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Analytical Methods

Immunoassay based on monoclonal antibody for aflatoxin detection in poultry feed

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ABSTRACT

An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on an anti-aflatoxin B_1 monoclonal antibody was standardised and validated for aflatoxin screening in poultry feed samples and its performance was compared to high-performance liquid chromatography (HPLC). The ic-ELISA showed good linearity ($r^2 = 0.994$) and detection limits of 1.25 ng g^{-1} for broiler feed and 1.41 ng g^{-1} for laying hen feed. Mean aflatoxin recovery rates by ic-ELISA were 102% (laying hen feed) and 98% (broiler feed). Aflatoxins were detected in 88.2% of the 34 broiler feed samples by ic-ELISA and HPLC at means of 10.48 ng g^{-1} and 8.41 ng g^{-1}, respectively, while 92% of laying hen feed samples ($n = 36$) showed aflatoxin contamination at means of 20.83 and 19.75 ng g^{-1}. The standardised ic-ELISA showed reliability and a high correlation with HPLC of 0.97 (broiler feed) and 0.98 (laying hen feed) indicating its potential for aflatoxin screening in poultry feed samples.

1. Introduction

Aflatoxins are a group of structurally related toxic metabolites produced mainly by *Aspergillus flavus* and *A. parasiticus* (Eaton & Groopman, 1993). The major naturally occurring aflatoxin analogues are B_1 (AFB_1), B_2 (AFB_2) G_1 (AFG_1) and G_2 (AFG_2). AFB_1 and AFB_2 are the most commonly detected analogues in agricultural commodities (Goldblatt, 1971).

These mycotoxins have been shown to cause mutagenic, teratogenic and hepatocarcinogenic effects (CAST, 2003). The International Agency for Research on Cancer (IARC, 2002) has classified naturally occurring mixtures of aflatoxins as carcinogenic to humans (Group 1). In poultry, they can cause an increase in liver and kidney weights, multifocal hepatic necrosis, biliary hyperplasia, diarrhoea, immunosuppression, decreased feed intake, and decreased weight gain and feather mass (Giacomini et al., 2006; Sklan, Klipper, & Friedman, 2001).

Aflatoxin contamination in poultry feed is a worldwide problem (Beg et al., 2006; Dalcero et al., 1998; Oliveira et al., 2006) and can cause serious economic losses, firstly due to increased mortality in farm animals and secondly due to grain downgrading as an animal feed and as an export commodity (Bennett & Klich, 2003). Furthermore, when metabolised by poultry, aflatoxins or their metabolites can occur in tissues, blood, breasts, gizzard, liver and eggs and are a potential threat to the human consumer (Cortés et al., 2010; Herzallah, 2009; Salwa & Anwer, 2009).

Aflatoxins are heat stable and cannot be removed by industrial processing, therefore carry-over of aflatoxin metabolites to meat and eggs can occur and increase human exposure. The most effective control measure depends on a rigorous program of monitoring the feed-producing chain using sensitive and reliable analytical methods in order to minimise health risks.

Aflatoxin determination in food and feed is currently performed by high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS) (CAST, 2003; Kolosova, Shim, Yang, Eremin, & Chung, 2006). Although most of these methods are sensitive and accurate, they are laborious, expensive, time-consuming and unsuitable for analysis of large number of samples and also require costly equipment and extensive clean-up procedures (Kolosova et al., 2006).

Fast, reliable and sensitive analytical methods are needed, due to the strict guidelines on mycotoxin contamination that have been imposed by importing countries. This demand has led to the development of quantitative or semi-quantitative methods for mycotoxin screening, based on immunochemical techniques, such as enzyme-linked immunosorbent assays (ELISAs), since they do not require costly instrumentation, are able to analyse a large number

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of samples simultaneously and require no sample clean-up (Krska et al., 2008; Li, Zhang, & Zhang, 2009; Zhang, Wang, & Fang, 2011). In general, ELISAs are rapid, simple, specific and sensitive, they can be used in the field and have become the most common rapid methods for mycotoxin detection in food and feed (Zheng, Humphrey, King, & Richard, 2005). Additionally, the detection limits of ELISA can be comparable with or even lower than those obtained by instrumental methods (Kolosova et al., 2006).

However, commercial ELISA kits are expensive which makes their inclusion in routine analysis in developing countries difficult (Devi et al., 1999); therefore investments in immunoreagent production are an alternative to reduce costs. Several researchers have reported the development of ELISA methods based on monoclonal antibodies for AFB_1 detection and their application to different matrices (Chun, Kim, Ok, Hwang, & Chung, 2007; Kolosova et al., 2006; Li, Zhang, Zhang, Zhang et al., 2009; Zheng et al., 2005).

In this study, an indirect competitive ELISA (ic-ELISA) based on an anti-AFB_1 monoclonal antibody was standardised and validated for aflatoxin screening in poultry feed samples and its performance was compared to that of HPLC.

2. Material and methods

2.1. Production of the anti-AFB_1 monoclonal antibody (mAb)

The AF2 hybridoma cell line secreting specific anti-AFB_1 mAb (IgG_1 lambda isotype), derived from the myeloma cell line Sp2/0-AG14 and the BALB/c splenic cell, was prepared at Kagawa University, Japan (Kawamura et al., 1988).

The AF2 hybridoma cell line was cultured in RPMI + 10% foetal bovine serum: H-SFM (hybridoma serum-free medium, Gibco Co., Paisley, UK) (25:75, v/v). Anti-AFB_1 mAb was precipitated with $(NH_4)_2SO_4$ at 50% saturation from the supernatant and stored at −80 °C. Before use, the precipitate was dissolved in 0.1 M phosphate buffered saline (PBS) pH 7.3 and then dialysed against PBS followed by ultra-pure water (4 °C, 32 h). Sodium azide 0.02% was added to the dialysed mAb and it was aliquoted (30 μL) and stored at −20 °C. The anti-AFB_1 mAb was used for aflatoxin determination by ic-ELISA. This mAb cross-reacted with AFB_1 (100%), AFB_2 (133%), AFG_1 (13.4%) and AFG_2 (14.7%), but it showed very low cross-reactivity against AFL_1, AFL_2, AFM_1, AFQ_1 and AFB_{2a} (Kawamura et al., 1988).

2.2. Sampling

Feed samples intended for broilers (n = 34) and for laying hens (n = 36), collected in 2010 from a poultry farm and from the State University of Londrina Experimental Farm, respectively, Northern Paraná State, Brazil, were evaluated for natural aflatoxin contamination. Feed intended for the broilers belonged to four feed types (pre-starter, starter, grower and finisher) and were pelleted, while the feed intended for laying hens was mashed. For aflatoxin determination, 200 g of each sample were ground to 50 mesh and stored at −20 °C.

2.3. Aflatoxin analysis by ic-ELISA

2.3.1. Aflatoxin extraction

Aflatoxin extraction was performed according to Kawamura et al. (1988). An aliquot of feed sample (2 g) was shaken for 10 min at 150 rpm with 10 mL methanol:water (70:30, v/v). The crude extract was then filtered through Whatman No. 1 filter paper and diluted in PBST (PBS + 0.05% Tween 20) for ic-ELISA determination.

2.3.2. ic-ELISA

Aflatoxins were determined by a monoclonal antibody-based ic-ELISA according to Kawamura et al. (1988). Polystyrene microtitre plate wells (Corning, New York, NY) were coated with 100 μL AFB_1-BSA (bovine serum albumin) in PBS (0.015 M, pH 7.3) at 4 °C for 18 h. The microtitre plates were washed five times after each incubation step with PBST. In order to minimise non-specific binding, the wells were blocked with 200 μL 0.1% ovalbumin in PBS at 37 °C for 1 h. After the washing step, 50 μL anti-aflatoxin B_1 monoclonal antibody and 50 μL AFB_1 standards (0.05–10 ng ml^{-1}) or feed extracts were added and incubated at 25 °C for 1 h. Following a washing step, 100 μL horseradish peroxidase labelled goat anti-mouse IgG were added and incubated at 25 °C for 1 h. The microplates were washed again, and 100 μL substrate solution (3,3′,5,5′-tetramethylbenzidine/H_2O_2) were added. After 20 min the reaction was stopped by adding 50 μL 1 M H_2SO_4. The absorbance was measured at 450 nm in an ELISA microplate reader (ELX800; Bio-Tek Instruments, Winooski, VT). The average absorbance was calculated from the individual absorbances obtained from triplicate wells and the results were expressed as percentage of binding:

$$\text{Binding}\,(\%) = (A^+/A^-) \times 100$$

where A^+ is the mean absorbance in the presence of the aflatoxin standard or feed extract sample and A^- is the mean absorbance in their absence.

An in-house validation was applied, and the parameters evaluated were linearity, detection limit (*LOD*), quantification limit (*LOQ*), accuracy, precision and specificity. The linearity was assessed according to the linear regression analysis of seven calibration curves of the AFB_1 standard at concentrations ranging from 0.05 to 10.0 ng mL^{-1} (INMETRO, 2007). The *LOD* and *LOQ* were calculated, respectively, as 3-fold and 5-fold the standard deviation of absorbance from three replicate wells of unspiked samples of each matrix analysed on seven different days (INMETRO, 2007). A method blank was prepared in order to verify that none of the solvents, reagents, or instrumentation added any detectable positive biases to the toxin concentrations.

Accuracy and precision (repeatability and intermediate precision) were based on relative standard deviations (RSD%) of the aflatoxin recovery tests. Feed samples intended for broilers and laying hens with non-detectable aflatoxin levels by HPLC were artificially spiked with aflatoxins at concentrations of 10, 25, 50 and 100 ng g^{-1} (sum of the four analogues) and were maintained at 4 °C for 18 h before the extractions. Accuracy was assessed by aflatoxin recovery from two determinations (two extractions) in triplicate. Repeatability was evaluated by one determination (one extraction) of each concentration analysed in seven replicates on the same day, while for intermediate precision three determinations were performed by different analysts and on three different days (INMETRO, 2007).

The method specificity was evaluated by the interference of each matrix, analysing samples without contamination (INMETRO, 2007). The matrix interferences of feed intended for broilers and laying hens were analysed by testing 10-fold to 500-fold dilutions. Additionally, matrix interference was determined by comparing a standard curve prepared in PBS with a calibration curve added with a blank of the sample extract.

2.4. Aflatoxin determination by HPLC

2.4.1. Extraction and clean-up

Aflatoxin extraction and clean-up were carried out using Afla-Test® immuno-affinity columns (Vicam; Waters, Milford, MA), according to manufacturer's instructions for feed samples. An

aliquot of feed sample (20 g) added to 2 g NaCl was shaken at 150 rpm for 10 min with 40 mL methanol:water (80:20, v/v). The extract was filtered through Whatman No. 1 filter paper. The filtrate (10 mL) was diluted with 40 mL ultra-pure water and then filtered through a glass microfibre filter. Then, a 10-mL aliquot was applied to an immuno-affinity column at a flow rate of 1–2 drops/s. The column was washed twice with 10 mL ultra-pure water. Finally, aflatoxins were eluted with 1 mL methanol. The eluate was evaporated to dryness under a stream of nitrogen at 45 °C.

2.4.2. HPLC analysis

The aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) were analysed according to Miyamoto, Hamada, and Kawamura (2008). The dried samples were derivatised with 100 μL trifluoroacetic acid (TFA), mixed for 30 s, sonicated for 5 min and incubated at 25 °C for 15 min in the dark. Then 900 μL acetonitrile:water (1:9, v/v) were added, mixed for 15 s and an aliquot (20 μL) was injected into the HPLC.

The aflatoxins were analysed by a reversed-phase isocratic HPLC system (Shimadzu LC-10 AD pump and RF-10A XL fluorescence detector; Shimazu, Kyoto, Japan), using a C-18 Luna Phenomenex column (250 × 4.6 mm, 5 μm; Scharlau, Barcelona, Spain). Excitation and emission wavelengths were 365 and 450 nm, respectively. The mobile phase was $CH_3CN:H_2O$ (25:75, v/v) and the flow rate was 1.2 mL/min.

The detection and quantification limits were defined as three and five times, respectively, the area standard deviation of seven spiked standards at lower concentration (2 ng g^{-1}) detectable by HPLC on different days. Detection limits for HPLC were 0.13 ng g^{-1} (AFB_1), 0.03 ng g^{-1} (AFG_1), 0.59 ng g^{-1} (AFB_2) and 0.22 ng g^{-1} (AFG_2). The LOQs were 0.32 ng g^{-1} (AFB_1), 0.15 ng g^{-1} (AFG_1), 1.09 ng g^{-1} (AFB_2) and 0.48 ng g^{-1} (AFG_2).

The method accuracy was evaluated by the aflatoxin recovery rates from feed samples artificially spiked with aflatoxins at concentrations of 10, 25, 50 and 100 ng g^{-1} (sum of the four analogues) obtained from two determinations of each concentration (INMETRO, 2007). Recovery rates for total aflatoxins ranged from 84% to 109% with mean of 102% (RSD 0.29–13.96%) while the mean recovery rates for each analogue spiked at concentrations from 2.5 to 25 ng g^{-1} were 96% for AFB_1, 113% for AFG_1, 92% for AFB_2 and 102% for AFG_2 (mean CV 12.1%).

2.5. Correlation analysis of ic-ELISA and HPLC

The aflatoxin levels of positive samples detected by ic-ELISA and HPLC were compared using the Pearson correlation test (software Statistica 7.0, Tulsa, OK).

3. Results and discussion

In this study an ic-ELISA based on a monoclonal antibody was standardised for aflatoxin detection in naturally contaminated poultry feed samples. The optimised coating AFB_1-BSA concentration, anti-AF mAb and anti-IgGHRP were 250 ng mL^{-1}, 1:10,000 (corresponding to 173 ng mL^{-1} protein concentration), and 1:7000, respectively. Intra-laboratory validation of the ic-ELISA was based on the following parameters: linearity, detection limit (*LOD*), quantification limit (*LOQ*), precision, specificity and accuracy.

Fig. 1 shows the ic-ELISA standard curve for aflatoxins (0.05–10.0 ng mL^{-1}) and the linear regression analysis. A linear range was obtained between 0.05 and 10.0 ng mL^{-1} with a good coefficient of determination ($r^2 = 0.994$). The coefficient of linear correlation ($r = 0.997$) was higher than the minimal acceptable ($r = 0.99$) (INMETRO, 2007). The *LOD* and *LOQ* were, respectively, 0.036 and 0.041 ng mL^{-1}, corresponding to 1.25 and 1.43 ng g^{-1} for feed intended for broilers and 0.040 and 0.050 ng mL^{-1} (1.41 and 1.75 ng g^{-1}) for feed intended for laying hens. These results were similar to those reported by Li, Zhang, Zhang, Zhang et al. (2009) but lower than those reported by Zheng et al. (2005). Li, Zhang, Zhang, Zhang et al. (2009) obtained an *LOD* of 0.06–0.09 ng mL^{-1} for a monoclonal antibody-based ELISA developed for peanuts. Zheng et al. (2005) evaluated an ELISA AgraQuant® for total aflatoxin detection in cereals and derivatives and obtained a *LOD* of 2.5 ng g^{-1} for corn.

The precision of the ic-ELISA method was evaluated for repeatability and intermediate precision in terms of relative standard deviations (RSD) calculated as percentages (Table 1). The repeatability and intermediate precision showed RSDs ranging, respectively, from 4.26% to 10.13% (mean = 8.03%) and 9.95% to 13.63% (mean = 11.58%) for broiler feed and from 5.92% to 8.47% (mean = 6.94%) and 7.28% to 15.96% (mean = 12.49%) for laying hen feed. All the results were below the RSD values recommended by the Commission of the European Communities (2006), i.e., ⩽10.56% for repeatability and ⩽16% for intermediate precision for analysis at ng g^{-1} concentrations.

Specificity was evaluated by the interference of each matrix without contamination. Matrix interferences in ic-ELISA were tested using feed samples intended for broilers and laying hens with non-detectable aflatoxin levels by HPLC. Matrix interferences in ic-ELISA can result from non-specific interaction caused by protein, pigments, fat and solvents, or steric hindrance, which would overestimate the real toxin level. The matrix effect could be minimised by sample dilution prior to the ELISA method (Ono, Kawamura, Ono, Ueno, & Hirooka, 2000). For the two types of feeds, 30-fold and 40-fold dilutions showed lower percentages of matrix interference. In addition, a calibration curve added by a blank of the sample extract (diluted 35-fold) was compared to a standard curve prepared in PBS: methanol (9:1) and the two curves were superimposed, indicating that the matrix effect was minimised (Zhang, Wang, Fang, Wang, & Fang, 2009). Taking into account that 35-fold is an intermediate dilution between 30-fold and 40-fold and that in commercial ELISA tests 35-fold dilutions are used, this dilution was selected for the standardised ic-ELISA in this study.

Table 2 shows the aflatoxin recovery rates from feed intended for broilers and laying hens. In the laying hen feed, aflatoxin recovery by ic-ELISA ranged from 98% to 103% (mean = 102%; RSD 6.21%–11.90%) and from 90% to 107% (mean 98%; RSD 3.47%–

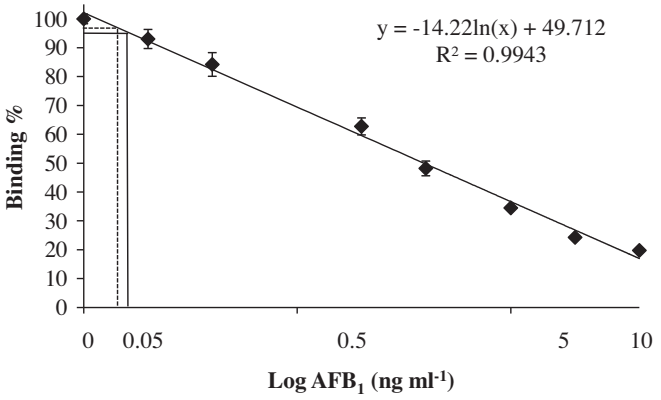

Fig. 1. Standard curve for AFB_1 detection by ic-ELISA. Circles represent the mean binding from seven standard curves performed on different days. Bars represent standard deviations. The detection limit was 0.036 ng mL^{-1} (1.25 ng g^{-1}) for feed intended for broilers (dashed line) and 0.040 ng mL^{-1} (1.41 ng g^{-1}) for feed intended for laying hens (continuous line), corresponding to the minimum concentration over 3 and 5% inhibition, respectively (mean minus 3-fold SD of 0 ng ml–1 AFB1) detected by ic-ELISA.

Table 1
Repeatability and intermediate precision of ic-ELISA for aflatoxin determination in laying hen feed and broiler feed.

Feed	Total AF (ng g^{-1})	Recovery					
		Repeatability			Intermediate precision		
		Mean ± SD	RSD_r (%)	RSD_r mean (%)	Mean ± SD	RSD_R (%)	RSD_R mean (%)
Broiler	10	10.2 ± 0.4	4.26	8.03	10.2 ± 1.1	10.46	11.6
	25	24.3 ± 2.3	9.55		25.3 ± 3.4	13.63	
	50	51.2 ± 5.2	10.1		50.6 ± 6.2	12.29	
	100	104.4 ± 8.5	8.19		102.5 ± 10.2	9.95	
Laying hen	10	10.2 ± 0.9	8.47	6.94	10.6 ± 1.7	15.96	12.5
	25	25.5 ± 1.9	7.36		26.0 ± 2.4	9.02	
	50	48.6 ± 2.9	5.92		49.6 ± 3.6	7.28	
	100	97.5 ± 5.9	6.01		98.9 ± 10.4	10.47	

RSD: relative standard deviation.
Recommended values according to the Commission of the European Communities (2006): RSD_r = 10.56% (0.66 times precision RSD_R at the concentration of interest); RSD_R = 16% calculated by the formula $RSD_R = 2^{(1-0.5\log C)}$ (C = 0.000001 ng g^{-1}) derived from Howirtz equation.

Table 2
Accuracy of ic-ELISA evaluated by total aflatoxin recovery from feed samples intended for broilers and laying hens.

Feed	Total aflatoxin added (ng g^{-1})	Recovery (%)[a]	RSD[b]	Mean ± SD
Laying hen	10	103 ± 12.2	11.90	102 ± 2.6
	25	98 ± 8.5	8.65	
	50	103 ± 6.4	6.21	
	100	103 ± 7.2	7.01	
Broiler	10	97 ± 14.3	14.79	98 ± 7.0
	25	90 ± 7.9	8.75	
	50	107 ± 14.5	13.50	
	100	99 ± 3.4	3.47	

Critical values for AFB_1, AFG_1, AFB_2 and AFG_2 recovery (%) according to the Commission of the European Communities (2006): 1–10 ng g^{-1} = 70–110%; >10 ng g^{-1} = 80–110%.
[a] Each result represents the mean ± SD of six determinations (duplicate spiking and triplicate analysis).
[b] Relative standard deviation (RSD): calculated as standard deviation/mean × 100.

14.79%) for the broiler feed. These results (Table 2) were similar to those reported for AFB_1 recovery (94–113%) from rice samples spiked with 10–500 ng g^{-1} (Kolosova et al., 2006), but higher than aflatoxin recovery (87.5%) in peanut spiked with 4.0 ng mL^{-1} total aflatoxins (Li, Zhang, Zhang, Zhang et al., 2009).

According to the Commission of the European Communities (2006), the critical values for recovery of AFB_1, AFG_1, AFB_2 and AFG_2 are 70–110%, for concentrations between 1 and 10 ng g^{-1}, and 80–110% for concentrations higher than 10 ng g^{-1}. Therefore, the standardised ic-ELISA showed adequate accuracy because the recovery rates remained within the recommended values.

Table 3 shows the aflatoxin levels in feed samples intended for broilers (n = 34) and intended for laying hens (n = 36) analysed by ic-ELISA and HPLC. The analysis of broiler feeds (n = 34) by HPLC detected AFB_1 in 88.2% and AFB_2 in 26.5% of samples. AFG_1 and AFG_2 were not detected in any sample. Considering total aflatoxins, 88.2% samples were contaminated at levels ranging from 0.79 to 60.80 ng g^{-1} (mean = 8.41 ng g^{-1}) by HPLC, while analysis carried out by ic-ELISA detected 88.2% aflatoxin-positive samples at levels ranging from 2.20 to 60.45 ng g^{-1} (mean = 10.48 ng g^{-1}). Aflatoxins were not detected by ic-ELISA in four samples, but were detected by HPLC, probably because the levels were close to the *LOD* of ic-ELISA. However, aflatoxins were not detected by HPLC in four samples but were detected by ic-ELISA. The linear coefficient of correlation (r) was 0.97 between HPLC and ic-ELISA (Fig. 2).

The laying hen feeds (n = 36) assessed by ic-ELISA showed aflatoxin contamination in 92% samples at levels ranging from 2.90 to 96.80 ng g^{-1} (mean = 20.83 ng g^{-1}). HPLC analysis detected AFB_1 in 89.7%, AFB_2 in 35.9% and AFG_1 in 2.6% samples. Regarding total aflatoxins, 92% samples were contaminated at levels ranging from 1.03 to 91.04 ng g^{-1} (mean = 19.75 ng g^{-1}) by HPLC. Aflatoxins were not detected by either method in one sample. Aflatoxins were not detected by HPLC in one sample but were detected by ic-ELISA. The linear coefficient of correlation (r) was 0.98 between HPLC and ic-ELISA (Fig. 2). These differences were probably due to the use of an immuno-affinity column for clean-up prior to HPLC analysis, which could minimise matrix interferences (Krska et al., 2008). However, high correlation coefficients were obtained regardless of the method or the type of feed. Zheng et al. (2005) reported a similar coefficient of correlation (0.95) between AgraQuant® ELISA and HPLC for corn matrix.

The ic-ELISA/HPLC ratio for feed samples intended for broilers and laying hens ranged from 0.65 to 3.69 and from 0.49 to 4.27, respectively, but most of samples (52.9% and 61.1%) showed ratios between 0.81 and 1.8 (Table 4). The overestimation of mycotoxin levels by immunoassay has been reported previously (Chinaphuti, Trikarunasawat, Wongurai, & Kositcharoenkul, 2002; Zheng et al., 2005). In immunoassays, the sample matrix may contain compounds with similar chemical groups which could also bind to the antibodies and can lead to underestimation or overestimation of the mycotoxin concentrations in commodity samples (Zheng et al., 2005).

In Brazil, the maximum allowed limit for aflatoxins in any product intended for animal feeding is 50 ng g^{-1} (sum of the four analogues) (Brasil, 1988). For mature poultry, 100 ng g^{-1} (sum of the

Table 3
Natural aflatoxin occurrence in feed samples intended for broilers and laying hens evaluated by ic-ELISA and HPLC.

Feed	n	Aflatoxins (ng g^{-1})					
		ic-ELISA			HPLC		
		Positive samples (%)	Mean	Range	Positive samples (%)	Mean	Range
Broiler	34	30 (88.2)	10.48	2.20–60.45	30 (88.2)	8.41	0.79–60.80
Laying hen	36	33 (92.0)	20.83	2.90–96.80	33 (92.0)	19.75	1.03–91.04

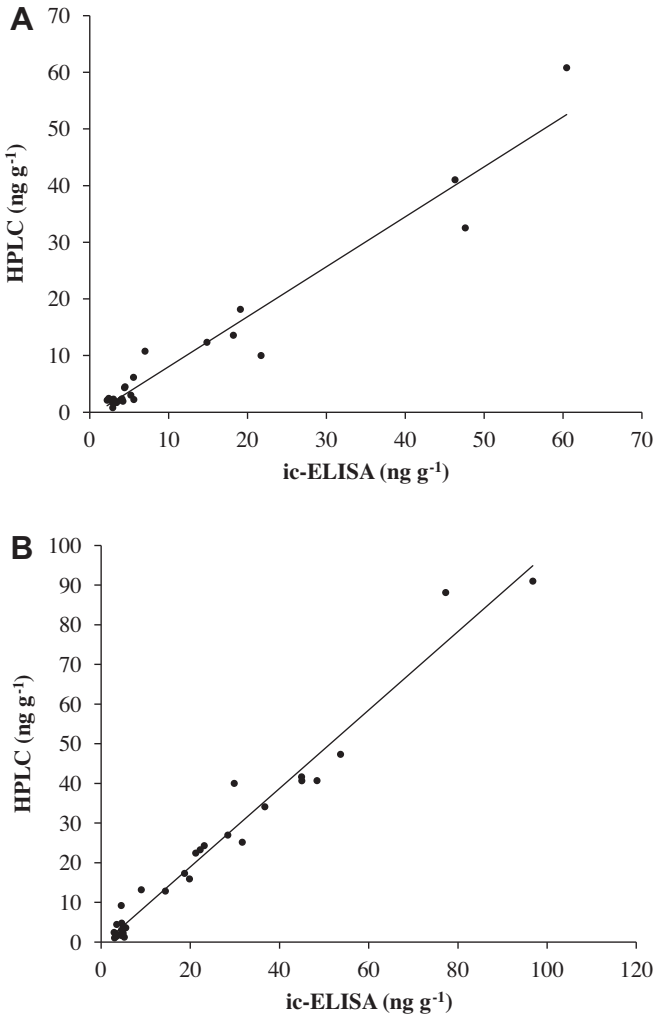

Fig. 2. Correlation between ic-ELISA and HPLC data for aflatoxin determination in naturally contaminated feed samples intended for broilers (A, $n = 34$) and for laying hens (B, $n = 36$). The linear regression equation $y = -0.791 + 0.8827x$ was obtained with correlation of 0.97 (broiler feed) and $y = -0.824 + 0.9892x$ with correlation of 0.98 (laying hen feed); x and y represent aflatoxin concentration determined independently by ic-ELISA and HPLC.

Table 4
ic-ELISA/HPLC ratio in feed intended for broilers ($n = 34$) and laying hens ($n = 36$) from Northern Paraná State.

ic-ELISA/HPLC ratio	Broiler feed *n* (%)	Laying hen feed *n* (%)
ND–ND		2 (5.56)
ND–D	4 (11.77)	1 (2.78)
D–ND	4 (11.77)	1 (2.78)
0.40–0.6	–	1 (2.78)
0.60–0.8	1 (2.94)	3 (8.33)
0.81–1.0	4 (11.77)	5 (13.89)
1.01–1.2	7 (20.59)	10 (27.78)
1.21–1.4	2 (5.88)	3 (8.33)
1.41–1.6	1 (2.94)	3 (8.33)
1.61–1.8	4 (10.26)	1 (2.78)
1.81–2.0	3 (8.82)	–
2.01–2.2	2 (5.88)	1 (2.78)
2.21–2.41	–	2 (5.56)
2.5–2.92	1 (2.94)	2 (5.56)
3.7–4.3	1 (2.94)	1 (2.78)

n = number of samples.
D = detected ($\geqslant LOD$).
ND = not detected (less than *LOD*).

four analogues) are the maximum allowed level in the United States of America (USA) while in European Union (EU) it is 20 ng g^{-1} (AFB_1) (FAO, 2004). Therefore, in the present study 86.7% and 93.3% (ic-ELISA) and 90% and 96.7% (HPLC analysis) of the feed samples intended for broilers showed aflatoxin levels below the maximum allowed levels in the EU and Brazil. For laying hen feed samples these rates were 60.6% and 90.9% (ic-ELISA) and 60.6% and 93.9% (HPLC analysis), respectively. No sample of either feed type showed levels above those permitted in the USA legislation.

The standardised ic-ELISA showed linearity, precision, accuracy, high sensitivity and high correlation coefficient with HPLC, indicating its potential for aflatoxin screening in poultry feed samples, with advantages such as simplicity, reduction of organic solvents and analysis of a large number of samples which reduces the cost of analysis.

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