Mycotox Res (2011) 27:63–72
DOI 10.1007/s12550-010-0077-0

ORIGINAL PAPER

Development of a multiplex flow cytometric microsphere immunoassay for mycotoxins and evaluation of its application in feed

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Received: 30 July 2010 / Revised: 5 November 2010 / Accepted: 8 November 2010 / Published online: 26 November 2010

Abstract A multi-mycotoxin immunoassay—using the MultiAnalyte Profiling (xMAP) technology—is developed and evaluated. This technology combines a unique color-coded microsphere suspension array, with a dedicated flow cytometer. We aimed for the combined detection of aflatoxins, ochratoxin A, deoxynivalenol, fumonisins, zearalenone and T-2-toxin in an inhibition immunoassay format. Sets of six mycotoxin-protein conjugates and six specific monoclonal antibodies were selected, and we observed good sensitivities and no cross-interactions between the assays in buffer. However, detrimental effects of the feed extract on the sensitivities and in some cases on the slopes of the curves were observed and different sample materials showed different effects. Therefore, for quantitative analysis, this assay depends on calibration curves in blank matrix extracts or on the use of a suitable multi-mycotoxin cleanup. To test if the method was suitable for the qualitative detection at EU guidance levels, we fortified rapeseed meal, a feed ingredient, with the six mycotoxins, and all extracts showed inhibited responses in comparison with the non-fortified sample extract. Contaminated FAPAS reference feed samples assigned for a single mycotoxin showed strong inhibitions in the corresponding assays but also often in other assays of the multiplex. In most cases, the presence of these other mycotoxins was confirmed by instrumental analysis. The multiplex immunoassay can be easily extended with other mycotoxins of interest, but finding a suitable multi-mycotoxin cleanup will improve its applicability.

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Keywords Multiplex · Immunoassay · Flow cytometer · Microspheres · Mycotoxins · Feed

Introduction

Mycotoxins are secondary metabolites produced by fungi and often co-occur. Their presence in food and feed are serious threats to the health of humans and animals and monitoring is vital. The most common assayed mycotoxins are aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (F), zearalenone (ZEA) and T-2-toxin (T-2). Many methods are available for the detection of mycotoxins and, mainly, they can be divided into immunochemistry- and chromatography-based techniques, with enzyme-linked immunosorbent assay (ELISA) and liquid chromatography combined with mass spectrometry (LC-MS/MS) being the most popular. For the execution of legal tasks, often requiring high specificity, accuracy, sensitivity and good reproducibility, mycotoxins are frequently determined by LC-MS/MS. The simultaneous detection of several mycotoxins is a major advantage of this technique. Recently, a multi-mycotoxin LC-MS/MS-based method for the simultaneous detection of 23 mycotoxins was described (Monbaliu et al. 2009). However, this technique is less suitable for rapid and high throughput testing. It needs skilled personnel to handle the sophisticated machines and often requires sample cleanup, by the use of immunoaffinity (for specific mycotoxins) or solid phase extraction columns (Krska et al. 2008). Therefore, LC-MS/MS methods are laborious and time-consuming and less practical for on-site testing.

Nowadays, ELISA is the most common immunoassay format used. ELISA test kits for the detection of the major mycotoxins are widely available on the market (Goryacheva

et al. 2007). They allow easy and fast quantitative detection with good sensitivities and are suitable for the high throughput screening of samples and for on-site testing. For some sample materials, additional sample cleanup is necessary to avoid under or overestimates by disturbing matrix effects (Zheng et al. 2006). Other low-cost rapid immunoassay formats used are strip tests (Shim et al. 2009) and fluorescence polarization (Maragos 2009). A major disadvantage of these rapid immunoassay formats is that they are not suitable for the simultaneous detection of several mycotoxins, although a duplex strip test (Shim et al. 2009) and a duplex microarray assay (Lamberti et al. 2009) have recently been described.

A new platform for robust multiplexed immunochemical detection is the MultiAnalyte Profiling (xMAP) technology from Luminex (Austin, TX, USA). It is an emerging technology that uses small carboxylated polystyrene microspheres which are internally dyed with a red and an infrared fluorophore (Dunbar et al. 2003). By varying the ratio of the two fluorophores, up to 100 different color-coded microsphere sets can be distinguished, and each microsphere set can be coupled with a different biological probe. The microspheres are detected and characterized by a dedicated flow cytometer (Early et al. 2002), using a red laser (635 nm) for excitation and emission wavelengths are measured between 645 and 669 nm and >712 nm. After the microspheres are classified, the reporter signal is measured. The general reporter molecule used is R-Phycoerythrin (R-PE) which is excited by a green laser (532 nm) and the emission is measured at 580 nm (Peters et al. 2007). This creates the possibility to simultaneously measure up to 100 different biomolecular interactions in a single well.

There are different types of microspheres available such as the xTAG® microspheres for DNA purposes, the generally used MicroPlex® and the SeroMap™ microspheres with altered surface for problematic assays. All these varieties have a size of 5.6 μm. The superparamagnetic MagPlex® microspheres (6.5 μm) used in this research are available in 80 unique colour codes. Scattered over the microsphere there are magnetite particles for response to a magnetic field which simplifies the work with food or feed samples.

The microspheres can be coupled with a wide range of biomolecules like nucleotides, peptides, proteins, antibodies, receptors, polysaccharides and lipids (Kellar and Ianonne 2002). The xMAP technology is already used in many fields and the number of applications is growing rapidly (http://www.luminexcorp.com/bibliography, 2010). The main xMAP applications are dedicated kits for medical testing like respiratory viruses, cytokine profiling, and neonatal screening (http://www.luminexcorp.com/products/assays/overview.html, 2010). However, the xMAP technology also allows you to develop your own customized assays. This has already been done in the field of nucleic acids (Dunbar 2006), food proteins (Haasnoot and du Pré 2007), antibiotics (de Keizer et al. 2008) and polycyclic aromatic hydrocarbons (Meimaridou et al. 2010), and whole bacteria (Peters et al. 2007) and plant viruses (Bergervoet et al. 2008) have also been assayed using this technology. More recently, an xMAP duplex immunoassay has been developed for the detection of the mycotoxins OTA and fumonisin B_1 (FB_1) in grain products (Anderson et al. 2010). In other assays, the xMAP technology proved to be as sensitive as ELISA in comparative testing and is less labor-intensive and reduces costs (Biagini et al. 2003)

In our multi-mycotoxin flow cytometric immunoassay xMAP approach, mycotoxin-BSA conjugates of aflatoxin B1 (AFB_1), OTA, FB_1, DON, ZEA and T-2 Toxin (T-2) are coupled to the carboxylated paramagnetic microspheres. These six coupled microspheres and all six specific monoclonal antibodies (Mabs) against the same toxins are added to the sample. The free mycotoxins from the standards and/or sample extracts will inhibit the binding of the Mabs to the mycotoxin-BSA conjugates on the microspheres. After a magnetic capturing step, a secondary anti-mouse antibody coupled with R-PE is added as a detection molecule. This mixture containing six different microspheres will eventually pass through the flow cell and, upon laser illumination, the microspheres will be classified and its mean surface reporter signals (mean fluorescence intensities; MFIs) will be quantified.

After extensive testing for optimum dilutions/responses, sensitivity, specificity and cross-interactions, six Mabs were selected for this multiplex assay. We tested calibration curves for each mycotoxin in buffer and in an extract of a "blank" rapeseed meal (a feed ingredient). To see if the assay was able to perform at the EU guidance levels for feed, we fortified rapeseed meal with the pure mycotoxins prior to sample extraction. Other sample materials (feed and some feed ingredients) were also tested for their matrix influence on the multiplex assay. Finally, contaminated FAPAS reference feed samples were tested and the results were compared with instrumental analysis data.

Materials and methods

Instrumentation

The xMAP assay measurements were carried out on a Luminex 100 IS 2.2 system, consisting of a Luminex 100 analyzer, a Luminex sheath delivery system and a Luminex XY Platform, which is programmed to position a 96-well plate, using StarStation System software from Applied Cytometry Systems (ACS, Dinnington, Sheffield, UK). All washing steps were carried out on a Bio-Plex™ Pro II Wash

Station (Bio-Rad Laboratories, Veenendaal, the Netherlands) using a magnetic plate carrier. Plates were incubated on a Dynatech microtiter vari-shaker (Alexandria, VI, USA). During the coupling procedures, the paramagnetic microspheres were captured using the DynaMag-2™ magnet stand (Invitrogen Dynal, Oslo, Norway). Mixing for sample extraction was done in a REAX2 end-over-end shaker (Heidolph, Schwabach, Germany). All centrifuge steps were done in an Eppendorf 5810 R centrifuge using the A-4-62 rotor (VWR International, Amsterdam, the Netherlands).

Materials, reagents and standards

MagPlex microsphere sets with numbers 036, 038, 054, 086, 090, 100 and Sheath Fluid were purchased from Luminex. Monoclonal antibodies (Mabs) against AFB_1 (6G4), FB_1 (1D6), OTA (5E2), ZEA (88) and T2 (8H2) were obtained from Soft Flow Biotechnology (Gödöllö, Hungary) and against DON (AB0222) from Aokin (Berlin, Germany). From Sigma-Aldrich (Zwijndrecht, the Netherlands), the AFB_1-BSA conjugate (A6655), 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), 2-(N-morpholino)ethanesulfonic acid (MES) and BSA were purchased. FB_1-BSA was a kind gift from Jules Beekwilder of Plant Research International (Wageningen, the Netherlands). BSA conjugates of OTA (CON003), DON (CON002), ZEA (CON005) and T-2 (CON004) were from Biopure (Tulln, Austria) as well as the solid standards of the mycotoxins AFB_1, OTA, DON, ZEA, FB_1 and T-2. Goat anti-mouse IgG-R-Phycoerythrin conjugate was obtained from Prozyme (Hayward, CA, USA). Skimmed milk powder (blotting grade blocker, non-fat dry milk) was from Bio-Rad Laboratories. Acetonitril was purchased from Biosolve (Valkenswaard, the Netherlands). Greiner Cellstar 96-well microtiter plates were used for all assays. All other chemicals were ordered from VWR. The "blank" rapeseed meal and other sample materials were supplied previously to and analyzed by RIKILT. Reference feed samples were ordered from FAPAS® (York, UK).

Coupling of mycotoxin-BSA conjugates to the microspheres

Each microsphere stock suspension (1.25×10^7 microspheres/ml) was vortexed vigorously for 5 min. From each stock, 500 µl (approximately 6 million microspheres) was taken and transferred to an Eppendorf tube. This tube was placed in the magnetic stand and beads were allowed to settle for 5 min. The supernatant was removed and 500 µl of 100 mM monobasic sodium phosphate pH 6.2 was added with the tube still in the magnetic stand. After 2 min of settling, the supernatant was removed again and the microspheres were resuspended in 80 µl of the same sodium phosphate buffer. To this microsphere suspension, 10 µl of 50 mg/ml Sulfo-NHS and 10 µl of 50 mg/ml EDC were added. The suspension was incubated in the dark at room temperature for 20 min. During this incubation, the microsphere suspension was mildly vortexed every 5 min. After incubation, the tube was placed in the magnetic stand and microspheres were allowed to settle for 1 min. The supernatant was removed and the microspheres resuspended in 250 µl of 50 mM MES buffer pH 5.0 by pipetting up and down several times. The microspheres were captured again and the washing step with the same MES buffer was repeated. A previously prepared 500-µl solution of the mycotoxin-BSA conjugate at a concentration of 125 µg/ml in MES was then added to the microspheres. This suspension was incubated for 2 h in the dark at room temperature while gently rotating. The microspheres were captured and washed 2 times with 500 µl PBS-TBN (Phosphate Buffered Saline containing 0.1% BSA, 0.02% Tween-20 and 0.05% sodium azide, pH 7.4). For storage, the microspheres were resuspended in 500 µl of the same PBS-TBN. After overnight storage at 4°C, the microspheres were ready for use in the assays. The final concentrations of the microspheres were determined in the Luminex by counting diluted portions for 60 s with a flow rate of 60 µl/min.

The xMAP immunoassay

Mycotoxin standard stock solutions were prepared in an acetonitrile/water mixture (80:20; v/v) at concentrations of 100–200 µg/ml. For the calibration curves, necessary standard solutions were prepared from these stock solutions by serial dilutions in water.

For the xMAP assay, 40 µl of standard solution or standard solution mixed (1:1; v/v) with sample extract (in the case of dose-response curves in sample extract) or 2 times in water-diluted sample extract was added to each well of a microtiter plate. Subsequently, 10 µl of concentrated PBM (5 times concentrated PBS containing 1% of skimmed milk powder) containing the Mabs of choice were added and incubated for 10 min on a shaker at room temperature. During the initial testing, each antibody was tested separately to check for cross-interactions. After this, the 6 microspheres sets coupled with different mycotoxins, around 1,000 microspheres of each set, were added in 10 µl of PBM and the assay was incubated for another 30 min at room temperature. The plate was then transferred to the washer for a total of 3 washing steps with PBS. The remaining volume after washing was approximately 30 µl. To each well, 70 µl of R-PE conjugated goat anti-mouse antibody (2.85 µg/ml in PBS) was added and the microspheres were brought back in suspension by pipetting up and down. The samples were incubated while shaking for

15 min. After incubation, the plate was again transferred to the washer for one final washing step with PBS. To each well, 70 µl of PBS was added and the microspheres were resuspended by pipetting up and down. The samples were measured in the Luminex analyzer for a total of 100 microspheres per set per sample with an average of 45 min for the measurement of a 96-well plate. During the measurement, the Luminex uses four detectors [three avalanche photodiodes (APDs) and one photomultiplier tube (PMT)]. Two APDs are used for the classification of the beads by measuring the emission signals (between 645 and 669 nm and >712 nm) from the two internal dyes excited by a red laser diode (633 nm). As shown in Fig. 1 (upper right part), the 6 microsphere sets are classified in the classifier plot. This laser diode is also used for the determination of side scatter signals of all the measured microspheres which correlates to the particle size. This side scatter signal is detected by the third APD, presented in the discriminator plot (Fig. 1, upper left part) and represents the total measured events. The PMT is used to measure the microsphere-bound reporter molecules after the excitation by a green laser (532 nm), and examples of the reporter signals are shown in Fig. 1 (the lower two parts) for the DON and ZEA assays in the multiplex format. The software calculates the median reporter signal for each measurement.

LC-MS/MS multi-mycotoxin method

The amounts of the mycotoxins DON, FB_1, T-2, HT-2, OTA, ZEA and in some cases AFB_1 were determined using an in-house validated and accredited LC-MS/MS based method. In short, 2.5 g of sample material was extracted with 10.0 ml of extraction solvent (acetonitril/water/formic acid: 84/16/1; v/v/v). The mixture was shaken for 2 h and then centrifuged. The supernatant was diluted with water (1:1), and filtered prior to LC-MS/MS analysis with 5-µl injections and eluted using a water (eluent A)/95% methanol/water (v/v) (eluent B) gradient, both containing 1 mM ammonium formate and 0.53 mM formic acid, at a column temperature of 35°C. The LC-MS/MS system consisted of a Shimadzu Prominence system, a Phenomenex

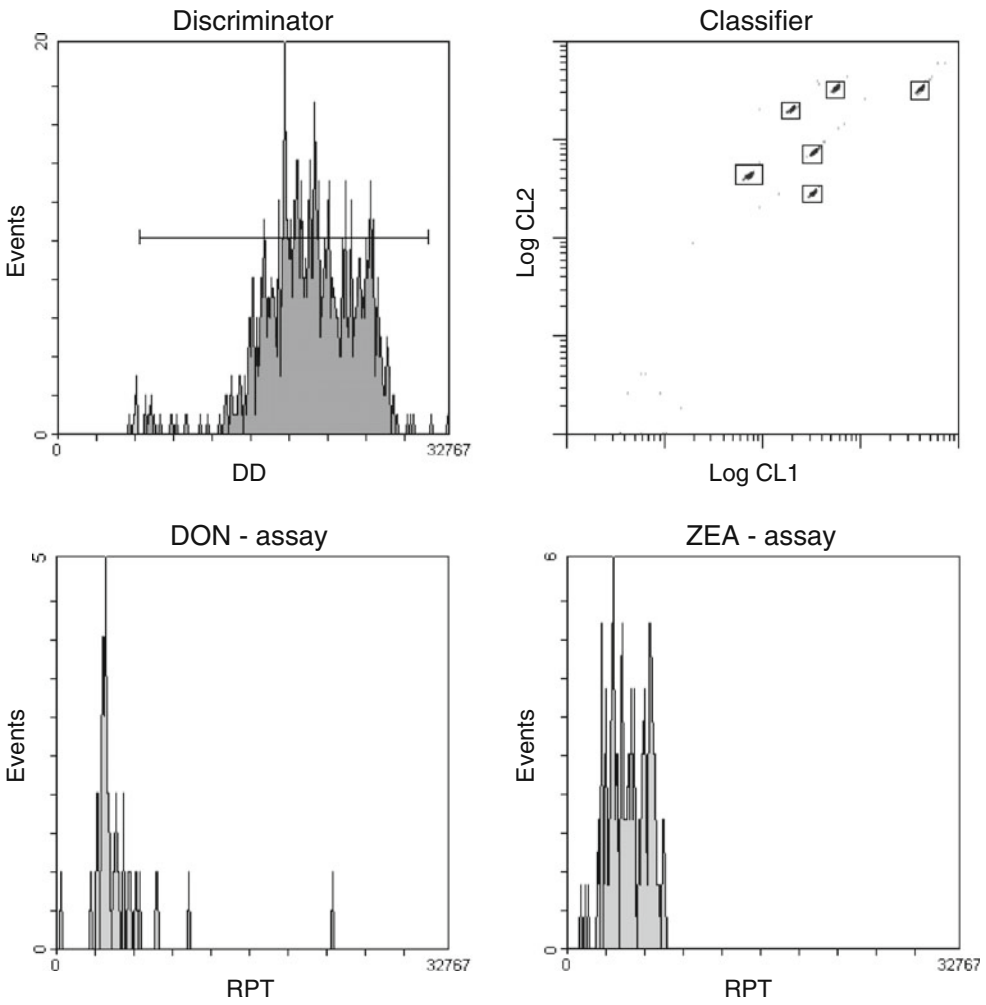

Fig. 1 Typical flow cytometric output of the StarStation software showing all microspheres in the Doublet Discriminator (*DD*) plot (*upper left part*), classification and counting of the microspheres based on the log CL1 (red) and log CL2 (infra-red) ratio in the Classifier plot (*upper right part*) and 2 of the 6 response plots (*lower two parts*) showing the reporter signals (*RPTs*) for the *DON* and *ZEA* assay in the multiplex flow cytometric immunoassay

Synergi 4 μ Hydro RP UPLC column (150 mm × 2 mm, 2.5 μm) and an AB SCIEX QTRAP® used in MS/MS-mode. The mycotoxin content was quantified with a standard addition procedure.

Fluorescent-HPLC (F-HPLC) AFB_1 detection

The AFB_1 content for some samples was previously determined in proficiency testing using an in-house validated and accredited HPLC-fluorescence-based method. In short, 20 g of sample material, 10 g of celite, 10 ml of water and 100 ml of chloroform were mixed for 30 min. After filtration, 2.0 ml of extract was evaporated until dryness. The residue was dissolved in 1.0 ml of methanol and the solution was diluted with 9.0 ml of water. The resulting solution was cleaned with Immuno Affinity Cleanup (IAC). The Fluorescent-HPLC system consisted of a Gilson pump and autoinjector, a Jasco fluorescence detector and a KOBRA-cell equipped with a Waters Symmetry C18 HPLC column (150×3.0 mm, 5 μm).

For analysis, 100-μl extracts or reference solutions were injected and eluted using a water/methanol/acetonitril eluent (130/70/40; v/v/v) containing 1 mM KBr and 1 mM HNO_3. Quantitative analysis was performed by calculation versus a calibration curve.

Extraction of feed matrix

Sample extraction was performed according to an in-house LC-MS/MS protocol that was in use for the simultaneous detection of several mycotoxins. For each sample, 2 times 2.5 g was weighed and transferred to a 50-ml tube. To the first tube, 10 ml of double distilled water was added. To the second tube, 10 ml of acetonitril/water (84/16; v/v) mixture was added. Both tubes were then incubated for 2 h at room temperature while gentle mixing using an end-over-end shaker. The tubes were centrifuged at room temperature for 10 min at 2,000*g* using a swinging bucket rotor. The supernatants were combined in equal volumes and incubated for 1 h at 4°C. After incubation, the mixed sample extracts were again centrifuged at the same speed. The supernatant was diluted twice and used directly in the assays. The dose-response curves were made with standard solutions diluted in water (see "The xMAP immunoassay") but also with mixtures (1:1; v/v) of the standard solutions and "blank" sample extract.

Results and discussion

Immunoassays for low molecular weight compounds use the direct (antibody-coated surfaces) or indirect (hapten-coated surfaces) competitive or inhibition assay formats. We have chosen for the indirect inhibition assay format in which the binding of the Mabs to the mycotoxin-coated microspheres is inhibited by the mycotoxins in solution. For the coupling of proteins to the xMAP microspheres, standard protocols are available (Luminex) and, therefore, BSA was used as the carrier protein for the mycotoxins during the microsphere coupling. The final selection of mycotoxin conjugates and Mabs was compiled after a previously performed large-scale screening of reagents obtained from different suppliers (data not shown) and was based on maximum responses, sensitivities of the dose-response curves, specificities (cross-reactions with other mycotoxins), and cross-interactions between the assays. The optimal coupling concentration for the mycotoxin–BSA conjugates to the microspheres proved to be 125 μg/ml. The addition of mycotoxin-specific Mabs, at optimized dilutions, and a secondary anti-mouse R-PE reporter antibody showed significant fluorescence responses for each mycotoxin-coupled microsphere set ranging from 3,000 to roughly 6,000 MFI in buffer (Table 1). The Mab stock solutions (1 mg/ml) were diluted from 600 to 30,000 times, resulting in final concentrations in the assay of 1.6 μg/ml for anti-AFB_1 and anti-FB_1, 0.83 μg/ml for anti-OTA, 0.67 μg/ml for anti-ZEA, 0.17 μg/ml for anti-DON, and 0.03 μg/ml for anti-T-2. The observed differences of signals depend on the coupling efficiencies of the mycotoxins to BSA and of the conjugates to the microspheres (influenced by the remaining free amino groups on the conjugates and the

Table 1 Average (n=2) maximum responses (MFI) obtained with the multiplex flow cytometric immunoassay in buffer, using the individual and the mixed antibodies

Antibody	Maximum responses (MFI) per assay[a]					
	AFB_1	OTA	ZEA	DON	FB_1	T-2
Mixed [b]	7,200±100	4,600±200	4,600±200	8,200±100	4,500±200	4,600±200
Anti-AFB_1	5,000±200	9	21	25	35	5
Anti-OTA	1	3,500±200	25	27	31	7
Anti-ZEA	13	15	5,800±300	33	25	3
Anti-DON	1	13	19	5,300±300	27	11
Anti-FB_1	5	7	29	27	3,200±200	7
Anti-T2	3	7	23	27	27	3,100±100

[a] All data were obtained in multiplex microsphere setting

[b] A mixture of the six Mabs

polar changes of the protein surface by the mycotoxin molecules) and on the dilutions and affinities of the different Mabs. All the individual mycotoxin-specific Mabs were tested with the complete mixture of six mycotoxin-specific microsphere sets to see whether cross-interactions between the assays could be observed. Table 1 shows that the final selection of reagents did not show any remarkable cross-interactions between the assays. However, except for the ZEA assay, the responses for each specific microsphere set increased when all six antibodies were used simultaneously (mixed) in the multiplex assay. It seems that the presence of higher concentrations of antibodies increase the responses, probably due to the non-specific binding of antibodies to each other in the multiplex assay. Fortunately, this presumed non-specific binding had no negative effects on the dose-response curves because full inhibitions were still obtained (Fig. 2a). The dose-response curves in buffer, measured in triplicate over a 3-day period, showed good sensitivities for all mycotoxins when measured in multiplex setting (Fig. 2a). The concentrations at 50% relative response [or at 50% inhibition (IC_{50} values)] of the dose-response curves in the different assays, were 0.29, 0.33, 0.39, 1.6, 2.2 and 6.7 ng/ml for OTA, AFB_1, ZEA, FB_1, T-2, and DON, respectively. Compared to the ELISA data supplied by the manufacturers, the IC_{50} values of the multiplex for OTA, ZEA and T-2 were comparable and were two and three times lower for FB_1 and DON, respectively, and four times higher for AFB_1. This indicates that the AFB_1 assay can probably still be improved, for instance, by modifying or changing the buffer. The small error margins show the high precision of the multiplex assay in buffer. Unfortunately, the curves for AFB_1, OTA, ZEA and T-2 are very steep and therefore have limited dynamic ranges. The assays were tested for the described mycotoxins only but will also detect derivatives and other forms of these mycotoxins as shown from the manufacturer's data sheets. According to the suppliers specifications, the anti-AFB_1 Mab was reported to have cross-reactivity with the aflatoxins B_2 (76 %), G_1 (55 %) and G_2 (6 %) and the anti-FB_1 Mab with the fumonisins B_2 (FB_2, 91 %) and B3 (FB_3, 209 %). The anti-ZEA Mab cross-reacts with zearalanon (138 %), α-zearalenol (91 %), β-zearalenol (21 %), α-zearalanol (69 %) and β-zearalanol (6 %) and the anti-T-2 Mab with acetyl-T-2 (12.3 %), HT-2 (3.4 %) and iso-T-2 (2.5 %). The anti-OTA and anti-DON Mabs had no reported cross-reactivities. The reported cross-reactivities might differ per type of assay and sample material and still need to be tested with relevant samples in the multiplex flow cytometric final format. In general, cross-reactivities may lead to overestimated concentrations of the assayed mycotoxin. For instance, guidance values for aflatoxins in feed are just set for AFB_1 and there is no guidance level for the total aflatoxins, as is the case for food. This means that our assay could give overestimated results for AFB_1 due to relatively high cross-reactivities with AFB_2 and AFG_1. If critical, samples tested positive for aflatoxins in the multiplex should always be checked for the actual concentration of AFB_1 using LC-MS/MS. Therefore, the AFB_1 assay is more qualitative than quantitative. The FB_1 antibody used in the assay has high cross-reactivities with FB_2 and FB_3. Like in food, there are combined guidance levels for FB_1 and FB_2 in feed. The concentration of FB_2 in feed is normally around 15–35% of the FB_1 concentration (Hascheck et al. 2001). The high cross-reactivity for FB_3 seems not to be a major problem since it is rarely present in feed (ingredients) as was shown by previous LC-MS/MS measurements within our institute (data not shown). Its occurrence seems related to the presence of high FB_1 concentrations. For ZEA, the appearance of high concentrations of its metabolites in feed are very unlikely (Vendl et al. 2010) and therefore will not contribute to substantial overestimations in this assay. In the case of T-2, the mentioned cross-reactions with HT-2 will slightly contribute to the total response when working with this Mab. If the simultaneous or single detection of HT-2 is desired another antibody is required or has to be added as a new parameter to the assay.

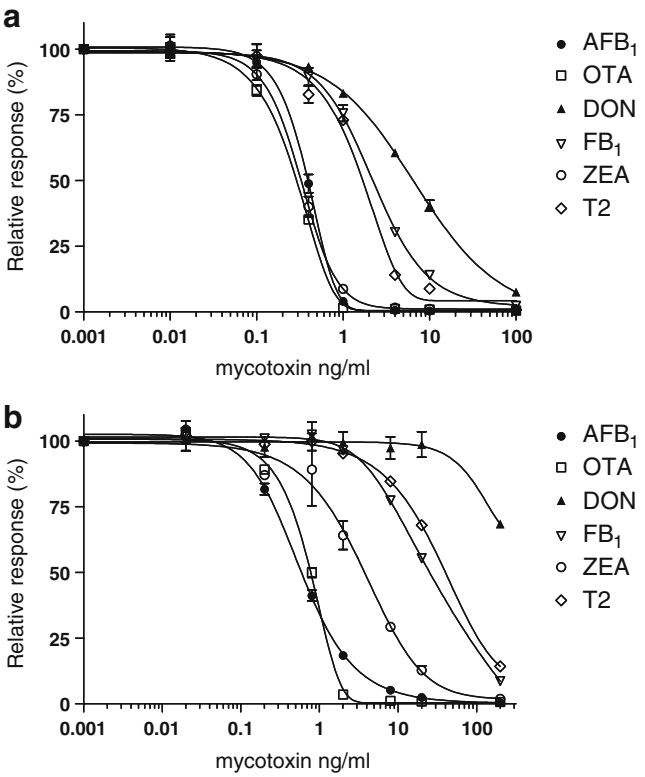

Fig. 2 Average dose-response curves (n=9) of the six mycotoxin assays in the multiplex microsphere inhibition assay format in buffer (**a**) and in two times diluted sample extract (**b**)

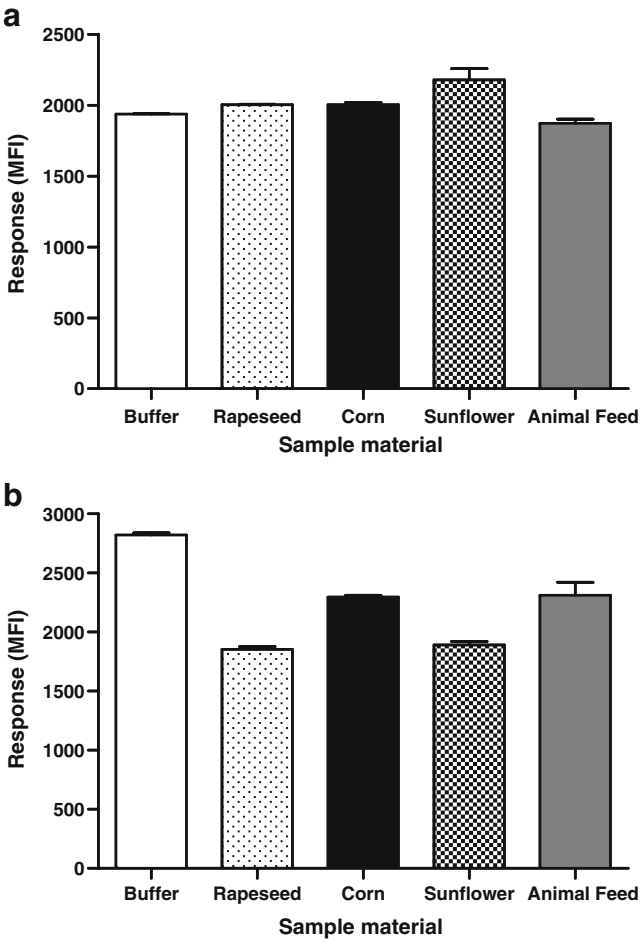

Fig. 3 Average (n=3) maximum responses (*MFI*) for the OTA (**a**) and AFB_1 (**b**) assays in different sample extracts and buffer

In this study, a rapeseed meal was chosen as the model "blank" feed sample material because, based on LC-MS/MS data available within RIKILT, mycotoxin concentrations were below the limits of detection (LODs) of this method for feed analysis (AFB_1 <0.005 mg/kg, DON <0.50 mg/kg, FB_1 <0.10 mg/kg, FB_2 <0.10 mg/kg, FB_3 <0.10 mg/kg, OTA <0.025 mg/kg, T2 <0.5 mg/kg, and ZEA <0.05 mg/kg). Therefore, it is still possible that this "blank" feed sample contains mycotoxins at levels below these LODs which might influence the screening assay. Measuring dose-response curves in the rapeseed meal extract showed significant decreases of the maximum responses, compared to the response in buffer, for most of the dose-response curves (up to 64%), and also had an effect on most of their sensitivities (Fig. 2b). The sensitivity for the DON curve was most influenced by the addition of the sample extract, and the addition of higher toxin concentrations will be necessary to produce a useful dose-response curve. The use of acetonitrile at a concentration of 14% showed no drastic influence on the total MFI for the DON assay when used as a blank sample. Also, for ZEA, a shift in sensitivity was observed and some toxin concentrations showed increased error margins. Besides a negative effect on the sensitivity and precision, the sample extract enlarged the dynamic range for ZEA. The same effect was seen for the AFB_1 curve. The OTA curve remained largely unaffected by the addition of sample extract. The effects on the maximum responses by the addition of different sample materials in the AFB_1 and OTA assays are shown in Fig. 3 which

Table 2 Average responses (MFI) obtained with the multiplex flow cytometric immunoassay (n=3) for extracts of a "blank" rapeseed meal fortified with mycotoxins at EU guidance levels (Directive 2002/32/EC 7 May 2002, Commission recommendation 2006/576/EC 17 August 2006)

Mycotoxin added	Level of addition (μg/kg)	Responses (MFI) for each assay					
		AFB_1	OTA	ZEA	DON	FB_1	T-2
None	0	2,100±50	2,800±100	3,600±200	980±50	1,600±50	2,000±50
AFB_1[a]	5	1,900±100	2,800±50	3,800±100	1,100±50	1,600±50	2,100±100
OTA[b]	50	2,100±50	30±3	3,700±200	980±50	1,500±50	2,000±100
ZEA[c]	100	2,200±50	2,800±100	420±20	1,000±50	1,700±50	2,100±50
DON[d]	900	2,300±100	2,900±200	3,900±200	460±50	1,600±200	2,200±200
FB_1[e]	5,000	2,300±50	2,800±100	4,000±100	1,000±50	39±3	2,300±50
T2[f]	10	2,300±100	2,900±200	3,800±200	1,100±50	1,700±100	1,400±100

Recommended guidance values of the EU:

[a] Lowest level for feed for dairy cattle

[b] Lowest level for feed for pigs

[c] Lowest level for feed for young pigs

[d] Lowest level for feed for pigs

[e] Lowest level for FB_1+FB_2 in feed for pig, horses, rabbits and pets

[f] The level for T2 was set at 10 μg/kg since there is no official guidance value

demonstrates that the AFB_1 assay is much more susceptible to matrix interference than the OTA assay. Each sample material shows a decrease of response compared to the response in buffer. The same negative effects were observed for the ZEA and FB_1 assays but were most severe for the DON assay. The T-2 assay, like the OTA assay, remained largely unaffected. Therefore, for quantitative analysis, this assay depends on calibration curves in blank matrix extracts, which is difficult because of the varying content of feed, or on the use of a suitable multi-mycotoxin cleanup.

To test if the method was suitable for the qualitative detection, the "blank" rapeseed meal was fortified with the 6 mycotoxins at EU guidance levels (Directive 2002/32/EC 7 May 2002, Commission recommendation 2006/576/EC 17 August 2006). All the fortified samples showed reduced responses (inhibition) in the specific assays when compared to the non-fortified controls (Table 2). The samples fortified with other mycotoxins could also be considered as negative controls for the specific assays and all the responses in the fortified samples were found to be significantly lower (lower responses compared to the average responses minus three times the SD). In the case of the OTA, FB_1, ZEA and DON assays, there are strong inhibitions of the responses (99, 98, 89 and 56 %, respectively), but for the AFB_1 assay, and to a lesser extent the T-2 assay, there is less inhibition (15 and 34 %, respectively) at these relevant concentrations. The AFB_1 assay certainly needs some improvement which is probably best done by changing one of the two essential reagents. For the T-2 assay, it is less urgent to make changes. The guidance value we chose was very stringent. For example, Liesener et al (2010) used a level of 250 μg/kg based on the comparison of the toxicity of T-2 to

Table 3 Comparison of the average responses (MFI, n=3) obtained with extracts of mycotoxin-spiked blank rapeseed sample (at guidance values) and with spiked extract of the blank sample, indicating extraction efficiencies of the different mycotoxins using the multi-mycotoxin extraction procedure

Mycotoxin	Average response (MFI)		Response ratio after and before extraction
	Fortified supernatant[a]	Fortified raw material[b]	
AFB_1	1,976	1,890	1.04
OTA	31	39	0.79
ZEA	529	459	1.15
DON	26	30	0.87
FB_1	284	418	0.68
T-2	691	1,376	0.50

[a] Blank rapeseed meal extract (supernatant) was fortified with the different mycotoxins at the 100% extraction efficiency levels

[b] Rapeseed meal was fortified before extraction at EU guidance levels

Table 4 FAPAS® reference samples[a] and the relative responses, based on rapeseed meal fortified dose-response curves, obtained with extracts in the individual assays of the multiplex flow cytometric immunoassay and instrumental analysis results

Sample No.	Assigned Mycotoxin	Assigned Value (μg/kg)	Percentage of inhibition (Luminex) and concentrations determined by instrumental analysis for the different mycotoxins											
			AFB_1			T-2		OTA		ZEA		FB_1		DON	
			Percentage inhibition	F-HPLC LC-MS data (μg/kg)		Percentage inhibition	LC-MS data (μg/kg)	Percentage inhibition	LC-MS data (μg/kg)	Percentage inhibition	LC-MS data (μg/kg)	Percentage inhibition	LC-MS data (μg/kg)	Percentage inhibition	LC-MS data (μg/kg)
T0470	AFB_1	11.8	90	11.9[b]		16	<10	8	<5	95	>200	37	34	64	1,440
T2225	ZEA	274	0	<1[c]		17	<10	48	<5	94	>200	84	147	55	920
T2230	DON	894	0	<1[c]		8	<10	5	<5	40	160	97	2,880	52	710
T04102	AFB_1	18.1	96	23.7[b]		0	<10	0	<5	5	<5	0	<20	10	<100
T0486	AFB_1	23.0	98	34.6[b]		1	<10	12	<5	77	<5	14	<20	32	<100
T0478	AFB_1	7.28	93	8.7[b]		35	<10	97	<5	88	52	84	55	52	200
T1748	OTA	8.03	0	<1[c]		23	<10	97	8	44	n.d.	82	77	32	140
T2240	DON	775	29	2.6[c]		50	47	19	<5	60	10	94	115	59	810
T1758	OTA	41.4	34	<1[c]		62	n.d.[d]	98	48	63	11	94	164	45	250

[a] Samples came from single mycotoxin proficiency testing. They were submitted for LC-MS/MS multi-mycotoxin analysis

[b] Previously determined values from FAPAS® proficiency testing for AFB_1 using F-HPLC

[c] Values determined using the LC-MS/MS multi-method

[d] The quantification of T-2 in this sample was hampered by an interfering peak but a high concentration of HT-2 (430 μg/kg) was detected

DON. Furthermore, some east European countries have set the guidance level for T-2 at 100 μg/kg. It also becomes clear from the response ratio in Table 3 that the extraction for T-2 is not optimal using the current protocol. This is not the case for AFB_1, the extraction of which seems to be optimal based on that ratio.

The contaminated FAPAS reference feed samples (fortified or naturally contaminated, which was not clear from the sample information) were investigated and the average maximum responses of the rapeseed fortified dose-response curves were used to calculate the percentages of inhibition (Table 4). All four samples that were assigned for the presence of AFB_1 (7–23 μg/kg) showed strong inhibitions (90–98 %) in the AFB_1 assay, but three of them also in the ZEA assay (77–95 %) and one (T0470) in the DON assay (64 %). With LC-MS/MS, ZEA was found in two of these samples (52 and >200 μg/kg) and the DON sample contained a high concentration (1440 μg/kg). The two OTA assigned samples showed strong inhibited responses in the OTA assay (97 and 98 %), but also in the FB_1 assay (82 and 94 %) and one (T1758) in the T2 assay (62 %). With LC-MS/MS, FB_1 was found (77 and 164 μg/kg) but T-2 could not be detected with LC-MS/MS in that sample because of an interfering peak. LC-MS/MS data showed a high concentration of HT-2 in this particular sample (430 μg/kg, data not published). The ZEA assigned sample (T2225) showed a strong inhibition in the ZEA assay (94%) but also in the FB_1 and DON assay in which the LC-MS/MS found a low concentration of FB_1 (147 μg/kg) and a high concentration of DON (920 μg/kg). The two DON-assigned samples showed strong inhibited responses in the DON assay (52 and 59 %) but with one sample (T2230) also in the FB_1 assay (97 %), in which the LC-MS/MS found 2,880 μg of FB_1/kg, and in the ZEA assay (40 %), in which the LC-MS/MS found 160 μg of ZEA/kg. The other sample (T2240) inhibited the T-2 assay (50 %), the ZEA assay (60 %) and the FB1 assay (94 %) and the LC-MS/MS found T-2 (47 μg/kg), ZEA (10 μg/kg) and FB_1 (115 μg/kg). These additional mycotoxins found in these reference feed samples, show the potential of the multiplex screening assay. However, one AFB_1 assigned sample (T0478) also caused strong inhibition in the OTA assay (97%) and another (T0486) in the ZEA assay, which could not be confirmed by LC-MS/MS.

The overall results of the qualitative multiplex immunoassay look promising and will be further exploited in new research. Unfortunately, this research cannot be extended with the same reagents, because of the discontinuation of the supply of the mycotoxin-BSA conjugates from Biopure. Home-made mycotoxin–protein conjugates and conjugates from other suppliers are now under investigation in combination with the described Mabs. The performances of indirect and direct inhibition assays will be compared, as well as improvements in the assay's protocols (incubation time, temperature and buffer composition). The multiplex immunoassay can be easily extended with other mycotoxins of interest, but finding a suitable multi-mycotoxin cleanup to remove matrix effects will improve its applicability.

Acknowledgement The authors wish to thank Jules Beekwilder (Plant Research International) for the kind gift of the FB_1-BSA conjugate.

References

Anderson GP, Kowtha VA, Taitt CR (2010) Detection of fumonisin B1 and ochratoxin A in grain products using microsphere-based fluid array immunoassays. Toxins 2:297–309

Bergervoet JHW, Peters J, van Beckhoven JRCM, van den Bovenkamp GW, Jacobson JW, van der Wolf JM (2008) Multiplex microsphere immuno-detection of potato virus Y, X and PLRV. J Virol Meth 149:63–68

Biagini RE, Schlottmann SA, Sammons DL, Smith JP, Snawder JC, Striley CAF, MacKenzie BA, Weissman DN (2003) Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. Clin Diagn Lab Immunol 10:744–750

Commission recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2, and HT-2 and fumonisins in products intended for animal feeding

de Keizer W, Bienenmann-Ploum ME, Bergwerff AA, Haasnoot W (2008) Flow cytometric immunoassay for sulfonamides in raw milk. Anal Chim Acta 620:142–149

Directive 2002/32/EC of the European Parliament and of the council of 7 May 2002 on undesirable substances in animal feed

Dunbar SA (2006) Applications of Luminex® xMAP™ technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta 363:71–82

Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, Jacobson JW (2003) Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP™ system. J Microbiol Meth 53:245–252

Early MC, Vogt RF, Shapiro HM, Mandy FF, Kellar KL, Bellisario R, Pass KA, Marti GE, Stewart CC, Hannon WH (2002) Report from a workshop on multianalyte microsphere assays. Cytometry 50:239–242

Goryacheva IY, De Saeger S, Eremin SA, Van Peteghem C (2007) Immunochemical methods for rapid mycotoxin detection: Evolution from single to multiple analyte screening: a review. Food Addit Contam 24:1169–1183

Haasnoot W, du Pré JG (2007) Luminex-based triplex immunoassay for the simultaneous detection of soy, pea, and soluble wheat proteins in milk powder. J Agric Food Chem 55:3771–3777

Haschecck WM, Gumprecht LA, Smith G, Tumbleson ME, Constable PD (2001) Fumonisin toxicosis in swine: an overview of porcine pulmonary edema and current perspectives. Environ Health Perspect 109:251–257

Keller KL, Iannone MA (2002) Multiplexed microsphere-based flow cytometric assays. Exp Hematol 30:1227–1237

Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C (2008) Mycotoxin analysis: an update. Food Addit Contam A Chem Anal Control Expo Risk Assess 25:152–163

Lamberti I, Tanzarella C, Solinas I, Padula C, Mosiello L (2009) An antibody-based microarray assay for the simultaneous detection of aflatoxin B1 and fumonisin B1. Mycotoxin Res 25:193–200

Liesener K, Curtui V, Dietrich R, Märtlbauer E, Usleber E (2010) Mycotoxins in horse feed. Mycotoxin Res 26:23–30

Maragos C (2009) Fluorescence polarization immunoassay of mycotoxins: a review. Toxins 1:196–207

Meimaridou A, Haasnoot W, Noteboom L, Mintzas D, Pulkrabova J, Hajslová J, Nielen MWF (2010) Color encoded microbeads-based flow cytometric immunoassay for polycyclic aromatic hydrocarbons in food. Anal Chim Acta 672:9–14

Monbaliu S, Van Poucke C, Detavernier C, Dumoulin F, Van De Velde M, Schoeters E, Van Dyck S, Averkieva O, Van Peteghem C, De Saeger S (2009) Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. J Agric Food Chem 58:66–71

Peters J, Sledz W, Bergervoet J, Wolf J (2007) An enrichment microsphere immunoassay for the detection of Pectobacterium atrosepticum and Dickeya dianthicola in potato tuber extracts. Eur J Plant Pathol 117:97–107

Shim W, Dzantiev B, Eremin S, Chung D (2009) One-step simultaneous immunochromatographic strip test for multianalysis of ochratoxin a and zearalenone. J Microbiol Biotechnol 19:83–92

Vendl O, Crews C, MacDonald S, Krska R, Berthiller F (2010) Occurrence of free and conjugated Fusarium mycotoxins in cereal-based food. Food Addit Contam 27:1148–1152

Zheng M, Richard J, Binder J (2006) A review of rapid methods for the analysis of mycotoxins. Mycopathologia 161:261–273