**Ph.D. THESIS**

# Multiplexed Solid Phase Immunoassay Platform for Poly-Mycotoxins: Beyond 20th Century Applications


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# 1. INTRODUCTION

Toxigenic fungi are constantly present and they can contaminate a wide range of agricultural products both before and after harvest. In 1960 the discovery of aflatoxins in turkey feed in the UK precipitated a rigorous study of mycotoxins and initiated global awareness of the magnitude of potential problems associated with mycotoxins. Various species of fungi that frequently contaminate grain can release significant quantity of harmful mycotoxins. The toxins are low-molecular weight secondary metabolic fungal products. Thus far over 300 mycotoxins have been identified, of which, a dozen groups receive international attention as potentially threat to human and animal health. Possible exposure to mycotoxins through the food chain represents a major 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 of the United Nations (FAO), world food production needs to double by 2050. With such dramatic increase in food production, in the next 35 years, significant innovative solutions are required to sustain globally toxin free grains.

Wide spread mycotoxin manifestation can occur whenever humidity and temperature are optimal for fungal growth (Rahmani et al., 2009). The six major mycotoxins of worldwide concern are: aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, and T-2. Pathological effects can range from immediate digestive toxic response, endocrine abnormalities, impaired immunity, and carcinogenic/teratogenic effects (Liu and Wu, 2010; Reddy and Bhoola, 2010). Factors, related to both health hazards risks and socio-economic nature, influence the establishment of regulations for mycotoxin limits in food and feed. By the end of 2003, approximately 100 countries had in place technologies to monitor maximum

acceptable limits for mycotoxins in food and feedstuffs (FAO, 2004). During late 20$^{th}$ century, in concert with the European Community and the Food and Drug Administration (FDA), FAO has introduced regulations to set maximum permissible concentration limits for six mycotoxins in food and feed. Risk assessment and monitoring of mycotoxins in feed and food products require appropriate analytical methods. Immunoassays were lunched in the mid 1950's and moved onto wide spread use by the 1980's. Since the mid 1990's, most industrialized counties have selected the competitive ELISA's for mycotoxins detection for regional labs (Barna-Vetro et al., 1994). The Association of Official Analytical Chemists International (AOAC) and the European Standardization Committee (CEN), the European equivalent of ISO, have a number of standardized methods of analysis for mycotoxins that have been validated with inter-laboratory studies, and the validation study numbers are growing. The latest edition of Official Methods of Analysis of AOAC contains approximately 40 validated methods for mycotoxin determination (Horwitz, 2000). Reviews have also been published about validation methods for mycotoxins (Gilbert and Anklam, 2002; FAO, 2004). Sensitive and accurate analytical methods based on chromatographic or immunochemical techniques are available for all major mycotoxins. Several other techniques have been proposed: including fluorescence polarization immunoassays, use of molecular imprinted polymers, infrared spectroscopy, capillary electrophoresis, surface plasmon resonance biosensors, thin layer chromatography (TLC), mouse bioassay, HPLC, gas chromatography (GC), and protein phosphatase inhibition. Most available ELISA assays in the field for mycotoxin detection are targeting contamination with a single mycotoxin (Magan and Olsen, 2004). Based on increasing number of reports about spontaneous co-infections, the need for multiplexed assay capacity is evident (Sulyok et al., 2007). The evidence of the poly-mycotoxic reality represents a more complex health risk challenge. More extensive surveillance of poly-mycotoxin contamination is needed. To address the emerging phenomenon of wide poly-mycotoxin infestations, competitive version of solid/liquid phase multiplexed microfluorosphere immunoassay was considered. In 2008, the first poly-mycotoxin assay with five-plexed technology using FC was introduced (ISAC; Czeh et al.; 2008). In 2010, Anderson also reported (Anderson et al., 2010) a competitive multiplexed mycotoxin assay with two-mycotoxins. In 2011, Peters and his colleagues reported an indirect inhibition immunoassay with a six-plexed configuration utilizing the x-MAP MultiAnalyte profiling system compatible with the Luminex 100 instrument (Austin, TX, USA) (Peters et al., 2011). The inhibition-based assay included carboxylated paramagnetic microspheres with a washing step. In 2011, as a direct response to the existence of multi-mycotoxins as a global

health challenge, the first commercial poly-mycotoxin multiplexed assay platform capable of detecting simultaneously six mycotoxins was introduced (Czeh et al., 2012). Mycotoxicology is a branch of biology that is committed to a global effort to eliminate significant danger posed by mycotoxins to human health. The demand for the multi-disciplinary approach is also supported by increasing number of observation of wide spread contamination of grain followed by bio-transfer through the food chain to humans (Fink-Gremmels, 1999; Peraica et al., 1999; Plestina et al., 1990; Wild and Gong, 2010). Bio-transfer to humans can be direct from contaminated grain product or indirect by consuming food derived from animal sources already tainted with mycotoxins. Most available ELISA assays in the field for mycotoxin detection from mammals, including humans, are targeting detection of contamination with a single mycotoxin (Magan and Olsen, 2004). Currently, rapid detection of exposure to poly-mycotoxins in the field is available only for plant products.

There are four methods available to mitigate mycotoxins production: (1) destruction of mycotoxins with environmentally friendly bacterial enzymes, (2) irreversible blocking of functionally toxic terminals by developing bio-degradable masking compounds or by blocking in vivo absorption of toxins, (3) cultivate species of grains that are naturally resistant to fungi, and (4) improve harvesting/shipping/storage technology for grain. In the past decade, due to rapid development of LC-MS/MS, frequent presence of poly-mycotoxins has been well documented. Better sample preparation protocols have also been developed (Berthiller et al., 2014). To describe the new merging sample preparation technology that is LC-MS/MS based, a new acronym has appeared; it is called QuEChERS, which is the short form for quick, easy, cheap, effective, rugged and safe sample preparation (Berthiller et al., 2014). CFIA as a multiplexed platform, which is well suited to address both newly established challenges: (1) the frequent presence of poly-mycotoxins and (2) the possibility of numerous masked versions (conjugates) produced by the same fungus. The ultimate objective is to develop pre-emptive strategies, by introducing mycotoxin-focused epidemiology called myco-epidemiology (MycoEpi). In the future, MycoEpi will be able to predict global zones at risk, discover and destroy mycotoxin-contaminated grain before it can enter the food chain. It will also, identify regions where mycotoxin bio-transfer most likely will or has already occurred.

## 2. MAIN OBJECTIVES – HYPOTHESIS DRIVEN EXPERIMENTS

Population growth requires increased production and distribution of food free of poly-mycotoxin. In 2012, world population was in excess of 7 billion (Worldometers, 2012). Currently, worldwide large-scale grain production is centralized in less than 10 regions

requiring massive international shipping and distribution networks. According to a 2009 report by FAO, humanity will need to produce 70% more food by 2050 to feed a projected 9.3 billion people (FAO - Economic and Social Development Department., 2008). Therefore, the aim of this thesis is to contribute to the elimination of large-scale catastrophes related to contaminated alimentation. Hazard reduction includes avoiding destruction of large existing food supplies in order to eliminate lethal consequences of delivering contaminated food. Distribution challenges are further aggravated with increasing manifestation of poly-mycotoxins in stored grains and other related food and feed by-products. This thesis is structured around two hypothesis driven scientific challenges. Consolidated effective solutions will generate synergy to strengthen global food supply management. The combined elements will help to identify health threats in advance and will help to eliminate tainted grain supplies from entering the global food chain. The overall focus is to design and test a robust immunoassay system to meet the emerging 21$^{st}$ century challenge represented by multiple variations of conjugated and native forms of poly-mycotoxins.

### 2.1. Hypothesis driven contributions are:

(a) Exploitation of superior binding kinetics potential of microfluorospheres to develop rapid and cost effective multiplexed solid microfluorospheres suspended in liquid phase environment as part of an analytical platform.

(b) Develop standardized competitive quantitative poly-mycotoxins immunoassay protocols with instrument independent cross calibration assuring compatibility of assay system with universal flow cytometry platform.

In the future, with the two above-mentioned elements forging a robust platform supports the introduction of the field of myco-epidemiology (MycoEpi). By embracing a new integrated monitoring, and detecting paradigm, MycoEpi will contribute to mycotoxin outbreak prevention. It will be a comprehensive risk management approach for global food supply protection. MycoEpi will apply traditional empirical principles of epidemiology integrated with 21st century biotechnology and telemetry. It will enhance the efficiency and the general strategy towards public health decision-making. MycoEpi based on multi-disciplinary evidence acquisition strategy. There is increasing evidence of co-occurrence of conjugated mycotoxins or secondary metabolites (Streit et al., 2013). The proposed strategy offers complex food safety risk analysis, i.e. quantified risk probability assessment pertaining to pockets of individuals or populations from the additive effects of the poly-mycotoxin contamination (European Commission, 2002; Merrill, 1997).

The first step is to improve robust analytical capacities and provide affordable global access to poly-mycotoxin detection. The second step is to provide practical and universal protocol and platform to cross calibrate various bench-top flow cytometers (b-FC's). This is to assure universal capacity to report quality mycotoxin concentrations. The current use of ELISA kits is already counter-productive. Therefore it is critical to approach the challenge with the best available analytical strategies to support the detection of poly-mycotoxins in grain. In the future, it will be possible to identify poly-mycotoxins or to detect multiple masked mycotoxin cluster contamination with integrated risk assessment/reduction strategy (IRAS).

### 2.2. Strategies

To accomplish stated the following strategy was developed.

1. With high affinity monoclonal antibodies had to be developed a competitive fluorescent microsphere immunoassay (CFIA) technology. It is based on solid phase surface binding in a liquid suspension environment to exceed ELISA's analytical limits.
2. The CFIA technology required a complementary assay; a unified single-step extraction protocol for specimens in order to handle up to six mycotoxins simultaneously.
3. Established a comprehensive validation protocol for the analytical platform with spiking experiments including parallel strength of agreement statistics (BA). The protocol needed to demonstrate effective elimination of matrix effects related to both multiplexed immunoassays and specimen preparation related interference of toxin release without the benefit of certified reference materials (CRM).
4. To assure that CFIA analytical platforms compatibility with a variety of b-FC's, instrument independent cross calibration (IICC) protocol was required. A quality control protocol system was developed to integrate the post-acquisition software with IICC.
5. The FCAP Array software required customization for the poly-mycotoxin analytical platform. The improved software provides a user friendly environment and will be a critical module, which will support in the future complex data management for global MycoEpi including bio-transfer diagnostics.

## 3. MATERIALS AND METHODS

The development of the poly-mycotoxin detection platform, required: various cereal grains, processed food; human and animal body fluids, and some related organ tissues. The assay platform was designed to ultimately address mycotoxin source diversity including: (1) grain,

(2) primary bio-transfer in biological fluids such as blood, urine and saliva, (3) body fluids and organs tissues including primary or secondary bio-transfer sources.

**Poly-mycotoxins detection with CFIA**

Data acquisition was performed with each b-FC's own software. Multiplexed CFIA protocol was previously published (Czeh et al., 2012). Post-acquisition software was customized as previously described to permit the operator to manage the assay with considerable transparency and ease. With CFIA platform and integrated software, results were generated in a consistent and uniform format regardless of the make of instrument used (Czeh et al., 2012).

**Extraction protocols from plant and animal species**

The efficacy of a robust one-step extraction was critical to the successful implementation of a multi-instrument poly-mycotoxin assay platform. The protocol for sample preparation and details of unified mycotoxin extraction for grains have been previously reported (Czeh et al., 2012). The unified extraction protocol had to be sensitive enough to detect up to six mycotoxins accurately and reproducibly around critical acceptable cut-off concentrations. A single step extraction protocol was developed for the side-by-side evaluation of seven instruments. Passing mycotoxins from grain to animals or humans through the food-chain can led to bio-transferred mycotoxicosis. All procedures described in this study for mycotoxin recovery from animals were conducted in accordance with the guidelines of the European Communities Council Directive (86/609 EEC) (European Commission, 1986) and approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine.

**Assay validation protocol with spiking experiments**

For validation of the poly-mycotoxin assay, all procedures were carried out at several concentrations in triplicates. Experiments included spiking of blank extracts at one concentration level after single step extraction. The objective was to differentiate between extraction efficiency and matrix effects induced signal suppression/enhancement (Sulyok et al., 2006). ISO standard 5725-5:1998 is one of the other recommended guidelines for method validation (Gilbert and Anklam, 2002; ISO, 1998; Sulyok et al., 2007).

**Instrument Independent Cross Calibration: evaluation of different b-FC's through synchronization of signal processing**

The objective was to determine whether there was sufficient compatibility across instruments to measure poly-mycotoxins. To address the wide functional diversity posed by seven b-FC's, a comprehensive multiplexed analytical platform protocol was required (Czeh et al., 2013).

**Post-acquisition software**

The post-acquisition software assured that poly-mycotoxins were evaluated with most available b-FC's. The modified software was designed to work with up to six mycotoxins. All features and options integrated into the post-acquisition software are described in some details in the results section.

## 4. RESULTS AND DISCUSSION

CFIA began as a multiplexed immunoassay to replace available ELISA. With a step-by-step strategy, an analytical platform was developed that includes 21st century biotechnology to combat poly-mycotoxins.

### 4.1. Enhanced multiplexed immunoassay

In the early 2000's, monoclonal antibodies available for ELISA were unsuitable for multiplexed analytical mycotoxin detection. Standard curves for competitive immunoassays follow a sigmoid shape (see Figure 1).

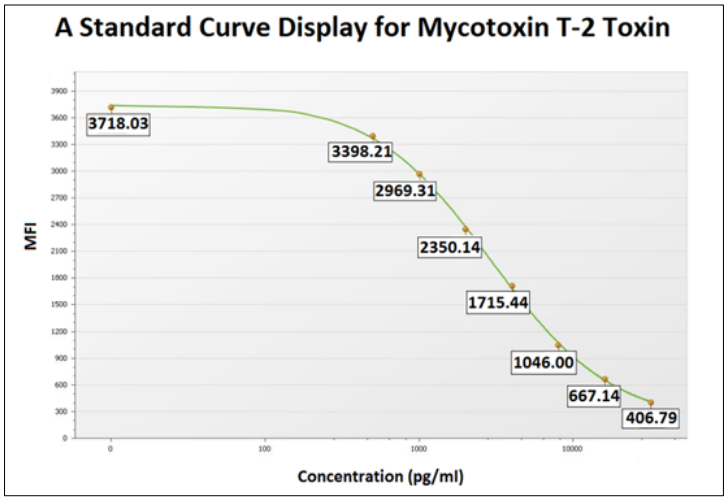


**Figure 1:** *Standard curve display for mycotoxin T-2 toxin. It illustrates the sigmoidal curve generated typically by a competitive immunoassay.*

With higher affinity reagents it is possible to stretch the linear segment of the curve (Barna-Vetro et al., 1994). The enhanced assay has two advantages: (1) often resolves problems

associated with the multiplexed matrix environment see Figure 2**,** and (2) makes it possible to adjust each standard curve to match EU cut-off points to be in the middle of the linear concentration range; see Figure 2.

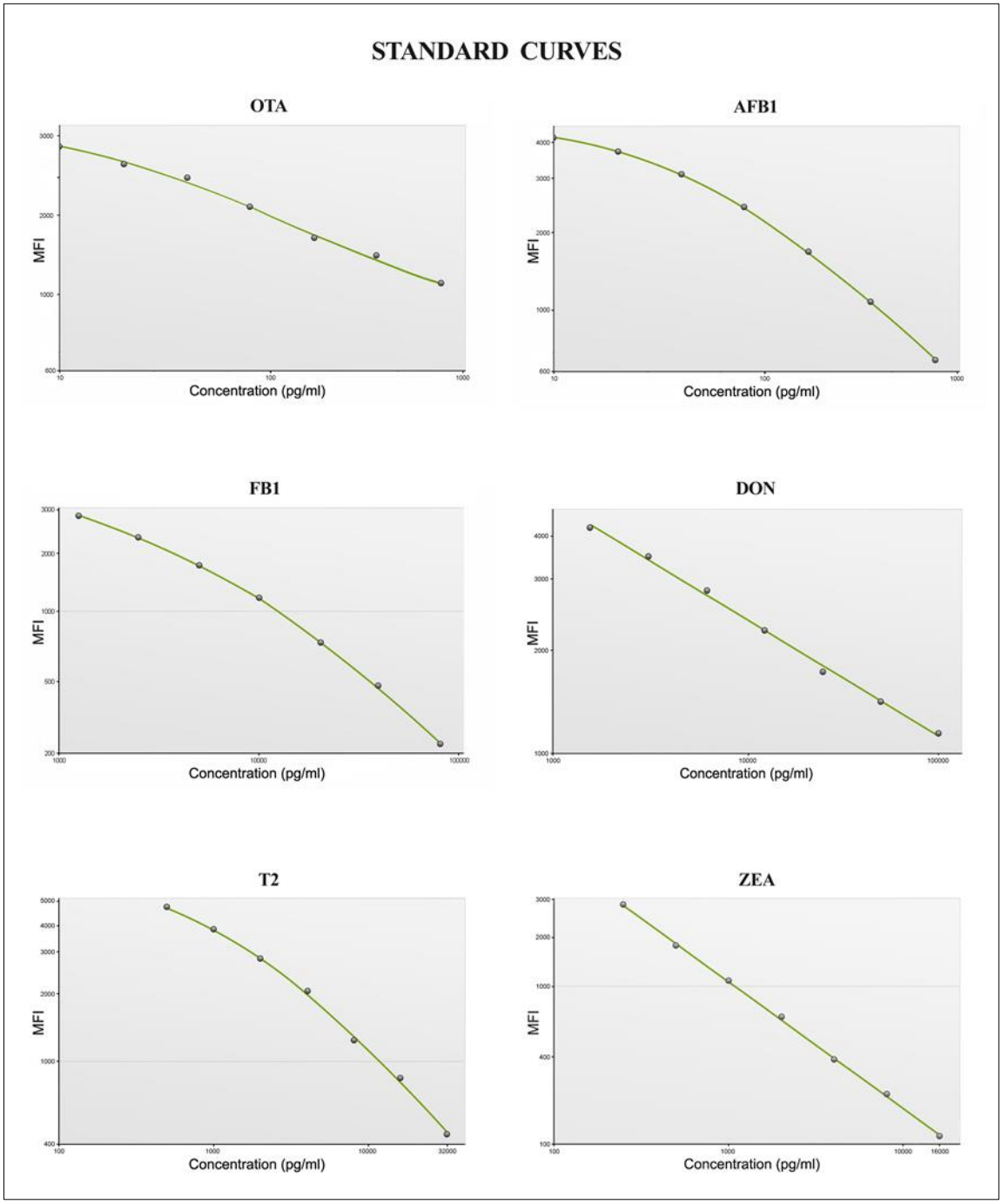


**Figure 2:** *Using high affinity MAbs multiplexed standard curves were generated using seven concentration points with Luminex100 b-FC. Please note that mid rage concentration are approximately where EU cut-off limits are.*

### 4.2. Unified poly-mycotoxin extraction

The development of the single-step extraction protocol required significant know-how experience to assure robust compatibility with the multiplexed assay conditions. The assay had to be robust regardless of single or up to six-plexed configuration. Figure 3. illustrates that the unified single-step extraction protocol was not introducing any poly-mycotoxin matrix effect.

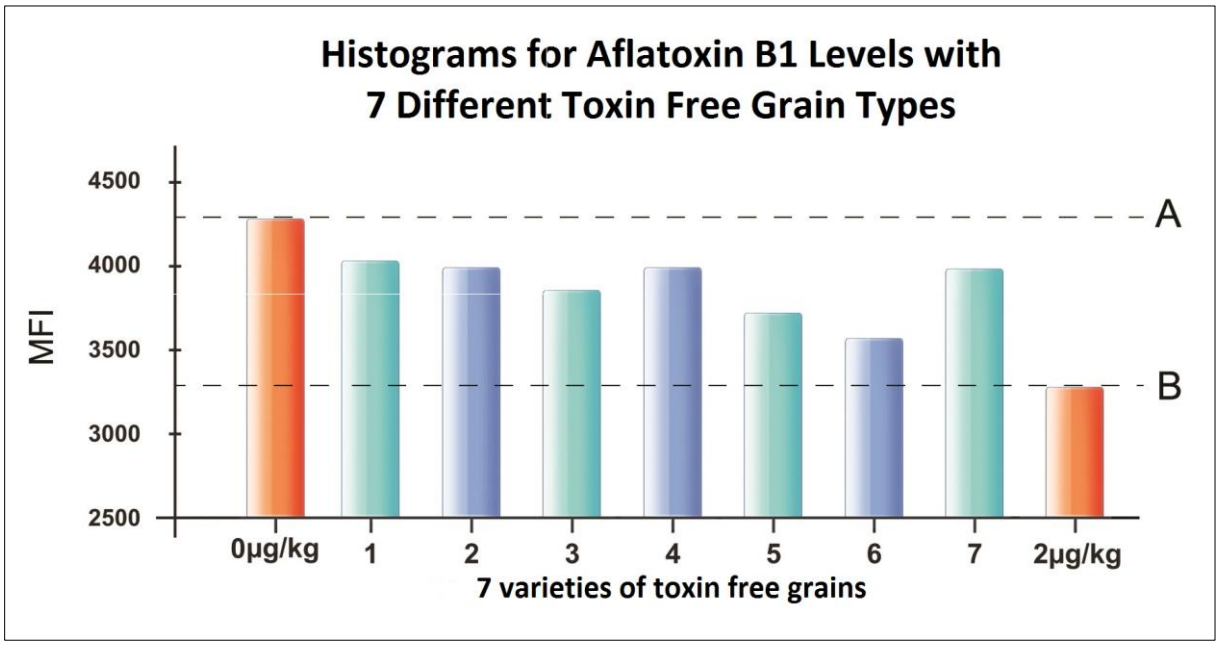


**Figure 3:** *Histograms for Aflatoxin B1 Levels with seven Different Toxin Free Grain Types*

*1 = wheat; 2 = pea; 3 = mung bean; 4 = white lupine; 5 = spring barley; 6 = chickpea; 7 = rye & pea feed mixture. In this histogram the first column (0µg/ml) illustrates the toxin free standard, the last one (2µg/kg) represents the maximum permitted toxin level for EU. The mean fluorescence intensity (MFI) readings of all 7 varieties of toxin free grains are all between A and B representing from zero standard and the MFI of the maximum permitted AFB1 concentrations.*

For illustration purposes, and to keep the presentation short, Aflatoxin B1 was selected from the six mycotoxins in Figure 3. Aflatoxin B1 represents the worst-case scenario. It has the overall lowest mycotoxin concentration among the six, thus requiring the highest analytical sensitivity for robust detection. It was critical to demonstrate that the single-step extraction was without any detrimental effect. Seven different grain specimens were prepared and assayed. As previously established with the reference method, all seven specimens had mycotoxin values bellow acceptable background limit (see Figure 3). When considering the critical cut-off points determined by EU, all results fall within acceptable error range. Acceptable levels of mycotoxin contaminations are illustrated in Figure 3. The objective was to demonstrate that the matrix effect does not have impact on results obtained with the seven

different specimens. Figure 3 indicates the outcome of an experiment where 7 different mycotoxin free grain preparations were assayed for AFB1. The MFI range between broken lines A and B represents acceptable levels of matrix effect. All seven combination of grain batches tested yielded matrix effects below the fluorescent signal generated by zero concentrations (Figure 3). To assure assay precision, spiked grain can provide additional benefits. From results of similar experiments depicted in Figure 3. It is clear that the MFI values of toxin-free samples remain between limits A and B for all six mycotoxins. A and B in Figure 3 represent zero standard and maximum permitted AFB1 concentrations respectively.

### 4.3. Validation protocols with spiking experiments and parallel statistical assessments to demonstrate elimination of multiplexed assay related matrix effects

As certified reference materials (CRM) were unavailable for all six mycotoxins, alternative methods were required to demonstrate assay sensitivity. One objective was to demonstrate that the sensitivity range of CFIA exceeds ELISA's performance for all six mycotoxins. It included systematic dissection of all non-specific binding related matrix effects, which is inherent to all multiplexed immunoassays. The superior performance of CFIA compared to ELISA is illustrated in Table 1. It lists the limit of detection for all six mycotoxins. CFIA poly-mycotoxin assays sensitivity was 1.1 to 8.3 times higher compared to ELISA; see Table 1.

| ***Mycotoxin*** | ***Median Fluorescence*** | ***CV %*** | ***LOD (µg/kg)*** | | **Δ (x)** | **Limits (*µg/kg*)** |
|---|---|---|---|---|---|---|
| | | | ***CFIA*** | ***ELISA*** | | |
| ***Aflatoxin B1*** | 4177 | 1.7 | 0.12 | 1.00 | 8.3 | 2 |
| ***Ochratoxin A*** | 3866 | 1.6 | 1.63 | 5.00 | 3.1 | 5 |
| ***Fumonisin B1*** | 2278 | 2.0 | 45.85 | 222.00 | 4.8 | 2000 |
| ***T-2 toxin*** | 4511 | 2.4 | 32.50 | 35.00 | 1.1 | 300 |
| ***Zearalenone*** | 5113 | 2.3 | 1.89 | 10.00 | 5.3 | 100 |
| ***DON*** | 4598 | 2.2 | 87.80 | 200.00 | 2.3 | 1750 |

**Table 1:** *Comparison of limit of detection (LOD) for CFIA and ELISA for all 6 mycotoxins.*

### 5.4 Findings of analytical fluorescence detection protocol across seven b-FC's

In order to achieve instrument independent PE fluorescence intensity standardization, the use of a single calibration microfluorosphere system was essential. The instrument independent cross calibration (IICC) assured that all seven instruments were synchronously calibrated for analytical fluorescence emission detection. The custom developed post-acquisition software

system provided uniform quality management for quantitative fluorescence detection regardless of instrument variation. The cross-calibration for all seven instruments is illustrated on Figure 4. With IICC, using QuantiBRITE fluorescent intensity, the equations were not synchronous for Luminex100 and Accuri b-FC's. Such anomaly was observed on two instruments because manual adjustment for PMT gain for PE fluorescence was unavailable for Accuri and Luminex100.

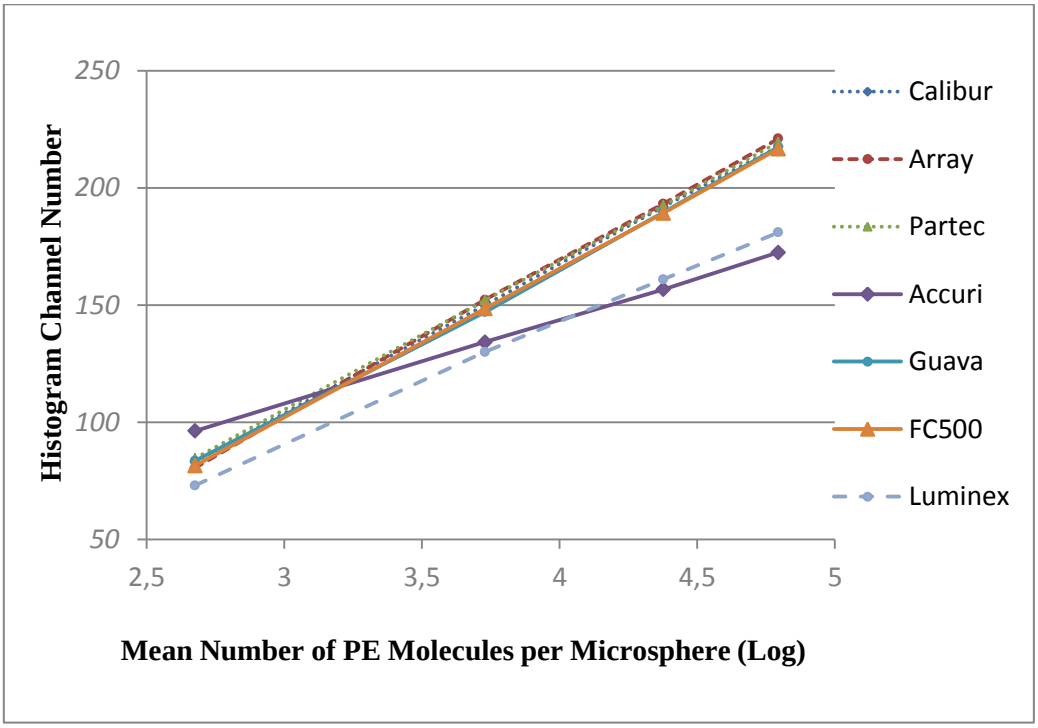


**Figure 4:** *Cross-calibration curves obtained with the IICC protocol using QuantiBRITE. Fluorescent intensity versus the PE molecules per QuantiBRITE PE microfluorospheres are shown for 7 different instuments.*

### 4.5. Quantification of solid phase target's dynamics in three-dimensional fluid space.

The results presented here support the freedom the CFIA's binding kinetics conditions render. There is significant difference between ELISA and CFIA antibody/antigen binding capacity is the significant restriction when solid phase binding is limited exclusively to the microtiter plate surface. To demonstrate the liberating impact of CFIA's free fluid space condition, binding kinetics measurements were made of the mycotoxin PE conjugates on microfluorosphere surface. This was accomplished by recording sequentially the deminishing reporter molecule related fluorescence intensity of mirofluorospheres over time. The initial study focused on the binding kinetics performance of CFIA and ELISA side-by-side. To permit comparison between optical density (OD) units of ELISA and mean fluorescence intensity (MFI) for CFIA, a unified optical scale was devised see Figure 5.

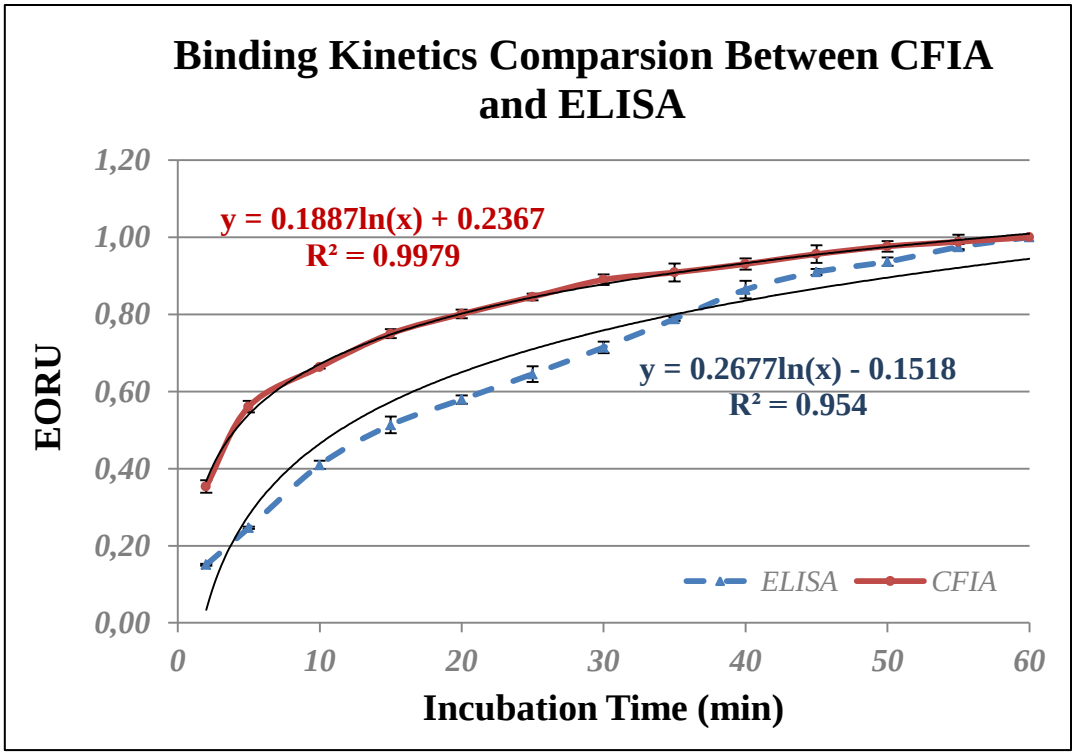


**Figure 5:** *Binding kinetics comparison between CFIA and ELISA. On the y-axis EORU's represent MFI and OD values unified by taking the maximum 60-minute values as unit 1. All other time points were adjusted accordingly as percentages of the unit.*

The two curves illustrate the difference in binding kinetics between the two types of immunoassays. The reaction rates are different until the 45 minutes mark. There is a significant acceleration of reaction rate observed with the CFIA assay; i.e. shorter incubation times. In Figure 5, the y-axis reads equivalent optical reading units (EORU) for both assays. The EORU's represented on the y- axis are obtained by taking the 60 minute reading of MFI and OD values as maximum or unit 1. The assumption was, that if antibody/antigen biding opportunities were more frequent for CFIA, the incubation times would be shorter. Binding kinetic equations were established for the two types of immunoassays. They are $y = 0.1887\ln(x) + 0.2367$ and $y = 0.2677\ln(x) - 0.1518$ with $R^2$ of 0.998 and 0.954 respectively for CFIA and ELISA. The equations were based on fitting triplicate measurements over sixty minutes using the aggregate mean for each time point; see Figure 5.

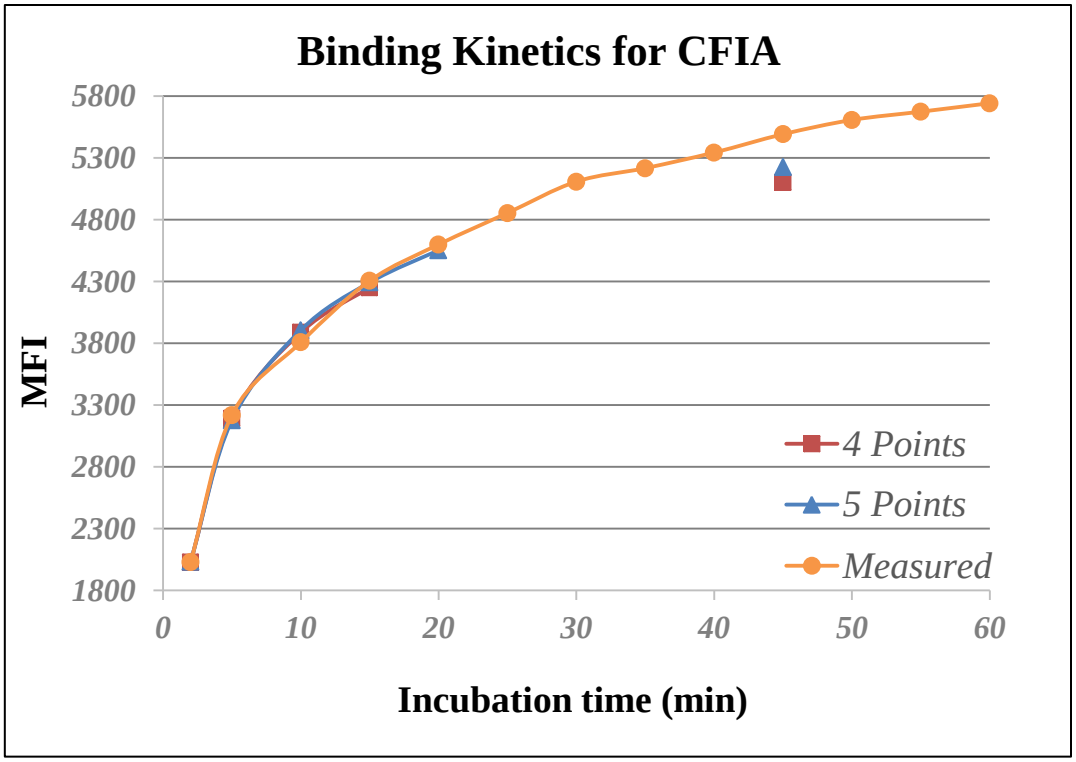


**Figure 6:** *Binding kinetics for CFIA using 15, 20, and 60 minutes incubation times. Lines with square and triangle are extrapolated based on 4 and 5 points measurements respectively. The line with circular shape included 12 measured points.*

DON toxin, was selected as to represent all six of them to make the point in a short presentation. The final step was to generate actual incubation curves with DON with one-hour incubation time and extrapolate binding saturation based on 15 and 20 minutes of incubation. The equations revealed a predictable relationship between the actual and projected binding curves for DON; see Figure 6. Figure 6 include actual readings (circular shape) and two extrapolated curves based on readings at 4 and 5 standard points, they are red and blue lines respectively.

### 4.6. CFIA analytical platform results from bio-transferred mammalian specimens.

The ultimate objective of this study was to integrate the CFIA analytical platform to include analysis of specimens of various animal origins for poly-mycotoxins. Thus far this has been achieved only for zearalenon (ZEA) in laboratory rats (see Figure 7) as a simplex assay. The preliminary results suggest that this analytical platform has capacities to detect bio-transferred mycotoxicosis. The customized post-acquisition FCAP Array software includes considerable number of options to support efficient data management for both plant and animal based analysis with the CFIA platform (see Table 2).

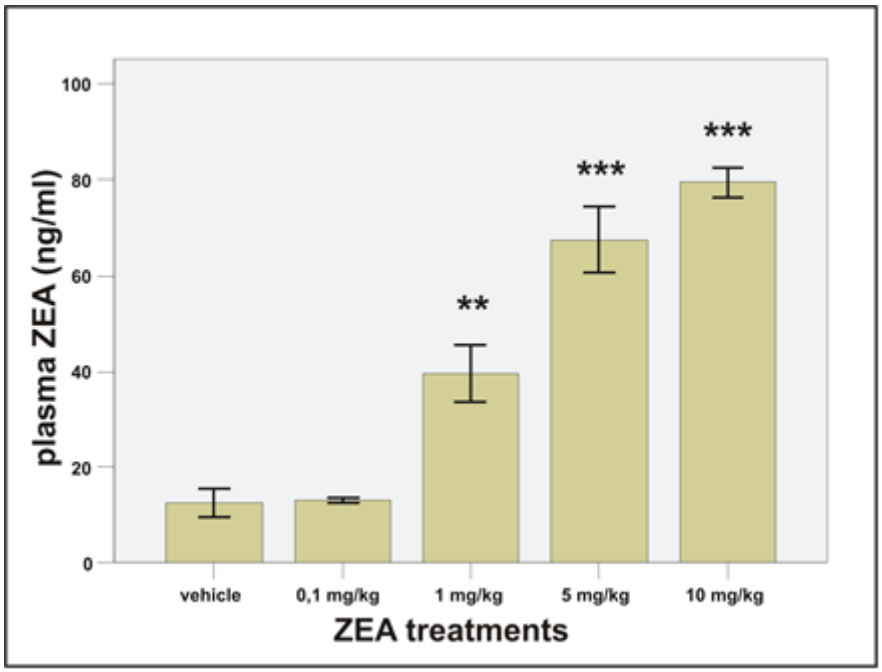


**Figure 7:** *Blood plasma ZEA concentrations. Blood plasma ZEA concentrations in immature female rats after 3-day exposure to different dosage of ZEA (0.1–10 mg/kg bw) were measured with CFIA. There was a dose dependent increase in plasma ZEA concentration following oral administration. Blood plasma levels showed strong positive correlation (R = 0.919) with uterus weight in a dose dependent manner. (**) Significantly different from control (p,0.001) (n = 6–7). (***) Significantly different from control (p,0.001) (n = 6–7).*

| | Software Features | Mycotoxins Measured | | | | | |
|---|---|---|---|---|---|---|---|
| | | **AB1** | **OTA** | **FB1** | **T-2** | **ZEA** | **DON** |
| **1** | **Indication of EU legal limits (µg/kg)** | *2* | *5* | *1400* | *300* | *100* | *1250* |
| **2** | **Optional local or national limits*** | *feasible* | *feasible* | *feasible* | *feasible* | *feasible* | *feasible* |
| **3** | **Child/adult/animal use** | | | | | | |
| **4** | **Plotted points on standard curves** | *6* | *6* | *6* | *6* | *6* | *6* |
| **5** | **Restriction of mycotoxins # in kit** | *no* | *no* | *no* | *no* | *no* | *no* |
| **6** | **Identification of available log scale** | *yes* | *yes* | *yes* | *yes* | *yes* | *yes* |

**Table 2**: *Features of post-acquisition software for multiplexed poly-mycotoxin assay management.*

## 5. NEW FINDINGS

The mission was from the beginning to deliver significant benefit to humanity through reduced costs of quality alimentation free of mycotoxins. Develop a new analytical platform previously unavailable in the agricultural sciences. Integrate both innovative applications from clinical field and include 21st century biotechnology.

1. Utilizing microfluorosphere based FC technology with simultaneous six-mycotoxin detection immunoassay from grain was developed. It is a competitive fluorescent multiplexed

immunoassay (CFIA). The hypothesis was based on the premise that suspended solid phase in liquid environment will accelerate binding kinetics and will give significant advantage to CFIA over traditional solid/liquid phase ELISA. Results will be provide evidence of inceased assay speed and sensitivity. The CFIA platform for six mycotoxins was introduced commercially. The development included some microfluorosphere solid suspended phase system modifications to achieve conjugation exceeding all previous ELISA performance. Developed a conversion technology from HPR conjugation used for ELISA to R-PE tagging which was required for CFIA protocol. Demonstrated that the modified conjugation technology did not compromise antibody performance. It was adjusted to remain stable during scale-up for commercial poly-mycotoxin assay production.

2. Developed a unique instrument independent cross-calibration (IICC) protocol for quantitative fluorescence detection of PE for a variety of flow cytometers. This calibration study was based on theoretical principles of biophysics and optics. The new instrument cross-calibration protocol permitted universal side-by-side evaluation of poly-mycotoxin assays kit utilizing most available bench-top FC's.

3. Developed a unified single-step extraction protocol, which handles up to six mycotoxins simultaneously without compromise in assay quality. The new protocol combined with IICC opened the possibility to develop a new analytical assay platform including agricultural use.

4. Assisted in development and evaluated post-acquisition software for b-FC's, which compensates for instrument design variations/deviations. With the help of the new software, it was possible to perform poly-mycotoxin assays, that are unaffected by different signal processing strategies and laser differences available on most b-FC's. Compensations protocols were imbedded in the software and are transparent to operators. Developed an instrument validation strategy to establish performance standards to meet EU acceptable mycotoxin limits. The customized quantitative assay protocol required implementation of performance evaluation with reference materials and with spiking experiments.

5. CFIA was further modified to be compatible with bio-transfer of toxins through the food chain. It is specifically for mammal and poultry specimens to detect victims of mycotoxicosis. Most of the pioneer work reported here was based on ZEA detection testing laboratory animals.

6. Future implementation of MycoEpi is proposed, based on the development of an analytical platform with biomedical potential. Such approach will have a significant role in the future development of a comprehensive global risk/hazard management paradigm. MycoEpi strategy will embrace health risk and hazard management based exclusively on evidence. Starting from crop growth, all the way to impact on consumers' though mycotoxin bio-transfer. It will monitor risk to health including prevention/intervention of direct or indirect contamination through the food chain.

## 6. APPLICATIONS OF RESULTS

In industrialized countries, the bread and cereal eaten and the coffee with milk consumed in the morning is much safer today compared to the late $20^{th}$ century. However, there are some significant challenges ahead to secure the safety of the entire global food chain. Only a multi-disciplinary approach can secure global food supply's safety in the future. MycoEpi is one critical step towards preventing future massive outbreaks with food poisoning. Poly-mycotoxin surveillance depends on the proposed biomedical platform. Combined with MycoEpi, it will monitor effectiveness of new crop growing techniques, environmentally sound neutralization of contaminated alimentation and other hazard avoidance management strategies. Such integrated holistic approach may extend the application of this analytical platform to include in the future a tool for clinical assessment of poly-mycotoxicosis.

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# 8. PUBLICATIONS SERVED AS BASIS FOR THE THESIS

**Publications related to the Thesis**

1. Czeh A, Schwartz A, Mandy F, Szoke Zs, Koszegi B, Feher-Toth S, Nagyeri Gy, Jakso P, Katona LR, Kemeny A, et al. Comparison and Evaluation of Seven Different Bench-Top Flow Cytometers With a Modified Six-Plexed Mycotoxin Kit. *Cytometry: Part A* 2013.

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2. Czeh A, Mandy F, Feher-Toth S, Torok L, Mike Z, Koszegi B, Lustyik G. A Flow Cytometry Based Competitive Fluorescent Microsphere Immunoassay (CFIA) System for Detecting Up to Six Mycotoxins. *J Immunol Methods* 2012;384:71-80.

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IF: 4.092; Citations: 7

**Conference presentations related to the Thesis**

1. **Czeh A,** Torok L, Gorombey P, Torok T, LantosE, Lustyik Gy. Development of Flow Cytometric Multiplexed Microbead Assay for Detection of Mycotoxin Contamination. Regional Biophysics Conference, Balatonfüred, Hungary, 2007.

2. **Czeh A,** Torok L, Torok T, Siklodi B, Lustyik Gy. Development of competitive multiplexed microbead array technology for simultaneous detection of mycotoxins in food and feed products. ISAC XXIV International Congress, Budapest, Hungary, 2008. - **Oral Presentation**

3. **Czeh A,** Toth Sz, Torok L, Torok T, Lustyik Gy. Development and validation of Multiplexed Microbead Assay for Detection of six different Mycotoxins in feed. CYTO Congress, Seattle, Washington, USA, 2010.

4. **Czeh A,** Toth Sz, Torok L, Torok T, Lustyik Gy. Side by side Comparison Between the Traditional ELISA and the New Fungi-Plex$^6$ Microbead Assay (MA) for Detection of six Mycotoxins. World Mycotoxin Forum, Amsterdam, Holland, 2010.

5. **Czeh A,** Toth Sz, Mike Z, Koszegi B, Mandy F, Lustyik Gy. Flow Cytometry is Reaching New Horizons In Disease Prevention: An Illustration How A Multiplexed Mycotoxin Kit Performs Using Several Types of Instrument with Either Analog or Digital Signal Processing. CYTO Congress, Baltimore, Maryland, USA, 2011.

6. **Czeh A,** Mandy F, Lustyik Gy. Flow Cytometry Based Myco-Epidemiology has Potential Impact on Mycotoxin outbreaks and Health: A Largely Ignored Global Concern A Harmonized Approach to Preventive Medicine. ESCCA Congress, Dublin, Ireland, 2011.

7. Székács A, Bánáti H, **Czéh Á**, Tóth Sz, Juracsek J, Darvas B, Székács I, Lustyik Gy. Detection of environmental and food contaminant mycotoxins with polymeric bead-based immune-detection in a multiplexed flow cytometry assay format. The 4th Annual NanoScience Technology Symposium, Miami, Florida, USA, 2011.

8. **Czeh A,** Feher-Toth Sz, Szoke Zs, Ferenczi Sz, Kriszt, R, Nagyeri Gy, Mandy F, Kovacs KJ, Lustyik Gy, A Microbead Assay Platform for the Detection of Zearalenone from rat blood and brain tissue. CYTO Congress, Leipzig, Germany, 2012.

9. Fehér-Tóth Sz, Kukolya J, **Czéh Á,** Török L, Lustyik Gy. A new fluorescent microsphere based assay for studying Aflatoxin B1 biodegradation by flow cytometry CYTO Congress, Leipzig, Germany, 2012.

Cumulative impact factor: 9,7 Sum of independent citations: 13