JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Rapid Aflatoxin B_1 ELISA: Development and Validation with Reduced Matrix Effects for Peanuts, Corn, Pistachio, and Soybeans

NANJU A. LEE,*,† SHUO WANG,†,§ ROBIN D. ALLAN,‡ AND IVAN R. KENNEDY†

Faculty of Agriculture, Food and Natural Resources and Department of Pharmacology, University of Sydney, NSW, 2006 Australia

Among the competitive ELISAs for aflatoxins that have been described, few have been adequately validated for reduced matrix effects. Using an aflatoxin B_1 (AFB_1)-specific polyclonal antibody (produced from AFB_1−oxime conjugated to bovine serum albumin (BSA)) and AFB_1− and AFB_2−enzyme conjugates, four direct competitive ELISAs based on 96-microwell plates (two standard assays and two rapid assays) were developed, paying special attention to producing a robust assay relatively free of interferences for a range of agricultural products. The antibody was AFB_1-specific, detecting only AFB_1 in a mixture of four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), but showed significant cross-reaction with AFG_1 (57−61%) when an individual compound was tested. Standard assays (long assays) exhibited higher sensitivities than rapid assays (short assays) with IC_{50} values of 12 ± 1.5 and 9 ± 1.5 μg/kg in sample (with 1 in 5 dilution of sample extract) for AFB_1 and AFB_2−enzyme conjugates, respectively. These assays have narrower detection ranges (7.1−55.5 μg/kg in sample) and required dilution of sample extracts to overcome solvent and matrix interferences, making these assays less ideal as analytical methods. Rapid assays exhibited IC_{50} values of 21.6 ± 2.7 and 12 μg/kg in sample for AFB_1− and AFB_2−enzyme conjugates, respectively. These assays have ideally broader detection ranges (4.2−99.9 μg/kg in sample) and showed no methanol effects up to 80% with significantly reduced matrix interferences as a result of the shorter incubation times and increasing the amounts of enzyme conjugate used. Therefore, the rapid assays were formatted to perform without a need for extract dilution. The rapid assays can be completed within 15 min, potentially suitable for receival bays where quick decision-making to segregate low and high contamination is critical. Further validation using the rapid assay with AFB_1−enzyme conjugate indicated relatively good recoveries of AFB_1 spiked in corn, peanuts, pistachio, and soybeans, which were free from significant matrix effects. It can be concluded that this rapid assay would be suitable for monitoring aflatoxin AFB_1 at current legal maximum residue limits of 10 μg/kg in food such as corn, peanuts, pistachio, and soybeans.

KEYWORDS: Aflatoxin B_1; ELISA; food; antibodies; carcinogen; monitoring; food safety

INTRODUCTION

Aflatoxins are toxic metabolites produced by fungi, mainly *Aspergillus flavus* and *A. parasiticus*. They are listed as group I carcinogens by the International Agency for Research on Cancer (IARC), primarily affecting liver (*1*). The LD_{50} of aflatoxins can be as low as 0.5 mg/kg body weight (*1*), which is significantly more toxic than most other known carcinogens. For these reasons, the presence of aflatoxins in food and animal feeds is potentially hazardous to the health of both humans and animals. Aflatoxin B_1 (AFB_1) has been shown to induce mutation at codon 249 in the tumor suppressor gene p53, which occurs in most heptatocarcinomas (*2*). Although some reports suggested that hepatocarcinogenesis in humans does not directly associate with aflatoxins (*3, 4*), the high incidence of liver cancer in South Africa (*5*), South-East Asia (*5*), Korea (*6*), Taiwan (*7*), and China (*8*) are still suspected to be linked to a combination of high dietary exposure to aflatoxins and hepatitis B viral infection. Furthermore, aflatoxin contamination affects the economic values of the crops as well as reduced efficiency of animal production, resulting in higher costs incurred by all sectors from production to consumption. The tolerance levels currently set by the regulatory bodies worldwide are typically 0.05 μg/kg for AFM_1 in milk, 10 μg/kg for AFB_1 and 20 μg/kg

* To whom correspondence should be addressed. Tel.: +61 2 9351 8710. Fax: +61 2 9351 5108. E-mail: a.lee@acss.usyd.edu.au.
† Faculty of Agriculture, Food and Natural Resources.
‡ Department of Pharmacology.
§ Current address: Faculty of Food Science and Bioengineering, Tianjin University of Science and Technology, Tianjin 300222, People's Republic of China.

10.1021/jf0354038 CCC: $27.50
Published on Web 04/20/2004

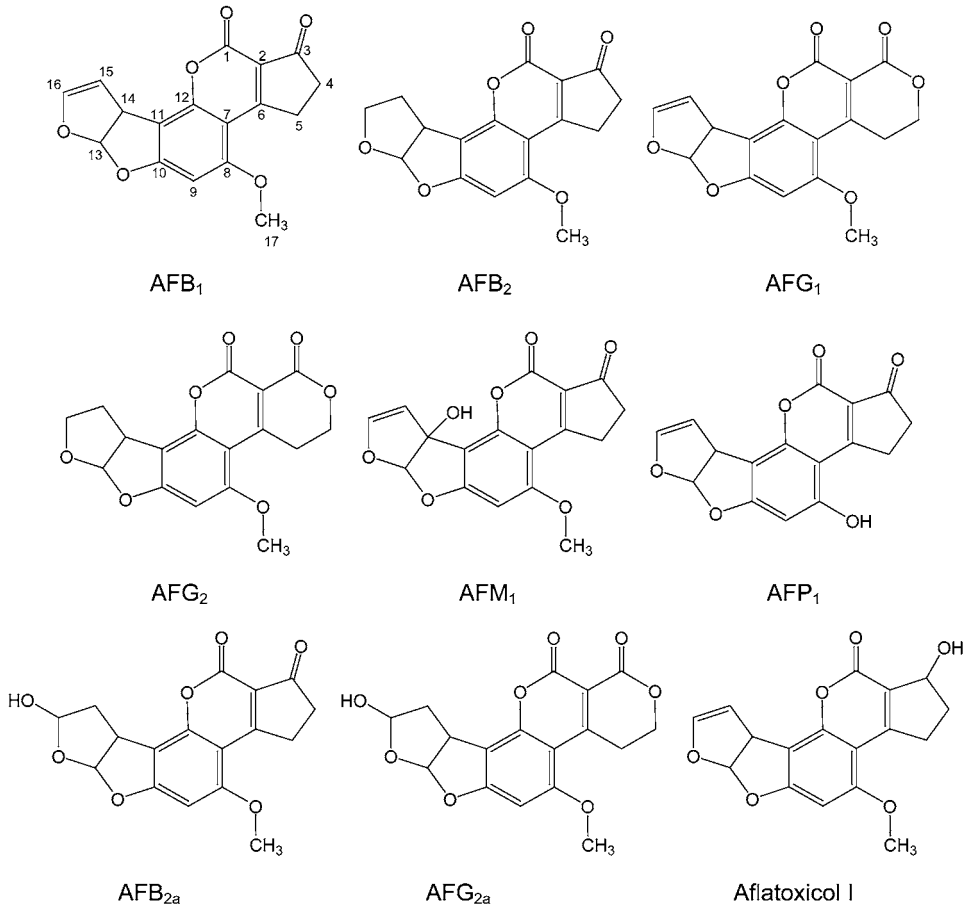


Figure 1. The structures of aflatoxins and their metabolites. The carbon atoms are numbered respectively on the AFB_1 structure.

for total aflatoxins in food intended for human consumption and 20−300 μg/kg for total aflatoxins in animal feeds (*9*). The European Commission is finalizing a proposal to set new tolerance levels at 2 μg/kg for AFB_1 and 4 μg/kg for total aflatoxins in certain species (*10*).

The aflatoxins commonly found are AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 (**Figure 1**). AFB_1 is the most potent of all aflatoxins known to date and is generally found in the highest concentration in food and animal feeds. Aflatoxin contamination is most frequently found in peanuts, corn, and oil seeds such as cottonseed. They have also been reported to contaminate wheat, sorghum, Brazil nuts, almonds, walnuts, pecans, dried fruits, legumes, peppers, potatoes, rice, copra, filberts, milk, and milk products. The contamination can occur in the field, during harvest and transportation, and during storage, under conditions where mold is allowed to grow. Since these toxins are heat stable, they are very difficult to destroy once formed. It is important to prevent mold growth through good agricultural, storage and manufacturing practices, with proper monitoring for possible contamination.

The analysis of aflatoxins in food and animal feeds is a difficult task for a number of reasons. First, aflatoxin contamination can be very unevenly distributed in food and feed samples. Obtaining a representative sample from a nonhomogeneous bulk lot is a challenge, and this is known to be the error-determining step in the whole analytical procedure (*11*). Second, different interfering substances co-extract from different food and feed matrixes. Multistep cleanup procedures are, therefore, commonly employed to remove the interferences prior to the actual analysis. Current analysis is accomplished by various methods including the minicolumn method (*12*), thin-layer chromatography (TLC) (*13*), high performance liquid chromatography (HPLC) (*14*), and enzyme-linked immunosorbent assay (ELISA) (*15*).

TLC analysis is a relatively economical method of aflatoxin measurement with little equipment but can be tedious and is time and labor consuming. Estimation by visualization inherently gives a higher variation between analyses. Chromatographic analysis is widely accepted as an official method for aflatoxin analysis. HPLC analysis requires an extensive cleanup procedure and derivatization to improve the detection sensitivity, needing specially trained personnel to perform it. ELISAs for aflatoxin and aflatoxin metabolites have been developed rapidly in the past two decades because of their simplicity, adaptability, sensitivity, and selectivity. To give a few examples, ELISAs specific to AFB_1 (*16*, *17*), AFB_2 (*18*), AFG_1 (*19*), total aflatoxin (*20*), and each of the major metabolites such as AFB_{2a} (*21*), AFQ_1 (*22*), and AFM_1 (*23*) have been reported. Only ELISAs for total aflatoxin and those specific to AFB_1 and AFM_1 are commercially available, mainly driven by the regulatory requirements. In some cases, descriptions of ELISAs based on AFB_1-specific antibodies (indicated by the cross-reaction information) were misleading as quantifying total aflatoxin in food samples without providing adequate validation data.

There has been an increase in demand for monitoring aflatoxins in developing regions such as South-East Asia, Middle East, and Africa, where high incidence of liver cancers prevail, to assess the health and economic risks posed by aflatoxin contamination in food and animal feeds. Consequently, simple, quick, reasonably accurate, specific, and cost-effective methods requiring little equipment are needed to suit the economic factors and infrastructure of these developing regions. The objective of this study was to develop a quick and effective ELISA test for measuring AFB_1 at the maximum residue limit (MRL) of

10 and 20 μg/kg for food and animal feeds, sufficiently robust for different food commodities and to validate its analytical parameters for matrix effects. Matrix effects from food and animal feeds are a common problem experienced by immunoassays, but little has been reported previously. This paper will describe the development of a rapid assay that can significantly reduce the matrix effects and validation of this assay with spiked samples. This ELISA would be a valuable analytical tool to apply to several research projects involving surveys, risk assessment, agronomic management, and biocontrol strategies to reduce the impact of aflatoxin contamination in Asia-pacific regions (*24*).

MATERIALS AND METHODS

Materials. Aflatoxin congeners and metabolites (AFB_1, AFB_2, AFG_1, AFG_2, AFB_{2a}, AFG_{2a}, AFP_1, and AFM_1) and other mycotoxins (cyclopiazonic acid, ochratoxin A and B) were purchased from Sigma (St Louis, MO). Fumonisins (B_1, B_2, and B_3) were purchased from the Program on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council (Tygerberg, South Africa). AFB_1−BSA, and other chemicals used in the hapten synthesis, immunization and in an ELISA were purchased from Sigma (St Louis, MO). Ovalbumin (OA), keyhole limpet hemocyanin (KLH) and *N*-(β-maleimidopropionic acid) hydrazide were purchased from Pierce (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Dako Corporation (Carpinteria, CA). Analytical grade methanol was obtained from Ajax Chemicals (Clyde, Australia). Protein A agarose and PD-10 desalting columns were from Pharmacia (Uppsala, Sweden). Silica gel 60 (70−230 mesh), TLC using silica gel 60 F_{256} precoated plates and preparative thin layer chromatography (PLC) using silica gel 60 F_{254} precoated plates (20- × 20-cm, 2-mm thick) were purchased from Merck (Darmstadt, Germany). Maxisorp polystyrene 96-microwell plates were purchased from Nunc (Rockilde, Denmark)

Instrumentation. Absorbances of microwells were recorded by a Labsystems Multiskan Ascent microplate reader (Labsystems, Helsinki, Finland) with dual-wavelength mode (450−650 nm). H^1 nuclear magnetic resonance (NMR) was recorded with a Varian Gemini 300 instrument (300 MHz) using $CDCl_3$ as a solvent. Where possible and necessary, chemical products were monitored by TLC using silica gel 60 F_{256} precoated plates with visualization under UV light.

Hapten Synthesis. AFB_1 and AFB_2 were converted to their respective AFB_1−oxime and AFB_2−oxime using a method described by Chu et al. (*25*) and Hastings et al. (*26*), respectively.

Preparation of Aflatoxin Oximes. A typical reaction for AFB_1 was conducted as follows (**Figure 2**, **Scheme A**). Carboxymethylhydroxylamine HCl (10 mg, 0.046 mmol) was added to a solution of AFB_1 (10 mg, 0.032 mmol) in methanol/water/pyridine (4:1:1) and the mixture was refluxed at 60 °C for 3 h. After keeping overnight at room temperature, the solution was concentrated under vacuum to produce the yellow residue. The residue was chromatographed on a silica column or on a 20- × 20-cm preparative silica 60 F_{254} TLC plate (chloroform/methanol, 95:5) to separate a fluorescent product, which was confirmed to be aflatoxin B_1−oxime by H^1 NMR when compared with the reported spectrum (*24*): TLC (chloroform/methanol, 63:37) R_f 0.24; ^{1}H NMR δ 6.81 (d, H13), 6.47 (t, H16), 6.40 (s, H9), 5.48 (t, H15), 4.81 (m, OCH_2CO), 4.78 (m, H14), 3.90 (s, OCH_3), 3.36 (m, H5), 3.00 (bs, H4).

AFB_2 oxime was prepared in a similar manner, but starting with smaller amount (5 mg). AFB_2 oxime was not confirmed by H^1 NMR due to insufficient quantity, but its R_f (0 with chloroform/methanol, 95:5) agreed with the published value (*26*).

Preparation of Enzyme and Protein Conjugates of AFB_1–Oxime and AFB_2-Oxime. To AFB_1−oxime in 3 mL of dry dichloromethane at 0 °C was added *N*-hydroxysuccinimide (NHS, 5.4 mg, 0.047 mmol) and 1,3-dicyclohexylcarbodiimide (DCC, 9.6 mg, 0.047 mmol), followed by 4-(dimethylamino)pyridine (DMAP, 5 mg). The mixture was stirred overnight, then filtered to remove the byproduct, cyclohexyl urea, and the solvent was evaporated. The residue was redissolved in 0.6 mL of dry dimethylformamide (DMF) for the conjugation to BSA, OA, KLH, and HRP using the methods described earlier (*27*).

Briefly, the active ester was slowly added to a pre-cooled buffer solution (50 mM K_2HPO_4, pH 9.1) containing the above protein and not more than 10% (v/v) DMF to maintain the solubility of the hapten in the coupling mixture. The mixture was kept at 4 °C overnight, and then the enzyme conjugate was desalted using a PD-10 column, eluting with phosphate buffer saline (PBS, 50 mM sodium phosphate, 0.9% (v/v) NaCl, pH 7.2). The BSA, OA, and KLH conjugates were extensively dialyzed against PBS.

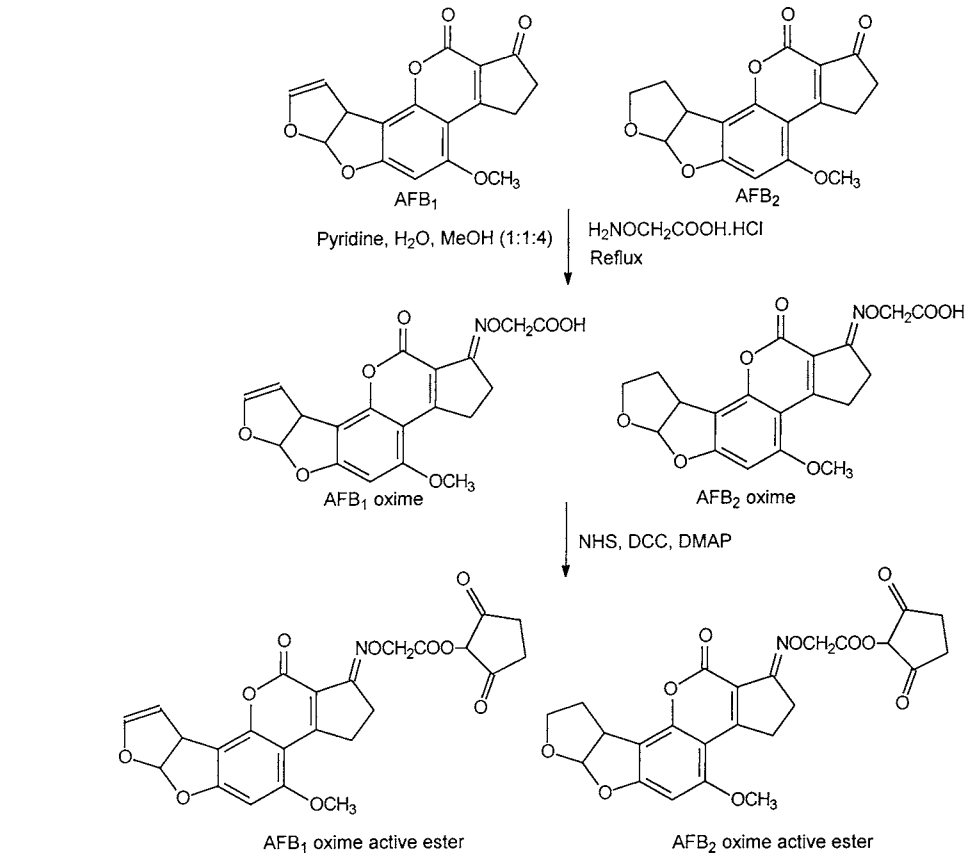
The enzyme and protein conjugates of AFB_2 oxime were prepared in a manner similar to yield AFB_2−HRP, AFB_2−OA, AFB_2−BSA, and AFB_2−KLH.

Preparation of the Phenolate Derivative of AFB_1 and AFB_1-BMPH-HRP. The phenolate of AFB_1 was prepared using a method by Ho and Wauchope (*28*) with modification to conjugate to HRP (**Figure 2**, **Scheme B**). AFB_1 (8.3 mg, 15.4 mmol) in 1 mL acetonitrile and 1 mL of 1M HCl was heated at 60 °C for 3 h. The hemiacetal form of AFB_1 (AFB_{2a}) was extracted with chloroform and confirmed on TLC (with B_{2a} as a standard). Chloroform was evaporated under vacuum, and the residue was redissolved in 0.5 mL of 20% methanol and 0.5 mL of 0.1 M sodium pyrophosphate to form the phenolate ion. To this solution was added *N*-(β-maleimidopropionic acid) hydrazide (BMPH, 13.8 mg, 23.1 μmol) in 110 μL dimethyl sulfoxide (DMSO), and the reaction was allowed to proceed at room temperature for 6 h in the dark. Sulhydryl-modified HRP was prepared by adding 10 mM 2-iminothiolane HCl to the HRP solution containing 4 mM ethylenediaminetetraacetic acid (EDTA). After 4 h of incubation, glycine was added to the reaction solution, and the protein solution was desalted by gel filtration using a PD10 column. After the free sulfhydryl groups were quantified using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sulfhydryl-modified HRP solution was added to the reaction mixture and it was stirred overnight at 4 °C. The HRP solution was dialyzed extensively against PBS.

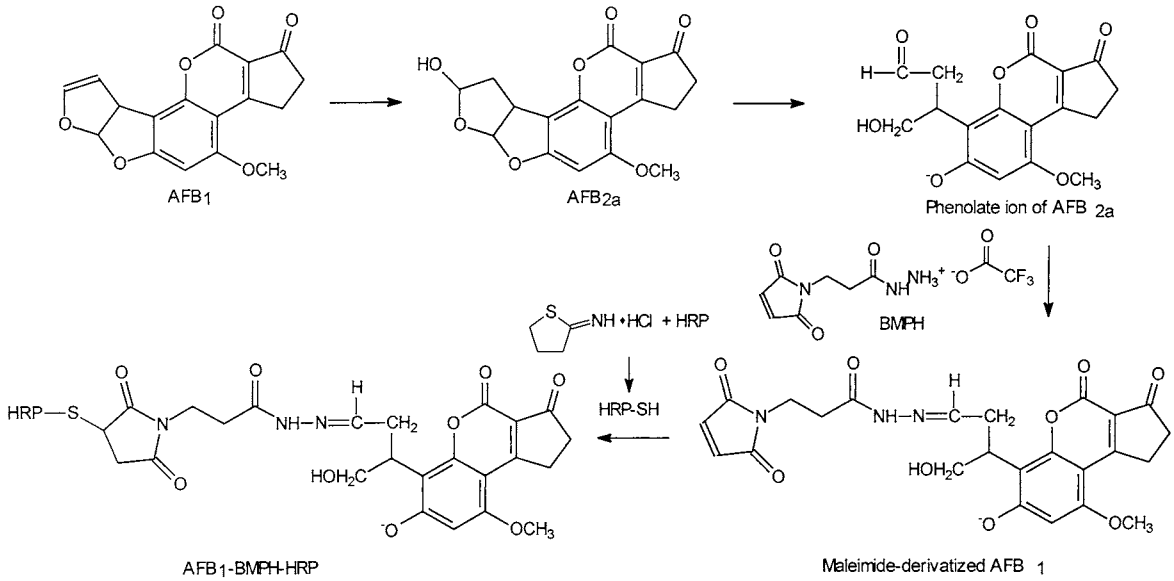
Antibody Production. Antibodies were raised by intradermal and intermuscular injections of BSA conjugates into New Zealand white rabbits, using a similar immunization approach to that described in Lee et al. (*26*), except that lower concentrations were used. The immunogen was diluted in 0.9% saline and emulsified in Freund's complete (for first immunization) or incomplete adjuvant (for subsequent immunization) to give 0.5−1 mg/mL (for first immunization) or 0.25−0.5 mg/mL (for subsequent immunization). After three initial injections at two-week intervals, booster injections were given monthly. Each immunization was given in a total volume of 1 mL. Blood was collected from the marginal ear vein 7−10 days after each booster injection. The titer for specific antibody was monitored by an indirect immunoassay using the immunized hapten conjugated to a protein different from that of the immunogen, such as AFB_1−KLH and AFB_1−OA. Antisera were purified by protein-A-agarose affinity chromatography (*29*). The purified antibodies were dialyzed against PBS.

Preparation of Aflatoxin Standards. The concentration of AFB_1 stock solution (5.8 mg/L in methanol) was established by following an AOAC official method 971.22 (*30*). UV spectrum of the stock solution was scanned from 200 to 500 nm against methanol as a reference solvent, and the concentration was calculated using molecular absorptivity (ε) of 21 500. The concentration of stock solution of AFB_2, AFG_1, and AFG_2 were established in a same manner. The AFB_1 working standards were prepared by diluting the stock solution in 80% methanol to obtain 100 μg/L of AFB_1. From 100 μg/L of AFB_1, 33.3, 11.1, 3.7, 1.2, 0.4, 0.14 μg/L were obtained by serial dilution in glass tubes. The standards in 16% methanol was prepared using the standards in 80% methanol diluted 1 in 5 with water. The standards for corn, peanuts, pistachio, and soybeans were prepared in the same manner using the respective sample extracts.

ELISA Protocols. *Antibody Coating Protocol*. Microwells were coated with anti-AFB_1 antibody at 10 μg/mL in carbonate buffer (0.05 M carbonate buffer, pH 9.6) overnight. After washing the wells with PBS containing 0.05% (v/v) Tween 20 (PBS/T), 1% fish gelatin hydrosate (FGH) in PBS were incubated for 1 h. The excess blocking solution was removed by washing with the PBS/T. All incubations were performed at room temperature.



Scheme A



Scheme B

Figure 2. Hapten synthesis. Scheme A is the syntheses of AFB_1– and AFB_2–oxime and active esters. Scheme B is the synthesis of the phenolate ion of AFB_1 and conjugation to a protein via a cross linker, BMPH.

Standard Assay. AFB_1 standard or diluted sample extract (100 μL) and HRP enzyme conjugate (100 μL, diluted in 1% BSA in PBS) were added to the antibody-coated wells, and the mixed solution was incubated for 60 min. After washing with PBS/T, substrate/chromogen solution (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in acetate buffer, pH 5.5, 100 μL) was added to all the testing wells, and the plate was incubated for 30 min. The plate was read at a dual wavelength mode (450/650 nm) after stopping the color development with 1.25 M sulfuric acid (50 μL). For control and blank wells, 16% methanol and diluted sample extract were used in their respective standard curves. Final absorbance was calculated by subtracting the absorbance of the corresponding blank wells (background color).

Rapid Assay. AFB_1 standard or undiluted sample extract (50 μL) and HRP enzyme conjugate (100 μL, diluted in 1% BSA in PBS) were

premixed in a mixing microwell plate. The premix solution (50 μL) was added to the antibody-coated wells, and the wells were incubated for 5 min. After washing with PBS/T, substrate/chromogen solution (100 μL) was added to all the testing wells, and the plate was incubated for 10 min. The plate was read in a usual manner after stopping the color development with 1.25 M sulfuric acid (50 μL). For control and blank wells, 80% methanol or undiluted sample extract was used in their respective standard curves. The calculation was performed in the same manner as that in the standard assay.

Sample Extraction for ELISA. Peanuts, corn, wheat, sorghum, barley, and soybeans were purchased from the local supermarket, and pistachio samples were a gift from Dr. Hassan Yazdanpanah of Beheshti University of Medical Science and Health Sciences, Tehran, Iran. Peanuts, corn, and pistachio were confirmed to contain aflatoxin at less than 0.1 μg/kg by HPLC performed by a National Association of Testing Authorities (NATA, Australia) accredited mycotoxin analytical laboratory, Agrifood Technology, Melbourne Australia, using the modified AOAC official method.

The efficacy of extraction solutions was studied by shaking a 25-g subsample of pistachio known to be contaminated with aflatoxin (analyzed by HPLC) with 75 mL of 55% methanol, 80% methanol, 80% ethanol, 80% ethanol with 2% Tween 20, and 2% cyclodextrin on a rotary shaker for 30 min and allowing the solutions to stand until the particles settled. The clear supernatants were transferred to glass vials for analysis. In an experiment to compare the efficiency of filtration using Whatman No. 1 filter paper, centrifugation at 10 000 rpm for 30 min, and filtration using a syringe filter with a 0.45-μm pore size nylon membrane for removal of the unsettled particles, sample extracted in 80% methanol was used.

For spike and recovery studies, food samples were finely ground to particles less than 1 mm and thoroughly mixed. A 25-g subsample in a glass jar lined with aluminum foil was extracted with 75 mL of 80% methanol (v/v) containing 4% (w/v) NaCl using a rotary shaker (IKA Labortechnik, Staufen, Germany) shaking at 250 rpm for 15 min, and the mixture was allowed to stand for 15−30 min to separate the supernatant. The clear supernatant was transferred to a glass vial for analysis by the rapid assay. For the standard assay, the supernatant was diluted 1 in 5 with PBS or 1% BSA-PBS prior to analysis.

Spiking Method. A dry spiking technique was used in all the spiking studies. In a typical spiking study, three lots of six 25-g samples were spiked with AFB_1 dissolved in methanol at three different levels (a total of 18 samples). The samples were thoroughly mixed with a stainless steel spatula, then left dark in a fume hood overnight for the methanol to completely evaporate. After mixing samples again with a spatula, three samples were extracted for immunoassay (fresh samples), and three samples were stored at room temperature in the dark for four weeks (aged samples) before analysis.

RESULTS AND DISCUSSION

Synthesis of Aflatoxin Haptens. The approach used by Chu et al. (*25*) to synthesize an AFB_1−carboxymethyl oxime with a terminal carboxyl group for conjugation was used (**Figure 2**, **Scheme A**). This was the most practical way to produce AFB_1 hapten with a relatively high success rate of achieving high yield. The oxime derivative can then be conjugated to a protein either by the synthesis of an active ester or direct conjugation using water soluble carbodiimide. This approach would direct the dihydrodifurano moiety of AFB_1 away from the point of conjugation and would allow production of antibodies able to detect dihydrofuran-containing compounds. As shown in the previous studies, the resulting antibodies were specific to AFB_1 with some degree of cross-reaction with AFG_1 in some cases, because of the dihydrodifurano moiety.

In general, heterologous competitive immunoassays provide higher sensitivity than homologous immunoassays for small analytes, by directing the antibody's binding affinity toward the free analyte in an assay. Assay sensitivity, therefore, can be improved by using a competitor, such as an enzyme-conjugate

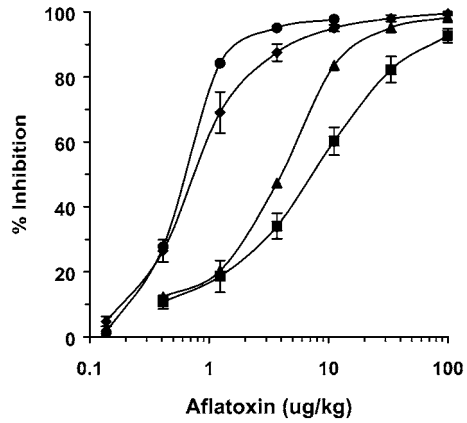


Figure 3. AFB_1 standard curves of standard assays using AFB_1–HRP (◆) and AFB_2–HRP (●), and of a rapid assay using AFB_1–HRP (■), and AFB_2–HRP (▲).

(in a direct assay) or a coating antigen (in an indirect assay), prepared from a hapten that is structurally similar but not identical with that used for antibody production.

Taking this approach, aflatoxin AFB_2−carboxymethyl oxime was synthesized with reaction conditions similar to those used for AFB_1−carboxymethyl oxime synthesis. AFB_2 is a dihydro derivative of AFB_1, lacking a double bond at C15−C16; therefore, it is a good candidate for the competitor. AFB_2 was conjugated to HRP as an enzyme conjugate, and OA and KLH as coating antigens after conversion to the oxime derivative.

Attempts were also made to link AFB_1 to an enzyme with a cross linker, BMPH, as illustrated in **Figure 2**, **Scheme B**. BMPH contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide group on the other end. Also, it is highly water soluble, which is an important factor for the subsequent conjugation to protein in aqueous conditions. BMPH was chosen as a cross-linker to join an aldehyde group of the phenolate ion (of AFB_{2a}) and a sulfhydryl group on HRP. AFB_1 was converted to a phenolate ion containing an aldehyde group for conjugation via a two-step reaction (from AFB_1 to AFB_{2a} and from AFB_{2a} to a phenolate ion). The resulting phenolate ion was then reacted with the hydrazide group of BMPH to form a maleimide-derivatized AFB_1. Sulfhydryl groups were introduced on HRP by reducing disulfide bonds with 2-iminothiolane HCl, and the result was quantified by using DTNB. Reacting the maleimide-derivatized AFB_1 with the sulfhydryl-modified HRP formed an AFB_1−BMPH−HRP as a yellow solution. This approach would keep the cyclopenenone ring of AFB_1 intact, and allows the conjugation to proteins to be achieved at the dihydrodifurano end.

Assay Optimization. The production of antibody specific to AFB_1 was confirmed by titration against AFB_1−KLH or −OA. For direct competitive immunoassay, optimum concentrations of enzyme conjugates were established by titrating three enzyme conjugates against antibodies coated on microwell plates at 2.5−10 μg/mL and by determining the concentrations producing an optical density of 1−1.5 unit. The enzyme conjugate with a lower hapten-to-enzyme ratio used in the conjugation gave a slightly better assay sensitivity (AFB_1 concentration giving 50% inhibition of color development (IC_{50}) at 0.5 μg/kg in 16% methanol) in a standard assay than did the enzyme conjugate with a higher ratio used in the conjugation (IC_{50} 0.8 ± 0.1 μg/kg in 16% methanol) (**Figure 3**). The difference in IC_{50} was not as prominent as those observed in immunoassays for other small molecules such as pesticides (*27*). The enzyme conjugate with the lower hapten-to-enzyme ratio used for conjugation was

less stable even at 4 °C storage, losing enzymatic activity even though the binding affinity remained the same. To improve the assay sensitivity, a hapten heterology approach was examined, using the AFB_1−antibody with AFB_2−enzyme conjugate in a competitive direct immunoassay. Only a slight improvement in sensitivity with an IC_{50} at 0.5 ± 0.02 μg/kg in 16% methanol was obtained (**Figure 3**), showing no significant advantages. Unfortunately, the use of AFB_1−BMPH−HRP conjugate in a heterologous competitive immunoassay was not practical due to high nonspecific binding from the required high enzyme concentration.

The indirect competitive assays using immobilized antigens (AFB_1−KLH or −OA and AFB_2−KLH) exhibited sensitivities significantly lower than that of the direct competitive assay format (IC_{50} > 100 μg/kg). The low sensitivities were due to a high nonspecific binding giving a high background and standard curves with low slopes (data not shown). This was therefore not pursued further, even though the expected matrix interferences could be lower with the indirect approach.

A rapid assay was developed for a number of reasons, but the main one leading to this choice of format was the need to overcome the matrix interferences by increasing the enzyme conjugate concentration in an assay. Inhibition of the enzyme conjugates was found to be the major effect of the matrix interferences in the standard assays. The standard curves for the standard assay and rapid assays using AFB_1−HRP and AFB_2−HRP are shown in **Figure 3**. The rapid assays were able to withstand up to 80% methanol without affecting the assay performance. The standard curves for rapid assays shown in **Figure 3** are prepared in 80% methanol. By contrast, the standard curves of the standard assays were prepared in 16% methanol to reflect a 1 in 5 dilution of sample extract with water needed to reduce solvent and matrix effects. The IC_{50} values were reduced at least 7-fold from 0.8 ± 0.1 μg/kg in a standard assay to 7.2 ± 0.9 μg/kg in a rapid assay using AFB_1−HRP and from 0.6 ± 0.02 to 3.5 μg/kg using AFB_2−HRP (**Figure 3**). This phenomenon has been observed previously with the diflubenzuron immunoassay (*31*), but in this case, without a need for sample extract dilution. The limit of detection in an assay was calculated as a concentration that gives 20% inhibition of color development (IC_{20}), which was approximately the lowest part of the linear portion of the standard curve. For the standard assay, the limit of detections in an assay were 0.5 ± 0.1 and 0.4 ± 1.2 μg/kg for AFB_1− and B_2−HRP, respectively. The respective limit of detection in a sample would be 7.1 ± 2.1 and 6.0 ± 1.5 μg/kg for AFB_1− and AFB_2−HRP respectively, when a sample extract was prepared according to the extraction protocol described in the method section. For the rapid assay, the limits of detection in an assay were 1.4 ± 0.4 and 1.2 μg/kg for AFB_1− and AFB_2−HRP, respectively. The respective limit of detection in a sample, when extracted according to the extraction protocol, would be 4.2 ± 1.2 and 3.6 μg/kg for AFB_1− and AFB_2−HRP. There was a concern that the narrow detection ranges (7.1−55.5 μg/kg for AFB_1−HRP and 6−18 μg/kg for AFB_2−HRP) and the steep slopes of the standard curves for the standard assays may not be ideal for quantification. Narrow detection range has been one of the shortcomings that analysts perceive about immunochemical methods. For the rapid assays, the detection ranges were broader and the slopes of the standard curves were more ideal for analysis (4.2−99.9 μg/kg for AFB_1−HRP and 3.6−33.3 μg/kg for AFB_2−HRP).

The assay using the AFB_2−HRP experienced even greater matrix interferences than that using AFB_1−HRP, leading to greater reduced color development. Therefore, the immunoassay using AFB_1−HRP was chosen for further validation.

Table 1. Cross Reactivity of Standard and Rapid Assays for Aflatoxins and Metabolites

	standard assay		rapid assay	
compound	IC_{50} (μg/kg)[a]	%CR[b]	IC_{50} (μg/kg)	%CR
	aflatoxins			
AFB_1	0.8	100.0	6.5	100.0
AFB_2	13.5	5.9	55.0	11.8
AFG_1	1.4	57.1	10.8	60.5
AFG_2	50.1	1.6	83.0	7.8
	metabolites			
AFM_1	>250	<0.3	>250	<2.6
AFB_{2a}	>250	<0.3	>250	<2.6
AFG_{2a}	2267.9	0.04	2698.8	0.2
AFP_1	>250	<0.3	638.5	1.0
aflatoxicol I	107.1	0.7	257.3	2.5

[a] IC_{50} is a concentration of the test compound giving 50% of color inhibition. [b] %CR is determined as IC_{50} (AFB_1)/IC_{50} (test compound) × 100. No inhibition of color development was observed for cyclopiazonic acid, ochratoxins A and B, and fumonisins B_1, B_2, and B_3 at 10 mg/kg.

Assay Specificity. The assay specificity was determined for aflatoxin congeners (AFB_1, AFB_2, AFG_1, and AFG_2), the known metabolites (AFM_1, AFP_1, AFB_{2a}, AFG_{2a}, and aflatoxincol I), and other mycotoxins (cyclopiazonic acid, ochratoxins A and B, and fumonisins B_1, B_2, and B_3) could be found in the same food commodities. For aflatoxins, the antibody was relatively specific to AFB_1, as indicated by the cross reactivity studies shown in **Table 1**. No cross reaction was observed with other mycotoxins such as cyclopiazonic acid, ochratoxins A and B, and fumonisins B_1, B_2, and B_3 at 10 mg/kg, some of which could coexist in food. AFG_1 cross-reacted at 57−61% relative to AFB_1, when the IC_{50} values were compared. The cross reaction for AFB_2, AFG_2, and other metabolites were below 6 and 12% for the standard and rapid assays, respectively. The cross reactivity was in the order of AFB_1 > AFG_1 > AFB_2 > aflatoxicol I, AFG_2 > AFM_1, AFB_{2a}, AFG_{2a}, and AFP_1. The cross reactivity pattern remained relatively unchanged between the standard assay and the rapid assay, indicating assay time did not affect the relative binding affinity of the antibody in this format.

From this specificity, it can be concluded that the antibody's affinity was mainly directed toward the dihydrodifurano ring and the methoxyl group at C17. The strong binding affinity toward dihydrodifurano was evident in the cross reaction with AFG_1 and not other compounds with modified dihydrodifurano moiety. For example, the hydroxyl group on C14 of AFM_1 significantly reduced the antibody binding, giving less than 3% cross reaction relative to AFB_1, despite the presence of dihydrodifurano ring. The weak but significant binding affinity toward the methoxyl group at C17 was evident in the cross reaction with AFP_1, in which the methoxyl group was replaced with the hydroxyl group at C17, resulting in less than 1% cross reaction relative to AFB_1. The cross reaction for AFB_1 and AFB_2 suggested that the antibody was also exhibiting weak affinity toward the cyclopentenone ring. However, the cyclopentenone ring alone did not induce significant binding, which was evident by the cross reactivity for AFM_1, AFP_1, and AFB_{2a}. Evidently, the ketone group at C3 was also essential for the overall antibody interaction, as shown in the cross reaction for aflatoxicol I, which differs from AFB_1 only in the functional group at C3 (hydroxyl group instead of ketone group). The AFB_1 oxime derivative, which was used for the antibody production, seems to effectively

retain the structural and electrostatic properties of AFB_1. There has been some concern that aflatoxin antibodies could potentially interact with unknown compounds of similar structures in the food sample, leading to false positives. However, any compound with slight dissimilarity in the structure from AFB_1 seems to affect the apparent binding, as shown in the cross reaction. Therefore, it can be concluded with adequate reasons that both the standard and the rapid assays are AFB_1 specific, suitable for quantifying the most potent toxin AFB_1 in naturally contaminated food and feed samples.

Matrix Interferences. One of the common challenges of immunoassay for food analysis is matrix interference, causing false positives by lowering the color development. This occurs when either (1) the enzyme activity is inhibited by the presence of inferences in the sample extracts, (2) the interaction between the antigen/analyte (AFB_1) and the antibody is hindered, or (3) both of these phenomena has occurred concurrently in an immunoassay. Matrix interference is a common problem for all aflatoxin-specific immunoassays, which could cause false positives. The reported effects were either inhibiting enzyme activity only (*32*−*34*) or inhibiting both enzyme activity and antibody binding (*35*, *36*).

These matrix interferences can be reduced by a number of ways, such as dilution of sample extract or removal of interferences by sample cleanup procedures using solid-phase extraction or addition of heavy metal salts for precipitation of certain interferences. Dilution is a commonly used procedure to reduce the interferences (*37*−*39*), but this procedure would also reduce the quantifiable sensitivity. This approach works well with immunoassays exhibiting very high sensitivity able to accommodate the dilution factors and still maintain the detection limit at legal requirements. However, a common error occurring in an immunoassay is dilution error if the dilution factor is too great. When the second approach is used, sample cleanup procedure is generally kept as simple as possible to sustain the advantage of immunoassay as ease of use. Interferences in a form of particles can be removed by centrifugation or filtration, and many sample preparation protocols have incorporated one of these procedures to remove the interferences.

Peanuts, corn, pistachio, sorghum, soybeans, wheat, barley, tea and coffee were chosen as test samples to study the matrix interferences. **Table 2** lists the % color reduction when compared with methanol and IC_{50} for these test samples. For standard assays, the sensitivities were not affected at all or only slightly, but the color development was significantly reduced when compared with 16% methanol. This suggested that the sample co-extractants were mainly interfering with the enzyme activity and not the antibody's binding ability, inhibiting only the color development and not the percent inhibition. This would lead to false positives of noncontaminated samples and also in overestimating AFB_1 contents in the contaminated samples. The degree of enzyme interference varied with different food samples tested (**Table 2),** indicating individual validation and optimization of the extraction protocol would be necessary for each sample type. It was decided that less than 10% color reduction by sample matrixes when compared to 16% methanol could be considered as acceptable.

For peanuts, the problem was minimized by simply diluting the extract with 1% BSA−PBS instead of water, reducing the color difference between control and sample extract to less than 10%. The protein in the diluent seemed to act like a stabilizer to protect the enzyme from the interfering materials. This approach, however, did not overcome the interferences with other sample matrixes. Peanut extracts stored frozen for more than a week produced additional matrix interferences inhibiting color development as much as 50%, but the antibody binding was not affected. Thus, peanut extracts were best analyzed when freshly extracted, if possible, without prolonged storage. Other samples were not affected by prolonged storage.

Table 2. Matrix Interferences Measured as % Control Color Reduction and IC_{50} Values on the Standard and Rapid Assays

	standard assay		rapid assay	
sample type	% control color reduction	IC_{50} (μg/kg)	% control color reduction	IC_{50} (μg/kg)
methanol[a]	0.0	1.0	0.0	6.1
barley	8.8	0.9	3.7	6.0
sorghum	19.6	1.1	9.7	5.6
wheat	53.9	0.8	5.5	6.4
peanut	45.8	0.9	9.0	6.6
corn	4.0	0.9	4.2	6.4
soybeans	5.0	0.8	5.2	6.4
pistachio	17.6	1.0	9.2	5.9
tea	42.2	1.0	-	-
coffee	84.3	1.5	-	-

[a] For the standard assay, 16% methanol was used and for the rapid assay, 80% methanol was used. % Control color reduction was calculated as $(1 - A_{sample}/A_{methanol}) \times 100$, where A_{sample} is the absorbance (at 450 nm) of test sample extract with AFB_1–HRP conjugate (maximum color), and $A_{methanol}$ is the absorbance (at 450 nm) of methanol with AFB_1–HRP conjugate. Samples were extracted by shaking for 15 min on the rotary shaker, and the extracts were diluted 1 in 5 with water for the standard assay analysis. For the rapid assays analysis, the sample extracts were used directly.

Filtration using Whatman No. 1 filter paper was only effective in removing large particles. Centrifugation at 10 000 rpm for 30min was more effective in removing the finer particles than the filtration. It was found that some corn, wheat, and barley extracts resulted in fine particles that even centrifugation could not completely remove. Filtration using a syringe filter with 0.45-μm pore size nylon membrane was effective in removing the fine particles that the centrifugation was not able to remove, but also removed AFB_1, probably by adsorption onto the membrane. It was finally decided that reducing % color reduction to less than 10% would not be possible without a further cleanup step or extensive dilution for the standard assay.

The rapid assay was generally less affected by matrix interferences (**Table 2**). There were some solvent effects from 80% methanol increasing the maximum absorbance by 5−10%. To keep the consistency in the color development, the sample extract was diluted in 80% methanol instead of water or 1% BSA−PBS, as was used for the standard assays, if further dilution for analysis was required. No significant color reduction (<10%) and % inhibition by the test matrixes (peanuts, corn, pistachio, wheat, sorghum, barley, and soybeans) in the rapid assay was observed, as shown in **Table 2** and **Figure 4**. The superimposition of these standard curves indicated that these test matrixes did not significantly affect the assay sensitivity, and analysis could be performed using a standard curve prepared in 80% methanol.

To study the extraction efficacy, the recovery rates of 80% methanol, 55% methanol (a protocol suggested by a commercial ELISA kit), 80% ethanol, 80% ethanol containing 2% Tween 20, and 2% cyclodextrin were compared using two pistachio samples known to be naturally contaminated with aflatoxin at 42 and 141 μg/kg as analyzed by HPLC. Methanol (80%) gave the best recoveries of AFB_1 in these samples with the rapid extraction protocol as described in the method section. Ethanol (80%) recovered an average of 85% relative to the recovery of

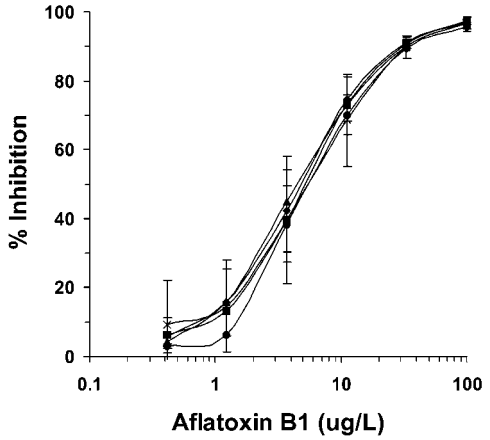


Figure 4. The rapid assay standard curves for AFB_1 in 80% methanol (◆), peanuts (■), soybeans (▲), pistachio (●), and corn (x).

80% methanol. Lowering the methanol content from 80 to 55% generally reduced the extraction efficiency, but the recovery was better than ethanol. The addition of detergent (2% Tween 20) to the extraction solvent reduced the recovery to an average of 49% compared to 80% methanol. In general, the extraction efficacy for a higher aflatoxin concentration (141 μg/kg) was slightly lower with any extraction system than for lower aflatoxin concentration (42 μg/kg) but still within the acceptable range of greater than 80% recovery.

The extractions by 3-min blending in a Waring blender and 15-min shaking at 250 rpm on the rotary shaker were also compared for matrix interferences. The 3-min blending, as expected from the previous studies with pesticide ELISAs (*31*), produced greater matrix interferences in the immunoassay, lowering the maximum color development even in a rapid assay. The extraction efficacy between these two methods, however, was not significantly different. Interestingly, for peanut samples, addition of water prior to methanol when extraction is performed significantly reduced co-extraction of interferences from the sample. This method, however, did not affect the extraction of interferences from other samples. From these results, the 80% methanol with 15-min shaking at 250 rpm, followed by a period of resting for particle sediment or filtration, was chosen for the validation studies described below.

Validation Studies. Initial validation for both standard and rapid assays to establish the assay specificity for AFB_1 was conducted using spiking of 80% methanol with various concentrations of AFB_1, AFB_2, AFG_1, and AFG_2. Methanol (80%) was used instead of sample extract in this study, to avoid obscure results resulting from the matrix interferences and the low natural contamination of aflatoxins in the sample. The amount of each aflatoxin spiked in 80% methanol and recovered by the standard assay and the rapid assay are illustrated in **Figure 5**. Both assays gave good correlation with the AFB_1 concentration spiked in 80% methanol and the respective *R*-values of 0.98 and 0.99 for the standard and rapid assays. Both assays were able to specifically detect AFB_1 in solutions containing four aflatoxin congeners at different concentrations. There was, however, a tendency for a significant overestimation that occurred when AFG_1 was present in a quantity greater than AFB_1 (SP2 in **Figure 5**). In this instance, the standard and rapid assays overestimated the spiked concentration, giving 190 and 142% recoveries, respectively. However, in areas where only *A. flavus* is known to prevail, such as most countries in SE Asia (*40*), these assays would be a valuable screening tool for AFB_1 contamination.

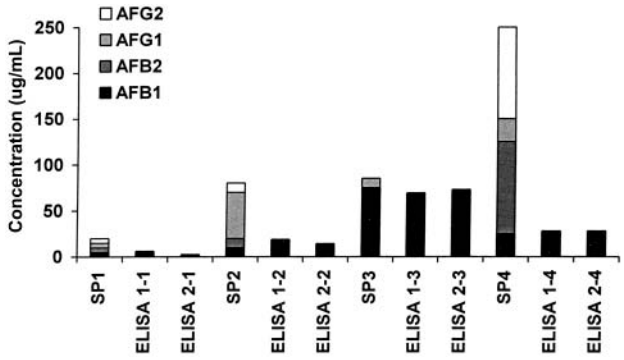


Figure 5. Aflatoxin spiked with different combination of AFB_1, AFB_2, AFG_1, and AFG_2 in 80% methanol (SP1–SP4) and analyses by the standard assay (ELISA 1) and the rapid assay (ELISA 2).

Table 3. Spike and Recovery of AFB_1 in Various Sample Types by the Rapid Assay Using AFB_1–HRP

sample type	spike level (μg/kg)	mean[c] ± SD (μg/kg)	%CV	% mean recovery	regression equation
peanut	5	5.0 ± 1.3	26.0	99.0	$y = 0.78x + 1.18$
(fresh)[a]	15	13.1 ± 1.9	14.5	87.3	($R^2 = 0.99$)
	50	40.3 ± 8.8	21.8	80.5	
peanut	5	5.9 ± 0.4	6.8	118.3	$y = 0.84x + 2.65$
(aged)[b]	15	16.5 ± 1.5	9.1	110.0	($R^2 = 0.99$)
	50	44.5 ± 7.9	17.8	89.0	
corn	5	5.0 ± 1.4	28.0	99.7	$y = 0.67x + 2.04$
(fresh)[a]	15	12.6 ± 2.6	20.6	84.0	($R^2 = 0.99$)
	50	35.4 ± 3.2	9.0	70.8	
corn	5	3.6 ± 1.0	27.8	71.0	$y = 1.17x - 3.07$
(aged)[b]	15	13.5 ± 1.5	11.1	90.0	($R^2 = 0.99$)
	50	55.7 ± 1.2	2.2	111.3	
pistachio	5	6.1 ± 0.7	11.5	121.8	$y = 0.87 + 1.32$
(fresh)[a]	15	12.5 ± 2.6	20.8	83.6	($R^2 = 0.99$)
	50	42.0 ± 5.5	13.1	84.0	
pistachio	5	3.9		78.0	$y = 0.81 + 1.32$
(aged)[b]	15	12.3		82.0	($R^2 = 0.99$)
	50	36.0		72.0	
soybeans	5	4.1 ± 0.3	7.3	82.3	$y = 1.02 - 1.84$
(fresh)[a]	15	12.3 ± 1.1	8.9	81.7	($R^2 = 0.99$)
	50	49.2 ± 2.5	5.1	98.3	

[a] Fresh samples were those spiked and let stand overnight at room temperature before the extraction. [b] Aged samples are those spiked and stored at room temperature for four weeks before the extraction. [c] Data shown are means of three six-replicate samples, except for pistachio (aged), which is a single sample analysis.

Prior to the spike and recovery studies, each test sample was verified to contain aflatoxin at less than 0.1 μg/kg, by HPLC. The triplicate 25-g subsamples were spiked at three levels: (1) low (near the detection limit, 5 μg/g), (2) medium (15 μg/g), and (3) high (50 μg/g). The recovery was assessed using the rapid assay for two separate events (fresh samples and aged samples). Data shown in **Table 3** represent the recovery means of three subsamples spiked at each level, except for those aged pistachio samples, which are single-subsample analyses. In general, the recovery of AFB_1 for both fresh and aged samples was within the acceptable range of greater than 80%, with only a few slightly lower ones. The recoveries tended to be higher at lower spike concentration than those at higher concentrations, suggesting the extraction efficiency could be concentration dependent as discussed in the previous section. Unlike the previous studies by Figueira et al. (*37*), the recoveries between fresh samples (24 h at room temperature) and aged samples (4 weeks at room temperature) were similar or only slightly lower in aged samples, as expected, but still within the acceptable range. No degradation of AFB_1 was observed in food during

the long storage. The average recoveries determined from the gradients of the regression equations for peanuts, corn, pistachio, and soybeans were 78, 67, 87, and 100%, respectively, when extracted 1 day after spiking. For the aged samples, the recoveries were 84, 117, and 81% for peanuts, corn, and pistachio, respectively. For the latter samples, the *y*-intercept values of the regression equations indicated a tendency to overestimate. Further validation with naturally contaminated samples is needed to confirm this point.

Precision of the Immunoassays. The intra-assay reproducibility and interassay reproducibility were determined to study the assay precision. The variation of percent inhibition for 100, 33.3, 11.1, 3.7, 1.4, and 0.4 ng/g of AFB_1 in 80% methanol tested four times on the same day were 2.5, 7, 3.7, 12.5, 29.8, and 40.3%, respectively. Assays of the same material run over 9 days gave a deviation from the mean values of 0.6, 1, 1.1, 3, 9.2, 13.1, 32.3, and 67.9% for each of the respective concentrations. The tendency for increased deviation with concentration was mainly due to the serial dilution. This suggests that either extra caution should be taken in the standard curve preparation or the same standard solutions should be used for all the analysis, as the precision of the standard curve will influence the precision and accuracy of the analysis. In an ELISA kit where the standard solutions are provided, the deviation at low concentrations would be significantly improved. For the fresh spikes (5, 15, and 50 ng/g), the average percent coefficients of variation were 20.8, 19.2, 15.1, and 7.1% for peanuts, corn, pistachio, and soybeans, respectively. For the aged spikes, they were 11.2 and 13.7% for peanuts and corn, respectively. It can be concluded from these data that the spiking, extraction method, and the analysis by the rapid assay of peanuts, corn, pistachio and soybeans gave acceptable reproducibility.

In summary, four AFB_1-specific direct competitive immunoassays based on microwell plates, and the use of AFB_1-HRP and AFB_2-HRP were developed. The advantages of the rapid assay using AFB_1-HRP compared to the standard assay are the simplicity of the protocol, lower matrix interferences for the majority of foods of concern, relatively low dilution error, reducing the analysis variance, and ease of adaptation for high throughput screening and analysis. With detection limits of 7.1 and 4.2 μg/kg for standard and rapid assay respectively, both assays would be able to detect AFB_1 at the maximum residue limit of 10 μg/kg for human consumption currently enforced in Australia and South East Asia. There was some concern that AFG_1 may interfere with AFB_1 estimation if present in high concentrations, although it is uncommon for AFG_1 contamination to exceed AFB_1 contamination in food and animal feeds. As we have shown in various spike and recoveries studies, the analysis by these immunoassays is AFB_1-specific regardless of the presence of other aflatoxin congeners. The recoveries of AFB_1 spiked in various food samples were within an acceptable range with deviations from a mean also within an acceptable range. It should be noted that the current study is only concerned with the extraction and analysis, assuming the sampling techniques for aflatoxins would conform with proven protocols. Provided the personnel running the analysis are well trained and proper care and control has been taken, and a proven sampling technique is used, these immunoassays can be just as reliable as HPLC methods for analyzing AFB_1 in soybeans, corn, pistachio, and peanuts, providing more data with greater efficiency.

SAFETY

Aflatoxins are classified as carcinogens and great care should be exercised to avoid personal exposure and potential risk of laboratory contamination. All handling of pure compounds and immunoassays were done in the fume hood with protective gear such as laboratory coat, adequate footwear, safety glasses, gloves, and an approved disposable face mask (if necessary). The microwell plates were washed using Labsystems Wellwash Mk 2 (Helsinki, Finland) to avoid direct exposure, and the waste was treated with hypochlorite and acetone before disposal, as adapted from the method by Official Methods of Analysis of the AOAC International (*30*).

ACKNOWLEDGMENT

We thank Dr. Hassan Yazdanpanah of Beheshti University of Medical Science and Health Sciences, Tehran, Iran, for pistachio samples, and Karen Jackson of Australian Wheat Board (AWB), for the HPLC analyses used in validation.

LITERATURE CITED

(1) International Agency for Research on Cancer. IARC monograph on the Evaluation of Carcinogenic Risk to Humans; IARC: Lyon, France, 1993; Vol. 56.
(2) Foster, P. L.; Eisenstadt, E.; Miller, J. H. Base substitution mutations induced by metabolically activated aflatoxin B_1. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2695−8.
(3) Campbell, T. C.; Chen, J. S.; Liu, C. B.; Li, J. Y.; Parpia, B. Nonassociation of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China. *Cancer Res.***1990**, *50*, 6882−93.
(4) Katiyar, S.; Dash, B. C.; Thakur, V.; Guptan, R. C.; LottSarin, S. K.; Das, B. C. p53 Tumor suppressor gene mutations in hepatocellular carcinoma patients in India. *Cancer* (*New York*) **2000**, *88*, 1565−1573.
(5) Wild, C. P.; Jiang, Y. Z.; Allen, S. J.; Jansen, L. A.; Hall, A. J.; Montesano, R. Aflatoxin-albumin adducts in human sera from different regions of the world. *Carcinogenesis* **1990**, *11*, 2271−2274.
(6) Choi, M. J.; You, Y. C.; Kim, H. S.; Lee, B. M. Immunological monitoring of urinary aflatoxins and estimation of liver cancer incidence in Koreans. *J. Biochem. Mol. Biol.* **1996**, *29*, 105−110.
(7) Sun, C. A.; Wu, D. M.; Wang, L. Y.; Chen, C. J.; You, S. L.; Santella, R. M. Determinants of formation of aflatoxin-albumin adducts: A seven-township study in Taiwan. *Brit. J. Cancer* **2002**, *87*, 966−970.
(8) Sun, T. T.; Wu, S. M.; Wu, Y. Y.; Chu, Y. R. Measurement of individual aflatoxin exposure among people having different risk to primary hepatocellular carcinoma. *Princess Takamatsu Symposia* **1985**, *16*, 225−235.
(9) U.S. Department of Agriculture. *Grain Fungal Diseases & Mycotoxin References*, 1999.
(10) Berg, T. How to establish international limits for mycotoxins in food and feed? *Food Control* **2003**, *14*, 219−224.
(11) Whitaker T. B. Standardization of mycotoxin sampling procedures: An urgent necessity. *Food Control* **2003**, *14*, 233−237.
(12) Official Method of Analysis of AOAC International, *Official Method 975.36*, 17th ed.; AOAC International: Gaithersburg, MD, 2002.
(13) Official Method of Analysis of AOAC International, *Official Method 968.22*, 17th ed.; AOAC International: Gaithersburg, MD, 2002.
(14) Official Method of Analysis of AOAC International, *Official Method 2000.16*, 17th ed.; AOAC International: Gaithersburg, MD, 2002.
(15) Official Method of Analysis of AOAC International, *Official Method 990.34*, 17th ed.; AOAC International: Gaithersburg, MD, 2002.
(16) Morgan, M. R. A.; Kang, A. S.; Chan, H. W. S. Aflatoxin determination in peanut butter by enzyme-linked immunosorbent assay. *J. Sci. Food Agric.* **1986**, *37*, 908−914.

(17) Chu, F. S.; Ueno, I. Production of antibody against aflatoxin B_1. *Appl. Environ. Microbiol.* **1977**, *33*, 1125−1128.
(18) Hastings, K. L.; Tulis, J. J.; Dean, J. H. Production and characterization of a monoclonal antibody to aflatoxin B_2. *J. Agric. Food Chem.* **1988**, *36*, 404−408.
(19) Chu, F. S.; Steinert, B. W.; Gaur, P. K. J. Production and characterization of antibody against aflatoxin G_1. *Food Safety* **1985**, *7*, 161−70.
(20) Zhang, G.; Chu, F. S. Production and characterization of antibodies cross-reactive with major aflatoxins. *Experientia* **1989**, *45*, 182−184.
(21) Gaur, P. K.; Lau, H. P.; Pestka, J. J.; Chu, F. S. Production and characterization of aflatoxin B_{2a} antiserum. *Appl. Environ. Microbiol.* **1981**, *41*, 478−482
(22) Fan, T. S. L.; Zhang, G. S.; Chu, F. S. Production and characterization of antibody against aflatoxin Q_1. *Appl. Environ. Microbiol.* **1984**, *47*, 526−532.
(23) Haltzapple, C. K.; Carlin, R. J.; Rose, B. G.; Kubena, L. F.; Stanker, L. H. Characterization of monoclonal antibodies to aflatoxin M_1 and molecular modeling studies of related aflatoxins. *Mol. Immunol.* **1996,** *33*, 939−946.
(24) Lee,N.; Hill A. S.; Bui, V. T.; Tran, V. A.; Le, V. T.; Kennedy, I. R. Monitoring Mycotoxins and Pesticides in Grain and Food Production Systems for Risk Management in Vietnam and Australia. In *Quality Assurance in Agricultural Produce*; Johnson, G. I., Le V. T., Nguyen D. D., Webb, M. C., Eds.; ACIAR: Canberra, ACT, Australia, 2000; ACIAR Proceedings 100, pp 496−500.
(25) Chu, F. S.; Hsia, M. T. S.; Sun, P. S. Preparation and characterization of aflatoxin B_1-1-(O-carboxymethyl) oxime. *J. AOAC Int.* **1977**, *60*, 791−794.
(26) Hastings, K. L.; Hagler, W. M., Jr.; Harris, T. M.; Voyksner, R. D.; Dean, J. H. Production and characterization of aflatoxin B2 oximinoacetate. *J. Agric. Food Chem.* **1989**, *37*, 393−400.
(27) Lee, N. J.; McAdam, D. P.; Skerritt, J. H. Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. *J. Agric. Food Chem.* **1995**, *43*, 1730−1739.
(28) Ho, J. A. A.; Wauchope, R. D. A strip liposome immunoassay for aflatoxin B_1. *Anal. Chem.* **2002**, *74*, 1493−1496.
(29) Goding J. W. Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods* **1978**, *13*, 215−226.
(30) Official Method of Analysis of AOAC International, *Official Method 971.22*, 17th ed.; AOAC International: Gaithersburg, MD, 2002.
(31) Wang, S.; Hill, A. S.; Kennedy, I. R. Rapid on-site immunoassay for diflubenzuron in grains. *Anal. Chim. Acta* **2002**, *468*, 209−215.
(32) Wilkinson, A. P.; Denning, D. W.; Morgan, M. R. A. An ELISA method for the rapid and simple determination of aflatoxin in human serum. *Food Addit. Contam.* **1988**, *5*, 609−619.
(33) Ramakrishna, N.; Lacey, J.; Candlish, A. A.; Smith, J. E.; Goodbrand, I. A. *Aspergillus flavus* colonization and aflatoxin B_1 formation in barley grain during interaction with other fungi. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 71−76.
(34) Li, S.; Marquardt, R. R.; Frohlich, A. A.; Xiao, H.; Clarke, J. R. The development of a quantitative ELISA for aflatoxin B_1 in ground peanuts using antibodies isolated from the yolk of a laying hen. *J. Food Prot.* **1994**, *57*, 1022−1024.
(35) Figueira, A. C.; Taylor, K. D. A.; Barlow, P. J.; Morgan, M. R. Anomalous aflatoxin B_1 recoveries from whole peanuts and Brazil nuts measured by enzyme-linked immunosorbent assay. *Food Agric. Immunol.* **1991**, *3*, 13−19.
(36) Dell, M. P. K.; Haswell, S. J.; Roch, O. G.; Coker, R. D.; Medlock, V. F. P.; Tomlins, K. Analytical methodology for the determination of aflatoxins in peanut butter: Comparison of high-performance thin-layer chromatographic, enzyme-linked immunosorbent assay and high-performance liquid chromatographic methods. *Analyst* **1990**, *115*, 1435−1439.
(37) Chu, F. S.; Fan, T. S. L.; Zhang, G. S.; Xu, Y. C.; Faust, S.; McMahon, P. L. Improved enzyme-linked immunosorbent assay for aflatoxin B1 in agricultural commodities. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 854−857.
(38) Figueira, A. C., Taylor, K. D. A.; Barlow, P. J. ELISA determination of aflatoxin levels in whole nuts. *Food. Agric. Immunol.* **1990**, *2*, 125−134.
(39) Ramakrishna, N.; Mehan, V. K. Direct and indirect competitive monoclonal antibody-based ELISA of aflatoxin B1 in groundnut. *Mycotoxin Res.* **1993**, *9*, 53−63.
(40) Pitt, J. I.; Hocking, A. D. Current Knowledge of fungi and mycotoxins associated with food commodities in Southeast Asia. In *Mycotoxin Contamination in Grains*; Highley, E., Johnson, G. I., Eds.; ACIAR, Canberra, ACT, Australia, 2000, ACIAR Proceedings 37, pp 5−10.

Received for review November 28, 2003. Revised manuscript received February 22, 2004. Accepted February 26, 2004. Financial support provided by the International Atomic Energy Agency, Vienna (IAEA) and the Australian Centre for International Agricultural Research, Canberra (ACIAR).

JF0354038