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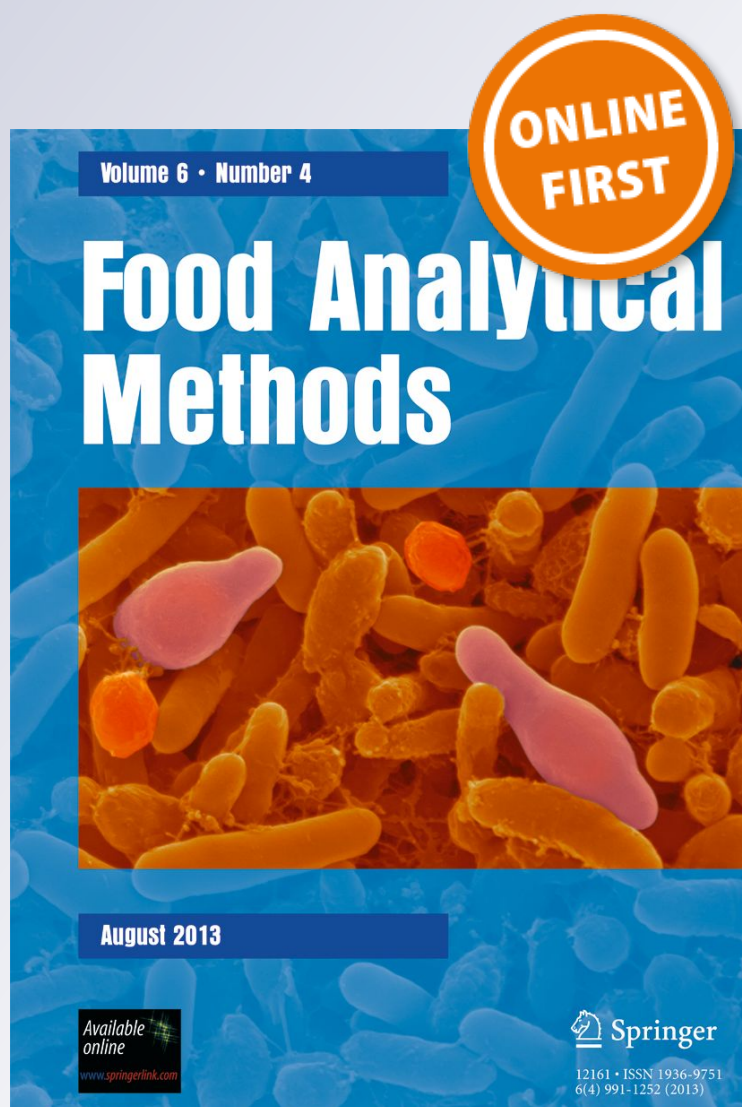
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Validation of a Biochip Chemiluminescent Immunoassay for Multi-Mycotoxins Screening in Maize (*Zea mays* L.)

Andreia Freitas[1,2] · Sílvia Barros[1] · Carla Brites[1,3] · Jorge Barbosa[1,2] · Ana Sanches Silva[1,4]

Abstract

A biochip chemiluminescent immunoassay was validated for multi-mycotoxins screening in maize. Screened mycotoxins were aflatoxins B$_1$ (AFB$_1$) and G$_1$ (AFG$_1$) ochratoxin A (OTA), zearalenone (ZEA), toxin T2 (T2), fumonisins (sum of FB$_1$ and FB$_2$) and deoxynivalenol (DON). The method included a single extraction step with acetonitrile:methanol:water (50:40:10, v/v/v). Spiked samples were fortified at 250 μg/kg for FB$_1$ + FB$_2$, 1 μg/kg for AFB$_1$ and AFG$_1$, 1.5 μg/kg for OTA, 50 μg/kg for ZEA, 25 μg/kg for T2 and 375 μg/kg for DON. The chemiluminescent signal of discrete test regions on the biochip is expressed in Relative Light Unit, and this value differs according to the mycotoxins' concentration. Threshold value and the cut-off were calculated. Low false results rate was achieved (< 5%) and the obtained precision data is in agreement with EU legislation performance criteria. This assay revealed to be a valuable and cost-effective screening method for simultaneous semi-quantification of mycotoxins.

Keywords Mycotoxins · Validation · Chemiluminescent immunoassay · Maize · Cereals · *Zea mays* L

Introduction

The word mycotoxin comes from the conjugation of the word "mykes", which in Greek means fungus and from the work "toxicum", which in Latin stands for poison. Mycotoxins are secondary metabolites of relatively small molecular weight (MW around 700) which can pre- or post-harvest contaminate a wide range of commodities from animal or plant origins (Chauhan et al. 2016). The most affected crops include cereals, like maize and wheat, and cereal-derived products, spices, coffee, cocoa, oil seeds, legumes, fruits, especially apples, and dried fruits like figs. Products such as eggs, dairy (e.g. milk and cheese) and meat can also be contaminated due to the ingestion of infected feed by livestock. On the other hand, wine and beer can be contaminated due to the colonization of grapes, barley or other cereals (Turner et al. 2009).

Mycotoxins can be produced by one or more fungal species. Aflatoxins (AFT) are generally produced by *Aspergillus flavus* and *Aspergillus parasiticus*, but *Aspergillus ochraceoroseus*, *Aspergillus bombycis*, *Aspergillus nomius* and *Aspergillus pseudotamari* can also produce AFT (EFSA 2013). AFT are carcinogenic to humans according to the International Agency for Research on Cancer (IARC 1993a).

Ochratoxins can be produced by fungi from genera *Aspergillus* (mainly *Aspergillus alutaceus*, formely so called *ochraceus*) and *Penicillium* (mainly *Penicillium verrucosum*) in a wide range of conditions, therefore, they are quite common and distributed (Sforza et al. 2006; Turner et al. 2009). Ochratoxin A belongs to the group 2B (Possibly carcinogenic to humans) according to IARC (IARC 1993b).

Fumonisins are derived from fungi from genera *Fusarium* and *Alternaria*. Most of the fumonisins present in maize are produced by *Fusarium verticillioides* (formerly *monoliforme*) and *Fusarium proliferatum* and fumonisins are also included in group 2B of IARC (Oliveira et al. 2015). FB1 and FB2 are the most common fumonisins in maize and also the most studied.

Trichothecenes like T2 toxin, HT-2 toxin (Type A trichothecenes) and deoxynivalenol (Type B trichothecenes) are

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produced by *Fusarium* species like *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium equiseti* (Sforza et al. 2006). *Fusarium graminearum* is the most distributed *Fusarium* species. Zearalenone (F-2 toxin), a phenolic resorcyclic acid lactone, is produced by several *Fusarium* species including *Fusarium graminearum* (EFSA 2011). T2 toxin and zearalenone are included in group 3 "Not classifiable as to its carcinogenicity to humans" according to IARC (2018).

Mycotoxins can survive to storage and processing even when food processing is carried out at high temperatures. Even if the mould is not visible in a product, this does not guarantee that it is free of toxins because the fungi may have already died but the toxin may be present and active. On the other hand, the presence of a fungus able to produce toxins does not mean that the food is contaminated with the toxin because there are factors involved in their production. Therefore, sometimes it is easier to prevent food contamination than to mitigate the problem of contamination (Turner et al. 2009).

One of the most important features in mycotoxin analysis is to carry out a representative sampling because the contamination is heterogeneously distributed in the food products. Most of the error associated with mycotoxins analysis is associated with sample collection. Therefore, the European Union has defined legislation laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (EC 2006a).

Due to the severe health problems associated with mycotoxin ingestion even in very low concentrations, such as carcinogenicity, hepatotoxicity, impairment of immune system, nephrotoxicity, teratogenicity, neurotoxicity and reproductive toxicity, it is of utmost importance to have sensitive and reliable methods for their detection in order to guarantee that food samples are in accordance with Commission Regulation (EC) No 1881/2006 of 19 December 2006 (EC 2006b), which sets maximum levels for certain contaminants, including mycotoxins, in foodstuffs (Table 1).

It is desirable to have a simple/easy-to-operate, fast and inexpensive method to screen samples regarding their level of contamination by mycotoxins. Immunoassays meet these requirements and are also sensitive and selective (Wang et al. 2013; Zhang et al. 2017a, b). Therefore, they are preferred as the first screening method (Anfossi et al. 2016). ELISA (Enzyme-Linked Immunosorbent Assay) kits have been used for this purpose and they are based on a competitive assay that conjugates a single antibody with a target molecule (direct ELISA) or requires two antibodies, a primary antibody and a complementary antibody, an enzyme-linked secondary antibody (indirect ELISA) (Turner et al. 2009; Pereira et al. 2014).

ELISA kits are commercially available for the regulated mycotoxins and can be an excellent tool to perform large and frequent surveys. Some authors have published papers describing the development of ELISA methods for the detection of mycotoxins in different matrices, namely in say and maize (Folloni et al. 2011), in rice (Li et al. 2014) and in bread and bran (Singh et al. 2018). Direct ELISA is rapid and eliminates, in some cases, cross-reactivity of secondary antibody but signal amplification may be due low to reduced immunoreactivity of the primary antibody. In the case of indirect ELISA, each primary antibody contains several epitopes that can be bound by the labelled secondary antibody. In this line, the signal is amplified and the method is more sensitive (Turner et al. 2009). The study of cross-reactivity of ELISA kits is important to avoid over-estimation of results of a specific mycotoxin. Berthiller et al. (2013) carried out an interesting review in which the cross-reactivities of commercial enzyme immunoassay kits and immunoaffinity columns are compared and showed great variability for masked mycotoxins. Nevertheless, in the case of risk assessment, it is important to know the target mycotoxin and the co-existing analogues. Positive results shall be confirmed, for instance by liquid chromatography coupled with mass spectrometry detection (LC-MS/MS).

Immunochromatographic methods have also been widely used for visual (yes/no) detection or semi-quantification of mycotoxins (Anfossi et al. 2013; Li et al. 2013a; Dzantiev et al. 2014; Song et al. 2014; Sun et al. 2014; Majdinasaba et al. 2015; Guo et al. 2017). The greatest drawback of

Table 1 Linear range, linearity, regulatory limit, precision and recovery of biochip chemiluminescent immunoassay for the simultaneous determination of multimycotoxins

Mycotoxin	Linear range (μg/kg)	Regulatory limit[b] (μg/kg)	r	Precision (%)	Recovery (%)
AFB1	0–14	5	0.9993	7.37	83.3
AFG1	0–75		0.9947	9.97	73.6
DON	0–7500	1750	0.9994	7.96	108.4
FB1 + FB2	0–300[a]	4000	0.9960[a]	21.2	85.6
OTA	0–60	5	0.9992	7.19	83.4
T2	0–300		0.9970	10.1	103.6
ZEA	0–150	350	0.9995	6.98	87.2

[a] For FB1

[b] Commission Regulation (EC) No. 1881/2006 of 19 December 2006, consolidated with Reg 1126/2007, maximum levels for unprocessed maize for human consumption

immunochemical based methods is the excessive selectivity that difficult the simultaneous determination of different mycotoxins. In this line, new developments are being made by immunochemical based tests (ICT). In fact, multiplex ICT strips were reported for simultaneous determination of mycotoxins, some of them allowing to detect mycotoxins from different classes (Li et al. 2013b; Song et al. 2014; Zangheri et al. 2015; McNamee et al. 2017).

The Evidence Investigator Biochip Array Technology (BAT) (Randox, UK) is used for the semiquantitative detection of multiple analytes in a single sample and, for instance, it has been applied to piperazines in urine (Castaneto et al. 2015), sulfonamides in honey (Popa et al. 2012), mycotoxins in feed samples (Plotan et al. 2016) among a long list of possible applications.

Biochips (9 mm × 9 mm) are solid state devices that contain a set of discrete test regions (DTR) with immobilized antibodies specific for mycotoxins. The semiminiaturized immunoassays are competitive chemiluminescent wherein the analyte competes with the conjugate for the binding sites and as a result the concentration of the analyte searched is inversely proportional to the light emitted (Plotan et al. 2016). Therefore, in this immunoassay, the light is produced via chemical reaction. The light signal (RLU) generated in each test region is detected through the use of digital imaging technology and the concentrations of mycotoxins are determined by comparison to the light signals that are stored and extrapolated in a calibration curve.

The aim of this paper is to validate an immunoassay for semi-quantitative screening of several mycotoxins in maize samples. Positive findings should afterwards be confirmed by a physic-chemical method, such as LC-MS/MS.

Materials and Methods

Chemicals and Reagents

Methanol and acetonitrile (both HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q plus system from Millipore (Molsheim, France). Mycotoxins standards, obtained from Sigma-Aldrich (Madrid, Spain) (Fig. 1), were prepared in the day of the analysis and dissolved in ultrapure water. They were immediately used, although they were stable for up to 14 days at − 20 °C.

Mixed work solution of mycotoxins (125 μg/kg for FUM (B1 and B2); 1.5 μg/kg for OTA; 1 μg/kg for AFB1; 1 μg/kg for AFG1; 25 μg/kg for T2; 50 μg/kg for ZEA and 375 μg/kg for DON) for the validation of the assay. Certified reference materials MA1750-1/CM and MA1764/CM from Test Veritas (Padova, Italy) were used to evaluate accuracy of the method.

Samples and Sampling Procedure

Samples of maize, kindly provided by InovMilho (Portuguese National Competence Center for Maize and Sorghum Cultures), were analysed for mycotoxins in this study. These samples were collected from the field experimentation trials located in the Coruche region of Portugal from September to October, 2018 and they were intended for human consumption. Each test portion (5 kg) corresponds to a trial modality and was hand collected after thorough mixing of several incremental samples taken from random field place locations according to the methodology described in the Commission Regulation (EC) No. 1881/2006 (EC 2006b) and in the European Regulation Commission Regulation (EC) No 401/2006 (EC 2006a) and their amendments, laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. The laboratory samples have been homogenized by grinding (Retsch rotor mill SK 300 with sieve of trapezoid holes of 1.00 mm) the entire test portion (5 kg) and the flours were mixed thoroughly for guarantee complete homogenisation as possible. From each sample, three sub-samples of 50 g each were placed in separate sterile sample collection tubes and preserved at − 20 °C until analysis. No further processing of the samples was done.

Analysis Procedures

Spiking Experiment

To determine the recovery of the target analytes, spiking experiments were performed. Blank samples of maize (5 g) were spiked with a multi-analyte standard solution, thoroughly mixed and kept at ambient temperature in the dark for 30 min. Afterwards extraction was performed as described in following sub-section.
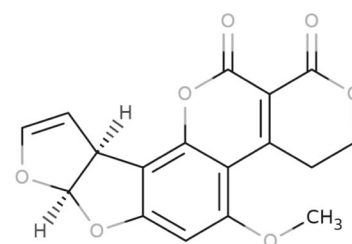
Extraction Prior Chemiluminescent Immunoassay

For the extraction procedure, 5 ± 0.05 g of homogenized sample was extracted with 25 ml acetonitrile:methanol:water (50:40:10, v/v/v). Then they were vortexed for 60 s and rolled for 10 min. After centrifugation (10 min at 3000 rpm), they were diluted with working strength wash buffer (included in the kit), 50 μl sample + 700 μl working strength (dilution factor, 75). The diluted sample was applied to the biochip following the instructions of the manufacturer for the assay Myco7 (Randox Laboratories Limited 2008). Randox can spot a total of 44 antibodies per biochip. In addition, there are reference and correction spots for internal quality control (3 total). For the array Myco7, Randox has 7 antibodies spotted.
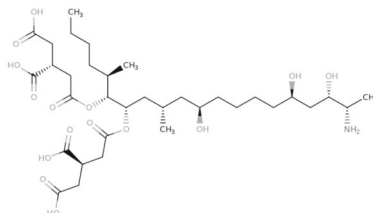
Fig. 1 Chemical structure of the mycotoxins validated by the biochip chemiluminescent immunoassay
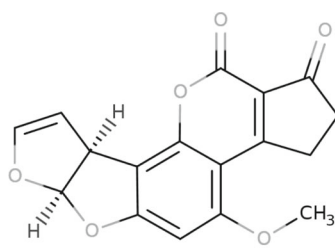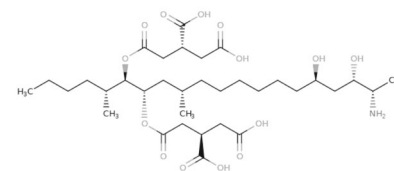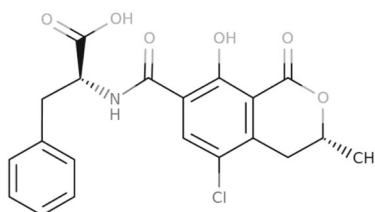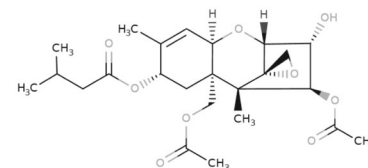

Aflatoxin B1

Aflatoxin G1
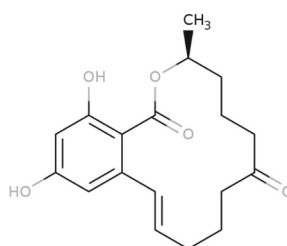
Fumonisin B1

Fumonisin B2
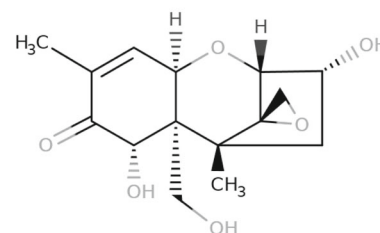
Ochratoxin A

T2 toxin

Zearalenone

Deoxynivalenol

Chemiluminescent Immunoassay Analysis

A competitive chemiluminescent immunoassay was employed for the determination of mycotoxins in maize samples. This immunoassay (Investigador™ EV 4065, Evidence Investigator Myco 7) bases on the Evidence Investigator Biochip Array technology and uses a Randox Biochip i.e. a solid-state with regions containing immobilized antibodies specific to mycotoxins. Increasing levels of mycotoxins yield decreased binding of the conjugate labelled with horseradish peroxidase (HRP) and thus a decrease in the emitted chemiluminescence signal, which is detected by digital imaging technology. The concentration of mycotoxins present in the sample is calculated from the calibration curves.

The kit is composed of six carriers composed of nine biochips each (54 biochips in total), nine calibrators of the mixture of mycotoxins in a range of concentrations, control, assay diluents, conjugate diluents, multianalyte conjugate, signal reagent, washing buffer, calibration compact disc and barcodes.

For the bioship assay, first, 150 µl of diluted wash buffer was pipetted to each biochip well. Then, 50 µl of calibrator or diluted sample was pipetted to the appropriate biochip wells. Afterwards, all edges of the handling tray were gently taped to mixed reagents. Then holding tray was incubated for 30 min at 25 °C and 370 rpm in a thermoshake. Finally, 100 µl of

working strength conjugate were pipette per biochip well and holding tray was again incubate for 60 min at 25 °C and 370 rpm.

For the capture of data by digital imaging technology, carriers are processed individually. After incubation, biochips are submitted to two quick wash cycles. Those waiting for imaging should be protected from light. The first carrier to be imaged is removed from the handling tray. Using a wash bottle with diluted wash buffer, approx. 350 µl, diluted wash buffer was added to each well, all edges of the handling tray are gently taped to release any reagents trapped below the biochip, and flick to waste with a sharp action. Then, four additional wash cycles are carried out. For each cycle, all edges of the handling tray shall be gently tapped for approximately 10 to 15 s, then biochips are left to soak in diluted wash buffer for 2 min. Finally, to remove any residues, diluted wash buffer is removed onto lint-free tissue. After tapping, 250 µl of working signal reagent is added to each well and they are covered to be protected from light.

After exactly 2 min (± 10 s), carriers are placed into Evidence Investigator™. In fact, the use of a timer is recommended to ensure that imaging occurs at the correct time. Capture of images is automatically initiated, and data is processed by Evidence Investigator™ (EV 3602; Randox Food Diagnostics, Crumlin, UK).

Evaluated Parameters

In the validation of the screening process it is only required the selectivity/specificity, to evaluate the limit of detection, and the applicability/robustness. However, precision and recovery of the method were also calculated.

Selectivity

Twenty blank samples from different origins were used for validation. Blank samples were spiked to a concentration of interest. Samples (both blank and spiked samples) were processed according the procedure described in "Evaluated Parameters". Samples were processed by different technicians, days and batches.

The chemiluminescent signal of discrete test regions (DTR) on the biochip is expressed in RLU, and this light intensity value differs according to the concentration of the mycotoxins detected. Threshold value (T) and the cut-off (Fm) were calculated according to the following equations (CRLs 2010):

$$T = B + 1.64 \times SD_B$$

Where B is the mean and SD is the standard deviation of the signal in RLU of the blank samples.

Table 2 Cross-reactivity of the biochip chemiluminescent immunoassay for the simultaneous determination of eight mycotoxins (Plotan et al. 2016)

Mycotoxin	Cross reactivity with	% cross-reactivity
AFB1	AFB1	100
	AFB2	30
	AFG1	17
	AFG2	4
AFG1	AFG1	100
	AFG2	56
	AFB1	9
	AFB2	4
DON	DON	100
	3-acetyldeoxynivalenol	844
	15-acetyldeoxynivalenol	10
Fumonisins	FB1	400
	FB2	70
	FB3	70
OTA	OTA	100
	OTB	2
T2	T2	100
	HT2 toxin	37
	T2 triol	3
ZEA	ZEA	100
	α-Zearalenol	112
	β-Zearalenol	64
	Zearalanone	59
	α-Zearalanol	45
	β-Zearalanol	47

$$Fm = M + 1.6 \times SD$$

Where: M is the mean and SD standard deviation of the signal in RLU of the spiked samples.

The mean signal of blank samples is, at least, ≥ 30% of the signal of spiked samples at the concentration of interest.

Linearity

A four-parameter curve fit method was used for the calibration by the Evidence Investigator system. Each of the nine calibration concentrations was read five times and the corresponding r values were obtained (Table 1). Acceptance criterion was r > 0.95.

Precision

The precision was calculated by analysing 20 fortified samples at the level of interest within the calibration range. Those levels were 250 µg/kg for the sum of FB$_1$ and FB$_2$, 1 µg/kg for

Table 3 Threshold value (*T*) and cut-off value (Fm) of the biochip chemiluminescent immunoassay for the different mycotoxins

	FB1+FB2		AFG1		Zea		OTA		AFB1		T2		DON	
	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked	Blank	Spiked
Spiking level (μg/kg)	–	250	–	1	–	50	–	1.5	–	1	–	25	–	375
Mean (RLU)	2441.3	139.6	5698.7	3175.4	3770.5	548.6	20,753.6	9897.3	8125.2	3420.1	6570.8	1622.3	12,538.3	5695.3
SD (RLU)	285.5	29.6	214.3	316.6	183.5	38.3	1981.8	2037.7	401.1	252.2	633.3	163.0	1002.6	453.1
T (threshold value) (RLU)	1973.1		5347.2		3469.5		17,503.4		7467.3		5532.1		10,894.1	
Fm (cut-off value) (RLU)	188.1		3694.5		611.3		13,239.1		3833.6		1889.6		6438.4	

AFB$_1$ and AFG$_1$, 1.5 μg/kg for OTA, 50 μg/kg for ZEA, 25 μg/kg for T2 and 375 μg/kg for DON. Results were given as CVs. The sum of FB1 and FB2 is constituted by half of FB1 and half of FB2. The selection of the concentrations was carried out taking in account the limits of quantification of the method for the confirmation of mycotoxins in maize by LC-MS/MS (Sanches-Silva et al. 2019).

Recovery

Recovery of the fortified samples was calculated according to the equation:

$$\text{Recovery (\%)} = \text{measured concentration/fortified level} \times 100$$

Data Analysis

In the blank samples, it should be verified that the result obtained was above than the cut-off value; in the fortified samples, the result obtained should be lower than the cut-off; in the duplicate sample, the results must be concordant, that is, it presents results above the cut-off value or results below the cut-off value and a deviation of the relative standard ≤ 10%. The cut-off value is used in effect, for decision of suspects.

Expression of results:

– Compliant: when the signal obtained exceeds the cut-off of the method (in RLU).

– Suspected of non-compliance: when the signal is less than or equal to the cut-off established in the validation. In this case, the result should be confirmed by another method.

For routine analysis, two negative quality controls (QC) and two positive QC shall be analysed each day, in parallel with unknown samples.

Results and Discussion

Validation of Chemiluminescent Immunoassay

The calibration curves (*n* = 5) for the FB1, OTA, AFB1, AFG1, DON, T2 and ZEA were obtained simultaneously and presented *r* values that met the acceptance criterion of *r* > 0.95, ranging from 0.9947 to 0.9995 (Table 1). Precision data (CVs) showed values lower than 10.1% for the simultaneous immunoassays, except for the sum of FB1 and FB2 which was 21.2%. However, these values are in agreement with performance criteria for fumonisin analysis according to Regulation EC no. 401/2006 (EC 2006a) and its amendments. Recovery was within 73.6% for AFG1 and 108.4% for DON (Table 1), which also meets the performance criteria for mycotoxins analysis according to Regulation EC no. 401/2006 (EC 2006a) and its amendments. Recovery of fumonisins was calculated taking into account the cross-reactivity (Table 2) indicated by the manufacturer. Plotan et al. (2016) validated the BAT immunoassay for feed samples and obtained a mean recovery of 109% for FB1 (10 μg/kg), 103% for OTA (2 μg/kg), 90% for AFG1 (2.5 μg/kg), 114%

Table 4 Comparison between the assigned contamination levels of the certified reference materials (maize) and the measured value by biochip chemiluminescent immunoassay

Certified control material	Mycotoxin	Assigned contamination level (μg/kg)	Satisfactory range (μg/kg)	Measured value (μg/kg)
MA1750-1/CM	AFB1	9.34	5.23–13.45	10.21
	AFG1	1.57	0.88–2.26	2.05
	FB1+FB2	3428.7	1714.35–5143.05	4487.3
MA1764/CM	ZEA	190.42	112.21–268.63	233.7

for DON (250 μg/kg), 110% for T2 (10 μg/kg), 96% for AFB1 (0.462 μg/kg) and 93% for ZEA (5 μg/kg).

According to Plotan et al. (2016), CCβ was calculated according to Commission Decision 2002/657/EC (2002) where at least 20 blank feed samples were tested. CCα corresponded to their mean concentration plus 2.33 times the SD of the within-laboratory R value and CCβ as CCα plus 1.64 times the SD of the fortified samples ($\beta = 5\%$). For the sensitive detection method (corresponding to dilution factor of 20) CCβ ranged between 0.21 μg/kg for OTA and 79.4 μg/kg for DON. These CCβ values ranged between 0.79 μg/kg for OTA and 298 μg/kg for DON for the dilution factor of 75.

Threshold value (T) and cut-off value (Fm) of the biochip chemiluminescent immunoassay for the different mycotoxins are compiled in Table 3 and the results of each of the 20 blank and of the 20 fortified maize samples is represented in Fig. 3. It was not found false positives for any of the mycotoxins and it was just found 5% of false negatives (corresponding to 1 sample in 20) for fumonisins, ZEA, AFB1, T2 and DON (Fig. 3).

In order to evaluate accuracy of the method, two CRM, from Test Veritas were analysed. Both CRM were maize samples, MA1750–1/CM was contaminated with AFB1, AFB2, AFG1, AFG2, FB1 and FB2, while MA1764/CM was contaminated just with ZEA. Comparison between the assigned contamination levels of the CRMs and the measured values by biochip chemiluminescent immunoassay is presented in Table 4 that shows results in agreement with the established data. The "satisfactory range" in this table is given by the provider of the Certified reference materials MA1750–1/CM and MA1764/CM, Test Veritas (Padova, Italy).

Anfossi et al. (2016) presented an excellent SWOT (Strengths, Weaknesses, Opportunities and Threats) analysis comparing immunochemical-based methods and chromatographic-based methods. According to this study the main strength of chromatographic-based methods are: the compliance of validation with legislation, to allow compounds identification and structural characterization of unknown and to be multi-target. On the other hand, immunochemical-based methods have the following strengths: are simple, cheap and portable, require limited sample treatment and allow managing a large number of samples. Due to these advantages, these methods are mainly applied for rapid on-site detection and routinely control (Anfossi et al. 2016).

There are critical steps in the chemiluminescent immunoassays. Therefore, it is important to take into consideration the following: (i) when pipetting solution into the wells of the biochips, one should be careful to avoid the formation of bubbles. Assay reagents and samples should be added with the tip of the pipette pointing towards the back of the biochip. 'X' marks the area optimal for sample addition (see Fig. 2); (ii) an appropriate number of washes shall be made to avoid background noise; (iii) with regard to imaging, it is important to not overfill wells during wash in order to reduce potential for well-to-well contamination; (iv) carriers should not be left to soak for longer than 30 min and (v) carriers awaiting imaging should be protected from light.

One of the most critical is the washing step(s). A significant background noise that prevents imaging can happen when washes are not enough.

Mycotoxins in Maize Samples

Fumonisins (FB$_1$ + FB$_2$) were detected in maize samples collected in Portugal between September and October 2018. Table 5 compiles the results of these samples for FB$_1$ and FB$_2$. All the samples were negative for the other mycotoxins under study. Moreover, all the samples presented a concentration for FB1 + FB2 lower than 300 μg/kg, except one sample. This sample should be further analysed by LC-MS or LC-MS/MS (Table 5).

French authorities informed the European Commission on 29 April 2014 that exceptional climatic conditions resulted in very high levels of mycotoxins (zearalenone, fumonisins and deoxynivalenol) on maize harvested in 2013 in Europe. In 2013, the growing conditions led to a higher carry-over of these mycotoxins into the maize flours causing a disruption in the maize milling supply chain for the rest of the season (until end of 2014). Therefore, French authorities stated at Commission one temporary derogation to the mycotoxin regulatory limits Commission Regulation (EC) No 1881/2006 of 19 December 2006 (EFSA 2014).

Soleimany et al. (2012) found FB1 in the range 48.2–209.3 μg/kg and FB2 was in the range 58.7–113.5 ng/g in maize meals from Malaysian markets ($n = 100$). Abia et al. (2013) have reported the occurrence of mycotoxins in different food commodities from Cameroon and found 508 μg/kg for FB1 and 149 μg/kg for FB2 in maize samples ($n = 37$) using a LC-ESI-MS/MS method.

X	X	X
X	X	X
X	X	X

Fig. 2 The optimal position for addition of assay reagents and samples in each well of the biochip is marked with an x
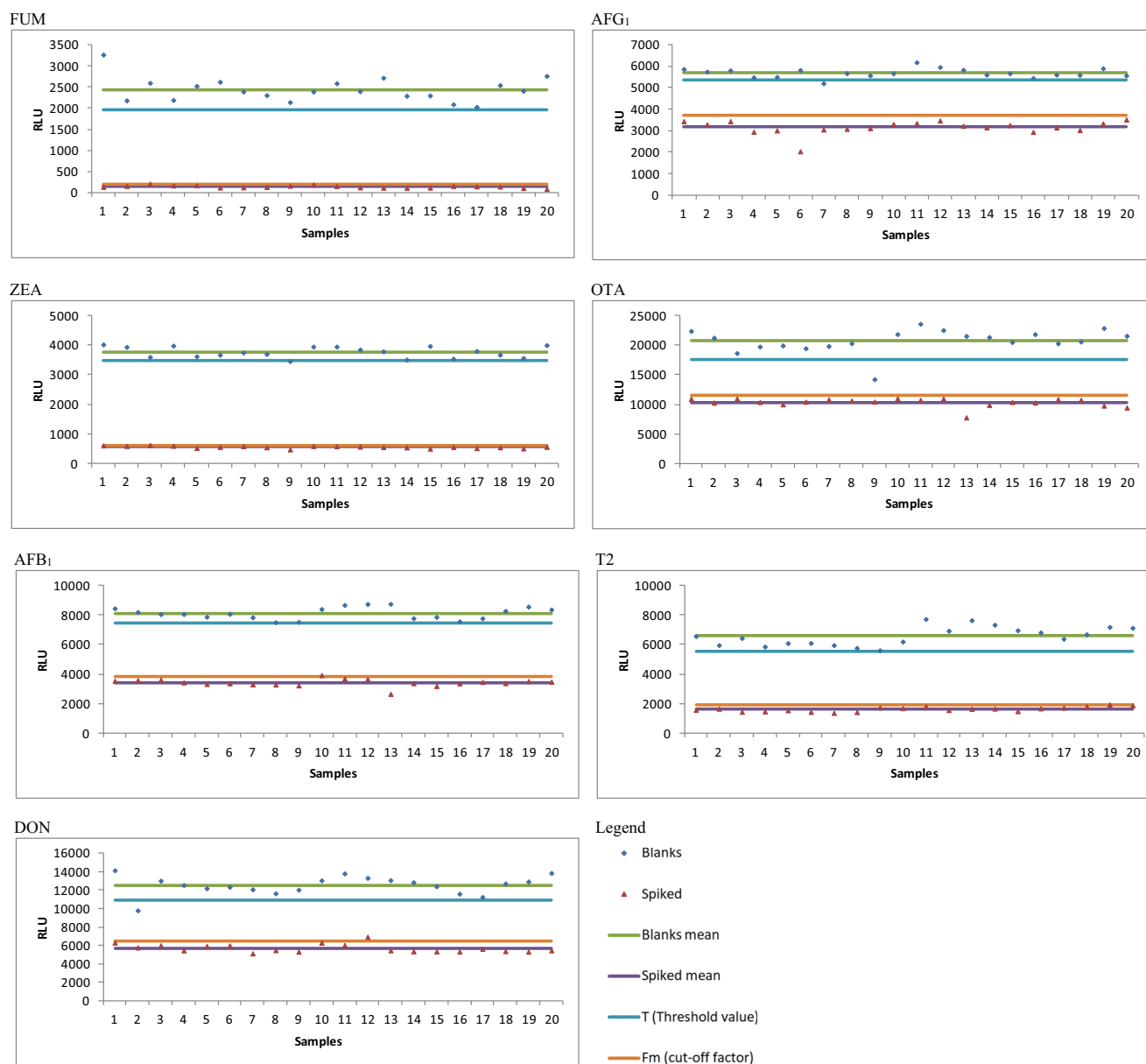
Fig. 3 Threshold value (*T*) and cut-off value (*Fm*) of each of the mycotoxins analysed by the biochip chemiluminescent immunoassay expressed in RLU (Relative Light Unit), for the 20 blank maize samples and for the 20 spiked maize samples at the level of interest

Murashiki et al. (2017) reported the levels of FB1 and AFB1 in maize meal and maize grain. Aflatoxin B1 ranging from 0.57 to 26.6 μg/kg was detected in 80 of 388 samples and FB1 was present in all samples analysed. FB1 levels were below the regulatory limits set by the European Union but the average probable daily intake (APDI) of fumonisin B1 estimated for the population of Shamva and Makoni districts in Zimbabwe were above the provisional maximum tolerable daily intake (PMTDI) of 2 mg/kg body weight/day for FB1, FB2 and FB3, independently or combined, recommended by The World Health Organization (WHO). PMTDI was calculated according to the dose of no observable adverse effect level of 0.2 mg/kg/day with a safety factor of 100 (WHO 2002).

The review of Oldenburg et al. (2017) put in evidence that the most prevalent occurring *Fusarium* mycotoxins in maize ears are the fumonisins (FB1 to FB4) produced by *F. verticillioides* and *F. proliferatum*. Visually asymptomatic maize kernels infected by *F. verticillioides* in endophytic state may contain toxins at rather low concentrations, but under stress conditions the fungus is switching to the pathogenic state and higher amounts of fumonisins may be produced. Therefore, the predicted global warming expected for temperate regions of Europe can increase the occurrence of mycotoxins, especially fumonisin-producing *Fusarium* species, being more evident in southern countries (Bryla et al. 2013).

Table 5 Samples content on the sum of fumonisins B1 and B2 by the validated chemiluminescent immunoassay

Samples #	Fumonisins B1 + B2 (µg/kg)
1	125.9
2	56.6
3	105.1
4	25.0
5	> 300[a]
6	41.2
7	8.1
8	3.1
9	190.5
10	46.7

[a] Suspected of non-compliance

Conclusions

The worldwide climatic changes (increase of global temperature and rainfall) caused by emission of greenhouse gases, will most probably be responsible for an increasing contamination of mycotoxins in food chain. In this regard, it is very important to implement capable control programmes as well as an established policy of risk assessment and management. These programmes can be assured by immunoassays. The validated BAT immunoassay is reliable, cost effective, rapid, semiquantitative and environmentally friendly and covers the regulated mycotoxins. Those characteristics were demonstrated during validation and through the analysis of the reference material.

Compliance with Ethical Standards

Conflict of Interest Andreia Freitas declares that there is no conflict of interest. Sílvia Barros declares that there is no conflict of interest. Carla Brites declares that there is no conflict of interest. Jorge Barbosa declares that there is no conflict of interest. Ana Sanches Silva declares that there is no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

Abbreviations AFT, aflatoxins; BAT, Biochip Array Technology; DTR, Discrete Test Regions; EC, European Commission; ELISA, Enzyme Linked Immunosorbent Assay; EU, European Union; Fm, cut-off; HRP, horseradish peroxidase; IARC, International Agency for Research on Cancer; ICT, immunochemical-based test; LC-MS/MS, liquid chromatography coupled with mass spectrometry detection; RLU, relative light unit; T, threshold value

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