A Direct Competitive Immunoassay for Fumonisins Detection in Maize Feed

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Abstract

The development of a direct competitive immunoassay formats for the Enzyme-linked immunosorbent assay (ELISA) method was undertaken for fumonisins (Fms) determination in feed**.** The use of polyclonal antibody was conducted on the microtitre plate for a rapid and sensitive detection. The direct format assay was based on the competition between Fms labelled with houseradish peroxidase (HRP) and unlabelled Fms for binding sites first of immobilised antibody (AbFms) followed by precoating of secondary anti-antibody IgG (Anti-IgG). A spectrophotometric assay was developed as a first step procedure for optimization of concentrations and conditions using micro-titre plates. Optimal parameters established for direct assay were 20 µg mL^{-1} of anti-IgG, 1:50 dilution of AbFms and 1:5 dilution of Fumonisins-HRP. The ELISA exhibited detection limit of 100 $\mu g \cdot L^{-1}$ fumonisins with a dynamic range from 100 - 2000 $\mu g \cdot L^{-1}$. The achieved detection range for Fms was within the required legislative limit of analyses. Samples analysis involved the rapid extraction (without clean-up) and pre-treatment using solid phase extraction (clean-up) before measuring using the developed ELISA. The results achieved were average of 97.6 % (clean-up extraction) and 77.9 % (without cleanup). The developed ELISA had a satisfactory agreement with HPLC (Confirmatory method). It also showed very high sensitivity and a potential method for a rapid, simple and sensitive detection of Fms in maize feed.

Keywords: **ELISA, direct competitive, anti-fumonisin antibody, mycotoxin, HPLC.**

Introduction

Fumonisins were first isolated from a culture of *Fumonisins verticillioides* (Gelderblom *et al.*, 1988). The isolation and characterisation of fumonisin came after active research into the causal agents for equine leukoencephalomalacia (ELEM), a syndrome characterised by liquefactive necrotic lesions in horse (Marasas, 2001), and human oesophageal cancer in some populations in the Transkei region of South Africa.

Fumonisins are categorised into A, B, C and P series of main groups (Wang *et al.*, 2006). The B-series of fumonisins (FmBs) are the most abundant toxic compounds produced mainly by *F. verticillioides* and *F. proliferatum* (Dutton, 1996). The major compounds of fumonisin are Fumonisin B_1 (FmB_1) and Fumonisin B_2 (FmB_2). The most abundant and toxic is FmB_1 followed by FmB_2.

These toxins are natural contaminants of cereal grains worldwide and are mostly found in maize and its products. Fumonisin-contaminated grains have been linked to various diseases: liver and kidney toxicity and carcinogenicity, pulmonary oedema, immunosuppression and neurotoxicity (Pagliuca *et al.*, 2005). It is also linked to the risk effect of oesophageal cancer in humans and possibly connected with neural tube defects (NTD) in the

Transkei region of South Africa, as well as in China and South Texas, USA (Shephard, 1996; Lino *et al*., 2006). According to the International Agency for Research on Cancer (IARC), FmB_1 is classified as a potential carcinogen (probable human carcinogen) (IARC, 2002).

Fumonisin (Fms) is a mycotoxin that is difficult to eliminate from the food chain. Normally, a program of monitoring the surveillance of fumonisin level is therefore necessary to ensure safe exposure, especially in the food chain. Analytical techniques for fumonisin detection are typically by chromatographic methods. This includes thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and post-hydrolysis gas chromatography, and these usually involve extraction procedures and clean-up processes (Sydenham and Shephard, 1996; Shephard, 1998). However, analyses by these methods are a difficult task and require a lengthy sample clean up especially when there are only trace amounts of fumonisins in matrix samples. Working with some chromatography methods also needs a form of chemical derivatization before detection is possible, with the exception of mass spectrometry.

The development of rapid and reliable methods must become a priority to comply with the level requirement. Because of this necessity, a number of fumonisin antibodies have been developed to allow for a faster analysis by immunoassay techniques. Immunoassay methods have been developed using polyclonal and monoclonal antibodies for fumonisin detection because of their adaptability, simplicity and selectivity (Wang *et al*., 2006). This method has been proven to be a sensitive analytical tool, obtaining low detection limits and offering reduced instrumentation costs.

In this study, the development of direct immunoassay using spectrophotometric method for fumonisin detection was investigated. The study described the immobilization of biological molecules (antibody receptors) on the solid surface by optimisation of ELISA test through checkerboard method on microwell plate was first investigated. The assay format was a direct system, using first immobilisation by precoating of anti-IgG before coating with anti-fumonisin antibody (polyclonal antibody) followed by competition of free fumonisins and HRP conjugated fumonisins.

The ELISA methods of analysis address two analytical concepts. First, the antibodies used as a recognition element are highly specific to their antigen (analyte). Secondly, the assay format will produce a more sensitive result for the analyte of interest. This is an important technique for optimisation and validation of the commercially available reagents on the microtitre plate. In this work indirect competitive assay format of test was examined. Optimisation of the assay was accomplished by changing the concentrations (reagents) and assay conditions (times and temperature), as well as other experimental parameters such as blocking agents, selection and washing reagents. This process was for the purpose of achieving maximum response for optimum assay performance which meant highly specific binding of the antibody-antigen and a higher signal:noise ratio.

In this work, ELISA tests were developed using a direct immunoassay format and commercially available reagents based on achieving lower detection limits, a wider dynamic range, and a higher signal:noise ratio and sensitivity. For the development of Fms ELISA tests, a monoclonal antibody against Fms (AbFms) was used. The direct format was carried out

through the competition between free Fms and Fumonisins label with HRP (Fms-HRP) for the binding sites of AbFms before binding to the immobilised anti-antibody IgG (Micheli *et al.,* 2005). The last stage was introducing the enzyme substrate to react with the enzyme labelled (indicating the presence of antigens) for the colour development. The absorbance reading was inversely proportional to the concentration of the toxin.

Materials and Methods

Chemicals and reagents

The polyclonal antibody against fumonisin (AbFms) was purchased from Abcam (Cambridge, UK). Standard solution of fumonisins, fumonisin labelled with HRP and chromogen/substrate was supplied from Veratox kit, Neogen Corporation. Affinity purified anti-antibody unconjugated, anti-mouse IgG (H+L) from goat for the direct format was obtained from Pierce (U.K) Ltd. Fumonisins standard solution (Fumonisin B_1 and Fumonisin B_2) were purchased from Sigma-Aldrich Co. Ltd (Gillingham, United Kingdom (UK). Other reagents – polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). TMB solution was purchased from Insight Biotechnology (UK).

Buffer Solutions

A 0.1 M carbonate buffer, pH 9.6 was used for the immobilization of anti-IgG unconjugated (precoating) on microplates. A 0.01 M phosphate buffered saline (PBS), pH 7.4, used for preparation of MAbFms standard, blocking solution, dilution of antibodies and washing solution were purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). The washing solution was prepared by adding 0.05% Tween-20 (v/v) to the PBS (PBS-T). The fumonisins standard solution was prepared by diluting the stock solution (in methanol) with PBS.

Apparatus

The micro well polystyrene plates, MaxiSorp (Nunc Immuno) was purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). The spectrophotometric analysis for colour ELISA development was performed by a BMG Fluorstar galaxy ELISA plate reader (Aylesbury, Fisher Scientific (Loughborough, UK).). Incubations for every step of the reactions were carried out by Labsystem iEMS incubator/shaker HT.

Preparation of stock solutions

Stock solutions of reagents for the immunoassay study of fumonisins analysis were prepared as listed in Table 1.

Table 1: Preparation of stock solutions of reagents for immunoassay study of fumonisins analysis.

Reagents	Stock solution concentration	Preparation
Goat anti-mouse (H+L)	100 µg mL^{-1}	25 µl in 475 µl of 0.1 M carbonate buffer IgG pH 9.6
Anti-fumonisin (AbFms)	1:5 dilution	100 µl of in 400 µl of 0.01 M PBS pH antibody 7.4
Free Fumonisins	6 µg mL^{-1} (ppm).	Standard solution was diluted in 0.01 M PBS pH 7.4.
Fumonisin-HRP conjugated	1:2.5 dilution	50 µl of in 75 µl of 0.01 M PBS pH 7.4

Preparation of standard solution

The Fms standard solution from Sigma was dissolved in methanol (HPLC grade) and stored at -18℃ in a tightly capped and dark bottle. A 6 µg mL^{-1} of stock solution of Fms was diluted to the concentration range of 0-3000 µg L^{-1} in 0.01 M PBS, pH 7.4 for a calibration standard of ELISA and immunosensor test.

Optimisation by ELISA Procedure (checkerboard method)

ELISA procedures were carried out first to evaluate and optimise the AbFms coating range (1:25 to 1:5000 dilutions) and commercial Fms-HRP (1 to 1:50 dilutions) using the checkerboard method. The tests were performed in a 96-microwell plate in accordance with a direct ELISA format based on the method described by Anna *et al*. (2005). The first antibody coating and binding sites reagents were incubated for 2 h at 37 ℃. The optimisation of precoating immobilization using goat anti mouse IgG (H+L) (IgG) was also investigated **(**Figure 1). Various concentrations of reagent from 0 to 50 µg mL^{-1} were immobilised for 18 h at 4℃ in the microwell plate before coating with optimal AbFms concentration (2 h at 37℃) and labelling by optimal Fms-HRP concentration.

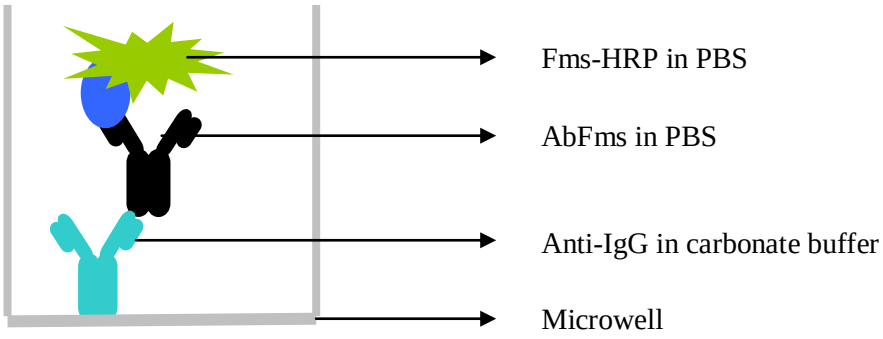


Figure 1: Precoating and coating step of capture antibody in direct immunoassay format using goat anti mouse IgG (H+L) and MAbFms, respectively.

Calibration curve of fumonisin developed by ELISA

Experiments were based on a competition between Fms-HRP and free Fms in a sample or standard for the binding sites of antibodies immobilised on the pre-coated well. The optimal concentration of reagents and conditions were used in producing a standard curve of Fms ranging from 0 to 3000 µg L^{-1}. The assay was done by following the procedure in Figure 2.

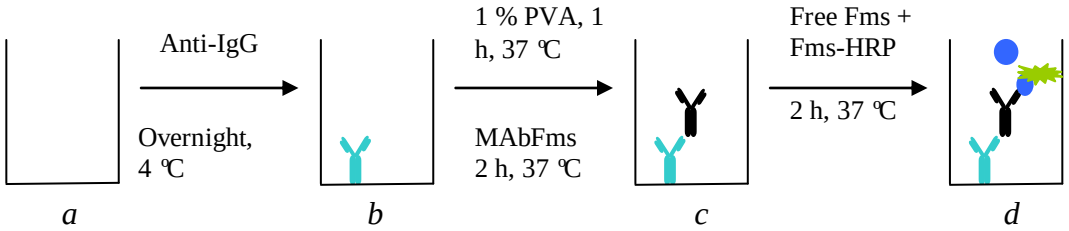


Figure 2: Schematic diagram of ELISA system for calibration curve of fumonisins detection until step (d).

The competitive assay was carried out using the following procedure: The microtiter Plate was pre-coated with anti-mouse IgG antibody (20 µg mL^{-1}, 50 µL/well) in 0.1 M carbonate buffer pH 9.6, for 18 h (overnight) at 4℃, followed by washing twice with 150µl/well phosphate buffered saline containing Tween 20 (PBS-T) and once with PBS alone. The plate was then blocked with 1% PVA (50 µL/well) and incubated for 1 h at 37 ℃. After washing as above, the plate was coated with anti-Fms monoclonal antibody (1:50 dilution, 50 µL/well) in PBS for 2 h incubation at 37 ℃, followed by washing. The competition solution was prepared by mixing 50 µL/well of free fumonisin B_1 (0–3000 µg L^{-1}) in PBS with fixed dilution of a solution of fumonisin-HRP conjugate (1:5 dilution) in PBS. The competition reaction was allowed to proceed for 30 min at 37 ℃ and then rinsed with PBS-T (twice), followed by PBS (once). Finally, the absorbance was measured by the addition of TMB substrate solution (50 µL/well) to each well and incubated at room temperature for 15 min before adding a 25 µL of stop reagent (H_2SO_4) and then measuring at 650 nm using the plate reader.

Maize feed samples analysis

Dried maize feed samples were collected from the United Kingdom and Malaysia. Samples were then ground using a food processor or blender at medium speed for 3-5 min until all the maize were blended. Samples were then packed properly in plastic bags and stored in a refridgerator (0-4℃) before use (to avoid contamination). For the spiked sample, a fumonisins solution (Neogen Kit) was spiked on the maize samples at concentrations of 50, 250, 500 and 2500 µg kg^{-1}. The mixture was manually shaken for 30 sec and then kept overnight to obtain a homogeneous mixture.

Extraction and immunoassay analysis without clean-up

Sample preparation and extraction were conducted following the procedure described by the Veratox ELISA kit (Neogen Corporation, UK). The samples or spiked samples of five g of ground maize was used, mixed with 25 mL of 70% methanol and 30% water and then shaken vigorously for 3 min. The extract was filtered (at least 5 mL) through a Whatman

#1 filter paper to remove the solid material and the filtrate was then collected and diluted with 1:10 of 0.01 M PBS pH 7.4 for analysis without further preparation. The assay procedure for immunoassay analysis was done by following the procedure in Figure 2.

Clean-up procedure and immunoassay analysis

For this procedure, a sample extraction was conducted similar to the procedure described above. Sample filtrates were collected and cleaned using C-18 SPE (Waters, Milford, MA) following the procedure accompanying the SPE columns. The C-18 SPE column was first conditioned by sequentially passing 5 ml methanol and 5 mL water through the column. A 4 mL of sample filtrate was then passed through the column, followed by 6.0 mL of deionised water. The fumonisin was then eluted from the SPE column by rinsing with 2.0 mL of methanol:water (70:30). The sample eluted was then collected and diluted with 1:10 of 0.01 M PBS pH 7.4 for analysis. The assay procedure for immunoassay analysis was done by following the procedure in Figure 2.

HPLC detection

The sample eluted was dried under nitrogen stream, and re-dissolved with 2 mL of acetonitril /water (1:1). Pre column derivatisation was first carried out before samples were then injected into the HPLC column. The process of derivatisation was started by transferring about 25 µl of samples or fumonisins standard in the small test tubes before the addition of 225 µl OPA reagent (OPA = 40 mg OPA in ml methanol, and diluting with 5 ml 0.1M disodium tetraborate solution (3.8g in 100 ml H_2O and 50 µl 2-mercaptoethanol). About 10 µl of the sample was then injected into the LC system within 1 min after the addition of the OPA reagent.

HPLC system

HPLC parameters for Fms measurement are described below using the isocratic system:

Mobile phase:
Methanol/0.1M sodium dihydrogen phosphate solution (15.6 g in 1 L H_2O) (77+23) and adjust pH to pH 3.3 using phosphoric acid.

Column:
C18 (Nucleosil 100 5 µm 15 x 0.46 cm)

Flow rate: 1.0 ml/min

Detector: Fluorescence detector (335 nm excitation and 440 nm emission)

Each experiment was carried out in triplicate and each value was the mean of three determinations.

Results and Discussion

The development of an immunoasssayr for Fumonisin detection was performed based on the direct immunoassay on the microtitre plate. Optimisation of the direct immunoreagent using the ELISA system with spectrophotometric detection was conducted. All the reagents used in this experiment were from a commercial supplier and some from the Veratox ELISA kit. A monoclonal mouse anti-fumonisins (AbFms) was chosen for the detection of total fumonisins in the sample. While Fumonisins labelled HRP (Fms-HRP) for enzyme reaction was selected from the Veratox kit (kit for total fumonisins) suitable for measuring total fumonisins. Our

study here was focused on the detection of total fumonisins in buffer and maize sample. The optimisation test was carried out by the checkerboard technique in a Nunc 96-well microplate based on the method described by Anna *et al*. (2005).

Optimisation of reagent by ELISA method

A checkerboard titration is a single experiment in which the concentration of two components is varied in a way that will result in a pattern. As shown in Figure 3 the anti-fumonisins antibody (AbFms) was serially diluted across the plate and the HRP-labelled fumonisin (Fms-HRP) was serially diluted down the plate. After adding the substrate, the colour was developed by the reaction of HRP. A high absorbance reading was obtained at range dilutions of 1:25 to 1:50 of MAbFms and dilutions of 1:1 to 1:10 of Fms-HRP, indicating high specificity of binding for the combination of both reagents. From this checkerboard matrix, the following curves were generated (Figure 4).

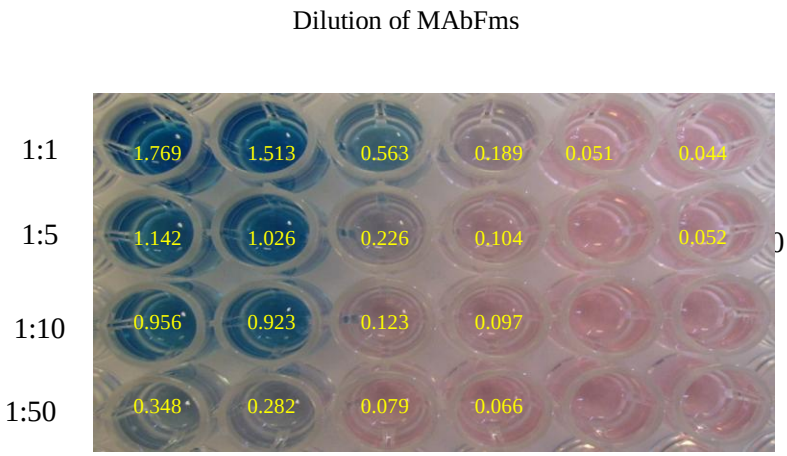


Figure 3: Checkerboard test for optimisation of two components: MAbFms and Fms-HRP. The yellow numbers represent the absorbance reading at 650 nm.

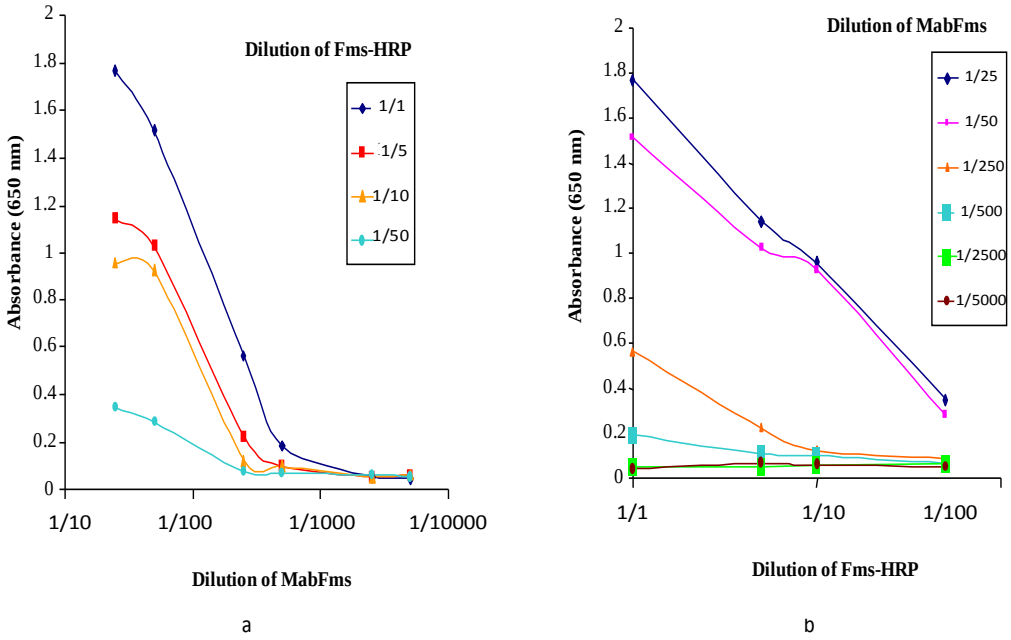


Figure 4: Optimisation of plate coating by a) AbFms and b) Fms-HRP dilution in the direct non-competitive checkerboard ELISA. Plates were coated with variable dilutions of both reagents.

As shown in Figure 4, increasing the combination of the AbFms and Fms-HRP concentration increased the absorbance reading (high specific binding). However, a high combination of concentration may also provide a high non specific binding that produces high background reading (less sensitivity) (Sadana and Chen, 1996). The maximum reading of 1.8 OD showed high affinity binding of Fms-HRP to MAbFms site on the well. However, the optimal specific binding by 1.02 OD with affinity concentration 1:50 and 1:5 dilution of MAbFms and Fms-HRP respectively, was selected. So, in this case, the absorbance reading was still obtained with a high specific binding at 1:5 of Fms-HRP and 1:50 of MAbFms and was chosen for the assay.

The use of anti-IgG to pre-coat solid face surfaces before immobilising the anti-capture antibody has been reported to increase the detection limit of aflatoxin M_1 (Micheli *et al*, 2005). Therefore, to maximize the ELISA signal, the use of pre-coated microtitre wells was investigated in this study. Then, the concentrations of reagents of an anti-mouse IgG antibody (IgG) were placed on the wells. The fixed amount of AbFms (1:50 dilution) and Fms-HRP (1:5 dilution) were used for the optimizing of the pre-coating reagent. Figure 5 shows the absorbance reading of the precoated IgG treated on the well. The signal was increased with the increasing of the anti-mouse IgG antibody (IgG) coated on the well, while a dramatic increase was achieved at a range between 1 to 20 µg mL^{-1}. A range from 5 to 20 µg mL^{-1} concentrations is suitable for assay development, and therefore 20 µg mL^{-1} was chosen for further experiments.

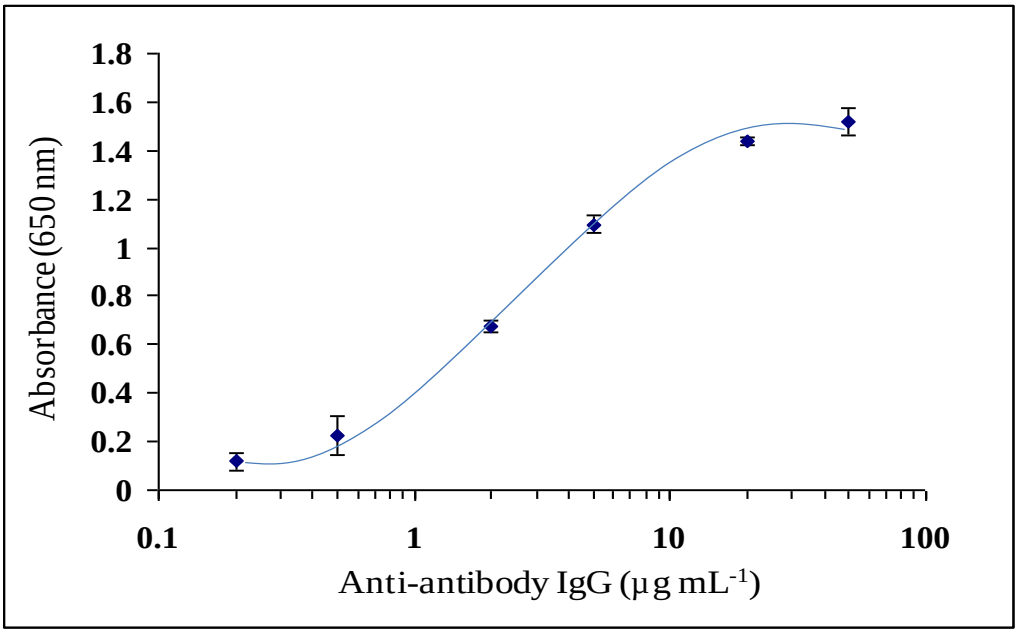


Figure 5: Optimisation of anti-antibody IgG (anti-IgG) concentration in a direct non competitive format. Plates were pre-coated by a variable concentration (0 - 50 µg/mL) of anti-IgG followed by coated 1:50 of AbFms and 1:5 of Fms-HRP. Error bar =SD, n=3.

The results obtained (Figure 6) show that the use of pre-coated wells produced a much greater signal than non-coated wells. The signal/background (S/B) was ~ 14 and 10 for pre-coated wells and non-coated wells, respectively, which was an increase of about 40% for the pre-coated wells compared to the non-coated wells. This indicates that the use of pre-coating of the anti-IgG in this system improved the signal reading in the assay. The increase in the signal indicated a better binding orientation of the antibodies in the assay and therefore it will be applied in future tests and sensor development. According to Micheli *et al.* (2005), pre- coating immobilisation will promote the binding of the antigen with its antibody in order to increase the sensitivity of the analysis.

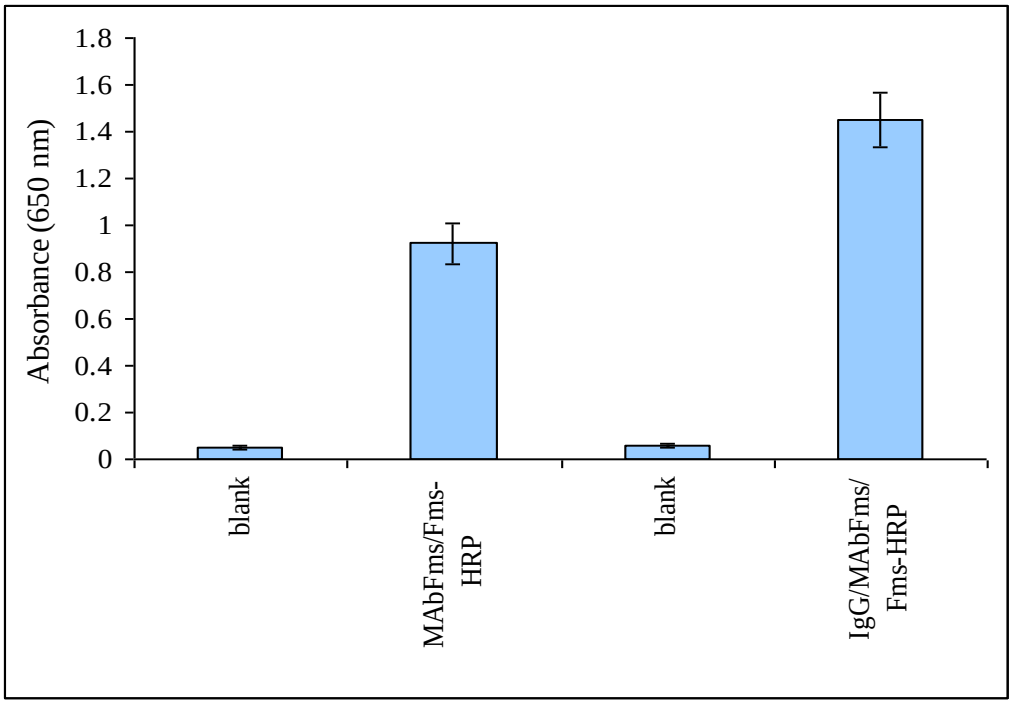


Figure 6: The different immobilizations of antibodies in a direct non-competitive format and comparison of absorbance readings between with and without anti-IgG immobilised on the micro-plate well. Error bar =SD, n=3.

Table 2 shows the optimal data of concentrations reagents and conditions obtained after the optimisation was completed.

Table 2: Optimal concentration/dilution of reagents and conditions used for the direct competitive assay of fumonisins detection.

Reagents	Concentrations/dilutions	Conditions
Anti-IgG antibody (anti-IgG)	20 µg mL^{-1}	overnight, 4 ℃
Anti-Fms antibody (AbFms)	1:50	2 h, 37 ℃
Fms-HRP conjugate	1:5	1 h, 37 ℃

Calibration curve for fumonisins detection using an ELISA method

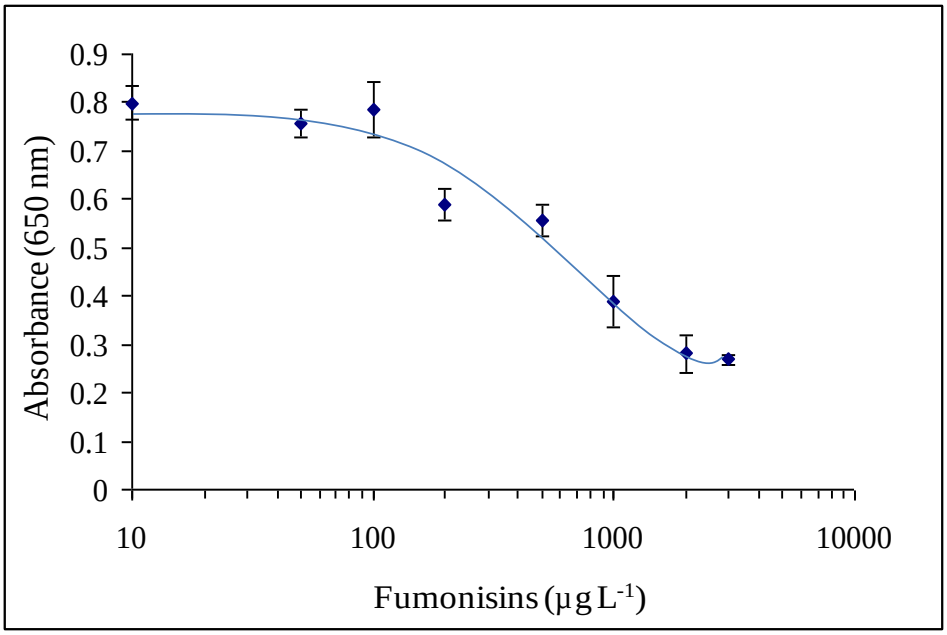
Utilizing the derived optimal concentrations and conditions for the competitive assay, a calibration curve for fumonisins was then carried out with free (0–3000 µg L^{-1}) and HRP labelled fumonisin in a buffer, mixed and added to the wells where they compete for the anti-fumonisin antibody active sites. From the result, shown in Figure 7, indicated that the signal decreased with increasing standard Fms concentrations. The signal dramatically decreased from 100 µg L^{-1} to 2000 µg L^{-1} of Fms. From the graph of Figure 7, the low signal at high concentration of Fms meant that the high amount of free Fms was bound to the specific antibody immobilised on the micro-plate well (less or no enzyme reaction). At the concentration less than 100 µg L^{-1} of Fms, the curve showed steadily in high absorbance reading meaning that the maximum amount of Fms-HRP was bound to the specific antibody immobilised (no or very low of free Fms was bound).

These calibration curves were fitted by using a 'non-linear regression plot' (Warwick, 1996). A dynamic range from 100 to 2000 µg L^{-1} (R^2 = 0.95) with a CV = 9.3% was achieved using the linear section of the curve. to calculate the values.

Assay response in extracted maize samples

To test the performance of the immunoassay in real sample matrix and conduct a recovery test for fumonisin, ground maize samples were spiked with various concentrations of fumonisins standard solution. The samples were first extracted using a methanol/water mixture (70:30) and then used without any further treatment and concentrated using a clean-up procedure (C-18 SPE column) for HPLC.

Other non-spiked maize samples were also extracted and determined in order to evaluate the possible matrix effect compared to fumonisins in buffer samples. For the preparation of ELISA standard curve, maize extract was redisolved with 1 ml of series free Fms standard solution in 0.01 M PBS buffer (Data not shown). For the non-spiked samples background signal was found to be similar to the signal normally achieved in buffer. This indicated that there was a minimum matrix effect on the background electrochemical signal of the immunosensor by following the procedure developed.



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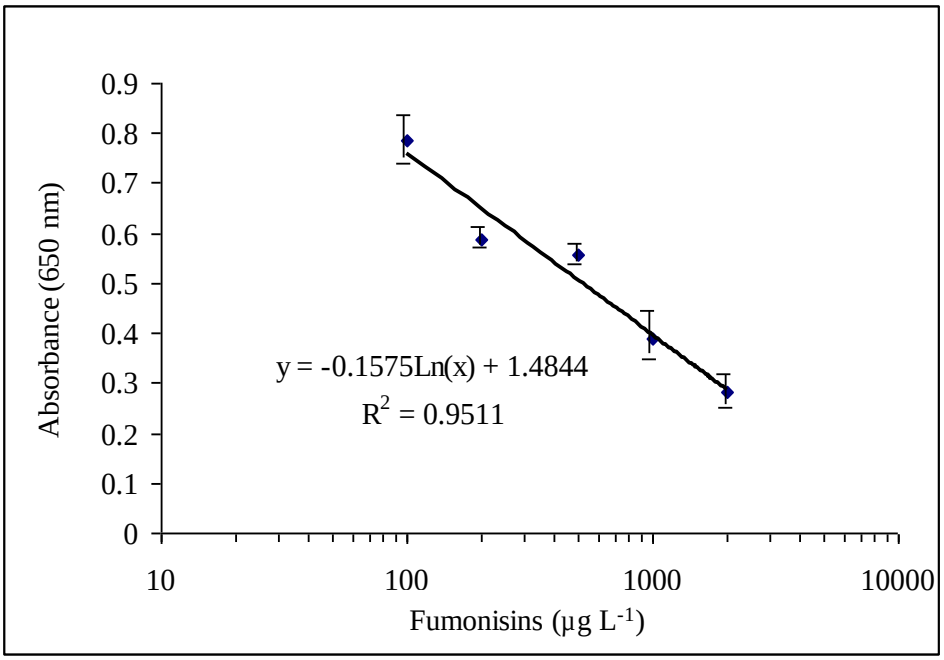

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Figure 7: (a) Spectrophotometric competitive immunoassay for calibration curve of fumonisins. Anti-IgG (20 µg mL^{-1}) and AbFms (1:50 dilution) were pre-coated and coated on the microwell plates, respectively, before adding a mixture of free fumonisins (0-3000 µg L^{-1}) and Fms-HRP (1:5 dilution). Blank: only 1% PVA and Fms-HRP (b) Linear regression a working range of 100 to 2000 µg L^{-1} ($(R^2=0.95)$ (CV= 9.3%). Error bars = SD, n=3

In the spiked maize samples, the recovery of the fumonisins was determined from the analysed concentrations of fumonisins in the maize samples, compared to the expected values spiked to the sample. Using a calibration curve of fumonisin in a non-spiked maize extract and C-18 cleaned samples, the obtained recovery results for spiked samples were calculated, and these are shown in Table 3.

When the two different extraction procedures were compared in terms of recovery, different percentages of recovery were found. The average recovery resulted for the extraction method without a clean-up step (rapid extraction technique) was much lower than the average recovery with a clean-up and concentration step (using C-18 SPE) (Table 3). The lower concentration of spiked fumonisins (250 µg kg^{-1}) obtained less recovery compared to the higher concentration (500 and 2500 µg kg^{-1}). This may suggest that substances in the samples containing low fumonisins inhibited the toxin signal response. By using a clean-up procedure (C-18 SPE column) which helped in removing these substances, the recovery of fumonisins increased due to their removal from the sample extract. According to Muscarella *et al*. (2008) and in agreement with regulation 401/2006/EC, a recovery in the range of 60–120% for fumonisins is expected for samples containing ~500 µg Kg^{-1}. From the results obtained in this study, the use of a clean-up procedure is recommended to improve fumonisin recovery especially at the low contamination level. Also conducting a standard curve for fumonisins using unspiked maize samples extracted and cleaned using C-18 may give very comparable results to fumonisin in the buffer analysis.

The analytical performance of developed ELISA was then compared to HPLC. In this case, the calculated recoveries for HPLC were in the range of 86 to 96.1% with an average value of 91.8%. This indicated the immunosensor having a satisfactory agreement with HPLC. The agreement was confirmed with the application of both methods on the detection of Fms in maize samples. However, in the HPLC system, less than 50 ug Kg^{-1} of Fms in spiked samples and non spiked (CTUK) was not detectable, because the lower limits of detection by HPLC were 50 µg kg^{-1} for FmB_1 and 75 µg kg^{-1} for FmB_2. Therefore, 2500 µg kg^{-1} FmB_2 in spiked maize showed a high recovery for HPLC and also with the ELISA.

Table 3**:** A comparison of results of maize samples and spiked maize samples (with fumonisins standard solution), and using two different extraction methods, where the samples were analysed using the developed ELISA and HPLC method

Samples	Fms added (µg kg^{-1})	Measure value (µg kg^{-1})								
		HPLC			Developed ELISA					
		Clean-up using 18			without clean-up			Clean-up using C18		
		Found	*V*	R	Found	*V*	R	Found	*V*	R
Non-infected	0	nd			< 100			< 100		
maize	50	nd			< 100			< 100		
	250	240.3 ± 10.8	*4.5*	96.1	173.5 ±7.9	*5.5*	69.4	190.1 ± 16.2	*8.5*	76.0
	500	466.3 ± 32.6	*7.0*	93.3	410.9 ± 24.2	*5.9*	82.2	560.3 ± 25.8	*4.6*	112.1
	2500	2150 ± 92.5	*4.3*	86.0	2055.8 ± 74	3.6	82.2	2616.1 ± 102	*3.9*	104.6
Average			*5.2*	91.8		*5*	77.9		5.7	97.6
MTUK unknown		nd			< 1			3.7±0.4	*11.1*	
MOMM unknown		1449.7±119	*8.2*		1034±126.5	*12.2*		1526.5±182.9	*11.9*	
FmB_2	2500	2337.2±217	*9.2*	93.4	2218.4±200	*9.0*	88.7	2533.5±250.2	*9.8*	101.3

V = %CV, R = % Recovery, MTUK = maize Tesco UK, MOMM = main open market Malaysia, FmB_2 = Fumonisin B2. *n*=3.

In the case of non spiked detection, the fumonisins in the COMM sample were in the range of 1034 to 1526 μg kg^{-1} and the lowest was obtained from immunoassay detection without clean-up extraction. This was because recoveries were also lower than the other methods but still reliable for the rapid detection method. The performance of the developed ELISA and the results achieved from analysing the maize samples indicated its useful application for the analysis of fumonisins in maize samples. This indicates the developed ELISA using monoclonal antibody detection is rapid and reliable for maize samples analysis.

Conclusions

In this study, we have developed a direct competitive ELISA using monoclonal antibody for the detection of fumonisins in buffer solution and matrix maize samples. There was better orientation of the antibody specific for the fumonisin by precoating the immobilisation antibody before it was coated with a monoclonal antibody on the ELISA plate. The establishment of the optimal parameters coated in the microwell plate were 20 μg mL^{-1} of ant-IgG, 1:50 dilution of monoclonal antibody and 1:5 dilution of Fumonisins-HRP. After optimisation of the reagents using the ELISA method, the direct competitive assay exhibited a linearity range between 100 to 2000 μg L^{-1}. The working range results for the ELISA (100 to 2000 μg L^{-1}) obtained for Fms detection is compatible for the analysis with the requirement of the EU legislation in food and feed (2 - 4 mg kg^{-1} (ppm)).

The assay was also examined in extracted and cleanup (using C-18 SPE columns) maize samples and showed very low matrix interference and high sensitivity. The high value of recovery of Fms in the spiked maize showed a better performance of the proposed immunoassay which was around 97.6 % and 77.9 % in average for clean-up extraction and direct extraction (rapid), respectively. The reliability of ELISA formats for the analysis of Fms in spiked or real samples was verified by comparison of the data with the fully validated confirmatory HPLC results.

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