Optimization of indirect immunoassay for aflatoxin B₁ detection

(Pengoptimuman immunoasai tidak langsung bagi penentuan aflatoxin B₁

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Abstract

The development of indirect competitive immunoassay formats for the Enzyme Links Immunosorbent Assay (ELISA) construction was undertaken for aflatoxin B_1 (AFB₁) determination. An indirect assay was based on the competition between an immobilised aflatoxin B_1 conjugated with bovine serum albumin (AFB₁-BSA conjugate) and the free AFB₁ for the binding site of monoclonal antibody against AFB₁ (MAbAFB₁). Then, the secondary anti-antibody IgG labelled with horseradish peroxidase (anti-IgG-HRP conjugate) was used as an enzyme label. A spectrophotometric assay using microtitre plate was used in optimizing the immuno-reagent used for competitive ELISA. The optimal conditions obtained for competitive ELISA were 1 µg/ml of BSA-AFB₁, 10 µg/ml of monoclonal antibody and 1 µg/ml of anti-antibody labelled HRP. The linear rage of standard curve (0.1 – 10 µg/litre) was achieved with a detection limit of 0.08 µg/litre. The achieved detection range for AFB₁ was within the European (2 – 4 µg/litre) and Malaysian (5 – 15 µg/litre) required legislative limit of analyses.

Introduction

Aflatoxins (AFs) are a group of highly toxic secondary metabolites produced by the fungi Aspergillus flavus and A. parasiticus particularly found in nuts (Ali 2000). The major occurring aflatoxins are aflatoxin B₁ (AFB₁), B₂ (AFB₂) (produced by A. flavus and A. parasiticus) and G_1 (AFG_1) and G_2 (AFG_2) (produced by A. parasiticus) (Davis 2001), plus two additional metabolic products, M_1 (AFM₁) and M_2 (AFM₂) (Khoury et al. 2008). Aflatoxin contamination is of global concern due to the acute and chronicle toxicity which produces 4 distinct effects: acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Bhatnagar et al. 2002). Meanwhile, AFB₁ is positively

associated with cell liver cancer and linked to human hepatocellular carcinoma (Bhatnagar et al. 2002). Therefore, concern has been expressed for the effect of this group of toxins on human health since AFB₁ is known to be the most predominant and most toxic (Ammida et al. 2004). To control and manage the level of aflatoxins, many countries worldwide have set permitted expose limits for direct human consumption, which vary from $1 - 20 \ \mu g/kg$ (ppb).

Analytical device for AFB_1 detection is an important tool in the control strategy to comply with AFB_1 legislation. Analytical methodology must allow determination of AFB_1 at least down to the specified regulatory level (2 – 4 ppb) (Dohlman 2006). Several techniques in routine analysis

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for identification and quantification of AFB₁ have been developed and reported such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) coupled with Immunoaffinity Column Assay (IAC) (Henry et al. 2000; Jamiez et al. 2000). However, some of the methods are very sophisticated and time consuming, thus, they are generally unsuitable for rapid or wide-scale monitoring programmes. In recent years, many research and development works carried out are focusing on rapid, easy to use and high sensitive techniques for large scale analysis such as immunoassay technique (Tothill and Turner 2003). In the case of ELISA method (immunoassay method), antibodies used as a recognition element are highly specific to their antigen (analyte). 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 study, ELISA tests were developed using an indirect immunoassay format and commercially available reagents based on achieving lower detection limits, a wider dynamic range, and a higher signal:noise ratio and sensitivity. To achieve maximum response for optimum assay performance, this process must have highly specific binding of the antibody-antigen and a higher signal:noise ratio. The sensitivity of the assay also depends on the concentrations of the antibody or antigen coating on the plate. Many researchers (Gascon et al. 1997; Grabowska et al. 2002; Oubina et al. 1999) have studied the effect of the incubation conditions such as temperature and time and their influence on the antibody or antigen immobilization activity using a competitive immunoassay. The correct time and temperature of incubation will achieve optimum coating and also increase the specific binding activity. The incubation steps of coating, binding and detecting are dependent on how many steps of assay are

used. However, if the incubation of the detection step using the enzyme labelling method is too long, then a high background signal will be found which can increase the non-specific binding (Tothill 2003). This will affect the sensitivity of the analytes detection limit.

For the development of AFB_1 ELISA tests, a monoclonal antibody against aflatoxin B_1 (MAbAFB₁) was used. The indirect format was carried out through the competition between the immobilised proteins-conjugated AFB_1 and the free AFB_1 in the sample for the binding sites of MAbAFB₁ (Ammida et al. 2004) The last stage is introducing the enzyme substrate to react with the enzyme labelled (indicating the presence of antigens) for the colour development. The absorbance reading is inversely proportional to the concentration of the toxin.

The objective of the study was to develop an indirect immunoassay method for the detection of aflatoxin B_1 . To achieve this objective, the work was focused on selecting the optimal antibody system and optimising the assay procedure (concentration of reagents and the condition of assay). The target response for optimum immunoassay performance is highly specific binding of the antibody-antigen and a higher signal:noise ratio.

Materials and methods Chemicals and reagents

All chemicals used were of analytical grade. Aflatoxin B_1 -BSA conjugate (AFB₁-BSA) and Aflatoxin B_1 (AFB₁) standard solutions were obtained from Sigma-Aldrich Co. Ltd (Gillingham, UK). The monoclonal antibody against AFB₁ (primary antibody) (MAbAFB₁), and rabbit anti-mouse IgG (H+L) labelled with horseradish peroxidase (HRP) (secondary antibody) were purchased from Abcam Ltd. (Cambridge, UK). Other reagents, polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), gelatine, potassium chloride and polyethylene glycol sorbitan monolaurate (Tween 20) were purchased from Sigma-Aldrich Co. Ltd (Gillingham, UK). Concentrated milk diluents (blocking solution) were obtained from KPL Ltd. (UK). A substrate of 3,3,5,5'-Tetramethylbenzidine (TMB) solution was purchased from Insight Biotechnology (UK).

Instrumentation

The micro well polystyrene plates, MaxiSorpTM (NuncImmuno) were purchased from Fisher Scientific (Loughborough, UK). Incubations for every step of reactions were carried out by LabsystemiEMS incubator/ shaker HT (UK). Spectrophotometric analysis of colour development was performed by a BMG Fluostar galaxy ELISA plate reader (Aylesbury, UK).

Buffer, blocking and standard solutions

A 0.1 M carbonate buffer, pH 9.6 (1.59 g Na₂CO₃, 2.93 g NaHCO₃) was prepared for the immobilization of AFB₁-BSA and anti-IgG unconjugated (precoating) on microplates. This was dissolved in 1 litre water and the pH value was adjusted to 9.6. A 0.01 M phosphate buffered saline (PBS), pH 7.4 was used for the preparation of AFB1 standard, blocking solution, dilution of antibodies and washing solution. PBS tablet was purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK). The washing solution was prepared by adding 0.05% Tween 20 to the PBS (PBS-T). The AFB₁ standard solution (in acetonitrile) was prepared by diluting the stock solution (1,000 µg/litre in PBS) with PBS to give a range of concentrations at 0, 0.001, 0.01, 0.1, 1, 10 and 100 µg/litre in PBS. A chemical polymer (1% PVA and 1% PVP) and protein polymer (1% gelatine and 1:20 milk diluents) in PBS, pH 7.4 were used as blocking reagents.

The AFB₁ standard solution in acetonitrile was diluted using PBS for stock solution (1 mg/ml) with pH 7.4 and stored at -18 °C in an amber bottle (dark) and tightly capped. From the stock, about 0.001 – 100 μ g/ litre of standard solution concentrations were prepared in PBS for standard curves of the ELISA system. Safety measures were applied such as wearing gloves, protection glasses and a mask when handling the chemical due to the potentially carcinogenic properties of aflatoxin. The toxin solution in acetonitrile was generally diluted in PBS buffer upon arrival and labelled as a stock toxic reagent before being stored in a locked fridge.

Indirect competitive ELISA

In initial experiments, the different concentrations range of AFB_1 -BSA conjugate, anti-aflatoxin B_1 antibody (monoclonal antibody) and anti-antibody labelled with HRP were determined by performing a checkerboard assay as detailed by Crowther (2001) (*Figure 1*). The optimization of different concentrations of reagents in different types of buffers, the condition of incubation times and the use of blocking agents in this experiment were followed as described by Ammida et al. (2004). The different volumes of reagents for coating the well plates were also investigated.

The ELISA system was also optimised by producing calibration curves using a series of standards of AFB_1 ranging from $0 - 100 \mu g/litre$. Three experiments were conducted i.e. the effect of incubation time during the competitive step from 0 to 120 min, the concentration of monoclonal antibody (MAbAFB₁) was varied at 5, 10 and 50 µg/ml, and the concentration of the HRP-labelled antibody was varied at 0.5, 1 and 5 µg/ml.





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Optimisation of reagents

Using the checkerboard method in the microwell plates, all the different concentrations of reagents and condition parameters were performed as presented in Table 1. The formation of non competitive assay was followed by immobilisation of AFB₁-BSA first, then blocking before coating with anti-aflatoxin B1 antibody and labelling with anti-antibody labelled with HRP. In each step of the assay, a 50 μ l/ well of reagents was added; the washing procedure was performed twice with a 150 µl/well phosphate buffered saline containing Tween 20 (PBST) and once with PBS alone. The colour development was initiated by addition of a TMB/substrate solution (50 µl/ well) to each well and incubation at room temperature for 15 min before measuring the absorbance at 450 nm in a plate reader.

Effect of coating buffers and blocking agents

A coating of AFB₁-BSA conjugate in 2 different buffers (0.1 M carbonate buffer pH 9.6 and 0.01 M PBS buffer pH 7.4) was added to the well of a microtitre plate. Four differenct blocking agents (1 - 3% PVA, 1 - 3% PVP, 1:10 to 1:40 milk diluents and 1 - 3% gelatine) were used for reducing the non-specific binding of the ELISA assay. They were added to the well after first coating the plates with the antibody. This experiment was performed before the assay was run.

Competitive assay preparation

A 50 µl/well of AFB₁-BSA conjugate (1 µg/ ml) in a coating buffer (0.1 M carbonate buffer pH 9.6) was added to the wells of a microtitre plate and incubated at 4 °C overnight (18 h), then followed by washing steps (same washing procedure as above). The plate was then blocked with a 1% PVA blocking solution (50 µl/well) before incubating at 37 °C for 1 h. The pre-incubation of AFB1 standard solutions $(0 - 100 \mu g/litre)$ with a fixed anti-aflatoxin antibody (MAbAFB₁) (10 µg/ml) in an eppendorf tube and incubated for 30 min at room temperature before transferring the mixture to the microtitre plate (50 µl/ well) and incubating at 37 °C for another 1.5 h. After the washing step, a 50 µl of anti-antibody labelled with HRP (anti-IgG-HRP) (1.0 µg/ml) in PBS was then added to the microtitre plate and incubated for 1 h at 37 °C.

The colorimetric reaction was initiated by the addition of a TMB substrate solution (50 µl/well) to each well and incubated at room temperature for 15 min. Then, the colour development reaction was stopped with H_2SO_4 (25 µl/well) before measuring the absorbance at 450 nm using a plate reader. The illustration of the schematic assay of the indirect competitive method used is shown in *Figure 2*.

Table 1. The parameters for optimisation of the ELISA test with different concentrations of reagents and conditions

Reagents/buffers	Concentrations	Conditions/Incubation
AFB ₁ -BSA conjugate in 0.1 M carbonate buffer pH 9.6	0 – 50 µg/ml	4 °C (18 h)
Blocking with PVA	1%	37 °C (1 h)
Anti-aflatoxin B ₁ antibody in 0.01 M PBS buffer pH 7.4	$0-10\ \mu\text{g/ml}$	37 °C (2 h)
Anti-antibody labelled with HRP 0.01 M PBS buffer pH 7.4	$0-10\ \mu g/ml$	37 °C (1 h)



Figure 2. Schematic diagram of indirect competitive ELISA format used in this study

Results and discussion Optimisation of anti-AFB₁ antibody (monoclonal antibody against AFB₁) and AFB₁-BSA conjugate

In this study, the spectrophotometric competitive enzyme-linked immunosorbent assay (ELISA) for AFB₁ detection was carried out. The tests were performed in a microwell titre with an indirect competitive format.The initial ELISA developments involved the optimization of the immunereagents used such as coating buffer and blocking reagent and also incubation times.

The performance of the specific binding of the monoclonal anti-AFB₁ antibody (MAbAFB₁) to the binding site of the AFB₁-BSA conjugate was tested through the checkerboard titration method. The analysis was conducted by immobilizing different concentrations of AFB_1 -BSA (0 – 50 µg/ml) and tested with various MAbAFB1 concentrations (0 -10 µg/ml). Figure 3 shows the different absorbance plots for different AFB1-BSA coating. All plots with AFB1-BSA coating increased with the increase of MAbAFB₁ concentration. However a different signal reading was clearly obtained between 1 and 10 µg/ml of MAbAFB1 in each AFB1-BSA concentration. This indicated that the

significant binding interaction between AFB_1 -BSA and $MAbAFB_1$ was in the range of $1 - 10 \ \mu g/ml$.

A high absorbance reading was achieved by 1 and 5 µg/ml of AFB₁-BSA when the MAbAFB₁ concentration was in the range of $5 - 10 \,\mu\text{g/ml}$. The result indicated that the optimal concentration for AFB₁-BSA for the specific binding of the antibody was in the range of $1 - 5 \,\mu\text{g}/$ ml (Figure 3). Hence, the smallest value (1 μ g/ml) that still produces optimum binding ability will be chosen for economical reasons to ensure competitive cost. Based on these results, a plate coating concentration of 1 µg/ml AFB₁-BSA and monoclonal antibody concentration of 10 µg/ml, will be used for further experiments. In this experiment, standard deviations cannot be generated since the experiment is not repeated; therefore error bars are not produced. Once the system is optimised, then the experiment can be repeated to assess the standard deviation and hence the repeatability.

The checkerboard titration for the optimisation of two reagents was suggested by Crowther (2001). First, there was the direct adsorption of an AFB₁-BSA to the support surface (96 well), and this approach

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Figure 3. Optimisation of plate immobilisation with different concentrations of AFB_1 -BSA conjugate and different concentrations of anti-aflatoxin B_1 antibody (MAbAFB₁) in an indirect format without using free AFB_1 (non-competitive)

of immobilisation was followed by other reseachers such as Butler (2000) and Ammida et al. (2004). Then the captured antibody of the monoclonal of anti-aflatoxin B_1 antibody (mouse monoclonal antibody) for the binding interaction from commercial site was selected. This is because using monoclonal antibodies (MAbs) enables the recognition of a specific epitope and there are no other antibodies present, specific or cross-reactive, to compete for binding to the adsorbed antigen and influence the measured antibody binding profiles (Giardina et al. 2003).

The parameters used as a standard method for ELISA development were also followed as indicated by Ammida et al. (2004). When the optimal concentrations of 2 reagents (AFB₁-BSA and MAbAFB₁) were achieved, an anti-IgG labelled enzyme, coating buffer, blocking agents and different conditions of assay were then optimised in the next set of experiments.

Optimisation of anti-mouse IgGhorseradish peroxidase conjugates (anti-IgG-HRP)

In the second experiment, different concentrations of anti-mouse IgGhorseradish peroxidase (anti-IgG-HRP) conjugate were optimised based on the indirect assay format. The labelled enzyme (horseradish peroxidase) is commonly used for catalytic reaction and produces a stable and sensitive product for measurement. The solution of anti-IgG-HRP conjugate was used as a tracer, and the TMB solution as the enzyme substrate. The concentration of the anti-IgG-HRP conjugate used in the immunoassay was optimized by performing a series of assays utilizing varying amounts of anti-IgG-HRP $(0 - 10 \mu g/ml)$ with a 1 h incubation time. The optimisation range of anti-IgG-HRP is shown in Figure 4. The trend was expected; the absorbance reading increased with the amount of anti-IgG-HRP used. This was because the enzyme reaction measured was proportional to the amount of specific binding of anti-IgG-HRP used to the fixed amount of MAbAFB₁ in the test solution.



Figure 4. Optimisation of anti-mouse IgG-HRP conjugate. Wells were coated with AFB₁-BSA (1 µg/ml), blocked with 1% PVA and followed by MAbAFB₁ (10 µg/ml) with an amount of anti-IgG-HRP (0 – 10 µg/ml). TMB solution as a substrate and H_2SO_4 was used to stop the reaction



Figure 5. Anti-AFB₁ antibody (MAbAFB₁) binding of AFB₁-BSA conjugate in two different coating buffers, 0.01 M PBS pH 7.4 and 0.1 M CB pH 9.6. Wells were coated with AFB₁-BSA (1 µg/ml), blocked with 1% PVA, followed by MAbAFB₁ (10 µg/ml) and continued with amount of anti-IgG-HRP (1 µg/ml). TMB solution as a substrate and H₂SO₄ was used to stop the reaction

The absorbance response dramatically increased between the concentrations of 0.05 and 1 µg/ml and was saturated after the maximum binding of 5 µg/ml (*Figure 4*). However, 1.0 µg/ml of anti-IgG-HRP was selected for future immunoassay tests. At a concentration of 1.0 µg/ml, a high signal was still obtained, which refers to the specific recognition capabilities of 10 μ g/ml of MAbAFB₁ concentration for the corresponding 1 μ g/ml AFB₁-BSA conjugate. The result still showed a high value of signal-to-background, which produced a high dynamic range in calibration curves of the competitive assay. The use of a low concentration of reagents signal can also reduce the cost of the analytical method developed.

Effect of coating buffer in indirect noncompetitive method

The adsorptive immobilisation of AFB₁-BSA to the plastic surface is dependent on the pH value of the coating buffer.Two different coating buffers (carbonate buffer, pH 9.6 and PBS, pH 7.4) were used for testing on the effect of immobilisation. These buffers with different pH values are able to influence the steric structure of protein which can influence the immobilisation system. The influences of the buffer at pH 9.6 and 7.4 response signals are shown in Figure 5. The adsorption of AFB₁-BSA on the plastic surface using the carbonate buffer pH 9.6 produced a higher reading compared to that of the phosphate buffer at pH 7.4. This result also showed the high ability for binding of the MabAFB₁ with AFB₁-BSAconjugate starting from $1 - 50 \mu g/ml$ before saturation occurs.

Results from Figure 5 suggested that the carbonate buffer in alkaline pH binding enhanced the immobilising adsorption of AFB₁-BSA on the polystyrene plates. Therefore, the carbonate buffer pH 9.6 was chosen as the coating buffer for this format and continues to be widely used in ELISAs. Previous ELISA studies for mycotoxin detection using direct and indirect assays have also proposed the use of a carbonate buffer (pH 9.6) for antibody or conjugated protein coating onto microwells as this produce a higher response (Alarcon et al. 2004; Ammida et al. 2004; Micheli et al. 2005; Piermarini et al. 2007). There was evidence that conjugated proteins when

exposed to high pH prior (in CB pH 9.6) to adsorption, displayed higher activity than when PBS pH 7.4 was used (Micheli et al. 2005).

Blocking agents

The use of blocking agents in an AFB₁ ELISA system is very important for investigating the blocking reaction on the surface of the wells. The main purpose is to reduce the amount of the non-specific binding of proteins as well as to produce low background readings in the system. Four types of blocking agents were chosen for testing in this experiment which used chemical polymers (PVA and PVP) and protein polymers (gelatin and milk diluents). These 4 types of blockers are frequently used as blocking agents in the ELISA system.

According to the study by Ammida et al. (2004) and Micheli et al. (2005), 1% PVA was used. In another case, PVP polymer was used and a low background result was obtained from the assay (Parker and Tothill 2009; Studentsov et al. 2002). Compared with protein polymers, milk diluents are common blockers applied in many ELISA test kits. In the past, gelatin was applied as a blocking agent (Crowther 2001). Thus, the 2 polymers and 2 proteinaceous blocking agents were performed and compared to each other to provide low background readings in this experiment. Each blocker, with a concentration of 1% PVA, 1% PVP and 1% gelatin as well as 1:20 milk diluent in PBS pH 7.4 was performed in replicate wells in the absence of free AFB_1 . Figure 6 shows the background readings obtained for each agent.

A high absorbance reading was obtained in the absence of a blocker, using PVP and gelatin. The presence of a high signal indicated a high amount of nonspecific binding of the antibody or enzyme labelled conjugate on the surface as well as providing a high background reading in the system. According to Chen et al. (2006), high background readings may possibly be caused by the stickiness of the antibodies to the well surface. Chen et al. (2006) also suggested that the addition of a blocking step after the first coating can eliminate or reduce non-specific binding on the surface. Figure 6 also shows that a low background reading similar to the control was also achieved by adding the milk diluents and PVA into the assay. This indicated that the most efficient blockers are milk diluents and PVA. These results would provide an indication of the ability of the blocker to reduce non-specific binding of antibodies to the surface which will provide low background readings that enhanced the signal/background (S/B) ratio for the assays. This phenomenon will give better specificity and sensitivity for the ELISA test developed in this work.

Milk diluents and PVA were chosen for further work to investigate their effectiveness as blocking agents to the unoccupied sites on the well surface. The test was carried out to determine the ability of blocking agents in the competition immunoassay which is exposed to 0 and 100 ng/ml of free AFB₁ concentration. The results in Figure 7 shows that at zero toxin concentration, a higher signal was obtained in the absence of a blocker and in the presence of PVA compared to milk diluents added. However, the results also showed high background readings in treatment without blocker as compared to the presence of blockers, PVA and milk diluents. So, the different values of signal (zero toxin) over background (S/B) of each treatment without blocker, with addition of PVA and with the presence of milk diluents were 1.6, 4.06 and 2 respectively.

A high S/B value in the presence of PVA would give an indication of the ability to prevent non-specific protein adsorption, which may enhance the sensitivity of the assay. The use of milk diluents in this assay had interference on the surface and this indicated that it may reduce the specific binding of antigen to the antibody site. The milk diluents may have a tendency to



Figure 6. Effect of various blocking agents on absorbance reading by 1% PVP, 1% PVA, 1% gelatin and 1:20 milk diluents. Wells were coated with AFB₁-BSA (1 μ g/ml), blocked, and followed by anti-IgG-HRP (1 μ g/ml). The control was coated with AFB₁-BSA (1 μ g/ml), without blocker and without anti-IgG-HRP



Figure 7. Effect of different blocking agents in the competitive method by 1% PVA and 1:20 milk diluents. Wells were coated with AFB_1 -BSA (1 µg/ ml), blocked, and followed by $MAbAFB_1$ (10 µg/ ml) and free AFB_1 before continuing with anti-IgG-HRP (1 µg/ml). Background was conducted using AFB_1 -BSA (1 µg/ml), blocked then with anti-IgG-HRP

deteriorate rapidly if not properly prepared and stored (unstable blocking agent) (Gibbs 1996). It may also exhibit little cross reactivity with typical immunoassay components such as antibodies and enzymes (Gibbs 1996). In this case, milk may also absorb mycotoxins, which is why it is not suitable for mycotoxin assays (Parker 2008).

From the two assayed blockers, PVA showed better results than milk diluents for this developed immunoassay. This is because PVA did not interfere with the antigen-antibody reaction and, at the same time, did not increase the background signal. The addition of PVA as a blocking solution in competitive immunoassay reduced the non-specific protein adsorption on the micro-plate surface. The results also gave a wide signal difference between zero and 100 μ g/litre of AFB₁ concentration when the assay was treated with PVA compared to the others. These results suggest that a high dynamic range of calibration curve can be produced using this system.

Competitive assay

The next experiment with the optimal parameters was to create a calibration curve of AFB₁ concentrations from $0 - 100 \mu g/$ litre with different volumes of reagents in the 96 NuncMaxsorp microwell plate. In this competition step, the free AFB_1 and the MAbAFB₁ were premixed in eppendorf tubes for 30 min at room temperature (25 °C), then transferred to the ELISA plate. This allowed the specific binding of MAbAFB₁ and free AFB₁ to react first before it was added to the wells coated with AFB₁ conjugate. A concentration of 100 and 50 µl of the reagents in each step of the procedure was used to compare the sensitivity of the results. Normally, these volumes added on the ELISA micro-plate are commonly used in the ELISA system.

The results for both concentrations used are presented in *Figure 8*. The absorbance reading decreased with increasing AFB₁ concentration for both effects. The response also showed a low background reading. This showed that with 100 μ l and 50 μ l concentrations of reagents, a high affinity for specific binding interaction was achieved. However, the non-specific binding reaction on the well



Figure 8. Calibration curves by competitive ELISA for AFB_1 with different volumes of reagent in the well plates. Wells were coated with AFB_1 -BSA (1 µg/ml), blocked with 1% PVA and followed by MAbAFB₁ (10 µg/ml) and free AFB_1 (0 – 100 µg/litre) before continuing with anti-IgG-HRP (1 µg/ml)



Figure 9. Effect of incubation times from 1 to 90 min at 37 °C in competition step of indirect format for AFB₁ detection. Wells were coated with AFB₁-BSA (1 µg/ml), blocked with 1% PVA and followed by MAbAFB₁ (10 µg/ml) and free AFB₁ (0 – 100 µg/litre) before continuing with anti-IgG-HRP (1 µg/ml)

surface was low. The percentage of relative binding (%B/B₀) (based on absorbance reading) of low to high AFB₁ concentration was found to be slightly similar for both volumes by 97% (highest) to 30 % (lowest). This observation suggested that 50 μ l could be used for further experiments and can be applied in 96 NuncMaxsorp microwell plates, thus reducing the use of expensive reagents.

Effect of incubation times in the competition steps

One of the most important parameters to influence the sensitivity of the assay is the incubation times. After a 30 min premix of the competition step, the plate was incubated at different times at 1, 30, 60 and 90 min at 37 °C. The purpose of this experiment was to investigate the optimal time needed to complete the competition reaction to determine the sensitivity of the assay.

Figure 9 shows that the signal obtained increased with incubation time. However, the noise with high SD also increased with the increase in the incubation time. The data from 30 - 90 min showed that there was a detectable difference in absorbance reading between low and high concentrations of AFB_1 . For these values, incubation at 30 min produced a low signal as compared to 60 and 90 min. A high background reading was obtained at 90 min incubation and the signals became unstable and flattened. The sensitivity of the assay may be increased with a longer incubation time at 37 °C, but the top of the standard curve may flatten out and become unstable, limiting the assay range and may increase the background reading. The best range was achieved at 60 min of incubation and this was therefore, chosen to continue with the investigation.

The data from all figures were observed and summarised in *Table 2*. The summary shows the selected concentration of reagents after optimisation in the presence of AFB_1 and using AFB_1 -BSA, antiaflatoxinB_1 antibody (mouse monoclonal antibody against AFB_1), anti-antibody labelled with HRP (goat anti-mouse IgG conjugate with horseradish peroxidase). Another factor, incubation time, was also found to have an effect on the sensitivity of spectrophotometric assay. The optimal incubation time in the competition step was attained after 60 min at 37 °C.

One of the critical parts that may cause a high background reading in the assay is high non-specific binding on the wells surface, which maybe from antibody

Reagents	Concentration	Incubation time
AFB ₁ -BSA conjugate	1.0 μg/ml	Overnight (12 h), 4 °C
Anti-aflatoxin B_1 antibody	$10 \mu g/ml$	1.5 h, 37 °C
Anti-antibody-HRP	$1.0 \ \mu g/ml$	1.0 h, 37 °C

Table 2. Selected concentrations of the different reagents used in the indirect format of the spectrophotometric immunoassay for aflatoxin B_1

conjugated enzyme binding. With a minor modification, the buffer solution of anti-IgG-HRP was mixed with 1% of PVA to avoid high non-specific binding in the wells. The best result of the calibration curve of AFB₁ was achieved. For the assay, a linear range from $0.1 - 10 \mu g/litre$ with the limit of detection was determined to be 0.08 $\mu g/$ litre. The ranges of detection limit for the calibration curve have to include the EU and Malaysian legislative levels. The maximum permissible limit for AFB₁ in foodstuffs is $1 - 20 \mu g/kg$ in more than 50 countries including the EU and Malaysia (15 $\mu g/kg$) (Abidin et al. 2003).

Conclusion

Indirect ELISA methods using monoclonal antibodies have been developed. The assays were optimised through changing reagents concentrations. The choice of blocking agent is also of vital importance for the signal/ background ratio, thus, the PVA block generally resulted in a better S/B ratio than the milk diluents. Incubation times, was also found to be another factor that has an effect on the sensitivity of spectrophotometric assay. The assays were able to detect AFB₁ to less than 1.0 µg/litre, which meets the legislative limits imposed by the European Union and most other countries around the world. For the indirect method, a working range of $0.1 - 10 \,\mu g/litre$ was achieved by optimal concentration of coating AFB₁-BSA, antibody binding MAbAFB1 followed by Anti-IgG-HRP.

References

- Abidin, H., Rosni, M. and Hasniza, A. (2003). Status of aflatoxin contamination in groundnut from five districts in Perak. J. Trop. Agric. and Fd. Sci. 3: 235 – 239
- Alarcon, S.H., Micheli, L., Palleschi, G. and Compagnone, D. (2004). Development of an Electrochemical Immunosensor for Ochratoxin A. Analytical Letter 37: 1545 – 1558
- Ali, N. (2000). Aflatoxins in Malaysian food. Mycotoxins 50: 31 – 35
- Ammida, N.H.S., Micheli, L. and Palleschi, G. (2004). Electrochemical immunosensor for determination of aflatoxin B₁ in barley. *Analytica Chimica Acta* 50: 159 – 164
- Bhatnagar, D., Yu, J. and Ehrlich, K.C. (2002). Toxins of filamentous fungi. In: *Fungal* allergy and pathogenicity (Breitenbach, M., Cremeri, R. and Lehrer, S.B., eds.), p. 167 – 206. New Orleans, USA: KARGER Medical and Scientific Publishers
- Butler, J.E. (2000). Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. *Methods* 22: 4 – 23
- Chen, G., Lin, Y. and Wang, J. (2006). Monitoring environmental pollutants by microchip capillary electrophoresis with electrochemical detection. *Talanta* 68: 497 – 503
- Crowther, J.R. (2001). *The ELISA guidebook*. Vienna, Austria: Humana Press
- Davis, P.J. (2001). Molds, toxic molds, and indoor air quality. Retrieved on 12 November 2006 from www.library.ca.gov/crb/01/notes/v8n1. pdf
- Dohlman, E. (2006). Mycotoxin hazards and regulations impacts on food and animal feed crop trade. Economic Research Service/ USDA. International Trade and Food Safety/ AER-828, p. 97 – 108. Retrieved on 12 November 2006 from www.ers.usda.gov/ publications/aer828/- aer828h.pdf
- Gascon, J., Oubina, A., Ballesteros, B., Barcelo, D., Camps, F., Marco, M.P., Gonzales-Martinez, M.A., Morais, S., Puchades, R. and Maquieira, A. (1997). Development of a highly sensitive enzyme-linked

immunosorbent assay for attrazine. Performance evaluation by flow injection immunoassay. *Analytica Chimica Acta* 347: 149 – 162

- Giardina, P.C., Evans, R.E., Sikkema, D.J., Madore, D. and Hildreth, S.W. (2003). Effect of antigen coating conditions on enzymelinked immunosorbent assay for detection of immunoglobulin G antibody to *Neisseria meningitides* serogroup Y and W135 capsular polysaccharide antigens in serum. *Clinical* and diagnostic laboratory immunology 10: 1136 – 1140
- Gibbs, J. (1996). Optimizing the separation step on 96-well plates. Corning Science Product Division, ELISA Technical. Bulletin 4: 1 – 6

Grabowska, K., Wang, X., Jacobsson, A. and Dillner, J. (2002). Evaluation of costprecision rations of different startegies for ELISA measurement of serum antibody levels. *Journal of Immunological Methods* 271: 1 – 15

- Henry, S.H., Whitaker, T., Rabbani, I., Bowers, J., Park, D., Price, W., Bosch, F.X., Pennington, J., Verger, P., Yoshizawa, T., van Egmond, H., Jonker, M.A. and Coker, R., (2000).
 Report 1012, Aflatoxin M₁ (WHO Additives, series 47.), Joint Expert Committee on Food Additives (JECFA)
- Jamiez, J., Fente, C.A., Vazquez, B.I., Franco, C.M., Cepeda, A., Mahhuzier, G. and Prognon, P. (2000). Application of aflatoxin by liquid chromatography with fluorescence detection in food analysis. *Journal of Chromatography* A 882: 1 – 10
- Khoury, E., Rizk, T., Lteif, R., Azouri, H., Delia, M.L. and Lebrihi, A. (2008). Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine-grapes and musts. *Food and Chemical Toxicology* 46: 2244 – 2250

Micheli, L., Grecco, R., Badea, M., Moscone, D. and Palleschi, G. (2005). An electrochemical immunosensor for aflatoxin M1 determination in milk using screen-printed electrodes. *Biosensors and Bioelectronics* 21: 588 – 596

- Oubina, A., Balleseros, B., Galve, R., Barcelo, D. and Marco, M.P. (1999). Development and optimisation of an indirect enzyme-linked immunosorbent assay for 4-nitrophenol. Application to the analysis of certified water samples. *Analytica Chimica Acta* 387: 255 – 266
- Parker, C.O. (2008). Development of an affinity sensor for the detection of aflatoxin M1 in milk. PhD Thesis, Cranfield University
- Parker, C.O. and Tothill, I.E. (2009). Development of an electrochemical immunosensor for aflatoxin M1 in milk with focus on matrix interference. Biosensor and Bioelectronics 24: 2452 – 2457
- Piermarini, S., Micheli, L., Ammida, N.H.S., Palleschi, G. and Moscone, D. (2007). Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B1 detection. *Biosensors and Bioelectronics* 22: 1434 – 1440
- Studentsov, Y.Y., Schiffman, M., Strickler, H.D., Ho, G.Y.F., Susana Pang, Y.Y., Sciller, J., Herrere, R. and Burk, R.D. (2002). Enhanced ELISA for detection of antibodies to viruslike particles of human papillomavirus. *Journal of Clinical Microbiology* 40: 1755 – 1760
- Tothill, I.E. (2003). On-line immunochemical assays for contaminant analysis. In: *Rapid* and on-line instrumentation for food quality assurance, (Tothill, I.E., ed.), p. 14 – 39. Cambridge, United Kingdom: Woodhead Publishing Ltd.
- Tothill, I.E. and Turner, A.P.F. (2003). Biosensors. In: *Encyclopaedia of food sciences and nutrition*, 2nd Edition, (Caballero, B.,Trugo, L. and Finglas, P., eds.), p. 20 – 28. Waltham Massachusetta, USA: Academic Press

Abstrak

Pembangunan immunoasai berdasarkan format persaingan tidak langsung bagi pembentukan *Enzyme Links Immunosorbent Assay* (ELISA) untuk penentuan aflatoxin B₁ (AFB₁) telah dijalankan. Asai tidak langsung ini berdasarkan persaingan antara kojugat AFB₁ tak gerak dengan albumin serum bovin (konjugat AFB₁-BSA) dengan aflatoxin B₁ bebas untuk tapak ikatan monoklonal antibodi terhadap AFB₁ (MAbAFB₁) . Kemudian anti-antibodi sekunder IgG yang dilabel dengan enzim 'horseradish peroxsidase' (konjugat anti-IgG-HRP) digunakan sebagai label enzim. Asai spektrofotometrik menggunakan plat mikrotiter telah dijalankan bagi pengoptimuman kepekatan immuno-reagen yang digunakan untuk ELISA saingan. Keadaan optimum bagi ELISA saingan ialah sebanyak 1 µg/ml BSA-AFB₁, 10 µg/ml antibodi monoklonal dan 1 µg/ml anti-antibodi berlabel HRP. Julat linear lengkungan piawai (0.1 – 10 µg/liter) telah tercapai dengan had pengesanan sebanyak 0.08 µg/liter. Julat pengesanan yang tercapai bagi analisis AFB₁ adalah dalam had perundangan yang diperakukan di Eropah (2 – 4 µg/liter) dan Malaysia (5 – 15 µg/liter).