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Research paper

A flow cytometry based competitive fluorescent microsphere immunoassay (CFIA) system for detecting up to six mycotoxins

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ABSTRACT

Background: Exposure to multiple mycotoxins through the food chain represents a major potential health hazard to both humans and livestock. They can cause a variety of severe acute as well as chronic diseases. Eliminating mycotoxins from various grain crops is a global health priority. According to the Food and Agriculture Organization (FAO), world food production needs to double by 2050. Innovative solutions will be required to sustain toxin free grain supplies worldwide.
Methods: A competitive flow cytometry based multiplexed assay with fluorescent microspheres has been developed. The new multiplexed method can analyze simultaneously any one or all six major mycotoxins. They include: Ochratoxin A (OTA), Aflatoxin B1 (AFB1), Fumonisin B1 (FB1), T-2 toxin (T-2), Deoxynivalenol (DON) and Zearalenone (ZEA), which are all potential human health hazards. The CFIA described here includes a simplified single extraction step for mycotoxins from specimens and a comprehensive post acquisition software module. The new assay system was developed with a FACSArray™ BD Bioanalyzer flow cytometer (BD Biosciences, Belgium).
Results: The CFIA performs favourably when compared to commercial ELISA. Sensitivity range with CFIA increased between 13% and 100% with an average improvement of 50% for the six mycotoxins.
Conclusions: The multiplexed assay presented here has the unique capacity to quantify up to six mycotoxins simultaneously from a single specimen extraction. CFIA's poly-mycotoxin detection sensitivity exceeds standard ELISA. CFIA may be part of a comprehensive assay system that will provide reliable and effective safeguard for agricultural commodities to be free of mycotoxins.

1. Introduction

Global corn production is over 700 million metric tons per year. Half of the world's corn is produced in: US, China, India and EU (USDA, 2010). Harmful mycotoxins are released and produced by various species of fungi that frequently contaminate grain (Table 1). The toxins are low-molecular weight secondary metabolic fungal products. Mycotoxins can cause a variety of acute and chronic diseases. They represent a major potential health hazard to both humans and livestock (Table 1). Pathological effects can range from immediate toxic response, endocrine abnormalities, impaired immunity, and carcinogenic and teratogenic effects (Liu and Wu, 2010; Reddy and Bhoola, 2010). In the agriculture commodities market, the elimination of mycotoxins from the food chain is a prime objective. In the future, flow cytometry based assays such as described below, may have a significant role safeguarding global food supplies. In the past 20 years, to enhance food safety, the European Commission, Food and Drug Administration of the USA (FDA),

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Food and Agricultural Organization (FAO) of the United Nations and other regulatory agencies have set maximum limits (ML's) tolerable for six of the mycotoxins in food and feed. Reference methods have been introduced for the validation of routine methods used for monitoring of these dangerous toxins (Krska et al., 2008). Competitive ELISA with relatively high throughput has proven to be a practical solution for the decentralized laboratory segment of the agricultural industry. Competitive ELISA's for mycotoxins determination have been available since the mid 1990s (Barna-Vetro et al., 1994). For most ELISA mycotoxin kits, a single extraction step is required (Krska and Molinelli, 2007) as it is optimized for the specific mycotoxins being analyzed. The reference method of choice for mycotoxin analysis is high performance liquid chromatography (HPLC) in combination either with mass spectrometry (MS) or with photochemical reaction using UV or fluorescence detection (Krska et al., 2008). After the extraction from a grain specimen, most reference methods require some clean-up step (Krska et al., 2008). The dual-tear combination with ELISA and HPLC at the regional and reference laboratory levels respectively, in the past, had served well the agricultural regulatory community. Recently there have been reports of frequent multiple contaminations with mycotoxins in grain (Krska et al., 2008; Wild and Gong, 2010). Shifting to a poly-mycotoxic environmental reality represents a newly emerging additional health risk to food and feed. Assay systems that can confront poly-mycotoxin contamination are needed. In the early 1990s Luminex Tech. (Austin, Texas, USA) introduced the first commercial multiplexed fluorescent microsphere (MFM) system. The MFM was promoted as an all-purpose immunoassay platform for simultaneous testing a large array of analytes (Vignali, 2000). The Luminex system is built around a compact, robust dual-laser flow cytometer with a 96-well plate processing front end. This multiplexed platform proved to be commercially successful (Elshal and McCoy, 2006). The competitive version of solid phase fluorescent microsphere immunoassay provides a practical multiplexed configuration to address the merging challenge of poly-mycotoxin infection. Fortunately, the competitive immunoassay method is compatible with the available multiplexed instrumentation. There are some other options to consider: Peters and colleagues have reported an indirect inhibition immunoassay with a six-multiplexed configuration utilizing the x-MAP Multi Analyte profiling system available from Luminex (Austin, Texas, USA) (Peters et al., 2010). The inhibition-based assay integrates carboxylated paramagnetic microspheres including a washing step. The same year, Anderson reported (Anderson et al., 2010) the first competitive multiplexed mycotoxin assay with a pair of mycotoxins developed also for the Luminex 100 flow cytometer. The CFIA reported here offers a unique configuration, which takes full advantage of the multiplexed immunoassay strategy (Czeh et al., 2010). This report describes a timely novel implementation of multiplexed flow cytometry. It has the potential to meet the poly-mycotoxin contamination challenge, which poses a potential threat to the global food industry. The new multiplexed assay integrates a single simplified sample extraction step. Also, for CFIA, post-acquisition software was developed. The software module is designed for universal flow cytometry file analysis, to provide rapid results simultaneously that are easy to interpret from one or up to six mycotoxins. The software automatically selects a display window with the appropriate dynamic range for the specific mycotoxins being analyzed.

2. Materials and methods

The CFIA multiplexed technology was developed while acutely aware of the potential compounded matrix effects problem that can manifest when combining multiple immunoassays in a single reaction chamber with simultaneous extraction of multiple mycotoxins from one specimen. For

Table 1
Summary of major fungi species, and corresponding mycotoxins including impact on humans and domestic animals.

Mycotoxins	Fungal species	Deleterious effects
Aflatoxins (B_1,B_2,G_1,G_2 and M_1)	*A. flavus* *A. parasiticus* *A. nomius*	Acut toxicity: LD_{50} values, 1.0–17.9 mg/kg BW (laboratory animals), 0.5 mg/kg BW (ducklings); hepatic lesions; teratogenic. Reduced feed efficiency, immune function and reproductive performance in ruminants. Carcinogenic in humans. Hepatotoxic and carcinogenic
Ochratoxin-A	*P. ochraceus* *A. alliaceus* *P. verrucosum* *P. nordicum*	Teratogenic, carcinogenic, decreased foetal weight, immunosuppressive, strong inhibition of protein synthesis, nephrotoxicity, hepatotoxicity, strong acute toxicity
Fumonisins	*F. moniliforme* *F. proliferatum* *F. subglutinans*	Hepatic lesions in pigs and cattle, Equine leukoencephalomalacia. Porcine pulmonary oedema. Implicated in oesophageal cancer in humans, hepato- and nephrotoxicity
Zearalenone	*F. culmorum* *F. graminearum* *F. sporotrichioides*	Infertility, reduced milk production and hyperoestrogenism in cows.
Deoxynivalenol (Vomitoxin)	*F. culmorum* *F. gmminearum* *F. sporotrichioides* *F. poae* *F. acuminatum*	Feed refusal, decreased weight gain and vomiting, teratogenic
T-2 toxin	*F. sporotrichioides* *F. poae*	Feed refusal, nervous system disturbances, diarrhoea, decreased milk production, acute toxicity, inhibition of protein synthesis, immunosuppressive

Text:
Table 1 identifies the six major fungi species and mycotoxins they produce. Some of the major deleterious health effects for both humans and animals are included (Barna-Vetro, 2002; Varga et al., 1996).

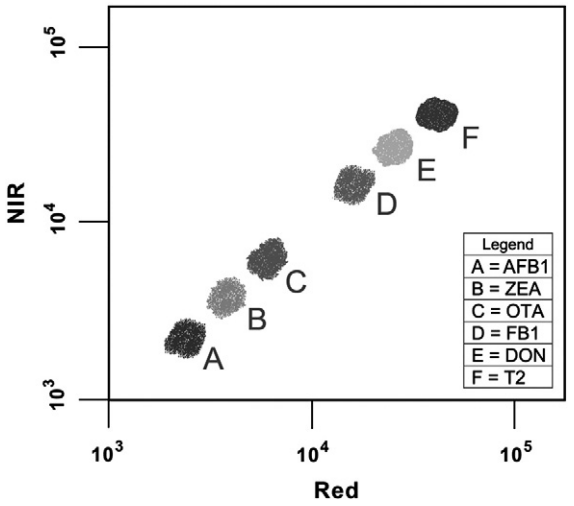

Fig. 1. Two dimensional dot plot display on the FACSArray™ for six mycotoxins. Text: Based on dual fluorescent scatter ploting, the 6 distinct microsphere populations are easily discriminated from each other.

this reason, the method section is divided into six parts. To deal separately with MAb labelling, unified extraction, matrix effect, quality management, and software integration and multiplexed assay management. All mycotoxins were obtained from Sigma Aldrich Ltd. (Budapest, Hungary). They were all dissolved in 84% acetonitrile (ACN). R-Phycoerythrin (R-PE) was obtained from Europa Bioproducts Ltd. (Cambridge, England). 5.1 μm diameter, carboxyl-modified polystyrene particles were obtained from Spherotech, Inc. (Lake Forest, IL, USA). All other chemicals were analytical grade and were supplied by Merck Ltd. (Budapest, Hungary). For standard calibration curves, a six-analyte containing stock solution was freshly prepared by mixing all six-reference materials together. While the eight different concentration levels for the mixtures were in eight separate tubes. MultiScreen HTS-BV 1.2 μm clear non-sterile filter plates, Heidolph Titramax 101 platform shaker and MultiScreen HTS Vacuum Manifold, were from Merck (Merck Ltd., Hungary). Throughout the evaluation a BD FACSArray™ Bioanalyzer with onboard acquisition software (both from BD Biosciences, Belgium) was used. It is a hybrid cytometer developed to analyze both cells and microspheres. The instrument has two lasers onboard (green: 532 nm, and red: 635 nm), detects two scatter signals and four fluorescence signals (yellow, red, far-red, and near-infrared). For the CFIA, the FACSArray™ flow cytometer, a dual red-color classification display was setup to identify 6 different microspheres representing up to 6 different mycotoxin species as distinct clusters in Cartesian space (Fig. 1). The instrument reads the reporter molecule, phycoerythrin (PE) fluorescence (570 nm), with the green laser. For automated sample handling, the FACSArray™ has a built-in 96-well plate loader.

2.1. Monoclonal antibodies, mycotoxin labeling and CFIA labeling with MAb

To improve assay performance, an antibody immune response enhancing step was included. The antigenicity enhancement step took place before immunization of the mice. It is accomplished by conjugating the antigen to a large protein molecule such as bovine serum albumin (Barna-Vetro et al., 2000; Gazzaz et al., 1992; Howanitz, 1988). In any competitive assay, the unlabeled mycotoxins (antigens) from a specimen compete with labeled ones that are included with the kit (Czeh et al., 2010). The labeling step for mycotoxins requires an added linker: either an -oxime or -hemisuccinate. For specific mycotoxin linkages see Table 2. A modified Kellar method was used to bind anti-mycotoxin antibodies (Kellar et al., 2006). They were conjugated to 5.1 μm diameter, carboxyl-modified, class specific/color-coded polystyrene microspheres from Spherotech, Inc. Lake Forest, IL, USA; next Kellar's shaking steps were performed (Kellar et al., 2006). Simultaneously up to six mycotoxins were assayed: Ochratoxin A (OTA), Aflatoxin B1 (AFB1), Fumonisin B1 (FB1), T-2 toxin (T-2), Deoxynivalenol (DON) and Zearalenone (ZEA). The six-plexed assay's performance characteristics were assessed for concentration of mycotoxin target values with six available reference materials (RM). It has been previously reported that small molecular structure based analytes such as ochratoxin A (OTA) can be detected using either displacement and/or competitive immunoassays (Ngundi et al., 2005). For OTA, the assay described here

Table 2
Six major mycotoxins and their corresponding radicals used for CFIA coupling.

Toxin	Converted to	Ref.	Modified
AFB1	AFB1-oxime	Chu et al., 1977	Amino-groups of R-PE react with AFB1-NHS-ester and forms stabile amid bonds between toxin and fluorescent dye.
OTA	OTA-active ester	Mezo et al., 2006	For labeling ochratoxin A (based on idea from Mezo et al., 2006) with R-PE first OTA was activated on his carboxilic group with BOP/HOBt/DIEA in molar ratio 1:1:1:2 in DMF. The active ester was drop by drop mixed with R-PE solution, incubated O/N with continuous stirring and the conjugate obtained was purified by dialysis in 0,1 M PBS.
ZEA	ZEA-oxime	Thouvenot and Morfin, 1983	The amino- groups of R-PE react with ZEA-NHS-ester and formed stabile amid bonds between toxin and fluorescent dye.
T-2	T-2 hemisuccinate	Chu et al., 1979	R-PE reacts with succinic-anhydride. The amino- groups of . R-PE reacts with T-2-NHS-ester and forms stabile amid bonds between toxin and fluorescent dye.
FB1		Pavliakova et al., 1999	R-PE-HS activated with NKS/EDAC and reacts with FBi solution. Reaction mixture was stirred 0/N at 4 °C and purified by dialysis in 0,1 M PBS.
DON	DON-hemisuccinate	Abouzied et al., 1991	The amino-groups of R-PE react with DON-NHS-ester and formed stabile amid bonds between toxin and fluorescent dye.

Text:
This table lists the six mycotoxins and the corresponding radicals used to conjugate them to fluorescent dye complexes. The references for methodology modifications required are also included (Chu et al., 1977, 1979; Mezo et al., 2006; Pavliakova et al., 1999; Thouvenot and Morfin, 1983; Abouzied et al., 1991).

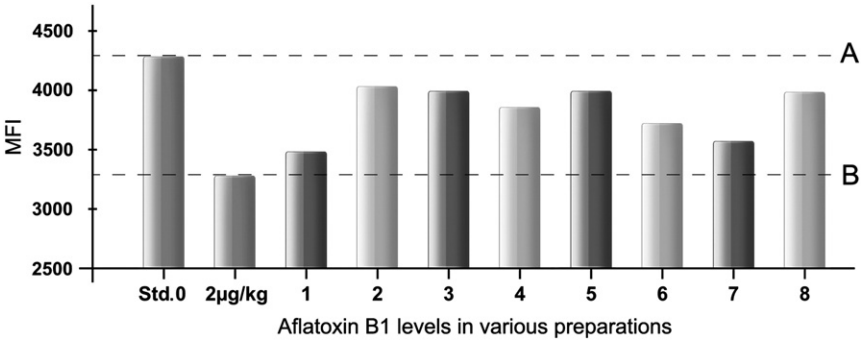

Fig. 2. Histograms for aflatoxin B1 levels with 8 different toxin free grain types. Legends. 1 = sunflower 5 = white lupine. 2 = wheat 6 = spring barley. 3 = pea 7 = chickpea. 4 = mung bean 8 = rye and pea feed mixture. Text: In this histogram the first column illustrates the toxin free standard, next the maximum permitted toxin level for EU, following with 8 varieties of grain illustrating the naturally existing levels that are all well below zero standard.

is similar to previously reported methodology and did not require any sample pretreatment (Ngundi et al., 2005).

2.2. Mycotoxin extraction from grain specimens

As there is considerable chemical diversity among analytes (Krska et al., 2007), both the optimization of the extraction solution and the duration of the extraction are critical steps to assure reproducible poly-mycotoxin detection. It is known that milling fractions with smaller particles release higher levels of *Fusarium* toxins when compared to fractions containing larger particles (European Commission, EC, 2007). In this study grain milling was in compliance with EC Commission Regulation No 1126/2007. Accordingly particles below 500 µm were removed by sieving. A single extraction protocol was utilized by empirically selecting different ratios of distilled water (DW), organic solvents (ACN), methanol (MetOH), and extraction times to determine the best overall condition (not reported here). The final selection for both wheat and maize extraction was 84:16 (v/v) ACN: DW. Samples were stored at 4 °C prior to extraction and protected from light exposure. 15 ml extraction solvent was added to 5 g of ground specimen. 10 min extraction was performed with a platform shaker (Heidolph Titramax 101) at 600 rpm and filtered with a Whatman No. 4 filter. After filtration, 10 µl of extracted specimen was diluted with 240 µl of buffer. Fig. 2 indicates the layout of an experiment where 8 different mycotoxin free grain preparations were assayed for AFB1. The objective was to demonstrate if the matrix effect impact is eliminated for all eight different grain specimens.

2.3. Matrix effect monitoring

In the case of competitive mycotoxin determination, there are four possible types of matrix effects: (1) ELISA related optical density interference (ODI), this matrix effect is unique to ELISA thus it is avoided with CFIA, (2) non-immune specific binding (NIS), (3) grain specific extraction related (GSE) and (4) multiplexed cross interference (MCI). These matrix effects are summarized in Table 3. The most significant step available to minimize matrix effect is the meticulous selection of high affinity antibodies. Once that is achieved, each remaining residual effect needs to be considered during the sequential adding of one mycotoxins at-a-time approach to the multiplexed assay system. Each matrix effect has some deleterious impact on quantitation of mycotoxins. In the case of ELISA, there is an additional source of error unique to the assay; the possibility of natural dyes released from various grains during extraction that may cause interference with optical density (ODI) (Table 3). All possible binding configuration combinations to specific mycotoxins are depicted in Fig. 3. Such matrix effect can occur in most types of solidphase immune assays. When unknown proteins cause none-specific blocking, it is called crossover-linking effect. All non-specific binding conditions may compromise assay results. Table 4 illustrates the protocol used to demonstrate the elimination of most matrix effects. The design included removal of one set of mycotoxin (one of six microsphere sub-classes) at a time from the six-plexed preparation. The first row values illustrate the aggregate matrix effect with all six mycotoxins. As CFIA is a competitive immunoassay, the fluorescent intensity values are at maximum intensity when the specimen's mycotoxin concentration is at zero level. From maximum fluorescence, with corresponding standards concentration increments the fluorescence intensity (MFI) declines.

2.4. Quality management: mycotoxin recovery from spiked specimen with precision and accuracy measurements

Spiking experiments were performed to evaluate assay reproducibility, precision, and accuracy. These experiments were also used to demonstrate that various matrix effects listed in Table 3 are all eliminated. It was critical to demonstrate that CFIA was effective at eliminating false positive results due to matrix effects. Eight different grain preparations were assayed. All of them had mycotoxin values below the acceptable background limit as previously established with the reference method (Fig. 2). An additional experiment was included to illustrate how well the recovery works with both wheat and pea preparations (Fig. 4). For all experiments the mycotoxins spiking preparations were from RM's. To determine the four test limits as suggested by Elshal and McCoy (2006), both spiked and naturally contaminated samples were analyzed. To demonstrate assay performance competence, most quality assurance programs use reference materials (RM). Ideally a national reference laboratory will have certified reference materials (CRM) available to confirm if the in-house method meets required precision and accuracy standards. For a central facility, it is preferable to evaluate assay performance with CRM

Table 3
Classification of matrix effects associated with immunoassays developed to detect mycotoxins.

MATRIX EFFECTS				
Type	ELISA	CFLA	All	All
		Multiplexed	Multiplexed	Immunoassays
Optical densityinterference (ODD	+	NR	NR	NR
None immune specific (NIS)	+	+	+	+
Grain specific interference (GSI)	+	+	+/−	NR
Multiplexed cross interference (MCI)	NR	+	+	NR

NR = not relevant
Text:
This table divides mycotoxin assay related possible matrix effects into four types:

- ELISA specific optical density interference (ODI),
- None immune specific (NIS),
- Grain extraction specific (GES),
- Multiplexed cross interference (MCI).

as part of an external quality assessment scheme. CRM's are prepared by the Institute for Reference Materials and Measurements (IRMM), and by the ex-BCR Bureau Communitarian de Reference, funded by the European Union as a Joint Research Centre located in Geel, Belgium. They are stable, homogeneous products with certified values for each mycotoxin. For the time being only two CRMs available out of the six mycotoxins of interest. It is quite challenging to demonstrate reliability and reproducibility without CRM's for all six mycotoxins. In situation such as described here, spiking and recovery assays are conducted to support method evaluation. The grain is spiked with a known concentration of a specific mycotoxin (Lee et al., 2004). It is recognized that grain spiked prior to milling, an extraction may bind to some of the added known concentration of mycotoxins and may reduce the total recoverable mycotoxin concentration (Barna-Vetro et al., 2000). To avoid this possible complication the unprocessed grain selected for spiking is from a stock that has been previously tested with the reference method (HPLC) to provide evidence of contamination free stock. Extraction was performed the same way as previously described for CFIA and took 100 μl of the diluted solution. Spiking was performed after extraction. The RM's are pure mycotoxin standards usually lyophilized or they are prepared in organic/aqueous format as working solutions ready for preparation of calibration curves. A RM grade mycotoxin level adjusted identical to the maximum concentration found on a standard curve is added to the 25 times diluted grain extraction. Both the spiked and un-spiked extractions are analysed.

2.5. Software integration: poly-mycotoxin software solution

Post-acquisition software was developed for CFIA. It is designed for generic flow cytometry file analysis, to deliver results simultaneously from one or up to six mycotoxins. The software selects a display window with the appropriate dynamic range for the specific mycotoxins being analyzed. The additional onboard software is called FCAP Array v3.0. FCAP Array v3.0 from Soft Flow Hungary Ltd. (Pecs, Hungary).

2.6. Multiplexed assay management

The combination of onboard FCAP Array v3.0 software and the customized post-acquisition software facilitates efficient operation of the multiplexed assay. The instrument operator empowered to a seamless management of the complex standard cure generation and interpretation/evaluation for the entire poly-mycotoxin immunoassay system.

3. Results

3.1. Sensitivity of mycotoxin detection and unified extraction protocol

The sensitivity range for CFIA exceeded ELISA was from 1.3 to 100 % higher (Table 5). This was achieved in part with the selection of improved affinity monoclonal antibodies (Barna-Vetro et al., 1994). Also by selecting the appropriate graphics to display the concentrations curves for each mycotoxin. The wide dynamic concentration range differences among the six mycotoxins demand such action as it is illustrated in Fig. 5. Concentrations can vary from a range of 0.64 to 100 ng/ml for OTA and DON respectively. There is always the possibility of increasing interference with an assay system that includes single extraction step for six mycotoxins. This situation is accentuated when involving specimens in various stages of processing. Fig. 4 illustrates how spike recovery for wheat and pea were contained below the critical maximum acceptable limits “M”. For all mycotoxins the recovery results were excellent. They were from 80 to 110% with target value at 100%. Another series of experiments were designed to provide evidence that small-observed matrix effects do not generate false positive signals. Eight different grains were assayed; all of them retained values below the acceptable background limit (Fig. 2). It is possible to follow how zero concentrations from each standard curve remained relatively constant as one mycotoxin at-a-time was removed from the six-plexed system (Table 4). It is reasonable to make side-by-side comparisons between CFIA and ELISA as a large percentage of in-the-field mycotoxin testing is performed with ELISA. Table 5 includes the results of such comparison. The ISO standard 5725-5:1998 is one of the available guidelines for method validation (Gilbert and Anklam, 2002; ISO, 1998; Sulyok et al., 2007). Besides trapping metabolic byproducts, in some specimens mycotoxins may be masked as glycosides. Such derivatives may escape the extraction step and interfere with the overall assay performance. This masking can manifest as an additional matrix effect

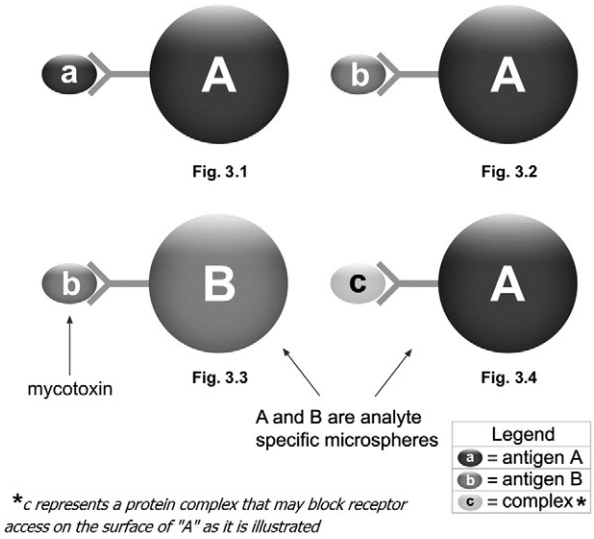

Fig. 3. Multiplexed cross interference (MCI). Text: Ideally, mycotoxin "a" and "b" should bind exclusively to "A" and "B" beads respectively as illustrated in Figs. 3.1 and 3.3. However there are exeptions. For example mycotoxin "a" may react with bead "B" as indicated in Fig. 3.2. This is an example of a matrix effect due to cross reaction between mycotoxin receptors. In Fig. 3.4. there is another type of non specific binding where some immune complex blocks a receptor on "A".

(Sulyok et al., 2006, 2007). However, in the experiments reported here, such masking effect was not observed. "The fitness for purpose of analytical methods" is another available document to guide to optimal performance (Eurachem, 1998). Because of the unavailability of CRM for all mycotoxins, RM's were used for this multiplexed mycotoxins evaluation. Beside RM's, it is possible to use batches of contaminated specimens and have them distributed to all participating laboratories during a proficiency testing period (Krska et al., 2007). In such cases, the survey coordinator will assign target values to the distributed "reference" materials, by taking the aggregate mean values from all participants. In this study spiking experiments were performed with RM's. Recovery results after a series of spiking experiments are included in Fig. 4.

3.2. Software integration: poly-mycotoxin software solution

The customized post acquisition software has the additional capacity to effectively and rapidly manipulate all data for up to six mycotoxins. In addition to assigning appropriate fluorescent log scale ranges for each set of reporter molecules, it is equally effective at identifying the suitable concentration scale for each mycotoxin standard curve (Fig. 5). Concentration scale ranged from 0.6 to 100 ng/ml depending on which mycotoxin is measured. As listed in Table 4, it is possible to follow how zero concentrations from each standard curve remained relatively constant as one mycotoxin at-a-time was removed from the six-plexed assay system. For each of the mycotoxin standard curves the control values were at zero MFI concentration.

3.3. Matrix effect monitoring: reproducibility, precision and accuracy

Globally, a large percentage of mycotoxin testing is performed with ELISA. In Table 5, there are some side-by-side comparisons between CFIA and ELISA. The parallel spiked and naturally contaminated sample analysis as suggested by Elshal and McCoy (2006) illustrated the excellent reproducibility of CFIA. Performance characteristics for assay validation include four parameters; limit of detection, limit of quantification (LOD/LOQ), linearity, and precision including blank controls. The average matrix interference was 452 units MFI. The starting point for the modified extraction protocol used was the Sulyok et al.'s (2006) publication. All the modifications were confirmed step by step with parallel analysis with both IAC and HPLC. All obtained values fall within acceptable error

Table 4
The breakdown of combined matrix effect when all 6-mycotoxins are integrated into a single assay.

	MFI on AFB1 bead	MFI on ZEA bead	MFI on OTA bead	MFI on FB1 bead	MFI on DON bead	MR on T2 bead
All six mycotoxins	5078.47	7003.04	5836.43	3503.16	6223.54	5699.79
Removed AFB1		7059.25	5804.95	3763.63	6194.72	5753.67
Removed ZEA	4760.62		5470.46	3339.62	5839.69	5337.68
Removed OTA	5130.91	7079.98		3745.93	6313.30	5709.02
Removed FB1	5057.76	7006.70	5809.00		6291.47	5718.06
Removed DON	4887.90	6424.77	5613.34	3362.89		5339.67
Removed T2	4958.79	6734.42	5654.72	3536.39	6132.60	

Text:
Matrix interference values are measured and tabled with all six-mycotoxin assays. In each row one of the six mycotoxins is absent as indicated. All values are at zero concentration measured in MFI. All data was collected reading standard curve at zero mycotoxin concentration.

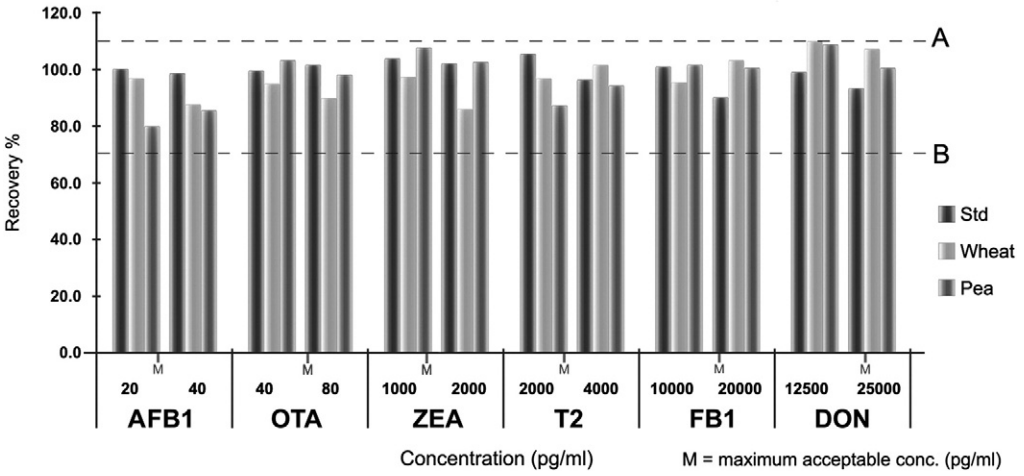

Fig. 4. Wheat and pea spiking recovery for all six mycotoxins. Text: These histograms illustrate the situation with six-plexed mycotoxin determination where two different grains are assayed (wheat and pea). They were spiked and mycotoxin recoveries are determined. For spiking, two concentrations were selected around the critical maximum permitted level of concentrations. The "M" letters designate the maximum tolerated level of mycotoxin contaminations. The numbers left and right of "M" represent the selected concentration ranges for each spiking experiments. The horizontal spaces between broken lines A and B represent the zone of acceptable concentration of mycotoxins according to EU regulations using the most conservative limits.

range when considering the critical cut-off points determined by EU. Currently acceptable levels of mycotoxin contaminations are illustrated in Fig. 2. The vertical space between broken lines A and B represent acceptable levels of matrix effect. All eight combination of grain batches tested yielded matrix effects below the fluorescent signal generated by zero concentrations (Fig. 2). In a poly-mycotoxin assay a variety of extraction methods would be impractical and economically counterproductive. The use of spiked grain provides additional benefits to assure precision. With freshly spiked grain previously free of toxins complications such as the effect of poly-mycotoxin contaminated stock are eliminated.

3.4. Post-acquisition software and multiplexed assay management

To effectively manage significant dynamic mycotoxin concentration differences, flexible, customized software is preferable when maximum acceptable concentration limits exceeds a span of 100 ng units. Specifically designed software provides automatic adjustments to graphical representation. With such dedicated software it is possible to auto-locate characteristic dynamic ranges for each of the six mycotoxin standard curves. A sigmoidal curve with an initial flat line is characteristic for a competitive immunoassay, as illustrated with 8 concentration points in Fig. 6 for OTA. The new software generates a seven point standard curve to read the entire unknown specimen concentration range. Such representative standard curves for all six mycotoxins are displayed in Fig. 7. In each case, the software automatically selected the best fitting curve for a given mycotoxin assay. The equations are selected to generate the most accurate and simple to interpret graphical representation for all standard curves. From the results depicted in Fig. 2, it is clear that the MFI values remain between limits A and B for all six mycotoxins. A and B in Fig 2 represent zero standard and maximum permitted AFB1 concentrations respectively. All values obtained fell between the limits and none of the MFI values were above the zero standard.

Table 5
Limit of sensitivity comparsion for six mycotoxins using CFIA and ELISA.

Mycotoxin	Median fluorescence	CV%	Maximum limits* (μg/kg)	Sensitivity (μg/kg)		Δ%
				Fungi-plex	Typical ELISA**	
Aflatoxin B1	4177	1.7	2	0.01	1.00	100
Ochratoxin A	3866	1.6	5	1.50	5.00	3.3
Fumonisin B1	2278	2.0	2000	35.93	222.00	6.2
T-2toxin	4511	2.4	300	27.27	35.00	1.3
Zearalenone	5113	2.3	100	1.55	10.00	6.5
DON	4598	2.2	1750	75.73	200.00	2.6

Text:
The smallest increment of improvement was 13%. This was achieved with T-2. The best performance improvement was 100% with Aflatoxin B1. For all assays performed the CV%'s remained under 2.5.

* The limits are the maximum permitted by the Council Regulation (EEC) No 315/93 and Commission Regulation (EC) No 1881/2006 in Europe.

** While all ELISA results surpassed minimum sensitivity requirements, they were inferior when compared with CFIA. When assay results were averaged by method, sensitivity of the CFIA exceeded ELISA performance by 50% combined for all six mycotoxins.

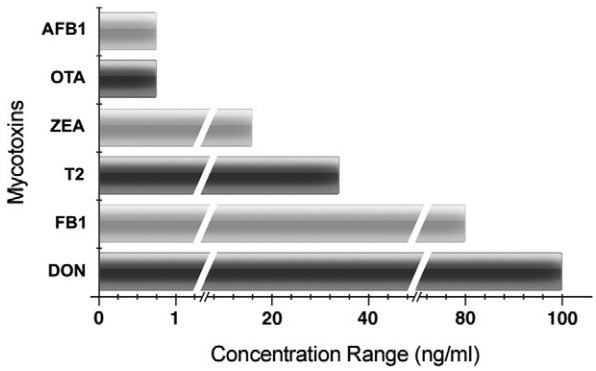

Fig. 5. Significant variation of dynamic range concentration for the six mycotoxins. Text: Mycotoxin co-infections can occur in grain. Because there are significant concentration range variations between the 6 toxins, often such multiple infections are difficult to detect. For example, the toxin levels can be below 0.6 ng/ml or above 100 ng/ml for OTA and DON respectively.

4. Conclusion and discussion

A conventional competitive immunoassay that was developed to work for two mycotoxins (Anderson et al., 2010) was modified and improved. MAb affinity (Barna-Vetro et al., 1994) to recognize poly-mycotoxins was enhanced and adjusted for the FACSArray™ BD Bioanalyzer, a hybrid flow cytometry platform. The multiplexed assay capacity was expanded to include all six critical mycotoxins. The newly developed CFIA system incorporates three innovative features: (1) a consolidated single extraction method, (2) a competitive immune assays series which are specifically tuned to be compatible and synchronous with the multiplexed assay format, and (3) a dedicated post-acquisition software with auto-adjust capacity to match six diverse dynamic concentration scales. CFIA's increased sensitivity is an obvious advantage when compared to ELISA (Table 5). The immune response enhancing step improved the performance of MAbs coupling to mycotoxins (Table 5). A series of experiments were performed to provide proof that small matrix effects observed do not generate false positive signals. This is a critical advantage for a multiplexed system that is dealing with up to six mycotoxins simultaneously. The enhanced sensitivity of CFIA extends system flexibility to integrate a dilution options should it be required in some special situations to resolve excessive matrix interference. The elimination of masking effect was further supported by alternative examinations with IAC and HPLC. All recovery results were within the limits set by the EU authority (European Commission, EC, 2006). The results illustrate that the assay system is robust; it is able to recover mycotoxins from spiked specimen at critical concentrations with precision that exceeds ELISA performance criteria (Table 5). It is important to note that a large percentage of in-the-field mycotoxin testing is performed with ELISA and the side-by-side comparison demonstrated that CFIA is a superior assay system (Table 5). The protocols with spiked grain illustrated some unique benefits. With freshly spiked grain previously free of toxins, some subsequent complications are avoided such as the possible

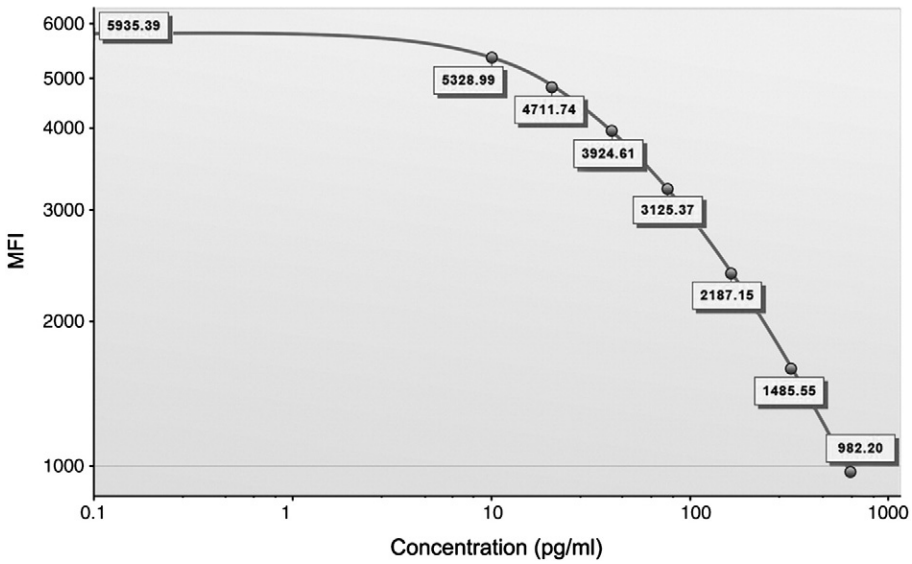

Fig. 6. A standard curve display for mycotoxin OTA. Text: This graph illustrates the typical sigmoidal standard curve generated by a competitive immunoassay with eight concentrations.

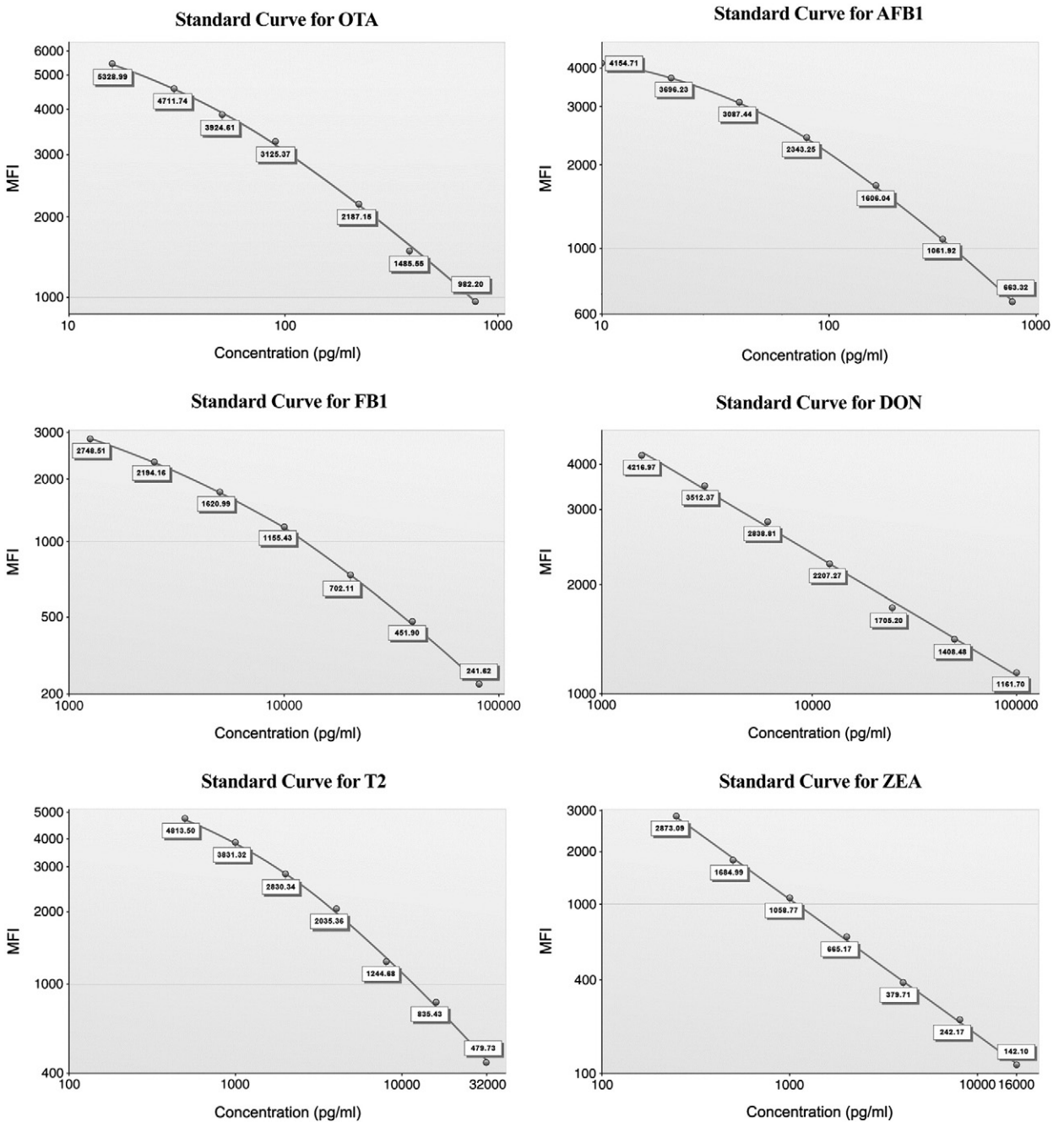

Fig. 7. Standard curve dispaly for all six mycotoxins with zero concentration adjustments. This graph illustrates the standard curves for all six miycotoxins as they are generated by the software. Only seven concentration points are used. By eliminating zero concentration, the flat line segment of the curve is eliminated.

effect of poly-mycotoxin contaminated stock. It is conceivable that naturally contaminated stock may contain metabolic derivatives including toxin products that would be unexpectedly recovered with the extraction process (Sulyok et al., 2006). With the new reality of frequent occurrences of poly-mycotoxin contamination, ELISA becomes an inappropriate choice in terms of cost, time investment and the potential risk of reporting false negative results (Krska et al., 2007; Sulyok et al., 2007). In some regions 75% of the infected feed specimens were contaminated with more than one mycotoxin (Krska et al., 2007; Sulyok et al., 2007). There is further evidence that some specific fungus can release different mycotoxins at different point in time during shipping and/or storage (Speijers and Speijers, 2004). Multiplexed flow cytometry may meet the recently identified additional challenges faced by the global food industry. In the future, to further advance effective food safety enforcement, there is a need for affordable certified reference materials (CRM) to include all six mycotoxins. Access to CRM's will play a significant role improving evidence-based quality testing worldwide. New affordable assays must exceed all current practices in

terms of reliability, comparability and traceability to secure the quality of global food market (Czeh et al., 2011). This study included only a modest panel out of food commodities available that are subject to mycotoxin infections in Europe. It did not include grains indigenous to Africa, Asia and the rest of the world. Extended evaluations are necessary for a comprehensive global multiplexed kit validation. It is likely that by the end of the second decade in the 21st century, flow cytometry will have a significant impact on food safety to protect public health on a global scale. Traditionally validation methods for mycotoxins are based on the principles of thin-layer chromatography (TLC), additional methods were developed and validated with HPLC and gas chromatography (GC). In the future, with flow cytometers getting considerably more compact, robust and cost effective, they may serve as provisional reference method candidates. The integrated multiplexed platform represented by CFIA will help to avoid false negative results that are virtually undetectable by current solo ELISA assays.

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