

Validation of analytical parameters of a competitive direct ELISA for aflatoxin B₁ in peanuts

N. A. LEE^{1,2}, N. C. RACHAPUTI³, G. C. WRIGHT³, S. KROSCH³, K. NORMAN⁴, J. ANDERSON⁴, S. AMBARWATI⁵, I. RETNOWATI⁵, O. S. DHARMAPUTRA⁵, & I. R. KENNEDY¹

¹Faculty of Agriculture, Food and Natural Resources, University of Sydney, New South Wales, Australia, ²University of New South Wales, School of Chemical Engineering and Industrial Chemistry, New South Wales, Australia, ³Queensland Department of Primary Industries and Fisheries, Australia, ⁴Peanut Company of Australia, Kingaroy, Australia, and ⁵SEAMEO BIOTROP, Bogor, Indonesia

(Received 20 January 2005; accepted 27 April 2005)

Abstract

Analytical validation of a competitive direct SUNQuik ELISA with a reference High Performance Liquid Chromatography (HPLC) method and other methods including a minicolumn method and the VICAM Aflatest[®] system for aflatoxin in peanuts was conducted. Both the ELISA and the VICAM Aflatest[®] system, using the same peanut extracts were analytically comparable with the HPLC method ($R=0.998$, $p < 0.000$). The minicolumn method was also found to be acceptable as a low cost rapid semi-quantitative test. Despite the large variation in sampling, the correlation between the SUNQuik ELISA and HPLC using the different peanut sub-samples was considered acceptable over the range of 0–1200 $\mu\text{g kg}^{-1}$ ($R=0.938$). No false negatives were found using the SUNQuik ELISA and false positives were either nil or negligible in all the studies conducted. The repeatability of the SUNQuik ELISA run on the same day was good with only $\pm 10\%$ deviation. The reproducibility of the SUNQuik ELISA between days was also acceptable, but with a higher deviation. Applying the SUNQuik ELISA for aflatoxin surveys of peanuts in Indonesia proved that the method can deliver high quality, cost- and time-effective analysis with very little establishment capital and maintenance.

Keywords: *Aflatoxins, human carcinogen, ELISA, immunoassay, high through-put analysis.*

Introduction

Aflatoxins are naturally occurring fungal toxins causing serious food quality and safety issues worldwide. They can affect a wide range of important agricultural produce such as nuts, cereals, dried fruits and oilseeds and their products such as beer (from barley), peanut products (such as Indonesian peanut sauce), cornflakes and breakfast cereals. The common species of fungi responsible for aflatoxin production in foods and feeds are *Aspergillus flavus* and *A. parasiticus*. *A. flavus* is generally considered as producing

Correspondence: N. A. Lee. University of New South Wales, School of Chemical Engineering and Industrial Chemistry, Sydney NSW 2052, Australia. Tel: +61 2 9385 4363. Fax: +61 2 93855966. E-mail: alice.lee@unsw.edu.au

aflatoxins B₁ (AFB₁) and B₂ (AFB₂) and is commonly found in tropical and subtropical regions of the world, including South-East Asia (Council for Agricultural Science and Technology [CAST] 2003). While *A. parasiticus* is generally considered as producing AFB₁, AFB₂, AFG₁ and AFG₂, and this strain is rare or absent in South-East Asia (CAST 2003, John Pitt, personal communication). Aflatoxins, in particular AFB₁, are recognized as potent human carcinogens, immunosuppressants, tetratoxins, hepatotoxins and mutagens, and can potentially cause severe health problems in human and animals and reduce animal productivity (International Agency for Research on Cancer [IARC] 1993). Continuous intake of highly contaminated foods and feeds may lead to death, but chronic low exposure can potentially cause various health complications by affecting the immune system (Fink-Gremmels 1999). The overall impacts of aflatoxins on human and animal diseases could be beyond our current imagination, because of lack of proven links between aflatoxin exposure and human diseases of unexplained causes.

Indonesia consumes around 800,000 tonnes of peanuts each year and is one of the world's largest peanut importers. Lubulwa and Davis (1994) estimated at least 22,000 deaths per annum in Indonesia are potentially caused by consumption of aflatoxin-contaminated food. This could result in potential economic losses of around \$332m AUD per annum. These authors also predicted an economic benefit in the order of \$452m AUD in Indonesia over a 30-year time frame, if a technology for reducing aflatoxin contamination in the food chain could be developed. Aflatoxin contamination also causes negative economic and trade impacts. The estimated total costs associated with losses of crop and livestock are over \$100m AUD per annum in Indonesia. In Australia, managing aflatoxin contamination in peanuts can cost the industry as much as \$4m AUD per annum in post-harvest processes such as blanching and sorting to ensure the final product for consumers is below the regulatory limit. (Hansen & Norman 1999, Ganzer 1999).

The Committee on Food Additives and Contaminants of the Codex Alimentarius Commission has recommended that the maximum level of total aflatoxins in peanuts intended for further processing be set at 15 µg/kg (Codex 1999). In Australia, the maximum allowable limit of total aflatoxin in peanut and peanut products is 15 µg/kg (Queensland Department of Primary Industry [QDPI] 2000). The National Agency for Drug and Food Control of the Republic of Indonesia set the maximum allowable limit of AFB₁ and total aflatoxin in peanut products to be 20 and 35 µg/kg, respectively. In the European Community, even more stringent regulations have been imposed, with lower maximum permissible limits of 2 and 4 µg/kg for aflatoxin B₁ (AFB₁) and total aflatoxin, respectively, in groundnuts, dried fruits, cereals and their processed foods for direct human consumption or as an ingredient in foodstuffs (European Community [EC] 1998). The decrease in maximum permissible levels for aflatoxin would have positive impacts on health risk, but significant negative impacts on economics and trades. As analysed by Otsuki et al. (2000), this could reduce by as much as 64% African exports to EC, which would be equivalent to \$670m USD export income.

Managing the risk associated with aflatoxin contamination is a complex issue, involving strategies for prevention, monitoring, management, good manufacturing practices and quality control in all stages of the supply chain (Food and Agricultural Organization [FAO] 2001). While prevention of fungal infection would be the best management solution, the unpredictable nature of aflatoxin production in the field

makes it difficult to completely prevent aflatoxin entering the food chain. An integrated management system such Hazard Analysis and Critical Control Point (HACCP) has been favoured for managing the risks posed by aflatoxin (FAO 2001). Establishing critical hazard points in the supply chain is one of the important components of HACCP, before appropriate management practices can be put in place.

Establishing sustainable analytical capability is critical for the development of an effective food safety program, especially in developing countries where high aflatoxin contamination seriously poses a health risk. Although the quality and defensibility of data is increasingly important in any analysis, a balance between the data quality and operational cost will dictate the choice of methods. ELISA is an analytical technique that is sensitive, specific, simple, fast and cost-effective. Despite ELISA having been successfully applied to a wide range of commodities for detection of aflatoxin, to date, with a few exceptions, ELISA has not been officially approved by national and international regulatory agencies as an analytical method, partly due to a lack of sufficient validation data proving analytical quality criteria have been met.

The SUNQuik AflaB₁ ELISA technique (Lee et al. 2004) was transferred to two agricultural research institutions in Indonesia as an international collaborative project which assessed the severity of aflatoxin contamination in Indonesian peanuts and identified critical hazard points in the Indonesian supply chain. To support the validity and reliability of the SUNQuik AflaB₁ ELISA, this paper presents an analytical validation of the ELISA with a reference HPLC method as well as other analytical methods including a minicolumn method, the VICAM Aflatest[®] system, currently employed by the peanut industry in Australia for quality assurance. The outcomes of the pilot survey based on ELISA analyses allowed the degree of severity and critical points of aflatoxin contamination in the local market supply chain in the Pati and Wonogiri regions of Java island (Indonesia) to be established quickly and cost-effectively (Dharmaputra et al. 2002).

Methods

Chemicals and reagents

AFB₁ and other chemicals used in the ELISA method were purchased from Sigma (St Louis, MO, USA). Analytical grade methanol was obtained from Ajax Chemicals (Clyde, Australia). Maxisorp polystyrene 96-microwell plates were purchased from Nunc (Rockilde, Denmark). Chemicals used in the Minicolumn and HPLC methods were obtained from Biolab (Clayton, Australia).

Instrumentation

Absorbances of the standard ELISA and the SUNQuik ELISA conducted in Australia were recorded by a Labsystems Multiskan Ascent microplate reader (Labsystems, Helsinki, Finland) in dual-wavelength mode (450 and 650 nm). Absorbance of the SUNQuik ELISA for the surveillance surveys was recorded by a portable miniphotometer Model 6 fitted with a 450 nm filter (Metertech Inc., Taiwan). The accuracy and precision of the miniphotometer was tested by comparing with the more sophisticated Multiskan Ascent microplate reader before being distributed to the collaborating Institutes in Indonesia. The performance of the two instruments

was comparable, but with slightly higher deviations with repeated reading given by the miniphotometer. Microwell plates were washed with Well Washer 4 Mk 2 (Labsystems, Finland). For VICAM Aflatest[®] affinity column method, Vicam Aflatest Series 4 fluorimeter was used to measure the total aflatoxin concentration in the sample. HPLC separation was performed using a Waters HPLC (Milford, MA). The minicolumns were visually evaluated under a UV lamp.

Sample extraction for the comparative validation study

For the comparative validation of the ELISA, HPLC, minicolumn and VICAM Aflatest[®] methods, four bulk peanut samples collected from the model farms in Kingaroy, Queensland during the 2002 harvest season were used. Each bulk sample was divided into three 250 g sub-samples and each sub-sample was extracted by blending with 500 mL of 80% methanol with 4% NaCl at a high speed for 2 min. The supernatant was collected after filtering through a Whatman No. 1 filter paper. Aliquots of 10–20 mL were supplied to three independent groups for analysis by different methods.

General sample extraction for ELISA methods

For the surveillance surveys and routine analysis, a 25 g sub-sample was extracted with 75 mL of 80% methanol by shaking either on a wrist shaker or on an orbital shaker at 150 rpm for 30 min and the extract was either filtered through Whatman No. 1 filter paper or allowed to stand until clear supernatant was formed. The sample extracts were used directly without further dilution in the SUNQuik ELISA and were diluted one in five with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 0.05 M sodium phosphate containing 0.9% NaCl, pH 7.2) for the standard conventional ELISA. Each sample was extracted in duplicate and analysis was carried out in duplicate wells.

Preparation AFB₁ for ELISA

For the SUNQuik ELISA, the standard solutions were prepared as follows. From the stock solution of 5.3 or 5.7 $\mu\text{g mL}^{-1}$ AFB₁ in methanol, which was calibrated as in the Association of Official Analytical Chemists (AOAC) official method 971.22 (AOAC 2002), 100 $\mu\text{g L}^{-1}$ in 80% methanol solution was prepared, and then other concentrations (33.3, 11.1, 3.7, 1.2 and 0.4 $\mu\text{g L}^{-1}$) were prepared by serial dilution in 80% methanol. These standard solutions were used directly in the SUNQuik ELISA.

For the standard ELISA, initial standard solutions of five-fold strength in 80% methanol were prepared to ensure the solubility of AFB₁. From these standard solutions, working standard solutions of 10, 3.3, 1.1, 0.3, 0.1 and 0.37 $\mu\text{g L}^{-1}$ were prepared by diluting one in five with 1% BSA-PBS. These standard solutions were used in the standard ELISA.

The SUNQuik AFB₁ ELISA method

Both the SUNQuik AflaB₁ ELISA and the standard conventional ELISA methods (Lee et al. 2004) were applied in the validation studies. All ELISA operations were

carried out at room temperature. The procedure for the SUNQuik AflaB₁ ELISA is as follows. Briefly, 50 µL of aflatoxin standard (in 80% methanol) or sample extract (80% methanol extract) was pre-mixed with 150 µL of aflatoxin-HRP conjugate diluted in 1% fish-gelatin hydrolysate in PBS containing 0.1% Tween 20 (PBS-T, PBS with 0.05% Tween 20, pH 7.2). The premix (50 µL) solution was transferred to each of the duplicate testing microwells pre-coated with AFB₁-specific antibody (1 µg per well). After incubating for 5 min, the plate was washed three times with 0.05% Tween 20 and dried by tapping on an absorbent paper towel. Colour was developed by adding enzyme substrate/chromogen solution (3,3',5,5'-tetramethylbenzidine/peroxide in acetate buffer, pH 5.5) at 100 µL per well and the microwell plate was allowed to stand for 10 min. The colour development was stopped by adding 1.25 M sulphuric acid at 50 µL per well and the plate was read using dual wavelength mode (450/650 nm) or the miniphotometer fitted with a 450 nm filter.

The standard conventional AFB₁ ELISA

Aflatoxin standard (in 16% methanol) or diluted sample extract and enzyme conjugate were added to microwells pre-coated with AFB₁-specific antibody as above. The reaction was incubated for 1h. As for the SUNQuik ELISA, the testing wells were washed with 0.05% Tween 20 in water and colour was developed by adding enzyme substrate/chromogen solution. After stopping the colour development with 1.25 M sulphuric acid (50 µL per well), the microwells were read using microplate reader as above.

VICAM AflaTest[®] Immunoaffinity chromatography

AflaTest[®] Immunoaffinity (VICAM) chromatography was performed according to the manufacturer's instructions. Briefly, 5 mL of diluted sample extract (2:3, extract:water) was passed through an immunoaffinity column at one drop per second. The column was washed with 10 mL of water by passing through the column at two drops per second. Aflatoxin was eluted from the column by passing through 1mL of methanol at one drop per second. Into the eluent was added 1 mL of methanol prior to reading using Vicam AflaTest[®] Series 4-fluorimeter.

HPLC analysis

A combination of the AOAC methods for aflatoxins in peanuts and peanut products (AOAC 1990, Method 968.22) and aflatoxins in cottonseed products (AOAC Method 980.20I) was used. HPLC analyses were performed on a Waters instrument, equipped with Model 515 pump, Model 717 autosampler and Model 474 fluorescence detector. Excitation and emission wavelengths were set at 365nm and 455nm, respectively. Data acquisition was achieved using a Model 746 Data Module. Chromatographic separations were conducted on a Nova-Pak[™] Phenyl radial compression column (100 mm x 8 mm I.D. 4 µm particle size) supplied by Waters (Milford, MA, USA), at a column temperature of 45°C. The flow rate was 2.0 mL min⁻¹ with an isocratic mobile phase composition of 20% (v/v) tetrahydrofuran (THF) in ultra-pure water (provided in-house by purifying distilled water, and filtered through a 0.22 µm filter, using the Millipore Simplicity[™] Q system (Millipore, MA, USA)). Post-column

derivatization was achieved by using a saturated solution of iodine in water at a flow rate of 0.9 mL min^{-1} at a post-column reaction temperature of 70°C .

Minicolumn method

The Holaday-Velasco method (AOAC method 979.18) was followed, with minor modifications. Briefly, 3 mL of benzene was added to 15 mL of sample extract and the mixture was shaken for 10 s. After layer separation was completed, 1 mL of benzene layer was loaded onto a minicolumn, followed by 1 mL of chloroform:acetone mixture (9:1 v/v). Concentration of the toxins was assessed by visually comparing the intensity of the fluorescent band in the minicolumn with a set of other columns containing standards with known aflatoxin concentrations, under an UV lamp. The peanut samples were segregated into four categories according to the aflatoxin contents judged by the minicolumn method. Segregation 1 is $>8 \mu\text{g kg}^{-1}$ aflatoxin, segregation 2 is $8 - <80 \mu\text{g kg}^{-1}$ aflatoxin, segregation 3 is $80 - <400 \mu\text{g kg}^{-1}$ aflatoxin and segregation 4 is $>400 \mu\text{g kg}^{-1}$ aflatoxin.

Analysis of AFB₁ in the peanut samples infected with toxigenic and non-toxigenic Aspergillus flavus and A. parasiticus

Peanut kernels were autoclaved (20 psi pressure at 121°C for 15 min) to deactivate any fungal or bacterial contamination and cooled down to room temperature before adding 1 mL of spore suspension of either toxigenic *Aspergillus flavus* (K4122) and *A. parasiticus* (K4165). The flasks were incubated at high humidity ($>90\%$) and 30°C for at least 10 days to induce aflatoxin production. After the 10-day incubation at 30°C , the flasks were transferred to an oven at 60°C for 2 days to kill the further fungal growth and aflatoxin production. A range of aflatoxin contamination levels were prepared by adding varied proportion of contaminated peanut kernels to uncontaminated peanut samples. The peanut samples were then ground and the peanut meal was used for the various experiments described herein.

Sampling strategies for surveillance surveys

Three large surveys of different local peanut supply chains were conducted in Indonesia to develop and implement integrated management packages to reduce the aflatoxin risk. The supply chain had been identified as having three levels, consisting of farmers, collectors and retailers. At the farmer level, 2 kg of wet raw pod peanuts were collected from 20 peanut plants randomly selected at each selected farm. At the collector level, three 2 kg-replicate samples of dry raw pod peanuts were collected randomly. Twenty-seven samples of raw kernels were collected from the traditional markets. Each peanut sample was divided into eight 250 g sub-samples using a sample divider and raw pods were then shelled manually. Each 250 g sub-sample was used to analyse for moisture content, the physical quality of kernels, mycological testing for *A. flavus* and AFB₁.

Aflatoxin analyses

AFB₁ contents in the kernels of wet and dry raw pods, and raw kernels collected in Indonesia were determined by SUNQuik ELISA in Indonesia. Two extractions were

performed for each peanut sample to examine the homogeneity of the ground samples and to ensure the validity of the data. About 10% of the samples were randomly selected from each batch collected for re-analysis by SUNQuik ELISA and the standard ELISA in another laboratory in Australia, and by HPLC in the Agrifood Technology Pty Ltd. and Peanut Company of Australia (PCA).

Safety

Aflatoxins are classified as carcinogens. All handling of pure compounds and immunoassays was carried out in the fume hood with protective gear such as laboratory coat, adequate footwear, safety glasses and gloves. 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 (AOAC International).

Results and discussion

Validation of analytical methods using the same set of sample extracts

Sampling/sub-sampling variation is a significant factor governing the accuracy of aflatoxin analysis (Whitaker 2003). To avoid variation due to sampling, common extracts of 12 samples of 250 g peanut samples were used for the comparative validation studies. The total aflatoxin concentrations in the samples ranged from 0–2710 $\mu\text{g kg}^{-1}$ (as analysed by HPLC), thus providing a wide range of aflatoxin contamination in peanuts for the study. The HPLC analyses revealed that the percentage distribution of AFB₁, AFB₂, AFG₁ and AFG₂ between the three sub-samples were similar. For example, the three sub-samples of Sample 1 consisted of $33.2 \pm 4.2\%$, $2.3 \pm 0.1\%$, $59.8 \pm 2.8\%$ and $4.7 \pm 1.2\%$ of AFB₁, AFB₂, AFG₁ and AFG₂ respectively. However, the concentrations of each aflatoxin congener between the sub-samples varied significantly. For example in the Sample 1, AFB₁ concentrations varied from 33–1030 $\mu\text{g kg}^{-1}$ (33.3, 37.6 and 1030 $\mu\text{g kg}^{-1}$ for sub-samples 1, 2 and 3 respectively) and AFG₁ concentrations varied from 68–1530 $\mu\text{g kg}^{-1}$ (67.8, 73.2 and 1530 $\mu\text{g kg}^{-1}$ for sub-samples 1, 2 and 3 respectively). Because of this variability, each sample was treated as an individual sample even though they may be three replicate sub-samples.

In preliminary studies, the performance of SUNQuik AflaB₁ ELISA was proven to deliver satisfactory results in a spike and recovery study (Lee et al. 2004). Using naturally contaminated peanut samples to further evaluate the performance of ELISA techniques, the correlation between SUNQuik ELISA data and HPLC analyses of AFB₁ was determined by a linear regression model. Each parameter in the linear regression equation was tested using the *t*-test to determine its significance and the *t*-test of the Y intercept (constant) was shown not to be significantly different from zero. Thus, the regression was performed by passing the least square fit line through zero to reflect this point. The linear regression equation of the least square fit, shown in Figure 1A, estimated an average recovery of 91% over the range between 0–250 $\mu\text{g kg}^{-1}$ (as determined from the slope), however, some variation would be expected at low AFB₁ concentrations.

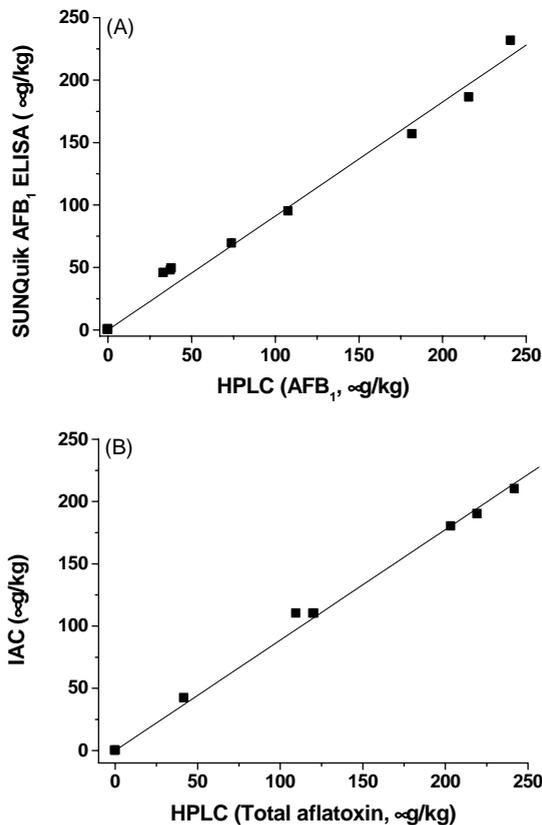


Figure 1. Relationship between the SUNQuik ELISA, the IAC (VICAM Aflatest[®]) and HPLC for aflatoxin analysis using the same sample extracts. (A) SUNQuik ELISA vs HPLC for AFB₁ analysis. The equation for the linear least square fit (straight line in A) is $Y = 0.91 \pm 0.03 * X$ ($R = 0.998$, $n = 11$, $p < 0.0001$). (B) IAC (VICAM Aflatest[®]) vs HPLC for analysis of total aflatoxin. The equation for the linear least square fit (straight line in B) is $Y = 0.89 \pm 0.01 * X$ ($R = 0.998$, $n = 10$, $p < 0.0001$).

The repeatability of the SUNQuik assay was tested by analysing the above sample extracts in two separate experiments in the same day. The relative standard deviation between the two ELISA analyses was 2.0% and the lowest and highest % coefficient of variation (%CV) were 0 and 5.7% respectively. The within-laboratory reproducibility of the SUNQuik assay was tested by analysing the same extracts five times over four days by different operators. The average %CV was 23.5% and the lowest and highest %CV was 5.8 and 37.4% respectively.

There was a significant positive correlation ($R = 0.998$) between the total aflatoxin content estimated by VICAM Aflatest[®] system utilizing the immunoaffinity assay and the HPLC (see Figure 1B). As for the regression between ELISA and HPLC, the Y-intercept of the regression between VICAM Aflatest[®] system and HPLC was found to be not significantly different from zero by the *t*-test, hence the data were treated as explained earlier. Even though the manufacturer's recommendation was not to exceed the $100 \mu\text{g kg}^{-1}$ loading capacity of the column, a linear relation with *R* value of 0.998 was obtained for the concentration range between 0–210 $\mu\text{g kg}^{-1}$, after omitting two

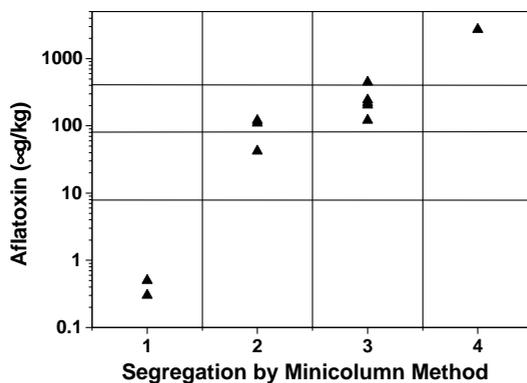


Figure 2. Segregation of peanut samples into four levels as estimated by the minicolumn method and their actual concentrations as analysed by HPLC. Segregation 1 is $>8 \mu\text{g kg}^{-1}$ aflatoxin, segregation 2 is $8 - <80 \mu\text{g kg}^{-1}$ aflatoxin, segregation 3 is $80 - <400 \mu\text{g kg}^{-1}$ aflatoxin and segregation 4 is $>400 \mu\text{g kg}^{-1}$ aflatoxin, indicated by the horizontal lines.

data points greater than $300 \mu\text{g kg}^{-1}$. The estimated average recovery from the regression equation of the least square fit was 89% for this testing system.

The correlation between the semi-quantitative minicolumn method and HPLC analyses were analytically acceptable as shown in Figure 2, however the minicolumn method had some tendency to underestimate the total aflatoxin contents in the test samples. For example, two of the three samples which were determined as Segregation 2 ($8-80 \mu\text{g kg}^{-1}$) by the minicolumn method were actually found by HPLC to be $>80 \text{ ppb}$ (110 and $120 \mu\text{g kg}^{-1}$ by HPLC) and hence in Segregation 3 ($<80-400 \mu\text{g kg}^{-1}$) category. In another example, one out of the five samples segregated into Segregation 3 by the minicolumn method should have been placed into the Segregation 4 ($>400 \mu\text{g kg}^{-1}$) category ($447 \mu\text{g kg}^{-1}$ by HPLC). This suggested that there was some scope for error by minicolumn method when judging borderline differences in aflatoxin levels.

Validation of analytical methods using peanut samples collected in Indonesia: Relationship between ELISA data obtained from two laboratories

For the AFB₁ analysis, the peanut samples from the surveillance survey were initially analysed in duplicate sub-sample by the SUNQuik ELISA in Indonesia. Sub-sampling variation in the current sampling protocol as measured by the variation in two ELISA analyses was generally good for most of the samples. Only 18% of the samples gave greater than 20% CV between the duplicate analyses. Around 10% of the samples were randomly selected for confirmation by HPLC in Australia for quality control purposes. These samples were also analysed by either the SUNQuik ELISA alone or both the standard ELISA and the SUNQuik ELISA prior to testing at HPLC laboratories in Australia. Figure 3 shows the linear relationship of the SUNQuik ELISA data obtained from two laboratories using sub-samples collected in two different seasons.

For the same reason as in the previous studies, the final regression was performed by passing the line of best fit through zero. Samples found to contain AFB₁ below the limit of quantitation (LOQ) of the ELISA as determined by the initial method

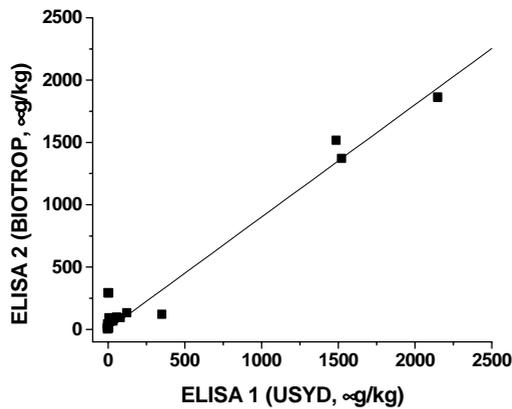


Figure 3. Relationship between the SUNQuik ELISA data obtained from two laboratories. The equation of linear least square fit is Y (ELISA 1, $\mu\text{g}/\text{kg}$) = $0.90 \pm 0.03 * X$ (ELISA 2, $\mu\text{g}/\text{kg}$) ($n=24$, $R=0.982$, $p < 0.0001$).

validation ($5 \mu\text{g kg}^{-1}$ in peanuts with 7% CV, Lee et al. 2004) were treated as zero in the regression calculation. Despite the expected high heterogeneity of the samples, the two sets of ELISA data had good agreement ($R=0.982$, $p < 0.0001$). The greater heterogeneity in the samples at lower concentrations and higher variation in immunoassay estimation at lower concentrations could contribute to some scattering of points at lower concentrations. As anticipated, ELISA inherently produced greater variation at the lower and upper ends of linear range of the standard curve than nearer to the middle point (IC_{50}) due to the sigmoidal dose-response effect. Thus overestimation of aflatoxin concentrations as a result of the greater variation could occur and care must be applied in interpreting and reporting data estimated at these extreme values. For any estimation closer to the upper limit of the linear range, extracts can be diluted for ELISA to provide estimations closer to the middle point. For any estimation closer to the lower limit of the linear range, re-analysis by either a more sensitive ELISA or HPLC analysis is recommended if more accurate data at low concentrations are required.

The SUNQuik ELISA vs the standard conventional ELISA

The antigen-antibody reaction time has a subsequent effect on the precision and accuracy of the assay and is one of the sources of errors in immunoassay. It is especially critical for rapid assays such as SUNQuik ELISA where the assays are not run under equilibrium conditions. To examine the effects of reaction time on the assay precision with naturally contaminated samples, the standard conventional ELISA where the binding reaction has sufficient time to reach equilibrium was compared with the SUNQuik ELISA where the reaction was stopped before equilibrium was reached. The study was conducted using the same sample extracts to avoid the complication of sub-sample variation. The regression tested with F -test ($p < 0.0001$) showed a good correlation between the two ELISAs with an R value of 0.993 (see Figure 4), indicating assay performance was not compromised in anyway by the short incubation time, as long as precise incubation time was kept.

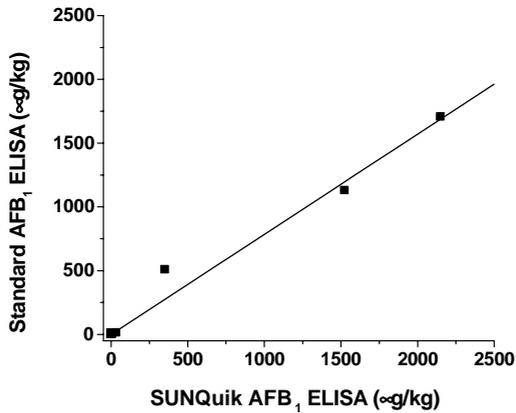


Figure 4. Relationship between the data obtained from the SUNQuik ELISA and the standard ELISA for the 23 peanut samples contaminated with aflatoxin. The equation of the linear least square fit is Y (Standard ELISA, $\mu\text{g}/\text{kg}$) = $0.79 \pm 0.02 * X$ (SUNQuik ELISA, $\mu\text{g}/\text{kg}$) ($n=23$, $R=0.993$, $p < 0.0001$).

Relationship between the SUNQuik ELISA and HPLC for the Indonesian peanut samples

Around 10% of the peanut samples from the surveillance surveys were re-analysed by two independent HPLC analytical laboratories in Australia as a quality control measure, as well as to examine the distribution of aflatoxin types. Greater than 90% of the *Aspergillus* population present in Indonesian peanuts is expected to be from infection of *A. flavus* (Dharmaputra & Retnowati 1996, Pitt et al. 1998), and hence AFB₁ should be the major contaminant representing >90% of the total aflatoxins. Thus, the surveillance surveys using AFB₁ analysis should be indicative of the total aflatoxin contamination for samples taken in Indonesia. The SUNQuik ELISA and HPLC analysis on different sub-samples were closely correlated ($R=0.938$, $p < 0.0001$, Figure 5), but not as well as when the same sample extracts were used in the comparison.

ELISA analysis was consistently slightly higher than HPLC (average 136% respective to HPLC). The overall difference between ELISA and HPLC analyses in this study was probably largely affected by sub-sampling variation even though the

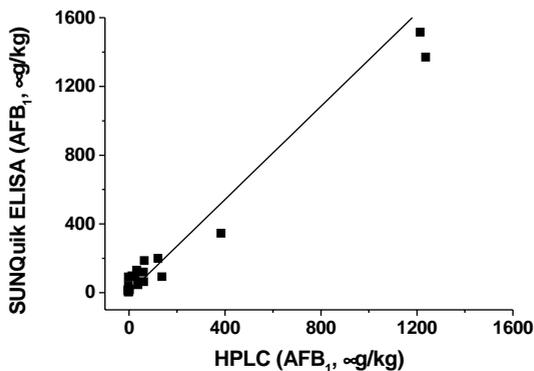


Figure 5. Relation between HPLC analysis of AFB₁ and ELISA data. The regression equation of the least square fit is Y (SUNQuik ELISA, $\mu\text{g}/\text{kg}$) = $1.36 \pm 0.09 * X$ (HPLC, $\mu\text{g}/\text{kg}$) ($n=30$, $R=0.938$, $p < 0.0001$).

sample size was relatively small (2 kg). The greater difference at lower concentrations by these two methods was probably a result of the combined factors of sub-sampling and variation in immunoassay as discussed earlier.

The SUNQuik ELISA performance parameters

The integrity of ELISA reagents is important, affecting the precision and accuracy of the test. Unstable reagents could lead to greater variation in data and result in lower precision. The assay precision was assessed over two years by monitoring several parameters of the standard curves such as maximum absorbance, IC_{50} , IC_{20} as a lower LOD and IC_{80} as an upper LOD. The results were calculated from 23 sets of assays. The average maximum absorbance was 0.876 ± 0.101 with %CV of 12%. The variability of absorbance as determined by %CV was dose-dependent as shown in Figure 6.

The %CV decreased as concentration decreased and ranged between 12–29%. After the dose-response was normalized by converting absorbance to % inhibition, the overall %CV of the standard curve was acceptably smaller and was not greater than 16%, thus considered acceptable in accordance with the EEC guidelines for precision, standardization and quality control for analytical methods (Krotzky & Zeeh 1995). The plot of IC_{20} , IC_{50} and IC_{80} values of 23 assays run over two years with upper and lower standard deviations and 95% confidence band are shown in Figure 7.

The deviations of IC_{20} and IC_{80} values as measured by %CV were 21% and 24%. The %CV for the IC_{50} where greatest precision was expected was 8%. These %CV values were within the recommended variation of 25%, 25% and 15% for low, high and midpoint of an ELISA standard curve respectively (Krotzky & Zeeh 1995). This implied that the stability of the reagents had been maintained over the testing period and the assay performance can be considered satisfactory.

It can be concluded that SUNQuik ELISA could be as precise and accurate as HPLC analysis for analysing AFB₁ in peanuts provided quality control has been implemented for ELISA to ensure the integrity of the reagents, proper preparation of standard solutions, and proper interpretation of data treated in the same manner as for

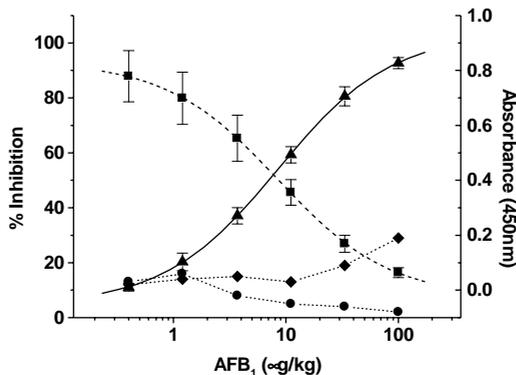


Figure 6. Standard curve of SUNQuik ELISA averaged of 23 assays run over two years. The square indicates absorbance against the aflatoxin B₁ concentration and the %CV for the absorbance is shown as diamond. The % inhibition (%I) is indicated by triangle and the %CV for the %I is shown as circles.

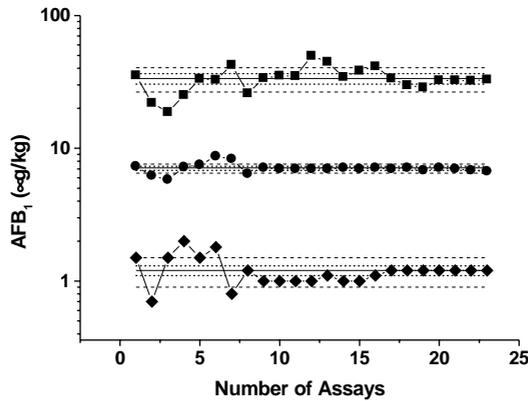


Figure 7. Precision of the SUNQuik ELISA determined as a variation of IC₂₀ (lower LOD of the standard curve), IC₅₀ and IC₈₀ (upper LOD of the standard curve) for 23 runs over two years. The dotted lines indicate 95% confidence band and the dashed lines are upper and lower standard deviations.

any analytical method. From these results, it is clear that provided due care is taken with quality control measures, the SUNQuik ELISA can be used as an accurate screening tool for segregating on the basis of aflatoxin contamination at the current maximum permissible limit of 15 µg/kg in peanuts. It is therefore a rapid, convenient and cost-effective method for use in surveillance surveys in Indonesia.

Analysis of biocontrol peanut samples inoculated with toxigenic and non-toxigenic A. flavus and A. parasiticus

Seventeen samples were obtained from a bio-control experiment for AFB₁ analysis by SUNQuik ELISA as part of the validation in a separate blind study. The study was

Table I. AFB₁ content in peanuts infected with toxigenic and non-toxigenic *A. flavus* and *A. parasiticus*, as analysed by the SUNQuik ELISA.

Sample treatment	AFB ₁ (µg/kg)
Non-inoculated peanut sample	0
1 x toxigenic <i>A. flavus</i> infected peanuts	162
2 x toxigenic <i>A. flavus</i> infected peanuts	223
3 x toxigenic <i>A. flavus</i> infected peanuts	416
1 x non-toxigenic <i>A. flavus</i> infected peanuts	0
2 x non-toxigenic <i>A. flavus</i> infected peanuts	0
3 x non-toxigenic <i>A. flavus</i> infected peanuts	0
1 x toxigenic <i>A. parasiticus</i> infected peanuts	234
2 x toxigenic <i>A. parasiticus</i> infected peanuts	64
3 x toxigenic <i>A. parasiticus</i> infected peanuts	574
1 x non-toxigenic <i>A. parasiticus</i> infected peanuts	0
2 x non-toxigenic <i>A. parasiticus</i> infected peanuts	0
3 x non-toxigenic <i>A. parasiticus</i> infected peanuts	0
1x non-toxigenic <i>A. flavus</i> + 1x non-toxigenic <i>A. parasiticus</i> infected peanuts	0
2x non-toxigenic <i>A. flavus</i> + 2x non-toxigenic <i>A. parasiticus</i> infected peanuts	0
1 x toxigenic <i>A. flavus</i> + 1x toxigenic <i>A. parasiticus</i> infected peanuts	408
2 x toxigenic <i>A. flavus</i> + 2x toxigenic <i>A. parasiticus</i> infected peanuts	294

1 x, 2 x and 3 x indicate the relative dose of infection.

conducted without the participants' foreknowledge of the kind of treatments given to these samples and the levels of contamination. As presented in Table I, the control sample and samples inoculated with non-toxicogenic strains were negative by ELISA.

Samples inoculated with toxigenic strains were positive and the levels of aflatoxin produced by the toxigenic strains were proportional to levels of inoculation received, with a couple of exceptions. For example, the samples inoculated with one, two and three levels of toxigenic *A. flavus* were found to have AFB₁ at 162, 223 and 416 µg kg⁻¹ roughly equivalent to the amount of inoculum received in each treatment. Evidently, the possibility for ELISA to produce false negative results is significantly lower. This further proved the reliability of the technique as an analytical tool as well as a cost-effective, high throughput-screening test for positives in similar studies.

Conclusion

The further validation of SUNQuik AFB₁ ELISA using naturally contaminated peanuts as the matrix of choice was undertaken to test its suitability as a decision-support diagnostic tool. These studies clearly demonstrate that ELISA is acceptable as an analytical method. A number of analytical methods currently used in research and by the Australian peanut industry were evaluated and found to be satisfactory in their performance. Despite the high expected sampling variation, an acceptable correlation between ELISA and HPLC for AFB₁ analysis was obtained when different sample extracts were used. More importantly, the accuracy of the SUNQuik ELISA was validated against a reference method using HPLC with fluorescence detection, and showed an exceptionally good correlation between ELISA and HPLC when the same sample extracts were used. The VICAM Aflatest[®] system also correlated very well with total aflatoxins (sum of AFB₁, AFB₂, AFG₁ and AFG₂) measured by HPLC. The minicolumn method also performed reasonably as a semi-quantitative screening test, but tended to skew towards concentrations slightly lower than HPLC. Its use as a rapid and low-cost screening test for use at receiving docks at shelling plants is probably justified. The rapid SUNQuik ELISA as compared with the standard conventional ELISA suggested that the rapid SUNQuik assay with the current operational protocol developed to maintain high operational precision was able to maintain the assay precision, acceptable as an analytical method. This was achieved by monitoring the variation in IC₂₀, IC₅₀ and IC₈₀ over two years and found they did not exceed the variation limits recommended by EEC guidelines for analytical methods (Krotzky & Zeeh 1995). Hence, it is important that all three parameters, as well as maximum absorbance, be monitored for quality control purposes. In summary, these validation studies strongly support the validity and reliability ELISA data generated from the pilot surveys conducted in Indonesia and the outcomes of these surveys in establishing the degree of severity and critical points of contamination in the local market supply chain in Pati and Wonogiri regions on the basis of current maximum permissible limit for aflatoxin (15 µg kg⁻¹).

Acknowledgements

The studies presented in this paper were supported by the Australian Government Centre for International Agricultural Research (Projects PHT1996/004 and

PHT1997/017). We thank Dr Victor Wong for his technical assistance during the validation study in Kingaroy Australia.

References

- AOAC International. 2002. Official methods of analysis of AOAC International, 17th ed. Gaithersburg, USA: AOAC International.
- Codex Alimentarius Commission. Food Standard Programme. 1999. Report of the 23rd session. Rome, 28 June–3 July 1999.
- Council for Agricultural Science and Technology (eds). 2003. Mycotoxins: Risks in plant, animal, and human systems. Task Force Report No 139, Chapter 2. Ames, Iowa, USA: CAST.
- Dharmaputra OS, Retnowati I. 1996. Fungi isolated from groundnuts in some locations of west Java. *Biotropia* 9:15–25.
- Dharmaputra OS, Retnowati I, Putri ASR, Ambarwati S. 2002. *Aspergillus flavus* and aflatoxin in peanuts at various stages of the delivery chain in Pati regency, Central Java. Paper presented at 21st ASEAN/3rd APEC seminar on post-harvest technology. Nusa Dua, Bali, Indonesia. 23–26 August 2003.
- European Community. 1998. Commission regulation (EC) No 152/98 of 16 July 1998 amending regulation (EC) No 194/97 of 31 January 1997 setting maximum levels for certain contaminations in food stuffs. *Off J Euro Communities*, July 17 1998, L 201/43.
- Fink-Gremmels J. 1999. Mycotoxins: Their implications for human and animal health. *Vet Quarterly* 21:115–120.
- Food and Agricultural Organization. 2001. Manual on the application of the HACCP system in mycotoxin prevention and control. Rome: Food and Agricultural Organization of United Nations.
- Ganzer C. 1999. Aflatoxin reduction in the blanching process. In: Dietzgen RG, editor. Elimination of aflatoxin contamination in peanut. Canberra, Australia: Australian Centre for International Agricultural Research. p 10–11.
- Hansen RB, Norman KL. 1999. Economic importance of aflatoxin to Australia Peanut Industry. In: Dietzgen RG, editor. Elimination of aflatoxin contamination in peanut. Canberra, Australia: Australian Centre for International Agricultural Research. p 7–9.
- International Agency for Research on Cancer. 1993. IARC Monograph on the evaluation of carcinogenic risk to humans. Vol. 56, IARC, Lyon, France.
- Krotzky AJ, Zeeh B. 1995. Immunoassays for residue analysis of agrochemicals: proposed guidelines for precision, standardization and quality control (Technical Report). *Pure Appl Chem* 67:2065–2088.
- Lee NA, Wang S, Allan RD, Kennedy IR. 2004. A rapid aflatoxin B₁ ELISA: Development and validation with reduced matrix effects for peanuts, corn, pistachio, and soybeans. *J Agric Food Chem* 52:2746–2755.
- Lubulwa ASG, Davis JS. 1994. Estimating the social cost of the impacts of fungi and aflatoxins. In: E. Highley et al., editors. Stored product protection. Proc 6th int working conf Stored-Product Protection. Wallingford, UK: CAB International. p 1017–1042.
- Otsuki T, Wilson JS, Sewadeh M. 2000. A race to top? A case study of food safety standards and African exports. Policy Res Working Paper. Washington DC: World Bank.
- Pitt JI, Hocking AD, Miscamble BF, Dharmaputra OS, Kuswanto KR, Rahayu ES, Sardjono. 1998. The mycoflora of food commodities from Indonesia. *J Food Mycol* 1:41–60.
- Queensland Department of Primary Industry. 2000. Aflatoxin in peanuts: Tips to reduce the risk. Crop Link. Queensland Department of Primary Industries, Farming Systems Institute, Kingaroy, QDPI publication.
- Whitaker TB. 2003. Standardisation of mycotoxin sampling procedures: An urgent necessity. *Food Control* 14:233–237.