Development of Antibody-based Kit for the Detection of Ochratoxin A in Grain Corn

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Abstract

The contamination of ochratoxin A (OA) in animal feed has become a huge threat to livestock and husbandry industries globally. Many countries had faced overwhelmed economic loss due to low feed and animal output productivities. This study reported the in-house production and characterisation of antibody IgG against OA. The performance of IgGs collected from two rabbits were investigated under titer and protein assay experimentation. Most IgGs exhibited good titer performance with high protein amount, especially during 2nd and 3rd bleeds. Further study was carried out using antibodies from the O1 3rd bleed. Direct competitive assay of OA antibody in phosphate buffer saline (PBS) and corn matrix system was conducted at different OA standard concentrations. Good linearity (R^2 = 0.9533 in PBS and R^2 = 0.9903 in corn matrix) with a broad working range were obtained from both systems. The sensitivity of the developed optical immunodiagnostic was confirmed with a low limit of detection (LOD) achieved, which was lower than the permitted limit of 5 ppb (LOD = 4.03 ppb in PBS and LOD = 2.70 ppb in corn matrix). Intra and inter-species cross-reaction showed that the produced OA antibody was highly selective towards its specific target analyte. The developed immunodiagnostic was verified with chromatographic analysis and the results from both detection methods complemented each other.

Keywords: Ochratoxin A, competitive ELISA, immunodiagnostic kit, grain corn

Introduction

Rapid development in livestock and husbandry industries has indirectly increased feed production and output, especially grain corn-based feed. However, due to certain factors, processed and non-processed grain corns can carry harmful mycotoxins along the production chain which can lead to serious health issues for livestock and human. *Aspergillus* and *Penicillium* species are potent fungi that can produce ochratoxins in most crops and also in the environment (Vamkudoth *et al*., 2020). The most frequently identified ochratoxins are ochratoxin A (OA), ochratoxin B (OB) and ochratoxin C (OC) (Figure 1). OA is found to be the most toxic and can seriously lead to harmful health effects on animals and humans. Storage environments such as poor aeration and high humidity, in addition to natural regional weather, are potential factors that can speed up the fungi growth and production of ochratoxins (Miraglia and Brera, 2002; Kumar *et al*., 2020). Contamination can also occur during processing and transporting due

to lack of good handling practice (Kaushik *et al*., 2013).

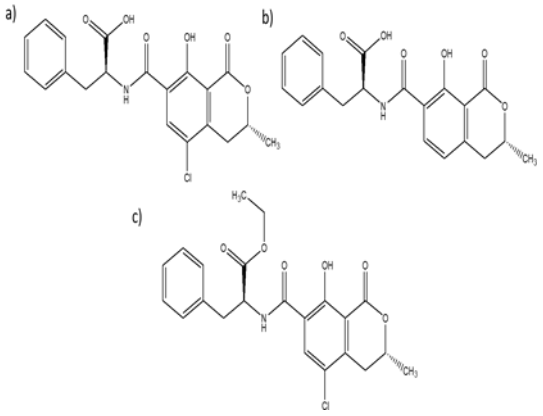


Figure 1. Chemical structure of commonly found ochratoxins; a) OA, b) OB and c) OC

The occurrence of OA in food and feed is a worldwide problem. Many countries are struggling with OA contamination in their crops, food products and feed (Kumar *et al*., 2020). Aflatoxins and fumonisins are usually dominant mycotoxins in grain corn samples (de Souza *et a*l., 2013; Shi *et al*., 2018; Udomkun *et al*., 2017), but recently the level of ochratoxins was frightening. Mycotoxin study in sub-Saharan Africa showed that corn was heavily contaminated with OA and affected children's health (James and Zikankuba, 2018). In addition, OA level in grain corn, corn, corn-based feed and corn silage samples collected around the globe was found to be over the permitted limit and the South Asia region displayed the most contaminated samples (Gruber-Dorninger *et al*., 2019; Anukul *et al*., 2013). In Europe, corn and feed samples also showed positive manifestation of the toxin (Streit *et al*., 2012). The concern on OA manifestation in feed is purely due to the alarming impact on animal health. OA is also known as nephrotoxins and is commonly associated with carcinogenic, hepatotoxic, immunotoxic, nephrotoxic and teratogenic effects on animals (Buszewska-Forajta, 2020). Due to its toxicity, the kidney, liver and intestine are the common target for OA and causes severe organs cancer in poultry (Yang *et al*., 2020). High ingestion of OA-contaminated feed results in low egg productivity and hatchability and low immune defence (Bryden, 2012). Consumption of affected animals can cause Balkan endemic nephropathy in humans (Luo *et al*., 2021).

An early ochratoxin detection method should be developed to screen the presence of the target analyte in grain corn before it can be marketed and consumed. The conventional OA detection methods such as chromatography and mass spectrometry are undoubtedly reliable and accurate. However, the procedure is time consuming, tedious and requires a large sample volume (Roseanu *et al*., 2010). A simple biosensor-accommodated-antibody method was the latest preferred OA detection technique in grain corn samples (Selvaraj *et al*., 2015). An antibody-based biosensor promises high sensitivity as the antibody is specifically developed for its target analyte and multi-devices application (Jia *et al*., 2021; Alhamoud *et al*., 2019). Aside from optical mean, a biosensor can also be applied to electrochemical interfaces.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), phosphate buffer saline (PBS) tablet, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), alkaline phosphatase yellow (pNPP) substrate, tetramethylbenzidine (TMB) substrate, aflatoxin B1 (AB1), ochratoxin A (OA), fumonisin B1 (FB1) and Ochratoxin BSA were purchased from Sigma. Ochratoxin KLH and ochratoxin horseradish peroxidase (HRP) were obtained from Creative Diagnostics. Aflatoxin B2 (AB2),

aflatoxin G1 (AG1), aflatoxin G2 (AG2), ochratoxin B (OB), fumonisin B2 (FB2) and fumonisin B3 (FB3) were purchased from Romer Labs. Ammonium sulphate [$(NH_4)_2SO_4$], sodium chloride (NaCl), sodium hydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate (Na_2HPO_4), acetonitrile (ACN, HPLC grade), glycine, methanol (MeOH), glacial acetic acid (GAA) and hydrochloric acid 37% (HCl) were acquired from Merck. Aflatoxin B2 (AB2), aflatoxin G1 (AG1), aflatoxin G2 (AG2), ochratoxin B (OB), fumonisin B2 (FB2) and fumonisin B3 (FB3) were purchased from Romer Labs. Pierce BCA reagent A and Pierce BCA reagent B were obtained from Thermo Scientific. Dialysis tubing and Tween 20 were purchased from Sigma-Aldrich. Aflatoxin BSA, nProtein A-Sepharose, dry milk, secondary antibody alkaline phosphatase (AP) conjugate, sodium hypochlorite and 95% ethanol (EtOH) were purchased from Fitzgerald, GE Healthcare, Santa Cruz, MP Biomedical, Clorox and HmbG Chemicals, respectively. Vacutainer (8.5 mL) for blood collecting was obtained from Becton, Dickinson and Company.

AKTAPRIME Protein Purifier (GE Healthcare) equipped with Primeview software was used for purifying of antibodies (separation of IgG). GE XK16 column was used for IgG purification. Multiskan Go ELISA Reader (Thermo Scientific) equipped with MultiskanGo software was used for absorbance reading. Rotina Refrigerated Centrifuge (Hettich) was used to separate serum from the blood sample and to separate antibodies from serum. Separation of extracted corn sample was conducted using refrigerated centrifuge 5810 R (Eppendorf). Venticell Incubator (MMM) was used during incubation for ELISA. Chromatographic determination of ochratoxins was conducted using UVE HPLC instruments (LCTech) equipped with Multi λ Fluorescence Detector 2475 (Waters).

Two New Zealand breed (Oryctolagus cuniculus) rabbits were purchased from a local university (Universiti Putra Malaysia) and denoted as O1 and O2. The rabbits' diet during the immunisation period included pellets and carrots. Rabbit pellet was purchased from a local company (D'Syira) and carrots were obtained at the local supermarket. Grain corn sample (GWG888) was obtained from a local source and was kept at room temperature.

Preparation of Solutions and Buffers

Primary and secondary injection solutions: Primary injection solution (PIS) was prepared by homogeneously mixing 2 mL 200 µg/mL ochratoxin BSA and 2 mL CFA using a syringe and then stirred at 4 °C overnight. The solution was mixed again using a syringe until an emulsion was formed. 0.5 mL emulsion then was transferred into two 1 mL syringes. Air bubbles were removed from the emulsion. Secondary injection solution (SIS) was prepared by substituting CFA with IFA.

1 M phosphate buffer (PB) and 0.01 M phosphate buffer saline (PBS, dialysis buffer): PB was prepared by mixing 100 mL 1 M $NaH_2PO_4.H_2O$ (acid) and 100 mL 1 M Na_2HPO_4 (base). The pH was adjusted to 7.4 with the base while stirring the solution. PBS was prepared by mixing 50 mL PB and 500 mL 1.5 M NaCl. Distilled water was added to make up to 5 L.

Affinity chromatography buffers: 0.1 M PB (pH 7) was prepared as a binding buffer. 0.1 M glycine (elution buffer, pH 3) and 1 M Tris buffer (pH 9) were prepared by adjusting the pH using HCl. 20% EtOH was used as washing buffer. All buffers were degassed prior purification process.

ELISA buffers: Dilution buffer was prepared by dissolving PBS tablet in deionised water (0.01 M, pH 7.4). Washing buffer (PBST) was prepared by mixing 1 L

0.01 M PBS (pH 7.4) with 0.5 mL Tween 20. Blocking buffer (0.05% dry milk) was prepared by dissolving dry milk in 0.01 M PBS (pH 7.4).

Production of Antibody

Immunisation: Polyclonal antibodies against OA were produced in-house by Biotechnology & Nanotechnology Research Centre (Animal Ethics Committee of MARDI approval number 20190215/R/MAEC00045). The rabbits were immunised for six months (February 2019 – July 2019). Firstly, blood from the rabbits was drawn once before injection with PIS two weeks later. After that, SIS was injected for 3 weeks before drawing the blood on the 4th week (1st bleed). Then, SIS injection and blood draws were alternated until 5th bleed.

Precipitation of serum and dialysis: After each bleeding, the blood samples were kept at room temperature for 2 hours. Then, the blood samples were spun (6000 rpm) in a centrifuge at 20 °C for 30 minutes. The obtained serum (yellowish solution) was transferred into a centrifuge tube and stored at -20 °C.

Frozen serum was thawed for 1 hour before being mixed with deionised water. While stirring the solution, saturated $(NH_4)_2SO_4$ solution was added drop by drop. The volume ratio of serum, deionised water and $(NH_4)_2SO_4$ was 1, 9 and 10, respectively. The solution was stirred continuously for 30 minutes. The solution was transferred into a 50 mL centrifuge tube and spun (6000 rpm) in a centrifuge at 15 °C for 30 minutes. The obtained white pallet was dissolved in 2-3 mL 0.01 M.PBS (pH 7.4). The solution then was transferred into a dialysis tube and secured tightly. The tube then was placed in dialysis buffer and dialysed at 4 °C with three times buffer changes (4-6 hours). The solution was collected and kept at -20 °C.

Affinity chromatography: The column's parts were immersed in 20% EtOH overnight and dried at room temperature. Protein A was poured into the column and attached to the AKTAPRIME instruments. The system wash was run using 20% EtOH and then followed by manual run using deionised water and 0.1 M PB (pH 7).

Titer Determination

A titer assay was conducted to evaluate the performance of antibodies from each bleed. 100 µL 65 µg/mL ochratoxin KLH was immobilised in a 96-well microplate (n=3) overnight at 4 °C. The plate was washed three times with 350 µL washing buffer (0.05 % Tween 20 in 0.01 M PBS). The plate then was filled with 250 µL blocking buffer (0.05 % dry milk in 0.01 M PBS) and incubated for an hour at 37 °C. The plate was washed again. 100 µL serial dilutions of OA antibody were immobilised in the well and incubated for 2 hours at 37 °C followed by washing the plate. 100 µL secondary antibody AP (1:1000 dilution) was added and allowed to incubate for 30 minutes at 37 °C. The plate was washed again before being filled with 100 µL pNPP substrate. After 30 minutes, the plate was read at 405 nm using an ELISA reader.

Protein Assay

BCA protein assay was carried out to determine the concentration of the developed OA antibody. BSA was used as the reference standard for mycotoxins antibody assay. 25 µL serial dilutions of BSA standard (0-4 mg/mL) was added into the microplate well (n=3). Two antibody dilutions (1 and 10x) were prepared and 25 µL of each dilutions was immobilised on the plate (n=3). 100 µL substrate (a mixture of Reagent A and Reagent B in 9:1 ratio) was added and the plate was incubated for 30 minutes at 37 °C before reading the plate at 560 nm using an ELISA reader.

Standard Curve Determination

OA standard curve was determined using competitive direct ELISA (Figure 2). 100 µL 1 mg/mL mycotoxin antibody was immobilised in a 96-well microplate (n=3) overnight at 4 °C. The plate was washed three times with 350 µL washing buffer (0.05 % Tween 20 in 0.01 M PBS). The plate then was filled with 250 µL blocking buffer (0.05 % dry milk in 0.01 M PBS) and incubated for an hour at 37 °C. The plate was washed again. 50 µL of serial dilutions of OA standard were immobilised in the well followed by 50 µL HRP conjugate. The mixture was allowed to incubate for 2 hours at 37 °C. The plate was washed again before being filled with 100 µL TMB substrate. The plate was read at 370 nm after 30 minutes of incubation using an ELISA reader.

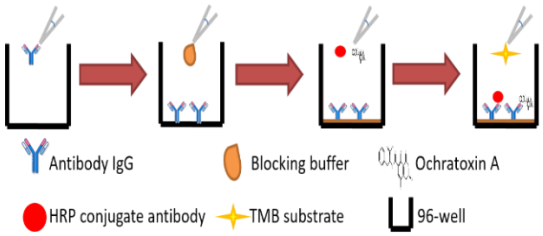


Figure 2. Direct competitive assay of OA in 96-well microplate.

Cross-reactivity

Cross-reactivity of each mycotoxin was conducted by introducing mycotoxins of the same and different groups into the assay. Mycotoxins used were AB1, AB2, AG1, AG2, OB, FB1, FB2 and FB3.

Real Sample Analysis

Grain Corn Health Test: Three non-sterile grain corn samples were put into a culture plate made of peptone sucrose agar. Two sets of sample plates were prepared. One set was set up at room temperature and the other set was set up in an incubator at 30 °C for five days. The fungus produced on the plate was then subcultured at room temperature for five days. In a sterile environment, two sets of sterilisation methods were conducted; i) Sodium hypochlorite and ii) Sodium hypochlorite with an autoclave. Three-grain corn samples from each method were put into a culture plate and then was set up at room temperature for five days.

Matrix Study of Grain Corn Sample (Dilution Factor): 3 g autoclaved grain corns were ground into powder and 15 mL extraction solvent (80 % ACN in deionised water) was added. The mixture then was shaken for 5 minutes and centrifuged at 3000 g for 10 minutes. The supernatant was collected and several dilutions were prepared. The competitive assay was conducted and the best dilution factor was determined by comparing the absorbance reading of each dilution with control (PBS).

Standard Curve in Corn Matrix: The same extraction procedure was conducted. Supernatant collected was filtered through filter paper. Several concentrations of OA standards were prepared using the diluted supernatant. The competitive assay was conducted to determine the standard curve.

Recovery Study: Ground corn was spiked with several concentrations of mycotoxins and then extracted using the same method. The competitive assay was repeated with extracted spiked samples.

HPLC Analysis

Prior to HPLC, mycotoxins first were extracted from grain corn samples. For ochratoxin analysis, 100 mL extraction solvent (80% MeOH) was added to 25 g ground corn and blended for 3 minutes or shaken for 60 minutes. 4 mL filtered extract was added into 12 mL PBS. Ochratoxin analyses were conducted using isocratic HPLC with fluorescence detection in reverse

phase analysis. Water:MeOH:ACN/GAA (40:55:5/1 v/v) was used as eluent. Injection of sample volume was 10-100 µL at a flow rate of 0.6 mL/min through RP C18 column at a column temperature of 40 °C. Fluorescence detector; Ex.: 395 nm, Em.: 440 nm.

Results and Discussion

Production of Antibody

After the bleeding process, the acquired blood samples further went through several processes to produce pure IgGs (antibodies) against the target mycotoxin from bulk antibodies. The affinity column was exclusively implemented to individually separate the IgGs. The serum was run through the protein A column and all IgGs presented were allowed to bind to protein A with the help of PB buffer while other antibodies were washed out of the system. Then IgGs were eluted out from the column using glycine HCl buffer by following the affinity principle. This process was all recorded in the form of a chromatogram where two peaks were observed. The first peak represented other proteins in the serum. The second peak was eluted IgG and samples from fraction 26 to 32 (under the peak) were collected.

Titer Determination

The performance of produced antibodies can be evaluated by reducing the antibodies into several dilutions followed by optical measurement. The purpose of titer is to determine which bleed of antibodies can produce the best and most reliable result with the lowest dilution. These antibodies then will be used for further analysis, thus this step is exceptionally important. In this study, OA antibody in all bleeds (except preimmune) showed relatively high absorbance at high antibodies concentration and the absorbance was gradually dropped as the concentration lowered (Figure 3). Antibodies from 2nd to 5th bleeds were fairly stable for several dilutions.

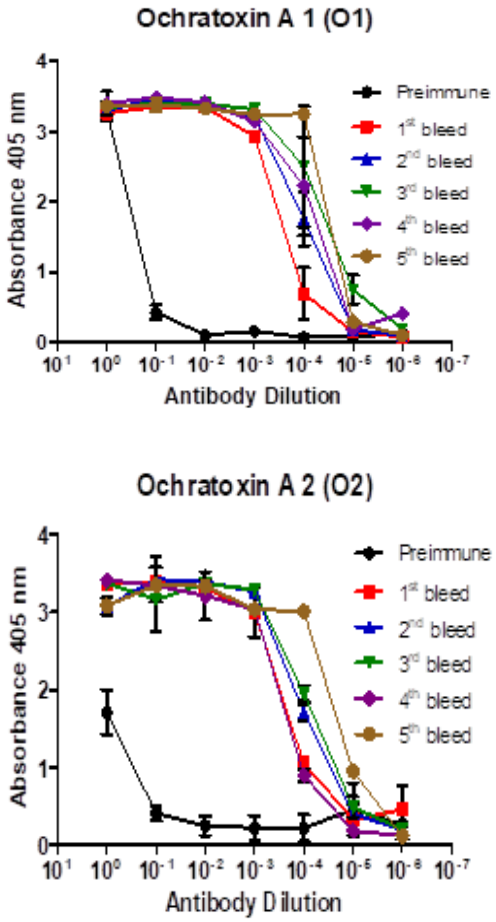


Figure 3. OA antibody performance at different serial dilutions at 405 nm.

Protein Assay

The concentration of the produced antibodies was determined from the BCA protein assay. Figure 4 displayed the calculated concentration of antibodies based on the BSA standard curve. Overall, the concentration was relatively low before the introduction of mycotoxin into the rabbits' immune system.

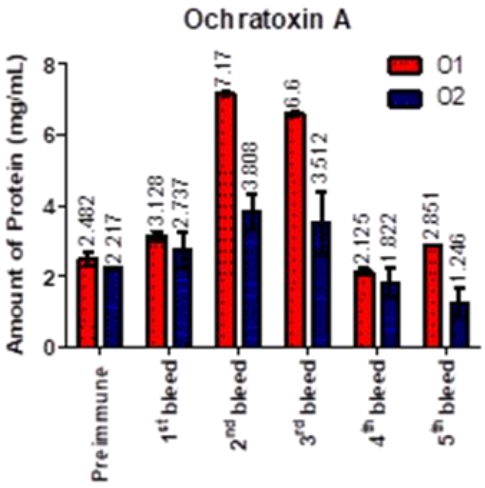


Figure 4. OA antibody (IgG) concentration found in collected rabbits' serum.

However, the concentration was gradually increased as the rabbits had built a protective immune barrier against the mycotoxins after several injections. Some rabbits maintained the amount of antibodies in their bodies and some rabbits showed low tolerance of mycotoxins after four injections.

Standard Curve Determination

Contrasting protein molecules, mycotoxins are considered small in molecular size. Thus, a highly sensitive detection technique should be applied in order to specifically capture the target analyte. Enzyme-linked immunosorbent assay (ELISA) was a reliable method for evaluating small molecules in samples. Competitive ELISA was particularly had been proven to provide a good optical response for mycotoxins detection, especially OA (Orlov *et al*., 2017; Kobun *et al*., 2019). Plastic substrate (microplate) provides strong antibody binding and the employment of specific and stable enzyme labels improves the absorbance signal of the competitive assay (Wu *et al*., 2019). In this study, antigen (OA) in several concentrations was allowed to compete with HRP label antibody to evaluate the sensitivity of the anti-OA antibody. As displayed in Figure 5, as the amount of OA in the system increased, the developed antibody lose the opportunity to bind with the HRP label and resulting in a low absorbance value. The assay also exhibited good linearity with a wide working range with a low limit of detection of 4.03 ppb (Table 4).

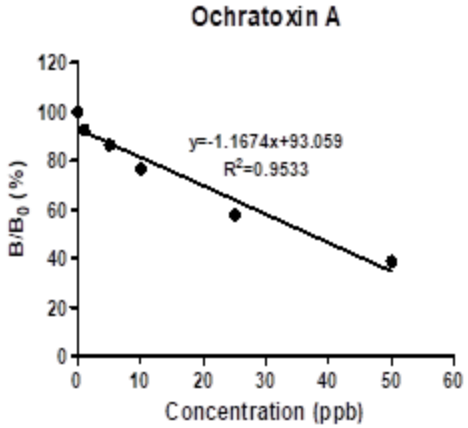


Figure 5. Performance of OA antibody in PBS system at 370 nm.

Cross-Reactivity

Competitive direct ELISA was conducted for cross-reactivity. OA antibody was cross-reacted with its corresponding group and other mycotoxins' group (Figure 6). OA displayed non-significant cross-reactivity towards all groups of mycotoxins. This is might be due to the massive difference in the chemical structure of mycotoxins' compounds. The performance of OA antibody in the competitive assay was highly selective towards its respective target analytes. OA displayed non-significant cross-reactivity towards all groups of mycotoxins. The performance of OA antibody in the competitive assay was highly selective towards its respective target analyte.

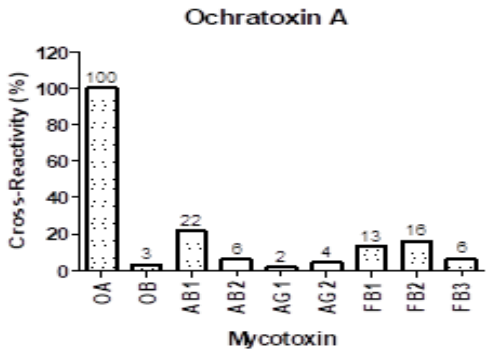


Figure 6. Intra and inter-species cross-reactivity of OA antibody.

Real Sample Analysis

Grain Corn Health Test: Table 1 showed the result of cultured non-sterile grain corn for five days at two different temperatures. Fungus (white and green) and mucous were observed for both room temperature and 30 °C. This proves that temperature does not significantly affect the growing of fungus on the corn samples. The produced fungus and mucous were individually subcultured to confirm the type of fungus grown on the corn. Table 2 showed the result of subcultured grain corn for five days at room temperature. It was confirmed that only two types of fungus were produced. Green fungus was suspected to be *Aspergillum sp*. which produces aflatoxins and ochratoxins. White fungus was suspected to be *Fusarium sp*. which produces fumonisins. However, the presence of fungi does not imply that mycotoxins were produced in the corn sample. Subcultured mucous was suspected to be one type bacteria.

Table 1. Culture of non-sterile grain corn for five day.

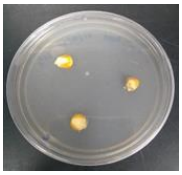
Temperature	Day 1	Day 5	Germination	Fungus	Mucous
Room (25 °C)			Yes	Yes	Yes
30 °C			Yes	Yes	Yes

Table 2. Subculture of non-sterile grain corn for five day at room temperature.

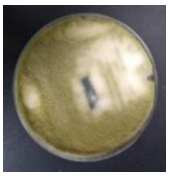
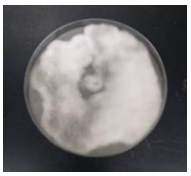
	Green fungus	White Fungus	Mucous
Day 5			
Fungus	Yes	Yes	No
Bacteria	No	No	Yes

Table 3. Culture of sterile grain corn for five day at room temperature.

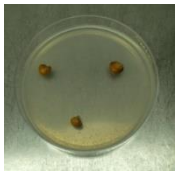
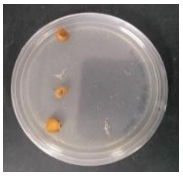
Method	Day 1	Day 5	Germination	Fungus	Mucous
Sodium hypochlorite			Yes	Yes (white only)	No
Sodium hypochlorite + autoclave			No	No	No

Table 3 showed the result of cultured sterile grain corn for five days at room temperature. Significant difference was observed for both sterilisation methods. After five days, the only white fungus was observed on corn sterilised with sodium hypochlorite. No green fungus or mucous was produced compared to non-sterilised corn. However, when grain corn was treated with both sodium hypochlorite and autoclaved, nothing was observed on the corn. The corn even did not germinate. This is due to the effect of high autoclave temperature which kills all potential bacteria, fungi and even nutrients in the corn. The grain corn treated with autoclave also looked a slight brownish compared to corn treated with sodium hypochlorite only.

Matrix Study of Grain Corn Sample (Dilution Factor): The performance of the OA antibody was tested with sample dilution from 0 to 150. The dilution factor was determined by comparing the absorbance reading of each dilution with control. Dilution with similar reading as control was selected for further assay experiment as it displayed zero matrix interference. After 15 minutes of TMB incubation, a matrix study of OA showed that dilution of 50 had similar reading as control (Figure 7).

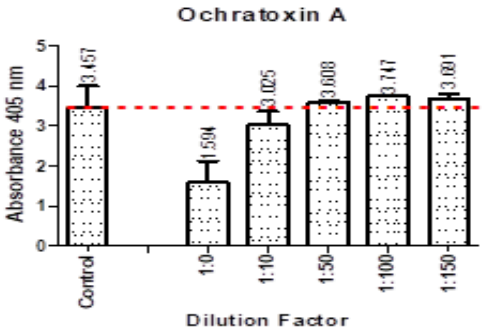


Figure 7. The matrix effect of corn sample towards OA antibody.

Standard Curve in Corn Matrix: The diluted corn sample extract was further spiked with several concentrations of OA to examine the matrix effect with the presence of a mycotoxin. The direct competitive assay was once again employed and the OA standard curve in the corn matrix was plotted (Figure 8). Good linearity was achieved at a wide working range. This proved that the matrix study was successful in eliminating possible interferences in the corn matrix. Table 4 summarised the comparison of the standard curve in PBS and corn matrix. The performance of antibody was better in corn matrix compared to PBS, as OA was detectable at much lower concentration (lower LOD and LOQ).

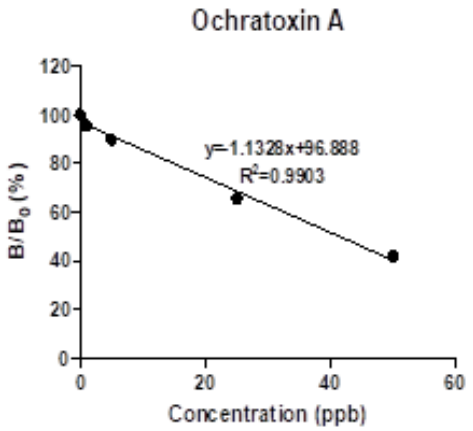


Figure 8. Performance of OA antibody in corn matrix at 370 nm.

Table 4. Comparison of OA competitive assay in PBS and corn matrix.

	R^2 value	LOD (ppb)	LOQ (ppb)
PBS	0.9533	4.03	12.21
Corn Matrix	0.9903	2.70	8.10

Recovery Study

Grain corn samples were spiked with several concentrations of OA and then were run for optical measurement. A recovery study is essential to demonstrate the efficacy of the mycotoxin extraction method from a grain corn sample. Different samples may require a different type of mycotoxin extraction method due to the nature and nutrient constitutions of the samples. In addition, the type of solvent and the ratio used also played an important role during extracting target mycotoxin. Most of the established extraction methods were applied for HPLC analysis and some of the methods were not suitable for optical sensor measurement. In this study, mycotoxins in grain corn samples were extracted using 80% ACN. A polar solvent such as ACN can work with a variety of mycotoxins, thus contributing to the success of mycotoxin extraction in corn samples (Nakhjavan *et al*., 2020). The combination of water and the organic solvent was proven in increasing polarity and helped in the effective extraction of solid samples (Iqbal, 2021). In addition, grain corn samples were also ground into small size to allow homogenous extraction (Munkvold *et al*., 2019). Taking into account all these parameters, samples were successfully extracted and resulted in good detection and recovery of the target mycotoxins (Table 5).

Table 5. Recovery of spiked grain corn samples using 80% ACN.

Spiked (ppb)	Found (ppb)	Recovery (%)
10	8.02	80.2
25	33.6	134.32

HPLC Analysis

The sensitivity of the developed optical immunodiagnostic for OA detection in grain corn was further verified using chromatographic analysis. From the results, the grain corn samples were heavily contaminated with OA (Table 6). Both developed immunodiagnostic and HPLC analysis methods detected the amount of OA which was over the MRL (5 ppb). These results validated the high recovery achieved from the previous section.

Table 6. Detection of OA in grain corn samples using immunodiagnostic and chromatography methods.

Detection Method	Found (ppb)
Ochratoxin A Immunodiagnostic Kit	25.60-37.60
High Performance Liquid Chromatography	25.82-27.02

Conclusion

A detection method of ochratoxin A in grain corns has been successfully developed using optical measurement involving biological reactions between antibody and ochratoxin A in ELISA protocol. This method has produced high selectivity, sensitivity and accuracy and can detect the presence of OA in low concentration in both PBS and corn matrix systems. In addition, this method has the potential to be applied to other feed sources such as nuts, paddy, wheat and grass.

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