solution and thus would greatly compromise its suitability to be used as a washing solution.

Due to the relatively high rigidity of the swollen Sepharose 4B Fast Flow, a stronger washing solution or detergent was therefore needed. Tween 20 was preferred to casein as additive. The lower viscosity of the PBS–0.05%Tween 20 solution also contributed to it been preferred over the PBS–0.05%casein solution. The PBS–0.05%Tween 20 detergent solution can easily flow-through the highly crossed linked Sepharose 4B

Fast Flow assay gel to eliminate excess of the unbound immunoreagents and other interfering substances which could affect the performance of the assay. This characteristic made it suitable for its purpose. Different percentages of Tween 20 (0.01%, 0.05%, 0.1%, 0.5% and 1%) in PBS were evaluated. PBS−0.05%Tween 20 gave very satisfactory results. Higher percentages of Tween 20 (0.1, 0.5 and 1%) in PBS were too strong to be used while lower percentages resulted in a high degree of false negatives. Finally, washing with 10 mL of PBS−0.05%Tween 20 proved optimal.

Effect of the thickness of the gel layer on the performance of the GBA

The use of a 10 mm thick gel resulted in a more rigid gel, which required large volumes of washing solution and/or several washing cycles during each of the assay steps. Insufficient washing cycles (volumes) resulted to insufficient removal of excess of mycotoxin-HRP conjugates and as a result led to unreliable results (higher false negative rate due to the persistent blue coloration). An additional problem encountered with the use of the 10 mm thick gel was the inhomogeneous distribution of the rabbit anti-mouse-anti-mycotoxin antibody coupled conjugate within the specific analyte gel layer. However, a 5 mm gel gave excellent and reproducible homogeneous gel layers. Thus, 5 mm was chosen as the optimal gel thickness. This was prepared by adding 25 μL of the coupled gel to 225 μL of the blocked gel.

Performance characteristics of the GBA

The assay cut-off limits were determined using the performance characteristic curve as described in the mention on method validation. The assay cut−off limits were 3 $\mu g\ kg^{-1}$, 1250 $\mu g\ kg^{-1}$, 200 $\mu g\ kg^{-1}$ and 1000 $\mu g\ kg^{-1}$ for OTA, FB_1, ZEN and DON respectively for the three different food matrices evaluated in this study. These cut−off limits were either below or at the maximum levels of these toxins in the different matrices evaluated as specified by the European Commission Decision 1187/2007 and 1881/2006. Especially for the control of exports of food commodities from low resource communities, assays with such cut−off limits will allow quick decisions to be taken in order to ascertain if the levels in the foodstuffs exceed the maximum permitted levels or not. Fig. 2 shows the results obtained with a typical multiplex

assay column after screening of a blank sample (column B) and a sample fortified above the respective analyte cut-off limits (column A).

Table 3 shows the qualitative performance characteristics obtained with maize samples for ZEN, DON, FB_1 and OTA. The results obtained indicated excellent specificity (100%) for all four analytes. Furthermore, the assays was more sensitive to DON and FB_1 (100% sensitivity) and less sensitive to ZEN and OTA (sensitivity 95 %). The low sensitivity for ZEN and OTA could be attributed to the low cut-off values for these analytes, causing fluctuations in the assay procedure to significantly influence the outcome of the results. On the other hand the false positive rate was 0% for all the analytes. Similarly, a 0% false negative rate was obtained for the analysis of DON and FB_1 whereas a 5% rate was obtained for ZEN and OTA. The positive predictive value was 100% for all the analytes evaluated. On the other hand the negative predictive value was 95% for ZEN and OTA and 100% for DON and FB_1. Similar results were obtained when the other matrices (peanuts, peanut cake and cassava flour) were evaluated. This observation further strengthen the fact that the different matrix types had little or no influence on the final results. Most probably, the influence of the different matrices on the assay result, was completely minimized after the sample extract was subjected to the rigorous but simple sample clean-up procedure described herein. Consequently, the assumption that the quality (specificity) of the immunoreagents used had a much greater influence on the final assay parameters can be very well valid for the screening assays reported herein.

As previously described, the performance characteristic curve was also used to determine the uncertainty region for the different analytes. The performance characteristic curve for OTA using fortified maize sample is as shown in Fig. 3. The shape of this curve could be described as sigmoid. C_0 is defined as the concentration at the point of intersection of the fitted sigmoid curve and the type 1 error line (αo) while C_1 is defined as the concentration at the point of intersection of the fitted sigmoid curve and the type II error line (βo). The interval C_0–C_1 represents the uncertainty region corresponding to false response rates. This region is specific for each analyte. For OTA detection, this region lies between 1.2 to 2.8 μg kg^{-1}. The values for the uncertainty regions for FB_1, DON and ZEN were also computed and are as shown in

Table 4. As can be seen the uncertainty range was broader for ZEN, FB_1 and DON in that order while OTA had the smallest uncertainty range. These differences could be attributed to the different properties of the anti-mycotoxin antibodies used to develop the multiplex assay.

Membrane-based assay

Two other membranes types commonly used to bind proteins (Biotrace NT and activated PES (ultrabind)), in blotting and dot binding assays were also investigated. These membranes did not offer any advantage over the Immunodyne ABC membrane. These membranes were less hydrophilic and as a consequence the flow of aqueous reagents (buffer solutions) through these membranes was greatly impeded which led to an increase in the total assay time (more than 1 h). Moreover, these membranes required longer incubation times for protein binding. Due to these shortcomings, both membranes were not further investigated. The Immunodyne ABC membrane was selected as the membrane of choice. However, different parameters which could influence the assay performance such as dilution factor of the sample extract with PBS was also evaluated and optimized.

Performance characteristics of the MBA

As previously described, the assay cut-off limits were determined using the performance characteristic curve. The assay cut-offs for the different mycotoxins were 3 μg kg^{-1}, 5 μg kg^{-1}, 700 μg $kg^{-1}$and 175 μg kg^{-1} for OTA, AFB_1, DON and ZEN respectively. These cut-offs were the same for all the matrices evaluated. Fig.4 shows the results obtained with the optimized MBA protocol. The top cassette shows the results obtained with a control blank (negative) sample while the bottom cassette shows the results obtained with a sample spiked at or above the assay cut-off value for the different analytes. As illustrated in Fig. 4, no background coloration was observed, which is an indication of the good sample preparation protocol. Furthermore, less background coloration implied easy interpretation of results, thus eliminating the aspect of individual subjectivity in evaluating the results. Table 5 shows the qualitative performance characteristics obtained from the validation experiments with maize, for all four mycotoxins. The specificity of the MBA was 100% for all the four

mycotoxins. Consequently, the probability of obtaining true negatives was 100% while the sensitivity was 100% for all four mycotoxins.

The percentage false negative rate was 0% for all the four mycotoxins, hence illustrating the excellent compliance of the assay with the recommendation of Commission Decision 2002/657. Finally, the positive and the negative predictive value were 100 % for all the analytes evaluated. Similar results were obtained with the other sample matrices (peanuts, peanut cake and cassava flour). Based on the excellent performance characteristic presented herein, the membrane-based flow-through assay could be considered as very reliable to screen for four targeted mycotoxins in four variety of food matrices.

Screening of naturally contaminated samples using the optimized MBA and GBA

In order to evaluate the applicability of both optimized assays, naturally contaminated samples were analysed in the laboratory and in the field. Naturally contaminated maize, peanuts and cassava flour samples were analyzed in triplicate. Table 6 shows the different results obtained with the naturally contaminated samples as well as the concentration of the different toxins measured in these contaminated samples following LC-MS/MS analysis. FB_1, DON and ZEN were not detected in peanut samples. Similarly, DON and ZEN were not detected in all the cassava flour samples analyzed. All peanut cake samples were contaminated with AFB_1 above 5 $\mu g\ kg^{-1}$ as revealed by both the LC-MS/MS and flow-through assays. Four peanut cake and all five cassava flour samples were OTA positive above the assays cut-off level (3 $\mu g\ kg^{-1}$). Results obtained with positive samples gave a very good visual contrast compared to those obtained with negative samples. In summary, both flow-through assays demonstrated excellent applicability as the results were in accordance with the LC-MS/MS results. Similarly, both MBA and GBA were field tested under tropical conditions in Cameroon, West Africa. A total of 60 (30 maize, 15 peanuts and 15 cassava flour) ground samples were screened. In summary, FB_1 and DON were the mycotoxins with a high frequency of occurrence in the maize samples (93% FB_1 and 37% DON). OTA and AFB_1 were detected in 67% and 82% respectively of the peanut total samples screened while AFB_1 was detected in 80% of the cassava flour samples. The LC-MS/MS results for these samples and others will be reported in subsequent

publications. A false negative rate of 3% was obtained for both tests. The results therefore revealed very good compliance of both assay types when used in tropical field conditions. However, it should be mentioned that all reagents were kept in ice prior to usage. All analyses were performed under the shadow in order to prevent protein denaturation by UV rays. The ice box could withhold ice for as long as 12 h with no significant increase in the inner temperature of the recipient. Without strict implementation of these conditions, the results reported herein would have been difficult to obtain in the field.

Comparison of both MBA and GBA formats

A successful screening method should be robust, sensitive with a high degree of flexibility, over a wide range of compounds. The assay protocol and results should be reproducible and the results obtained must be relevant and easy to analyze. For field work, the system should also be rapid and portable. Both assay types fulfilled all of the above cited criteria. Both assays also showed a much lower false negative rate when used under tropical field conditions, thus revealing excellent performance characteristics of such assays.

From a practical point of view, the MBA offers few advantages over the GBA. First and foremost, the preparation of the multiplex GBA column involved several steps, which if not carefully controlled can lead to serious consequences ranging from no signal generation to high false negative or positive rate. On the contrary, with the MBA, the assay steps are quite simple to follow from start to finish and did not require so much effort and concentration in order to achieve the desired results. Furthermore the preparation of the coupling support in the GBA requires approximately 6 h of laboratory time, unlike with the MBA whereby preparation of the assay membrane requires only 1 h of laboratory time, but can also be carried out under non laboratory conditions, which thus supports the applicability of such assay in low-income communities.

Conclusions

Two flow-through enzyme immunoassay formats for the detection of four mycotoxins in peanut cake, maize and cassava flour samples were successfully developed. Both assays did not make use of any equipment and consisted of limited operational steps, providing a yes/no response indicating whether the toxins were present or not above the maximum permitted levels of these analytes in the different matrices. Coupled to a simple and fast sample pretreatment the present approach allows to eliminate "compliant" samples from the sample set and helps prioritizing the "non-compliant" ones. Though these assays were originally developed for maize, peanut cake and cassava, other sample matrices such as unprocessed peanuts also gave very satisfactory results. Other sample matrices such as cornflakes and bread are also under investigation.

A regional experts meeting in 2006 on the mycotoxin problem with particular reference to resource limited communities reiterated the need for efficient, cost-effective analytical methods that can be used for the detection of mycotoxins in low-income communities [29]. Consequently, the availability of such multiplex assays for the screening of mycotoxins in a variety of food matrices may lead to safer consumer products in future, especially in low-income communities.

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References

1. Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C (2008) Food Addit Contam 25:152–163
2. Turner NW, Sabrahmanyam S, Piletsky SA (2009) Anal Chim Acta 2:168–180
3. Diana Di Mavungu D, Monbaliu S, Scippo ML, Maghuin-Rogister G, Schneider YJ, Larondelle Y, Callebaut A, Robbens J, Van Peteghem C, De Saeger S, (2009) Food Addit Contam 26:885-895
4. Krska R, Molinelli A (2009) Anal Bioanal Chem 393:67–71.
5. Van Emon JM, Gerlach CL (1998) J Microbial Methods 1:121-131.
6. Sibanda L, De Saeger S, Van Peteghem C (1999) J Food Microbiol 48:203–209
7. Sibanda L, De Saeger S, Barna-Vetró I, Van Peteghem C (2002) J Agric Food Chem 50: 6964-6967
8. De Saeger S, Sibanda L, Desmet A, Van Peteghem C (2002) Int J Food Microbiol 75:135–142
9. Paepens C, De Saeger S, Sibanda L, Barna-Vetró I, Léglise I, Van Hove F, Van Peteghem C (2004) Anal Chim Acta 523:229-235
10. Lobeau M, De Saeger S, Sibanda L, Baran-Vetró I, Van Peteghem C (2005) Anal Chim Acta 538 57-61
11. Goryacheva IY, De Saeger S (2007) Anal Chim Acta 590:118-124.
12. Saha D, Acharya D, Roy D, Shrestha D, Dhar TK (2007) Anal Chim Acta 584:343–349.
13. Schneider E, Usleber E, Märtlbauer E, Dietrich R, Terplan G (1995) Food Addit. Contam 12:387–393
14. Abouzied MM, Pestka JJ (1994) J. AOAC Int 77:495–501
15. Kolosova AY, De Saeger S, Sibanda L, Verheijen R, Van Peteghem C (2007) Anal Bioanal Chem 389:2103–2107
16. Goryacheva IY, De Saeger S, Delmulle B, Lobeau M, Eremin SA, Barna-Vetró I, Van Peteghem C (2007) Anal Chim Acta 590:118–124
17. Basova EY,. Goryacheva IY, Rusanova TY, Burmistrova NA, Dietrich R, Märtlbauer E, Detavernier C, Van Peteghem C, De Saeger S (2010) Anal Bioanal Chem 397:55–62

18. Beloglazova NV, Goryacheva IY, Rusanova TYu, Yurasov NA, Galve R, Marco MP, De Saeger S (2010) Anal Chim Acta 672:3-8
19. Devi KT, Mayo MA, Reddy KLN, Delfosse P, Reddy G, Reddy SV, Reddy DVR (1999) Lett Appl Microbiol 29:284–288
20. Burmistrova NA, Goryacheva IY, Basova EY, Franki AS, Elewaut D, Van Beneden K, Deforce D, Van Peteghem C, De Saeger S (2009) Anal Bioanal. Chem 395:1301–1307
21. Barna-Vetró I, Szabó E, Fazakas B, Solti L, (2000) J Agric Food Chem 48:2821
22. Ediage EN, Diana Di Mavungu J, Monbaliu S, Van Peteghem C, De Saeger S (2011) J Agric Food Chem 59:5173-5180
23. European Commission (2002), Commission Decision 2002/657/EC of 12 August 2002, Off J Eur Union L221:8–36
24. European Commission (2006), Commission Regulation of 23 February 2006 Off J Eur Union L70:12–34.
25. European Commission (2007), Commission Regulation (EC) No. 1126/2007 of 28 September 2007 amending Regulation (EC) No. 1881/2006 Off J Eur Union L255:14
26. European Commission (2006), Commission Regulation (EC) No. 1881/2006 of 19 december 2006 Off J Eur Union L364:5-24.
27. Zhengwei M, Haolan X, Dayong W (2010) Advanced Funct Materials 20:1053-1074
28. De Saeger S, Van Peteghem C (1999) J Food Protect 62: 65-69.
29. World Health Organization (WHO), 2006. Mycotoxins in African foods: implications to food safety and health. AFRO Food Safety Newsletter. World Health Organization Food safety (FOS), Issue No. July 2006.

Table 1

Percentage of each of the five mycotoxins trapped during different sample treatments

	Percentage of mycotoxin trapped (%)±SD	
[a]Mycotoxins	membrane filter (n=3)	custom made C_{18} SPE catridge (n=3)
AFB_1	0 ±0	20 ±0.5
DON	3 ±0.1	7 ±0.7
OTA	5.4 ±0.1	13 ±1
ZEN	0 ±0	6 ±0.4
FB_1	2.5 ±0.3	9 ±0.7

[a]spiked concentrations: AFB_1, 10 µg kg^{-1}; DON, 1000 µg kg^{-1}; OTA, 3 µg kg^{-1}; ZEN, 200 µg kg^{-1} and FB_1, 1250 µg kg^{-1}

SD: standard deviation

Table 2

Recovery data for the four different analytes using three different volumes of extraction solvent. Same extraction procedure was used for MBA and GBA.

	Recovery (%)±SD								
Mycotoxins	Maize			Peanut cake			Cassava flour		
	EV_1	EV_2	EV_3	EV_1	EV_2	EV_3	EV_1	EV_2	EV_3
AFB_1	54±1	90±8	96 ±	59±1	72±11	79±6	61±11	75±4	89±3
DON	60±4	79±6	88 ±7	51±7	65±3	73±4	59±5	68±0.1	75±8
OTA	47±2	90±18	107 ±8	48±7	61±6	71±16	54±7	66±17	82±7
ZEN	50±9	68±1	91 ±10	56±13	70±3	82±5	51±6	72±6	88±2
FB_1	57±3	81±4	95 ±4	41±6	51±8	76±5	38±2	63±2	71±9

SD: standard deviation. EV: extraction volume

EV_1: 2.5 mL, EV_2:5 mL, EV_3:10 mL

Table 3

Summarized performance characteristics for the GBA obtained with maize samples

[a]Performance parameters (%)	[b]Mycotoxins			
	ZEN	DON	FB_1	OTA
False positive rate	0	0	0	0
False negative rate	5	0	0	5
Sensitivity	95	100	100	95
Specificity	100	100	100	100
Positive predictive value	100	100	100	100
Negative predictive value	95	100	100	95

[a]40 samples were used to determine the performance characteristics: 20 control blank samples and 20 samples fortified at the assay cut-off limits for the different analytes.

[b]spiked level: 3 μg kg^{-1}, 1250 μg kg^{-1}, 200 μg kg^{-1} and 1000 μg kg^{-1} for OTA, FB_1, ZEN and DON respectively

Table 4

The uncertainty regions for FB_1, DON, OTA and ZEN obtained with the GBA

Uncertainty level (µg kg^{-1})	Mycotoxins			
	FB_1	DON	OTA	ZEN
C_0	400	250	1.2	100
C_1	900	900	2.8	177

Table 5

Summarized performance characteristics for the MBA obtained with maize samples

[a]Performance parameters (%)	Mycotoxins			
	AFB_1	DON	OTA	ZEN
False positive rate	0	0	0	0
False negative rate	0	0	0	0
Sensitivity	100	100	100	100
Specificity	100	100	100	100
Positive predictive value	100	100	100	100
Negative predictive value	100	100	100	100

[a]40 samples were used to determine the performance characteristics: 20 control blank samples and 20 samples fortified at the assay cut-off limits for the different analytes.

[b]spiked level: 3 $\mu g\ kg^{-1}$, 5 $\mu g\ kg^{-1}$, 700 $\mu g\ kg^{-1}$and 175 $\mu g\ kg^{-1}$ for OTA, AFB_1, DON and ZEN respectively

Table 6

Results obtained after screening (gel-based assay (GBA) and membrane based assay (MBA)) and confirmatory analysis (LC-MS/MS) of naturally contaminated samples

Sample matrices	Visual results[a]: GBA[b]				Visual results[a]: MBA[c]				LC-MS/MS results ($\mu g\ kg^{-1}$)				
	FB_1	DON	ZEN	OTA	AFB_1	DON	ZEN	OTA	AFB_1	FB_1	DON	ZEN	OTA
Maize 1	-	-	+	-	-	+	-	-	<LOQ	305	705	186	nd
Maize 2	+	-	-	-	-	+	-	-	nd	2623	990	50	nd
Maize 3	+	+	+	-	-	+	+	-	<LOQ	3258	2197	297	nd
Maize 4	-	-	+	-	-	-	+	-	<LOQ	1056	416	187	nd
Maize 5	+	+	+	-	-	+	+	-	nd	2156	1360	315	nd
Peanut cake 1	-	-	-	+	+	-	-	+	292	nd	nd	nd	4
Peanut cake 2	-	-	-	+	+	-	-	+	189	nd	nd	nd	3.7
Peanut cake 3	-	-	-	+	+	-	-	-	154	nd	nd	nd	2
Peanut cake 4	-	-	-	+	+	-	-	+	120	nd	nd	nd	3.6
Peanut cake 5	-	-	-	+	+	-	-	+	340	nd	nd	nd	15
Cassava 1	-	-	-	+	+	-	-	+	58	nd	nd	nd	3
Cassava 2	-	-	-	+	+	-	-	+	90	<LOQ	nd	nd	9
Cassava 3	-	-	-	+	+	-	-	+	160	nd	nd	nd	20
Cassava 4	-	-	-	+	+	-	-	+	41	<LOQ	nd	nd	12
Cassava 5	-	-	-	+	+	-	-	+	86	nd	nd	nd	6

nd: not detected, LOQ: limit of quantification

+: analyte concentration≥cut-off limit, -: analyte concentration<cut-off limit.

[a]all experiments were realized in triplicate

[b]GBA cut−off limits: 3 $\mu g\ kg^{-1}$, 1250 $\mu g\ kg^{-1}$, 200 $\mu g\ kg^{-1}$ and 1000 $\mu g\ kg^{-1}$ for OTA, FB_1, ZEN and DON respectively

[c]MBA cut-off limits: 3 $\mu g\ kg^{-1}$, 5 $\mu g\ kg^{-1}$, 175 $\mu g\ kg^{-1}$ and 700 $\mu g\ kg^{-1}$ for OTA, AFB_1, ZEN and DON respectively

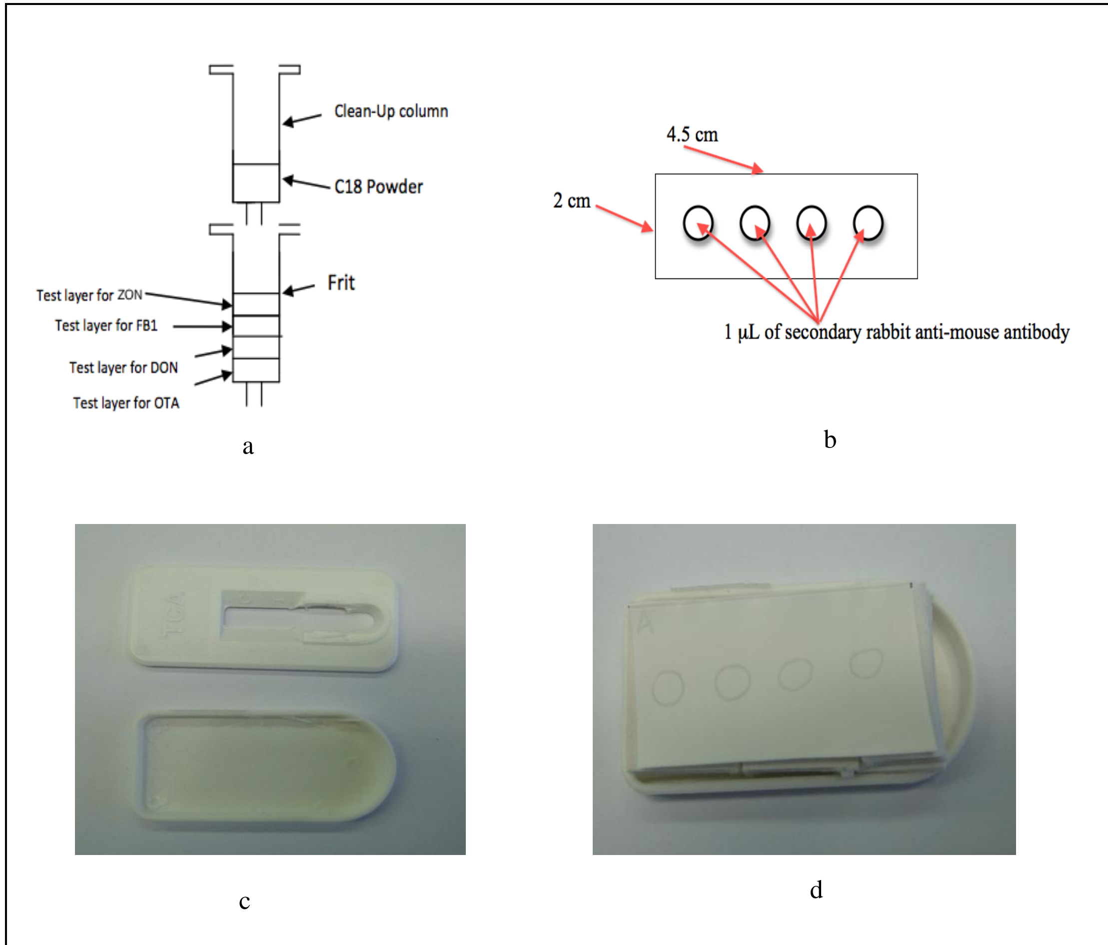
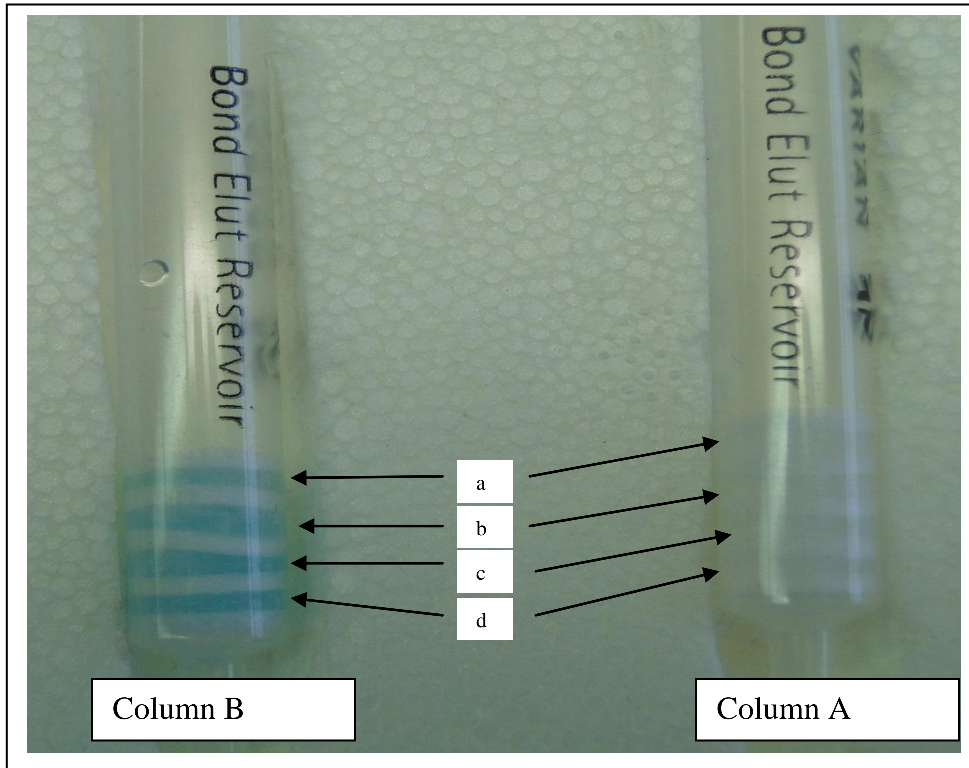


Fig. 1,



Test layers for; a:OTA, b:DON, c:FB_1, d:ZEN

Fig. 2

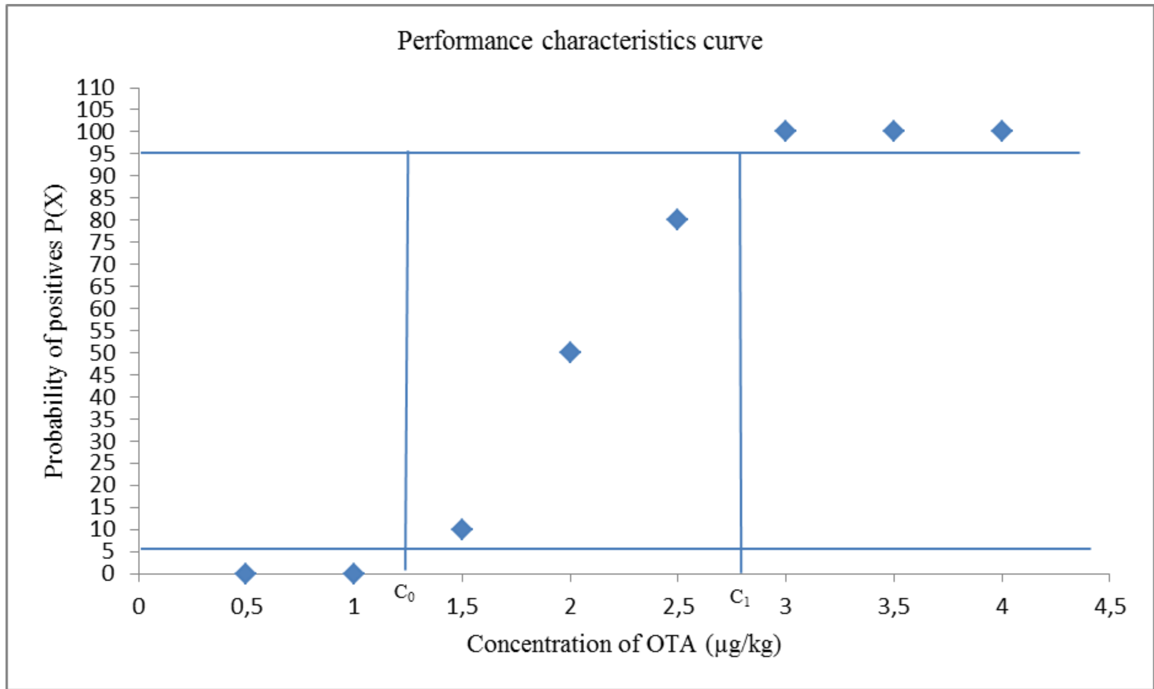


Fig 3

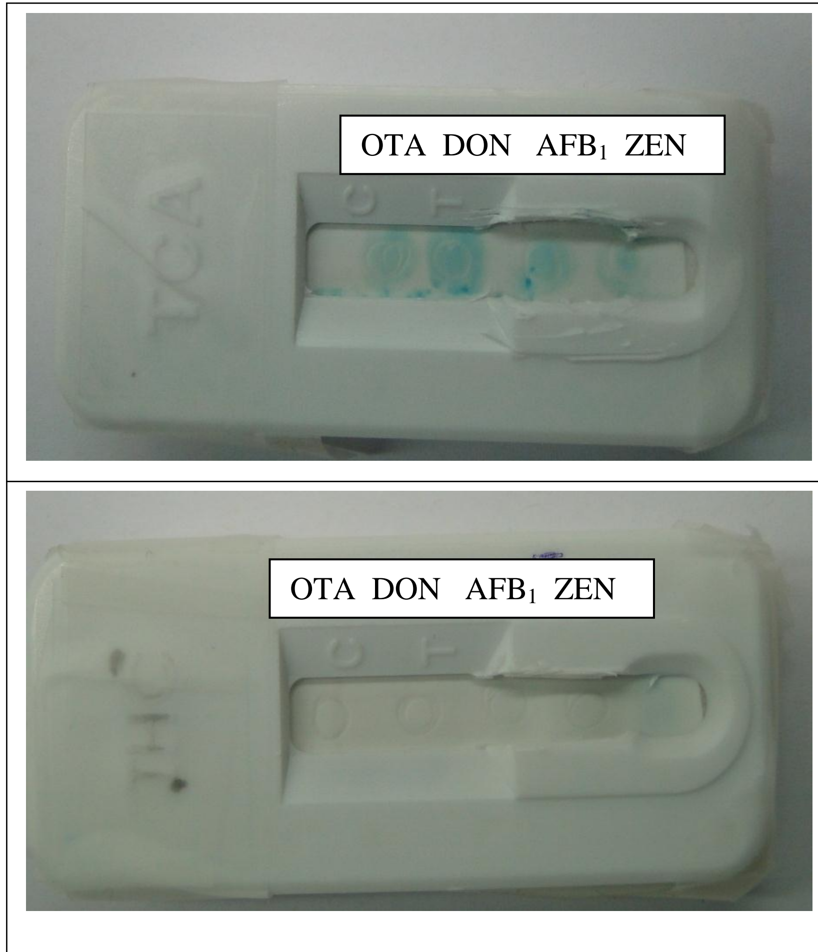


Fig 4:

Figure captions

Fig. 1:

(a) Schematic representation of a typical gel-based multiplex assay column, (b) Design of prepared assay membrane, (c) Top and bottom components of a typical cassette used for MBA, (d) Cassette filled with absorbent pads, on top of which is placed the assay membrane.

Fig. 2:

Results obtained with a multiplex GBA column after analysis of a blank sample (column B) and a positive sample (column A) contaminated with the four analytes above the assay cut-offs.

Fig. 3:

The performance characteristic curve for OTA as obtained with the GBA

Fig. 4:

Visual results with MBA assay conditions following analysis of a blank and spiked samples. Top figure: control blank; bottom figure: test sample spiked at the assay cut-off limit for the different analytes.